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Amino Acid-stimulated Ca^{2+} Oscillations Produced by the Ca^{2+}-sensing Receptor Are Mediated by a Phospholipase C/Inositol 1,4,5-Trisphosphate-independent Pathway That Requires G_{12}, Rho, Filamin-A, and the Actin Cytoskeleton*

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The G protein-coupled Ca^{2+}-sensing receptor (CaR) is an allosteric protein that responds to two different agonists, Ca^{2+} and aromatic amino acids, with the production of sinusoidal or transient oscillations in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Here, we examined whether these differing patterns of $[Ca^{2+}]_i$ oscillations produced by the CaR are mediated by separate signal transduction pathways. Using real time imaging of changes in phosphatidylinositol 4,5-biphosphate hydrolysis and generation of inositol 1,4,5-trisphosphate in single cells, we found that stimulation of CaR by an increase in the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) leads to periodic synthesis of inositol 1,4,5-trisphosphate, whereas L-phenylalanine stimulation of the CaR does not induce any detectable change in the level this second messenger. Furthermore, we identified a novel pathway that mediates transient $[Ca^{2+}]_i$ oscillations produced by the CaR in response to L-phenylalanine, which requires the organization of the actin cytoskeleton and involves the small GTPase Rho, heterotrimeric proteins of the G_{12} subfamily, the C-terminal region of the CaR, and the scaffolding protein filamin-A. Our model envisages that Ca^{2+} or amino acids stabilize unique CaR conformations that favor coupling to different G proteins and subsequent activation of distinct downstream signaling pathways.

The extracellular Ca^{2+}-sensing receptor (CaR),[1] a member of the family C of G protein-coupled receptors (GPCRs), has a major role in the adjustment of the extracellular Ca^{2+} concentrations ($[Ca^{2+}]_o$) by regulating parathyroid hormone secretion and the rate of Ca^{2+} reabsorption by the kidney (1, 2). Inactivating and activating mutations of the CaR in humans (3) and genetic disruption of the CaR gene in mice (4) established that the CaR functions in the control of Ca^{2+} homeostasis. Specifically, a major physiological role of the CaR is to correct small changes in $[Ca^{2+}]_o$ concentration by regulating parathyroid hormone secretion. The CaR is also expressed in the gastrointestinal tract, brain, pituitary, thyroid, skin, breast, pancreas, lung, bone, and heart (reviewed in Ref. 5), suggesting that this receptor plays additional, yet less well defined, physiological roles in the regulation of cell function (6, 7). Thus, the mechanisms that regulate CaR activity are attracting major attention.

Recent studies of CaR activation in individual living cells has shown that intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) oscillates upon stimulation of CaR by an elevation in $[Ca^{2+}]_o$ (8, 9). In addition to its role as sensor of $[Ca^{2+}]_o$, the CaR is also stimulated by aromatic amino acids (10) that, like $[Ca^{2+}]_o$, induce striking and lasting CaR-mediated $[Ca^{2+}]_i$ oscillations (9, 11). However, the patterns of $[Ca^{2+}]_i$ oscillations induced by these agonists are different. Aromatic amino acid stimulation of the CaR induces repetitive, low frequency $[Ca^{2+}]_i$ spikes that return to the base-line level, a pattern known as transient oscillations. In contrast, $[Ca^{2+}]_o$-elicited CaR activation produces high frequency sinusoidal oscillations upon a raised plateau level of $[Ca^{2+}]_i$ (9, 11). The amplitude, frequency, and duration of $[Ca^{2+}]_i$ oscillations are increasingly recognized as encoding important information for a variety of biological processes, and, consequently, there is intense interest in understanding the underlying mechanisms (12).

Our previous results produced several lines of evidence indicating that PKCs negatively regulate the frequency of $[Ca^{2+}]_i$ oscillations induced by activation of the CaR by increases in $[Ca^{2+}]_o$ (11). We hypothesized that periodic phosphorylation of the CaR by PKCs provides the negative feedback needed to cause $[Ca^{2+}]_o$-induced sinusoidal $[Ca^{2+}]_i$ oscillations. Intriguingly, the transient $[Ca^{2+}]_i$ oscillations produced by the CaR in response to amino acid stimulation appear to be mediated by a different pathway, but the mechanism(s) involved remained poorly understood.

In the present study, we examined whether sinusoidal and transient $[Ca^{2+}]_i$ oscillations produced by the CaR in response to Ca^{2+} or L-phenylalanine are mediated by different pathways. Using real time imaging of changes in phosphatidylinositol 4,5-biphosphate hydrolysis and generation of $Ins(1,4,5)P_3$ in single cells, we found that $[Ca^{2+}]_o$-induced CaR activation

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[1] The abbreviations used are: CaR, Ca^{2+}-sensing receptor; $[Ca^{2+}]_o$, extracellular Ca^{2+}; $[Ca^{2+}]_i$, intracellular Ca^{2+}; GPCR, G protein-coupled receptor; PKC, protein kinase C; PKD, protein kinase D; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; HEK, human embryonic kidney; MEF, murine embryonic fibroblasts; RK−/−, rhodopsin kinase knock-out mice; RPTEC, renal proximal tubule epithelial cells; RFP, red fluorescent protein; YFP, yellow fluorescent protein; GFP, green fluorescent protein; PHD, pleckstrin homology domain; PLC, phospholipase C; DAG, diacylglycerol.

leads to periodic synthesis of Ins(1,4,5)P_3, whereas L-phenylalanine stimulation of the CaR does not induce any detectable changes in the level of this second messenger. Furthermore, we identified a novel pathway that mediates transient $[Ca^{2+}]_i$ oscillations produced by the CaR in response to amino acids, which requires the organization of the actin cytoskeleton and involves the small GTPase Rho, heterotrimeric proteins of the G_{12} subfamily, the C-terminal distal region of the receptor, and the scaffolding protein filamin-A. Our model envisages that Ca^{2+} or amino acids induce distinct conformational states of the CaR, providing for the possibility of differential coupling to downstream signaling pathways.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—Human Embryonic Kidney (HEK-293) cells and murine embryonic fibroblasts (MEF), generated from doubled knock-out mice for rhodopsin kinase (RK) or $G_{12/13}-/-$ were maintained as described previously (11, 13). Primary renal proximal tubule epithelial cells (RPTEC) of human origin were obtained and maintained as suggested by Clonetics, CA. A cell line constitutively expressing the CaR was established by transfecting HEK-293 with an expression vector encoding the human CaR (pCR3.1-CaR), kindly provided by Dr. Allen Spiegel, NIDDK, National Institutes of Health. Transfections of the different plasmids were performed using Lipofectin or Lipofectamine Plus (Invitrogen) as described previously (14). Analysis of the cells transiently transfected were performed 24 or 72 (*in vitro* kinase assay) h posttransfection.

