The calcium-sensing receptor (CaR) is an allosteric protein that responds to extracellular Ca$^{2+}$ ([Ca$^{2+}]_o$) and aromatic amino acids with the production of different patterns of oscillations in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}]_i$). An increase in [Ca$^{2+}]_o$ stimulates phospholipase C-mediated production of inositol 1,4,5-trisphosphate and causes sinusoidal oscillations in [Ca$^{2+}]_i$. Conversely, aromatic amino acid-induced CaR activation does not stimulate phospholipase C but engages an unidentified signaling mechanism that promotes transient oscillations in [Ca$^{2+}]_i$. We show here that the [Ca$^{2+}]_i$ oscillations stimulated by aromatic amino acids were selectively abolished by TRPC1 down-regulation using either a pool of small inhibitory RNAs (siRNAs) or two different individual siRNAs that targeted different coding regions of TRPC1. Furthermore, [Ca$^{2+}]_i$ oscillations stimulated by aromatic amino acids were also abolished by inhibition of TRPC1 function with an antibody that binds the pore region of the channel. We also show that aromatic amino acid-stimulated [Ca$^{2+}]_i$ oscillations can be prevented by protein kinase C (PKC) inhibitors or siRNA-mediated PKCα down-regulation and impaired by either calmodulin antagonists or by the expression of a dominant-negative calmodulin mutant. We propose a model for the generation of CaR-mediated transient [Ca$^{2+}]_i$ oscillations that integrates its stimulation by aromatic amino acids with TRPC1 regulation by PKC and calmodulin.

The extracellular calcium-sensing receptor (CaR), a member of the C family of G protein-coupled receptors, is an allosteric protein that plays a major physiological role in correcting changes in extracellular concentration of Ca$^{2+}$ ([Ca$^{2+}]_o$) by regulating parathyroid hormone secretion (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. Interestingly, the CaR is also expressed in many other tissues, including the gastrointestinal tract, brain, pituitary, thyroid, skin, breast, pancreas, lung, bone, and heart (reviewed in Ref. 5), suggesting that this receptor plays additional, yet to be defined, physiological roles in the regulation of cell function. Indeed, recent evidence indicates that the CaR is involved in the control of intestinal fluid transport (6) and bone localization of hematopoietic stem cells (7) as well as the progression and spread of several cancers, including colorectal, breast, ovary, and parathyroid (8).

Recent studies of CaR activation in individual living cells have shown that the intracellular concentration of Ca$^{2+}$ ([Ca$^{2+}]_i$) oscillates upon stimulation of CaR by an elevation in [Ca$^{2+}]_o$ (9, 10). In addition to its role as a sensor of [Ca$^{2+}]_o$, the CaR is also stimulated by aromatic amino acids (11) that, like [Ca$^{2+}]_o$, induce striking and lasting CaR-mediated [Ca$^{2+}]_i$ oscillations (10). However, the patterns of [Ca$^{2+}]_i$ oscillations induced by aromatic amino acids and [Ca$^{2+}]_o$ which bind to topographically separate sites in the CaR (12, 13), are different (10). Aromatic amino acid stimulation of the CaR induces repetitive, low frequency, [Ca$^{2+}]_i$ spikes that return to the baseline 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$^{2+}]_i$. The amplitude, frequency, and duration of [Ca$^{2+}]_i$ oscillations are increasingly recognized to encode important information for a variety of biological processes, including gene expression and cell metabolism. However, the precise mechanisms responsible for the generation of different types of [Ca$^{2+}]_i$ oscillations remain incompletely understood (14–17).

Our previous studies indicated that activation of the CaR by increases in [Ca$^{2+}]_o$ promotes sinusoidal [Ca$^{2+}]_i$ oscillations through a phospholipase C/inositol 1,4,5-trisphosphate (Ins(1,4,5)P$_3$) cascade (18) and that protein kinase C (PKC) negatively regulates the frequency of [Ca$^{2+}]_o$-induced [Ca$^{2+}]_i$ oscillations (19). Intriguingly, the transient [Ca$^{2+}]_i$ oscillations elicited by the CaR in response to amino acid stimulation do not involve the PLC/Ins(1,4,5)P$_3$ cascade but a different signaling
pathway that requires the organization of the actin cytoskeleton and involves the heterotrimeric proteins of the G13 subfamily, the small GTPase Rho, the scaffolding protein filamin-A, and the cytoplasmic tail of the CaR (18). Although the precise mechanism(s) that generates the transient [Ca\(^{2+}\)], oscillations induced by the CaR has not been determined, we previously demonstrated that 2-aminoethoxydiphenyl borate, a modulator of cationic channels encoded by transient receptor potential (TRP) genes including TRPC1 (20, 21), abolished the transient, but not the sinusoidal, [Ca\(^{2+}\)], oscillations mediated by the CaR (10). The most frequently proposed TRPC1 activation signal in mammals is calcium depletion from intracellular stores (reviewed in Refs. 22 and 23). In addition, other mechanisms that do not involve store depletion, such as receptor activation and plasma membrane stretch, can also lead to TRPC1 activation (24–26). Furthermore, a new concept of multiplicity of activation, including receptor- and store-operated modes of activation in the same cell, is emerging based on recent studies with the TRPC1-related TRPC5 and TRPC7 channels (27, 28).