Single Cell $[Ca^{2+}]_i$ Imaging—$[Ca^{2+}]_i$ was measured in cells loaded with the calcium indicator fura-2 as described previously (11). Briefly, cells were incubated in saline solution (Hanks' balanced salt solution (Invitrogen) without phenol red, containing with 138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5 mM KCl, 0.3 mM KH_2PO_4, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 5.6 mM D-glucose, 20 mM HEPES, pH 7.4) containing 5 μM fura-2/AM for 45–60 min at 37 °C. The cells were then washed and placed in an experimental chamber, which was perfused with saline solution at 1.5 ml/min at 37 °C. The chamber in turn was placed on the stage of an inverted microscope connected to a digital imaging system. Ratios of images (340 nm excitation/380 nm excitation, emission filter 520 nm) were obtained at 1.5-s intervals. A region of interest covering 15 × 15 μm was defined over each cell, and the average ratio intensity over the region was converted to $[Ca^{2+}]_i$ using a calibration curve constructed with a series of calibrated buffered calcium solutions (Calcium Calibration Buffer Kit #2, Molecular Probes, OR). Identification of cells transiently expressing *Clostridium botulinum* C3 exoenzyme, Rho G14V, Fil-A14/15, CaRstop907, or CaR (see "cDNAs") was achieved by cotransfection with a plasmid encoding wild type green (GFP) or red fluorescent protein (RFP) (BD Biosciences). A minimum of 100 cells/experiment, each experiment done in at least duplicate or triplicate were used to measured $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$, Ins(1,4,5)P_3, and Diacylglycerol Imaging in a Single Live Cell—Simultaneous single live cell imaging of the fluorescent biosensors for $[Ca^{2+}]_i$ (PKCα-yellow fluorescent protein (YFP)), Ins(1,4,5)P_3 (GFP-PHD), and diacylglycerol (PKD-RFP) was achieved by emission fingerprinting with a confocal LSM 510 Meta microscope (Carl Zeiss, Germany) (nm excitation/nm emission PKCα-YFP: 514/527; GFP-PHD: 488/507; PKD-RFP: 543/583). Culture conditions for live cell imaging and quantitative analysis of the relative change in plasma membrane and cytosol fluorescence intensity of individual cells were performed as described previously (14), analyzing 50 cells/experiment, with each experiment done in at least duplicate or triplicate. The selected cells displayed in the figures were representative of 90% of the population of positive cells.

Western Blot, PKD Kinase Assays, and Exogenous Substrate Phosphorylation—Western blot analysis was performed as previously described (15). PKD autophosphorylation was determined in an *in vitro* kinase assay followed by SDS-PAGE analysis and quantification as described previously (13). Exogenous substrate syntide-2 phosphorylation by PKD was assayed and quantified by Cerenkov counting as previously published (16).

cDNAs—Primers for the synthesis of the cDNA encoding the pleckstrin homology domain (PHD) of human phospholipase C (PLC)-δ1 and human PKCα were designed with the Primer3 program (17). The sense primers for PHD and PKCα were 5′-CCGAATTCACGGCCTACAGGATGATGAG-3′ and 5′-CCGAATTCGGGGGGGGACCATGGCTGACGT-3′, respectively, whereas the corresponding antisense primers were 5′-CCGAATTCGGAAGTTCTGCAGCTCCTTG-3′ and 5′-CGGAATTCGCGCTGGTGAGTTTGCTACTGCACTCTG-3′. Total RNA extracted from human pancreatic cancer Panc-1 cells was employed as template for the synthesis of the cDNAs encoding the PHD and PKCα using reverse transcription-PCR (18). PHD and PKCα were cloned into the EcoRI site of vectors encoding the GFP or YFP, pEGFP-C1 or pEYFP-N1. A cDNA encoding human filamin-A, containing amino acids 1530 through 1875 (14 and 15 domains) (Fil-A14/15), was synthesized by reverse transcription-PCR using total RNA extracted form Panc-1 cells (see above) and the forward/reverse primers 5′-CTTAAGCTTCCATGGTACCCCGGAGCCCC-3′/5′-CGGAATTCCGAGGCCAGGCCCATAGGC-3′. The obtained cDNA was cloned into the EcoRI/HindIII sites of pcDNA3.1 *myc*-His C (Invitrogen). Site-directed mutagenesis to introduce a stop codon after amino acid 906 of the CaSR (CaR stop907) was performed as previously described (14) with the forward and reverse primers 5′-GGAGGCTCCACGTGATCAACCCCCTCCTCC-3′ and 5′-GGAGGAGGGGGTTGATCACGTGGAGCCTCC-3′, respectively. All of the constructs were verified by DNA sequencing and Western blot analysis. The vectors encoding the fusion protein between protein kinase D (PKD) and a red fluorescent protein (pPKD-RFP), untagged PKD and *C. botulinum* C3 exoenzyme were described previously (14, 16). The cDNA for the constitutively active human Rho G14V was obtained from the University of Missouri-Rolla cDNA Resource Center, MO.

Materials—[γ-^{32}P]ATP (370 MBq/ml) and horseradish peroxidase-conjugated donkey anti-rabbit IgG were from Amersham Biosciences. The anti-Ser(P)^{744}/Ser(P)^{748}, which specifically recognizes the phosphorylated state of those serines within the activation loop of protein kinase D (PKD), was obtained from Cell Signaling Technology. The anti-PKCμ antibody (C-20), which recognizes PKD, was obtained from Santa Cruz Biotechnology. The anti-RFP antibody was obtained from BD Biosciences. The anti-CaR was obtained from Affinity BioReagents. Fura-2/AM was purchased from Molecular Probes. All of the other reagents were the highest grade commercially available.

RESULTS

Differential Production of Ins (1,4,5)P_3 and Diacylglycerol (DAG) Mediated by the CaR in HEK-293 Cells Stimulated by L-Phenylalanine or $[Ca^{2+}]_e$—Several models have been proposed to explain the generation of $[Ca^{2+}]_i$ oscillations in response to GPCR activation, but definitive evidence identifying the mechanism(s) involved is available in very few instances. A major advance in this field has been the development of probes that allow the monitoring of the intracellular concentration of the two second messengers generated by PLC activation, Ins(1,4,5)P_3 and DAG. To determine whether $[Ca^{2+}]_o$ and aromatic amino acids differ in their ability to trigger the synthesis of these second messengers via the CaR, we used a set of biosensors to monitor, in real time and in the same cells, the synthesis of Ins(1,4,5)P_3 and DAG simultaneously with $[Ca^{2+}]_i$ oscillations. The synthesis of Ins(1,4,5)P_3 was monitored by examining the dynamic distribution of a fusion protein between GFP and the PHD of PLC-δ1 (GFP-PHD). This PHD binds phosphatidylinositol 4,5-biphosphate in the plasma membrane but translocates to the cytosol in response to PLC-mediated Ins(1,4,5)P_3 synthesis (19–22). DAG synthesis was monitored by imaging a fusion protein consisting of PKD and a red fluorescent protein (PKD-RFP) (14). PKD is a Ca^{2+}-insensitive serine/threonine kinase that rapidly translocates from the cytosol to the plasma membrane in response to PLC-mediated DAG production in response to GPCR stimulation (14, 23). As an indication of $[Ca^{2+}]_i$ oscillations, we monitored the cytosol to plasma membrane translocation of a chimeric protein between PKCα, a Ca^{2+}- and phospholipid-dependent PKC, and a yellow fluorescent protein (PKCα-YFP) (24).

To examine CaR-mediated changes in second messenger generation in single cells, HEK-293 cells stably expressing the human CaR, a cell system widely used to study CaR regulation (6), were cotransfected with plasmids encoding GFP-PHD, PKD-RFP, and PKCα-YFP. The cells were subsequently stimulated by increasing the $[Ca^{2+}]_o$ to 5 mM (Fig. 1*A*) or by addition of 5 mM L-phenylalanine (Fig. 1*B*). The intracellular distribution of the biosensors was monitored simultaneously in

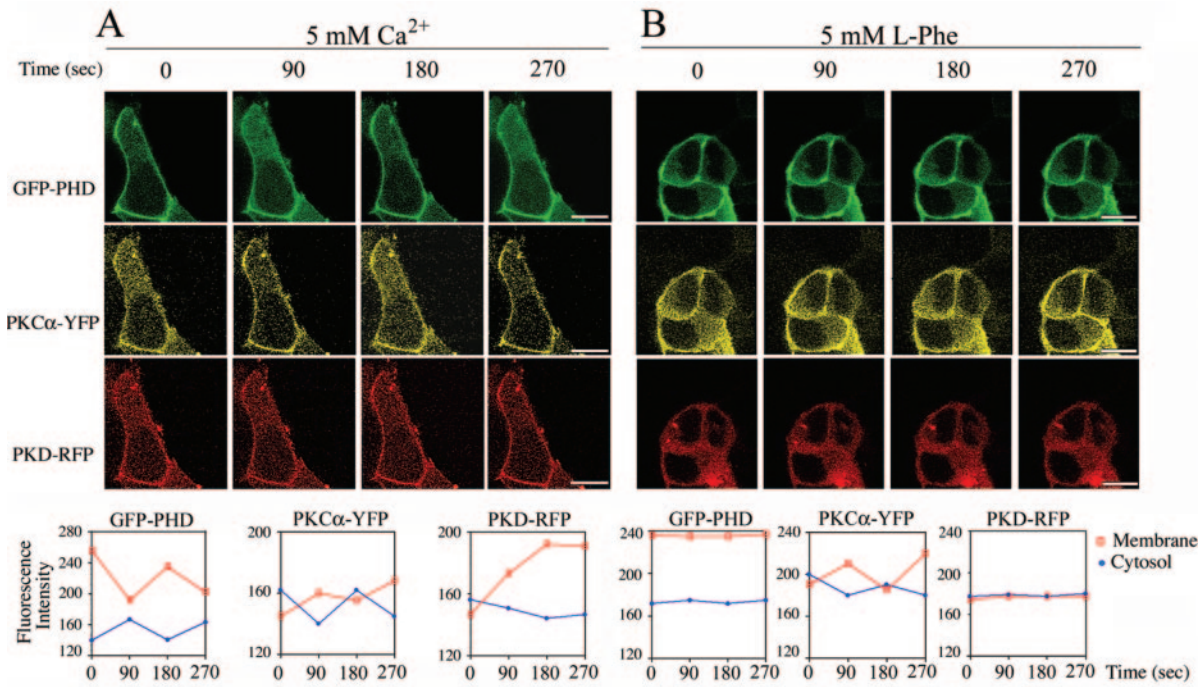

FIG. 1. **$[Ca^{2+}]_i$ oscillations and Ins(1,4,5)P_3/DAG synthesis in response to CaR stimulation.** HEK-293 cells constitutively expressing the CaR were transiently cotransfected with plasmids encoding sensors for Ins(1,4,5)P_3 (GFP-PHD), DAG (PKD-RFP), and Ca^{2+} (PKCα-YFP). The cultures were perfused with a saline solution (see "Experimental Procedures") containing 5 mM Ca^{2+} (*A*) or 5 mM L-phenylalanine (*L-Phe*) (*B*) during the indicated times and the intracellular distribution of the different sensors monitored by emission fingerprinting with a confocal microscope. Culture conditions for live cell imaging and quantitative analysis (*bottom panels*) of the relative change in plasma membrane and cytosol fluorescence intensity of individual cells was performed as described previously (14) analyzing 50 cells/experiment, with each experiment done at least in duplicate. The selected cells displayed in the figures were representative of 90% of the population of positive cells.

live cells using emission fingerprinting. In non-stimulated cells, the Ins(1,4,5)P_3 sensor GFP-PHD was predominantly localized at the plasma membrane, whereas the DAG sensor PKD-RFP was distributed throughout the cytoplasm but excluded from the nucleus. The distribution of PKCα-YFP was similar to PKD-RFP, *i.e.* throughout the cytoplasm, although in few cells PKCα-YFP was also present in the nucleus. GFP, RFP, or YFP alone localized both in the cytoplasm and nucleus (data not shown). $[Ca^{2+}]_o$-elicited CaR stimulation induced an oscillatory translocation of GFP-PHD from the plasma membrane to the cytoplasm, reflecting periodic production of Ins(1,4,5)P_3. These changes coincided with the oscillatory translocation of PKCα-YFP from the cytoplasm to the plasma membrane. DAG synthesis, monitored by the translocation of its sensor from the cytosol to the plasma membrane, peaked after 3 min. Within 5–6 min of decreasing the $[Ca^{2+}]_o$ to the basal level (1.5 mM), the sensors returned to the subcellular compartments they occupied before stimulation (data not shown).

In striking contrast to the results obtained with $[Ca^{2+}]_o$ stimulation, L-phenylalanine-elicited CaR activation did not induce any detectable synthesis of either Ins(1,4,5)P_3 or DAG, as revealed by the lack of redistribution of their corresponding sensors (Fig. 1*B*). The lack of translocation of the DAG sensor to the plasma membrane was not because of a low level of DAG accumulation. Inhibition of DAG conversion to phosphatidic acid by preventing its phosphorylation with 10 μM diacylglycerol kinase inhibitor I (IC_{50} = 2.8 μM) or II (IC_{50} = 120 nM) for 10 min, concurrently with 5 mM L-phenylalanine stimulation, failed to promote any plasma membrane translocation of the DAG sensor (data not shown). However, L-phenylalanine stimulation of the CaR induced a distinct and oscillatory translocation of PKCα-YFP from the cytosol to the plasma membrane (Fig. 1*B*). These results demonstrate that although both an increase in $[Ca^{2+}]_o$ or the addition of L-phenylalanine induce $[Ca^{2+}]_i$ oscillations via the CaR, only $[Ca^{2+}]_o$ induced periodic synthesis of Ins(1,4,5)P_3 and DAG production. These experiments provide strong support for the hypothesis that the CaR mediates sinusoidal or transient patterns of $[Ca^{2+}]_i$ oscillations in response to different agonists through different mechanisms.