In the present study, we examined the hypothesis that TRPC1, a highly conserved and widely expressed channel that allows the flux of sodium and calcium across the plasma membrane (29), is an essential element in the generation of transient [Ca\(^{2+}\)], oscillations in response to stimulation of the CaR by aromatic amino acids.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and cDNA Transfections—**Human embryonic kidney (HEK-293), obtained from American Type Cell Collection, and HEK-293 cells expressing the CaR (HEK-CaR) (18) were grown at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in a humidified atmosphere containing 10% CO\(_2\) and 90% air. cDNAs encoding wildtype calmodulin (CaM) and dominant-negative CaM (DN-CaM) were previously described (30). cDNA transfections were carried out in Opti-MEM using Lipofectamine Plus according to the manufacturer’s suggested conditions (Invitrogen).

**Immunocytochemistry, Confocal Microscopy, and Quantitative Imaging Analysis—**For immunocytochemistry, the cells were fixed in 10% buffered formalin phosphate for 15 min at 25 °C and then permeabilized with a solution of 0.4% Triton X-100-phosphate-buffered saline (PBS) for 5 min at 25 °C. After extensive PBS washing, the fixed cells were incubated for 18 h at 25 °C in blocking buffer (PBS-1% gelatin-0.05% Tween 20) and then stained at 25 °C for 60 min with the primary antibodies. Subsequently, the cells were washed with PBS-0.05% Tween 20 at 25 °C and then permeabilized with a solution of 0.4% Triton X-100-phosphate-buffered saline (PBS) for 5 min at 25 °C. After extensive PBS washing, the fixed cells were incubated for 18 h at 25 °C in blocking buffer (PBS-1% gelatin-0.05% Tween 20) and then stained at 25 °C for 60 min with the primary antibodies. Subsequently, the cells were washed with PBS-0.05% Tween 20 at 25 °C and stained at 25 °C for 60 min with Alexa Fluor-conjugated secondary antibodies diluted in blocking buffer and washed again with PBS-0.05% Tween 20. The samples were then mounted with a gelvatol-glycerol solution containing 2.5% 1,4-diazobicyclo-[2.2.2]octane.

Confocal microscopy was performed with a Zeiss PASCAL (Germany) laser-scanning microscope and a ×63 Zeiss Plan-Apochromat oil immersion objective (numerical aperture, 1.45). Alexa 488 was excited with 488 nm light, and its emission (green) was selected through a 505/530 band pass filter. Alexa 546 was excited at 543 nm, and its emission (red) was detected through a 560 long pass filter. Images were collected in multi-track mode using the same image acquisition settings with LSM5 PASCAL imaging system software version 3.2 SP2 (Zeiss).

Quantitative imaging analysis to assess the colocalization of the CaR and TRPC1 was performed using Manders’ overlap coefficient (31) as implemented by CoLocalizer Pro v. 1.4 software. The overlap coefficients generated by Manders’ equation have values between 0 and 1; a value of 0 indicates that there is no colocalization, whereas a value of 1.0 means there is complete colocalization. A total of 10 fields from different dishes, each containing at least 20 cells, were analyzed and the results obtained for a representative set of cells. Scattergram shows the distribution of pixels in the image according to the selected pair of channels (green and red) with colocalized pixels in white.

**Single Cell [Ca\(^{2+}\)], Imaging—**The cells were incubated in saline solution (Hanks Balanced Salt Solution; Invitrogen) without phenol red, containing 138 mM NaCl, 4 mM NaHCO\(_3\), 0.3 mM Na\(_2\)HPO\(_4\), 5 mM KCl, 0.3 mM KH\(_2\)PO\(_4\), 1.5 mM CaCl\(_2\), and 0.3 mM MgCl\(_2\).

**FIGURE 1. Expression and intracellular distribution of TRPC1 in HEK-CaR cells.** Equivalent amounts of HEK-293 and HEK-CaR cells lysates were analyzed by Western blot using an anti-TRPC1 antibody as described under “Experimental Procedures.” Confocal microscopy and quantitative imaging analysis of TRPC1 and CaR were performed as described under “Experimental Procedures.” Green fluorescence image of TRPC1 and red fluorescence image of CaR in HEK-CaR cells are displayed in panels A and B, respectively. C, colocalizations of TRPC1 and CaR fluorescence images are shown in white. D, simultaneous merging of total acquired green and red fluorescent images as well as calculated colocalized signals. Inset, scattergram showing the distribution of green and red pixels as well as the colocalized ones (white) corresponding to panel D. Bar, 10 μm.
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). A minimum of 100 cells/experiment, each experiment done in at least triplicate, was used to measure [Ca\(^{2+}\)\(_i\)].