Differential Regulation of PKD Activation Loop Phosphorylation and Kinase Activity in Response to CaR Activation by $[Ca^{2+}]_e$ or L-Phenylalanine—Recently, we demonstrated that the plasma membrane translocation of PKD, in response to GPCR-induced DAG synthesis, is necessary for PKCϵ-mediated phosphorylation of the activation loop of PKD, a critical step in the catalytic activation of this enzyme (23). In view of the results presented in Fig. 1, we predicted that $[Ca^{2+}]_o$-elicited CaR activation should lead to the PKC-mediated activation loop phosphorylation of PKD at Ser^{744} and Ser^{748}, leading to the catalytic activation of this kinase (25, 26). Fig. 2*A* shows that $[Ca^{2+}]_o$-elicited CaR activation induced a striking increase in the phosphorylation of Ser^{744} and Ser^{748} of PKD. In contrast, L-phenylalanine did not promote any detectable increase in the activation loop phosphorylation of PKD (Fig. 2*A*), even in the presence of diacylglycerol kinase inhibitor I or II at a 10 μM final concentration (data not shown). Further support for this conclusion was obtained by measuring PKD autophosphorylation. HEK-293 cells expressing the CaR stimulated with $[Ca^{2+}]_o$ or L-phenylalanine were lysed and PKD immunoprecipitated from the extracts. The immune complexes were incubated with [γ-^{32}P]ATP, subjected to SDS-PAGE, and analyzed by autoradiography to detect the prominent 110-kDa band corresponding to autophosphorylated PKD. The results presented in Fig. 2*B* show that $[Ca^{2+}]_o$, but not L-phenylalanine, induced a marked increase in PKD autophosphorylation activity. As illustrated in Fig. 2*C*, similar results were obtained when PKD activity in the immunocomplexes was determined by phosphorylation of syntide-2 (27, 28), a synthetic peptide previously demonstrated to be an excellent substrate for PKD (29). These results support the notion that an increase in $[Ca^{2+}]_o$ and amino

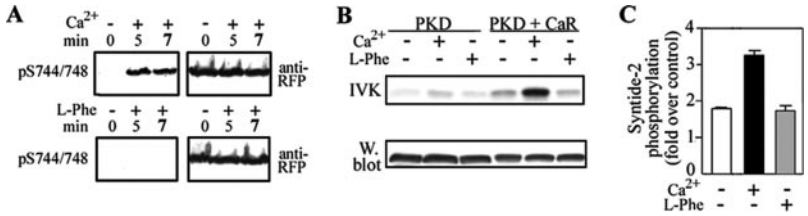

FIG. 2. **CaR regulation of PKD catalytic activation.** *A*, HEK-293 cells constitutively expressing the CaR and transiently transfected with a plasmid encoding PKD-RFP were stimulated with medium containing 5 mM Ca^{2+} or 5 mM L-phenylalanine (*L-Phe*) and lysed at the indicated times, and PKD phosphorylation was examined by Western blot using an antibody that recognizes the phosphorylation status of Ser^{744} and Ser^{748} within the activation loop of PKD (*pS744/748*). Anti-RFP was used as loading control for PKD-RFP. Results are representative of three independent experiments. HEK-293 cells cotransfected with plasmids encoding untagged PKD and the CaR were left unstimulated (−) or stimulated (+) with 5 mM Ca^{2+} or 5 mM L-phenylalanine for 10 min and lysed. PKD was immunoprecipitated from the lysates and its kinase activity determined by autophosphorylation activity (*IVK*) (*B*) and by phosphorylation of syntide-2, a PKD exogenous substrate (*C*). The autoradiogram shown in *B*, *top panel*, is representative of two independent experiments. Aliquots of total cell lysates were analyzed by Western blot (*W. blot*) to determine levels of PKD expression (*B*, *bottom panel*). *C*, graph represents the mean ± S.E. of syntide-2 phosphorylation obtained from two independent experiments, each performed in duplicate.

acids activate different intracellular signaling pathways via the CaR. Comparable results were obtained using COS-7 cells transiently expressing the CaR (data not shown).

Differential Intracellular Ca^{2+} Oscillations Mediated by L-Phenylalanine or $[Ca^{2+}]_e$ Stimulation of the CaR—The results presented in Figs. 1 and 2, and our previous studies (9, 11), support the notion that $[Ca^{2+}]_o$-elicited CaR activation leads to stimulation of the G_q/PLC pathway leading to the production of sinusoidal $[Ca^{2+}]_i$ oscillations. In contrast, the signaling pathways emanating from the CaR activated by L-amino acids that generate base-line $[Ca^{2+}]_i$ oscillations remain poorly understood. Consequently, we next focused our attention to the mechanism by which L-amino acids induce $[Ca^{2+}]_i$ oscillations through the CaR.

To examine CaR-mediated changes in $[Ca^{2+}]_i$ in single cells, HEK-293 cells stably transfected with human CaR cDNA were loaded with the fluorescent Ca^{2+} indicator fura-2 and imaged as described under "Experimental Procedures." As shown in Fig. 3*A*, intracellular Ca^{2+} imaging revealed that addition of 5 mM L-phenylalanine stimulated striking transient $[Ca^{2+}]_i$ oscillations that reached, on average, peaks of 300 nM $[Ca^{2+}]_i$ and that each $[Ca^{2+}]_i$ peak returned to base-line value (mean frequency of 1.3 ± 0.1 min^{-1}). In contrast, an increase in the $[Ca^{2+}]_o$ from 1.8 mM to 5 mM induced a rapid elevation in $[Ca^{2+}]_i$ to a new base-line level followed by oscillatory $[Ca^{2+}]_i$ fluctuations with a mean frequency of 3.8 ± 0.2 min^{-1}. These sinusoidal $[Ca^{2+}]_i$ oscillations ceased when $[Ca^{2+}]_o$ was returned to 1.8 mM.

We also examined whether the differential effects induced by L-phenylalanine or $[Ca^{2+}]_o$ on $[Ca^{2+}]_i$ oscillations are observed in cells exposed to other concentrations of these agonists. Addition of L-phenylalanine at 15 mM (rather than 5 mM) or a smaller $[Ca^{2+}]_o$ increment (to 2.25 mM instead of 5 mM) also elicited transient and sinusoidal $[Ca^{2+}]_i$ oscillations, respectively (Fig. 3*A*). However, an increase in $[Ca^{2+}]_o$ to 2.25 mM instead of 5 mM induced a smaller shift in the $[Ca^{2+}]_i$ base line followed by oscillatory $[Ca^{2+}]_i$ fluctuations that reached almost identical $[Ca^{2+}]_i$ peaks, *i.e.* 300 nM, to those induced by L-phenylalanine. We next determined the effect of a small increase in $[Ca^{2+}]_o$ or that of L-phenylalanine at higher concentrations on DAG accumulation and PKD phosphorylation. As shown in Fig. 3*B*, an elevation of $[Ca^{2+}]_o$ to 2.25 mM promoted both the translocation of the DAG sensor (*arrows*) as well as the PKC-dependent activation loop phosphorylation of PKD at Ser^{744} and Ser^{748}. In contrast, addition of 15 mM L-phenylalanine failed to elicit any detectable translocation of the DAG sensor or PKD phosphorylation at Ser^{744} and Ser^{748} (Fig. 3*B*). These results demonstrate that the difference in the pattern of signaling induced by L-phenylalanine and $[Ca^{2+}]_o$ via the CaR was maintained at different concentrations of these agonists, even when the concentrations of these agonists were adjusted to produce equivalent $[Ca^{2+}]_i$ peaks. Specifically, DAG synthesis and PKD activation loop phosphorylation occurred in association with sinusoidal but not transient $[Ca^{2+}]_i$ oscillations.

In addition to the parathyroid gland, the CaR has also been cloned from the kidneys of humans, rabbits, and rats (30–32). Within the kidney, several regions such as the proximal tubule express the CaR (33). Consequently, we obtained primary RPTEC of human origin to examine whether L-phenylalanine and $[Ca^{2+}]_o$ also induced different patterns of $[Ca^{2+}]_i$ oscillations in cells endogenously expressing the CaR. Western blot analysis of RPTEC showed that these cells express similar levels of CaR to HEK-293 ectopically expressing the same receptor (Fig. 3*C*, *inset*, *lanes 3* and *2*, respectively). No signal was detected in wild type non-transfected HEK-293 cells (Fig. 3*C*, *inset*, *lane 1*) or when the reactivity of the human CaR antibody was eliminated by preincubating the antiserum with the immunizing peptide (data not shown). Subsequently, RPTEC were loaded with the fluorescent Ca^{2+} indicator fura-2 and imaged as described under "Experimental Procedures." As shown in Fig. 3*C*, L-phenylalanine or $[Ca^{2+}]_o$ stimulation of RPTEC elicited $[Ca^{2+}]_i$ oscillations similar to the ones detected in HEK-293 cells ectopically expressing the CaR. Specifically, addition of 5 mM L-phenylalanine to RPTEC induced repetitive $[Ca^{2+}]_i$ peaks that returned to base-line value, whereas an increase in the $[Ca^{2+}]_o$ from 1.8 to 5.0 mM produced a rapid elevation in $[Ca^{2+}]_i$ followed by striking oscillatory fluctuations in $[Ca^{2+}]_i$ that did not return to base-line values, characteristic of sinusoidal oscillations. In further agreement with the results obtained with the HEK-293 cell model (Fig. 2*A*), $[Ca^{2+}]_o$ stimulation of RPTEC induced the phosphorylation of Ser^{744} and Ser^{748} within the activation loop of endogenously expressed PKD (Fig. 3*C*). In contrast, L-phenylalanine did not promote any detectable increase in the activation loop phosphorylation of PKD (Fig. 3*C*). These results demonstrated that the differential signaling evoked by L-phenylalanine and $[Ca^{2+}]_o$ are not restricted to cells transfected with constructs encoding CaR but can be also obtained in cells that endogenously express this receptor.

CaR-mediated Transient $[Ca^{2+}]_i$ Oscillations Require the Organization of the Actin Cytoskeleton—A number of studies have shown that the organization of the actin cytoskeleton plays a role in the generation of $[Ca^{2+}]_i$ oscillations in at least some cell types (34, 35). As a first step to elucidate the mechanism(s) involved in L-amino acid-induced $[Ca^{2+}]_i$ oscillations through the CaR, we determined whether disruption of the actin cytoskeleton differentially affects this type of $[Ca^{2+}]_i$ oscillations. We utilized the structurally unrelated agents cytochalasin D and latrunculin A, which induce actin cytoskeleton depolymerization through different mechanisms (36). Cytochalasin D binds to the growing end of actin filaments, leading to disrup-

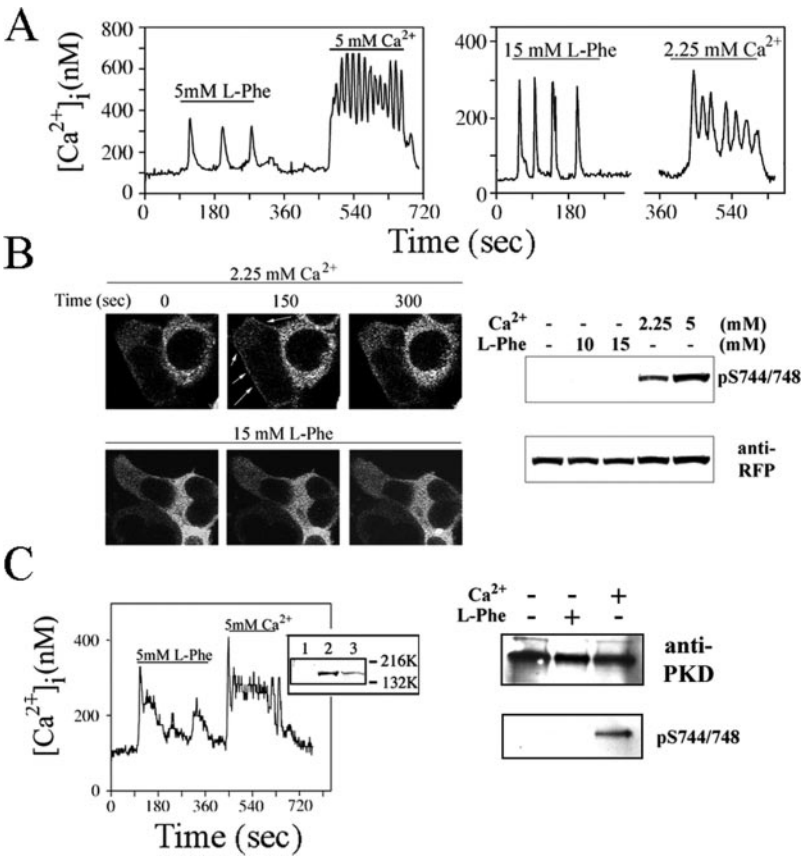

FIG. 3. CaR-mediated $[Ca^{2+}]_i$ oscillations in response to different concentrations of extracellular Ca^{2+} or L-phenylalanine (*L-Phe*) (*A*). HEK-293 cells constitutively expressing the CaR were perfused with saline solution containing the denoted concentrations of Ca^{2+} or L-phenylalanine during the times indicated by the horizontal bars and the $[Ca^{2+}]_i$ oscillations measured in individual cells as described under "Experimental Procedures." A minimum of 100 cells/experiment, done in at least duplicate, were used to measured $[Ca^{2+}]_i$. CaR-mediated PKD translocation and phosphorylation in response to extracellular Ca^{2+} or L-phenylalanine (*B*). HEK-293 cells constitutively expressing the CaR were transiently transfected with the plasmid encoding the sensor for DAG (PKD-RFP). The cultures were perfused with a saline solution containing 2.25 mM Ca^{2+} or 15 mM L-phenylalanine during the indicated times, and the intracellular distribution of the sensor monitored with a confocal microscope as described above. The selected cells displayed in the figures were representative of 90% of the population of positive cells. Alternatively, the cultures were stimulated with the indicated concentrations of Ca^{2+} or L-phenylalanine for 10 min and lysed, and PKD phosphorylation (*pS744/748*) was examined by Western blot. Anti-RFP was used as loading control for PKD-RFP. Results are representative of three independent experiments. $[Ca^{2+}]_i$ increments and DAG synthesis in response extracellular Ca^{2+} or L-phenylalanine stimulation of primary renal proximal tubule epithelial cells endogenously expressing the CaR (*C*). RPTEC endogenously expressing the CaR (*inset*) were perfused with saline solution containing 5 mM L-phenylalanine or 5 mM Ca^{2+} during the times indicated by the *horizontal bars* and the $[Ca^{2+}]_i$ oscillations measured in individual cells as described under "Experimental Procedures." A minimum of 50 cells/experiment, done in duplicate, were used to measured $[Ca^{2+}]_i$. Alternatively, the cultures were stimulated with 5 mM Ca^{2+} or 5 mM L-phenylalanine for 10 min and lysed, and PKD phosphorylation (*pS744/748*) was examined by SDS-PAGE and Western blot. Anti-PKD was used as loading control for total PKD. Results are representative of two independent experiments. *Inset*, wild type HEK-239 cells (*lane 1*), HEK-239 cells constitutively expressing the CaR (*lane 2*), and RPTEC (*lane 3*) were lysed, and the expression of the CaR protein was determined by SDS-PAGE and Western blotting using an anti-CaR antibody. Results are representative of two independent experiments.

tion of actively turning over actin stress fibers. Latrunculin A sequesters actin monomers and effectively disrupts both actin stress fibers and cortical actin filaments, which are more resistant to cytochalasin D.

As shown in Fig. 4, treatment of the cells with either cytochalasin D or latrunculin A abolished the generation of transient $[Ca^{2+}]_i$ oscillations elicited by addition of L-phenylalanine but did not prevent the sinusoidal $[Ca^{2+}]_i$ oscillations evoked by an increase in $[Ca^{2+}]_o$. In contrast, treatment of HEK-293 cells expressing the CaR with nocodazole, an agent that prevents the formation of microtubules by binding to tubulin monomers, did not interfere with the production of either transient or sinusoidal $[Ca^{2+}]_i$ oscillations induced by 5 mM L-phenylalanine or 5 mM Ca^{2+}, respectively. These results show that the organization of the actin cytoskeleton is necessary for the production of transient $[Ca^{2+}]_i$ oscillations in response to L-phenylalanine but it did not did appear to play a significant role in the generation of the sinusoidal $[Ca^{2+}]_i$ oscillations evoked by an increase in $[Ca^{2+}]_o$.

CaR-mediated Transient $[Ca^{2+}]_i$ Oscillations Require Functional Rho and the Interaction of Filamin-A with the CaR—The small GTP-binding proteins of the Rho family, including Rho, Rac, and Cdc-42, play a critical role in regulating the organization of the actin cytoskeleton (37). In view of our results showing that disruption of the actin cytoskeleton by cell treatment with either cytochalasin D or latrunculin A prevented the production of transient $[Ca^{2+}]_i$ oscillations by the CaR in response to L-phenylalanine, we examined whether Rho GTPases are required for the selective generation of transient $[Ca^{2+}]_i$ oscillations. Treatment of HEK-293 cells expressing the CaR with *Clostridium difficile* toxin B, which inactivates Rac, Cdc-42, and Rho (38, 39), or with *C. botulinum* C3 exoenzyme, which specifically inactivates Rho by ADP-ribosylation (40), did not affect the $[Ca^{2+}]_i$ response mediated by $[Ca^{2+}]_o$ stimulation of the CaR (Fig. 5*A*). In striking contrast, the same toxins abrogated the transient $[Ca^{2+}]_i$ oscillations evoked by L-phenylalanine (Fig. 5*A*), indicating that Rho is a downstream effector in a signaling cascade triggered by L-phenylalanine stimulation of the CaR.

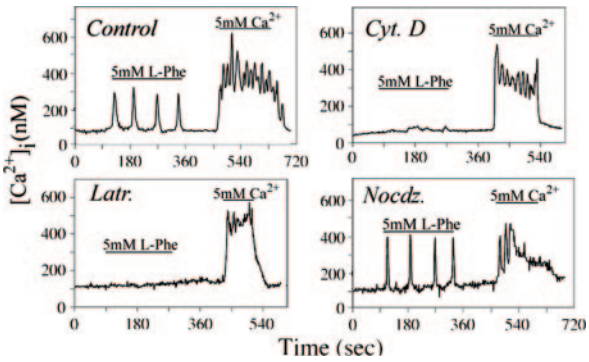

FIG. 4. **Effects of cytoskeleton disruption on CaSR stimulation.** HEK-293 cells constitutively expressing the CaR pretreated with cytochalasin-D (*Cyt. D*) (2 μM) for 1 h, latrunculin A (*Lantr.*) (2 μM) for 30 min, or nocodazole (*Nocdz.*) (0.1 μg/ml) for 1 h were perfused with saline solution containing the indicated concentrations of Ca^{2+} or L-phenylalanine during the times indicated by the *horizontal bars* and the $[Ca^{2+}]_i$ oscillations measured in individual cells as described under "Experimental Procedures." A minimum of 100 cells/experiment, each experiment done in at least duplicate, were used to measured $[Ca^{2+}]_i$.

To further examine the role of Rho in the production of $[Ca^{2+}]_i$ oscillations, we transfected wild type or CaR-expressing HEK-293 cells with a constitutively active mutant of Rho (Rho G14V). As shown in Fig. 5*A*, activated Rho induced striking $[Ca^{2+}]_i$ oscillations from a $[Ca^{2+}]_i$ base-line in HEK-293 cells expressing the CaR. In contrast, $[Ca^{2+}]_i$ oscillations were not detected in HEK-293 cells expressing Rho G14V alone (Fig. 5*A*) or Rho G14V in conjunction with the bombesin GPCR instead of the CaR (data not shown). This result indicates that activated Rho promotes transient oscillations in a CaR-dependent manner and suggests a novel role for the CaR in the generation of intracellular Ca^{2+} signaling in response to Rho activation.

Filamin-A, a large scaffold protein that interacts with the C-terminal region of the CaR (41–43), has been implicated in CaR-mediated Rho signaling (43). In view of the results shown in Fig. 5*A*, we examined whether this interaction played a role in the production of transient $[Ca^{2+}]_i$ oscillations mediated by L-phenylalanine-stimulated CaR. We transiently expressed the 14 and 15 domains of filamin-A (Fil-A14/15) to disrupt the interaction between endogenous filamin-A and the CaR (41–43) and determined the $[Ca^{2+}]_i$ oscillations in response to L-phenylalanine or $[Ca^{2+}]_o$ stimulation. As shown in Fig. 5*B*, expression of Fil-A14/15 selectively inhibited L-phenylalanine-induced transient $[Ca^{2+}]_i$ oscillations but did not prevent the sinusoidal $[Ca^{2+}]_i$ oscillations induced by an increase in $[Ca^{2+}]_o$. These results suggest that the interaction of the C terminus of the CaR with filamin-A plays a role in the generation of differential Ca^{2+} signaling by this receptor. Further support for this conclusion was obtained by expressing a truncated CaR (CaR-stop907) that does not interact with filamin-A (41, 42) in HEK-293 cells. As shown in Fig. 5*B*, L-phenylalanine failed to evoke transient $[Ca^{2+}]_i$ oscillations in HEK-293 cells transfected with the truncated CaR. This mutated form of CaR was functional, because an elevation in $[Ca^{2+}]_o$ induced a marked increase in $[Ca^{2+}]_i$ characterized by a peak and plateau, a response similar to that obtained with another CaR mutant in which threonine 888, the major site of PKC phosphorylation, was converted to non-phosphorylatable alanine (11).

$G\alpha_{12/13}$ Mediate CaR-induced Transient $[Ca^{2+}]_i$ Oscillations—Recently, the G_{12} subfamily has been implicated in pathways leading to activation of the low molecular weight G proteins of the Rho subfamily (44–49). In view of the results presented here implicating the actin cytoskeleton and Rho in the generation of transient $[Ca^{2+}]_i$ oscillations via the CaR, we hypothesized that the G_{12} subfamily mediates this type of oscillation. Most cell types express both $G\alpha_{12}$ and $G\alpha_{13}$ with overlapping functions, thus rendering it difficult to analyze the contribution of these G proteins to GPCR signaling. To circumvent this problem, we examined the $[Ca^{2+}]_i$ oscillations elicited by L-phenylalanine or an increase in $[Ca^{2+}]_o$ in MEFs generated from doubled knock-out mice for both $G\alpha_{12}$ and $G\alpha_{13}$ (referred as $G_{12/13}-/-$) (13) transiently expressing the CaR. MEF lacking rhodopsin kinase (RK−/−) served as a control. In agreement with the results obtained with RPTEC and HEK-293 cells, L-phenylalanine or $[Ca^{2+}]_o$ stimulation of MEF RK−/− evoked transient and sinusoidal $[Ca^{2+}]_i$ oscillations, respectively (Fig. 6). These results further demonstrate that these agonists elicit a different pattern of $[Ca^{2+}]_i$ oscillations in a variety of cell types.

Next, we determined the effect of L-phenylalanine or $[Ca^{2+}]_o$ on $[Ca^{2+}]_i$ oscillations in MEF $G_{12/13}-/-$ cells. As shown in Fig. 6, $[Ca^{2+}]_o$ stimulation of MEF $G_{12/13}-/-$ elicited sinusoidal $[Ca^{2+}]_i$ oscillations, in agreement with the notion that these types of oscillations are generated by the CaR via G_q and involve periodic production of Ins(1,4,5)P_3 and feedback inhibition by PKC (11). In striking contrast, the addition of L-phenylalanine to MEF $G_{12/13}-/-$ failed to promote any $[Ca^{2+}]_i$ oscillations (Fig. 6). These results demonstrate that the transient $[Ca^{2+}]_i$ oscillations mediated by the CaR in response to L-phenylalanine, but not the $[Ca^{2+}]_i$ signals elicited by $[Ca^{2+}]_o$, require a functional $G_{12/13}$. These results are in line with the well established role of $G_{12/13}$ in mediating Rho activation and organization of the actin cytoskeleton (50) and with our previous results implicating Rho and the actin cytoskeleton in mediating CaR-induced transient $[Ca^{2+}]_i$ oscillations. Collectively, our results are consistent with the hypothesis that $[Ca^{2+}]_o$ and L-phenylalanine induce different types of $[Ca^{2+}]_i$ oscillations by stimulating different G protein pathways through the CaR.

CONCLUSION

The CaR is an allosteric protein that responds to two different agonists, Ca^{2+} and aromatic amino acids, with the production of differing patterns of $[Ca^{2+}]_i$ oscillations. As shown here and in our previous studies (9, 11), aromatic amino acid stimulation of the CaR induces repetitive $[Ca^{2+}]_i$ spikes that return to the base-line level (transient oscillations), whereas $[Ca^{2+}]_o$-elicited CaR activation produces sinusoidal oscillations upon a raised plateau level of $[Ca^{2+}]_i$. The central hypothesis tested in this study is that CaR mediates sinusoidal or transient patterns of $[Ca^{2+}]_i$ oscillations in response to different agonists through different mechanisms.

Most models proposed to explain the mechanism by which $[Ca^{2+}]_i$ oscillations are generated in response to GPCR activation via the intracellular second messenger Ins(1,4,5)P_3 can be broadly divided in two major classes, depending on negative feedback effects of PKC on the production of Ins(1,4,5)P_3 or on the regulatory effects of $[Ca^{2+}]_i$ on the Ins(1,4,5)P_3 receptor (12). In the first case, the levels of Ins(1,4,5)P_3 change in a cyclical fashion thereby driving the $[Ca^{2+}]_i$ oscillations, whereas in the second case, $[Ca^{2+}]_i$ oscillations take place in cells with a steady state increased level of Ins(1,4,5)P_3 via a Ca^{2+}-induced Ca^{2+} release process mediated by the Ins(1,4,5)P_3 receptor. The recent development of techniques to image real time changes in the generation of Ins(1,4,5)P_3 and DAG in single cells has provided a valuable approach to distinguish between these models (24).

Our results demonstrated a dramatic difference in the ability of the CaR agonists to induce phosphatidylinositol 4,5-biphosphate hydrolysis and the corresponding generation of Ins(1,4,5)P_3 and DAG in single cells ectopically (HEK-293) or endogenously

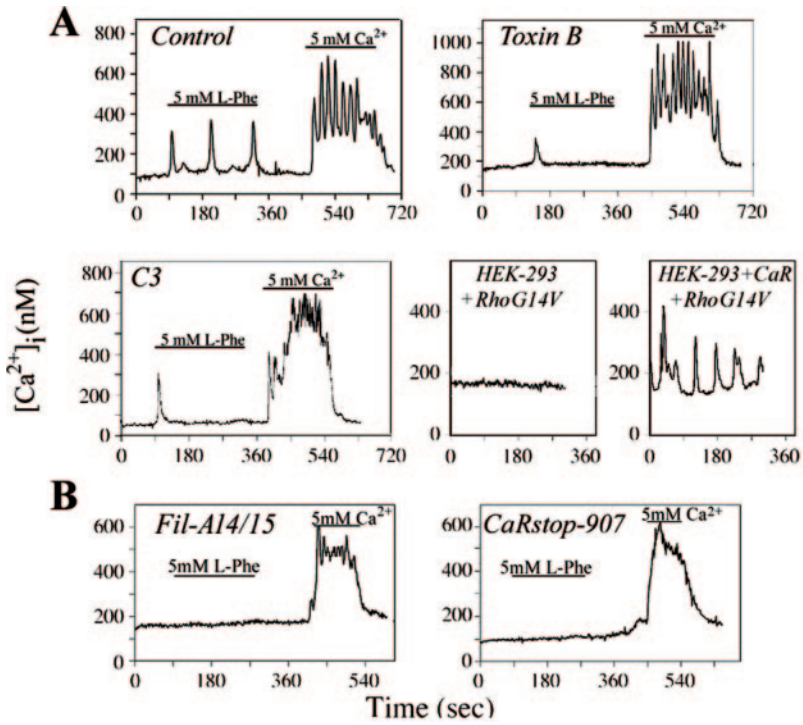

FIG. 5. **Rho GTPase, filamin-A, and the C-terminal distal region of the CaR mediate the production of transient $[Ca^{2+}]_i$ oscillations.** *A*, HEK-293 cells constitutively expressing the CaR pretreated for 1 h with *C. difficile* toxin B (*Toxin B*) (40 ng/ml) or transiently expressing *C. botulinum* C3 exoenzyme (*C3*) for 24 h were perfused with saline solution containing 5 mM Ca^{2+} or 5 mM L-phenylalanine during the times indicated by the *horizontal bars* and the $[Ca^{2+}]_i$ oscillations measured in individual cells. HEK-293 wild type cells (*HEK-293*) or HEK-293 cells constitutively expressing the CaR (*HEK-293+CaR*) were transiently transfected with a plasmid encoding a constitutively active Rho (+*RhoG14V*) and the $[Ca^{2+}]_i$ oscillations measured in individual cells. (*B*), HEK-293 cells constitutively expressing the CaR were transiently transfected with a plasmid encoding the 14 and 15 domains of human filamin-A (*Fil-A14/15*) or HEK-293 wild type cells transiently expressing a mutant CaSR with a stop codon after residue 906 (*CaRstop-907*) were perfused with saline solution containing 5 mM Ca^{2+} or 5 mM L-phenylalanine during the times indicated by the *horizontal bars* and the $[Ca^{2+}]_i$ oscillations measured in individual cells. *Control*, untreated HEK-293 cells constitutively expressing the CaR. A minimum of 100 cells/experiment, each experiment done in at least duplicate, were used to measured $[Ca^{2+}]_i$ as described under "Experimental Procedures."

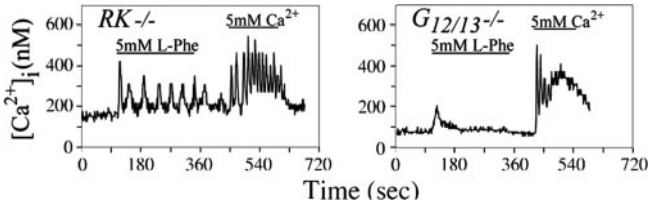

FIG. 6. **$G\alpha_{12/13}$ mediate CaR-induced transient $[Ca^{2+}]_i$ oscillations.** Murine embryonic fibroblasts generated from double knock-out mice for RK−/− (control) or for $G\alpha_{12/13}$−/−, transiently expressing the CaR, were perfused with saline solution containing 5 mM Ca^{2+} or 5 mM L-phenylalanine during the times indicated by the *horizontal bars* and the $[Ca^{2+}]_i$ oscillations measured in individual cells. A minimum of 100 cells/experiment, each experiment done in at least duplicate, were used to measured $[Ca^{2+}]_i$ as described under "Experimental Procedures."

(RPTEC) expressing the CaR. Specifically, $[Ca^{2+}]_o$-elicited CaR stimulation induced oscillatory translocation of the Ins(1,4,5)P_3 biosensor (GFP-PHD) from the plasma membrane to the cytoplasm, reflecting phosphatidylinositol 4,5-biphosphate hydrolysis in the plasma membrane and periodic synthesis of the diffusible second messenger Ins(1,4,5)P_3. These changes coincided with the oscillatory translocation of the Ca^{2+}-sensitive PKCα-YFP from the cytoplasm to the plasma membrane. In striking contrast, parallel experiments demonstrated that addition of L-phenylalanine, at concentrations that induced the production of robust and lasting transient $[Ca^{2+}]_i$ oscillations as revealed by the plasma membrane translocation of the Ca^{2+}-sensitive PKCα-YFP sensor, did not stimulate any detectable phosphatidylinositol 4,5-biphosphate hydrolysis or Ins(1,4,5)P_3 synthesis. These findings lead to the important conclusion that L-phenylalanine does not stimulate any detectable increase in PLC activity through the CaR and thus imply that aromatic amino acids induce transient $[Ca^{2+}]_i$ oscillations via the CaR through a different pathway.

The notion that the CaR acts trough PLC-dependent and PLC-independent pathways was further substantiated by the striking difference in the ability of the CaR agonists to induce translocation of PKD-RFP to the plasma membrane, indicative of DAG production. Furthermore, we found that $[Ca^{2+}]_o$-elicited CaR activation induced a prominent increase in the phosphorylation of PKD at Ser^{744} and Ser^{748}, the key residues located in the activation loop of this enzyme that are phosphorylated by novel PKCs and promote the catalytic activation of PKD (25, 26, 51). These results provide persuasive evidence demonstrating that the sinusoidal $[Ca^{2+}]_i$ oscillations induced by $[Ca^{2+}]_o$-elicited CaR activation involve PLC and Ins(1,4,5)P_3 and differ from the pathway leading to transient $[Ca^{2+}]_i$ oscillations initiated by L-amino acids through the same receptor. This conclusion prompted us to identify molecular steps involved in the signal transduction pathway that mediates CaR-induced transient $[Ca^{2+}]_i$ oscillations.

The results presented in this study demonstrate, for the first time, that L-phenylalanine-elicited CaR-mediated base-line $[Ca^{2+}]_i$ spiking was selectively abolished by multiple approaches. These include pharmacological disruption of the actin cytoskeleton using the structurally unrelated agents cytochalasin D and latrunculin A, inactivation of Rho GTPases with toxin B and C3, interference with the binding of filamin-A to the CaR, expression of a CaR lacking the binding region of filamin-A, and by expressing the CaR in cells derived from genetically modified mice lacking the α subunits of both G_{12} and G_{13}. In each case, we demonstrated suppression of $[Ca^{2+}]_i$ signaling in response to amino acid stimulation but retention of

$[Ca^{2+}]_i$ signaling induced by an elevation of $[Ca^{2+}]_o$. Reciprocally, activated Rho induced striking $[Ca^{2+}]_i$ oscillations from a $[Ca^{2+}]_i$ base-line in cells expressing the CaR, suggesting a novel role for this receptor in the generation of intracellular Ca^{2+} signaling in response to Rho activation. We concluded that L-phenylalanine-elicited CaR activation promotes baseline $[Ca^{2+}]_i$ spiking through a novel signal transduction pathway involving the heterotrimeric proteins of the G_{12} subfamily, the scaffolding protein filamin-A, the small molecular weight GTPase Rho, and the intactness of the actin cytoskeleton. Although the precise mechanism and components mediating the generation of transient $[Ca^{2+}]_i$ oscillations requires further investigation, it is tempting to speculate that the cytoplasmic tail of the CaR acts as a "nucleation" component around which Rho, filamin A, microfilaments, and possibly other proteins are recruited in response to L-phenylalanine stimulation of this GPCR. In this regard, it was recently demonstrated that thrombin-mediated activation of RhoA regulates Ca^{2+} entry by a mechanism involving a complex among activated RhoA, the transient receptor potential channel 1, the inositol triphosphate receptor, and an intact cytoskeleton (52). RhoA activation not only mediated the formation of this complex but also its translocation to the plasma membrane (52).

Upon agonist binding, a change in the GPCR conformation facilitates the activation of heterotrimeric G proteins, which in turn activate downstream signaling pathways depending on the specific type of G protein to which the receptor is coupled. This simple GPCR active state model is being reviewed in the context of recent theoretical and experimental evidence showing that the binding of different agonists to the same receptor can lead to the activation of different G proteins by inducing distinct GPCR conformations (reviewed in Ref. 53). Although evidence supporting this model of agonist-specific trafficking of receptor signaling, *i.e.* agonist trafficking (54, 55), has been found for a limited number of GPCR using mostly synthetic agonists, the importance of this effect under physiological conditions has not been clearly established (53). Our model is that the physiologically relevant agonists, Ca^{2+} and amino acids, induce distinct conformational states of the CaR, providing for the possibility of differential coupling to downstream signaling pathways.

In conclusion, our results demonstrate that the CaR mediates sinusoidal or transient patterns of $[Ca^{2+}]_i$ oscillations in response to different agonists through different mechanisms. Transient oscillations are produced via a PLC/Ins(1,4,5)P_3-independent pathway that involves Rho, filamin-A, and the organization of the actin cytoskeleton. Therefore, a conceptual model for the CaR could be that Ca^{2+} or amino acids induce distinct conformational states of this allosteric GPCR, providing for the possibility of differential coupling to G proteins and thus to downstream signaling pathways.

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