Small Inhibitory RNA (siRNA) Transfections and Western Blots—SMART pool siRNA duplexes for human TRPC1 (GenBank™ accession number NM_003304), PKCα (GenBank™ accession number NM_002737), and PKCβ1 (GenBank™ accession number NM_002738) were purchased from Dharmacon. Individual TRPC1 siRNA duplexes (sense sequences), also purchased from Dharmacon, were: oligo 1, GGACUACGGUGUCAGAAAUU, and oligo 2, GAGAA-GAACUGAGCUCCUUU. HEK-CaR cells plated at a density of 3 × 10⁵ cells/35-mm dishes were transfected with siRNA using TransIT-TKO Reagent (Mirus) as recommended by the manufacturer. Control transfections were carried out with TransIT-TKO alone or with Dharmacon siCONTROL non-targeting siRNA (catalog number D-001206-13). siRNA-transfected cells were used 48 h post-transfection. Transfection efficiency was examined with a non-targeting fluorescein-labeled siRNA (Cell Signaling).

Western Blot Analysis—Samples of cell lysates were directly solubilized by boiling in SDS-PAGE sample buffer. Following SDS-4.5–15% PAGE, proteins were transferred to Immobilon-P membranes (Millipore Corp.) and blocked by overnight incubation with 5% nonfat dried milk in PBS-0.05% Tween 20 (PT). Membranes were then incubated at room temperature for 3 h with the primary antiserum diluted in PT. Immunoreactive bands were visualized using horseradish peroxidase-conjugated IgGs and enhanced chemiluminescence Western blotting ECL reagent (Amersham Biosciences).

Analysis of [Ca\(^{2+}\)\(_i\)] Amplitude Response to [Ca\(^{2+}\)\(_o\)] after TRPC1 Down-regulation—HEK-CaR cells transfected with non-targeted siRNA (50 nM), pooled TRPC1 siRNA (50 nM),
TRPC1 siRNA1 (50 nM), or TRPC1 siRNA 2 (50 nM) were challenged 48 h post-transfection with 5 mM Ca\(^{2+}\) and the average amplitude of the \([Ca^{2+}]\) change determined in individual cells. We performed six separate experiments, examining 100 cells/experiment. In order to combine the data from the separate experiments, we normalized for each experiment the \([Ca^{2+}]\) response by dividing the average amplitude from each of the TRPC1 siRNA-transfected cells by the ones transfected with non-targeted siRNA, e.g. pooled TRPC1 siRNA/non-targeted siRNA. The normalized responses from each of the six experiments were used to obtain a cumulative mean normalized response for each siRNA treatment.

Materials—Antibodies were obtained from Santa Cruz Biotechnology, Inc. (anti-PKCα, anti-PKCβ1); Affinity BioReagents (murine monoclonal anti-CaR); Alomone Labs (rabbit polyclonal anti-TRPC1); Abcam (murine monoclonal anti-α-tubulin and anti-actin); Molecular Probes (anti-rabbit Alexa 488 and anti-mouse Alexa 546); and Invitrogen (anti-Myc). Fura-2 AM was purchased from Molecular Probes. All the other reagents were the highest grade commercially available.

RESULTS AND DISCUSSION

Expression and Intracellular Distribution of TRPC1 in HEK-293 Cells—HEK-293 stably expressing the human CaR, referred to as HEK-CaR, is a cell system widely used to study the regulation of the CaR (32). Initially, we verified that HEK-CaR express TRPC1, and subsequently we examined the intracellular distribution of this channel and the CaR. As shown in Fig. 1, top panel, HEK-CaR cells, as well as the parental HEK-293 cell line, express similar levels of TRPC1 as demonstrated by Western blot analysis. Immunocytochemistry and confocal microscopy of HEK-CaR cells co-stained with rabbit anti-TRPC1 and mouse monoclonal anti-CaR revealed TRPC1 immunoreactivity throughout the cytosol and a punctate distribution pattern in the plasma membrane (Fig. 1A), whereas CaR immunoreactivity was most prominent at the plasma membrane (Fig. 1B). No immunoreactivity was detected in HEK-CaR cells when the antibodies used against CaR or TRPC1 were preabsorbed with their corresponding immunizing peptides or in HEK-293 cells incubated with the anti-CaR antibody (data not shown). Quantitative imaging analysis of the distribution of TRPC1 and the CaR revealed that both proteins colocalized in discrete regions of the plasma membrane (Fig. 1C) with overlap coefficients of 0.014 ± 0.013 (mean ± S.E.) and 0.25 ± 0.013 for HEK-293 and HEK-CaR cells, respectively, as calculated by Manders' equation (31). The scatter gram (Fig. 1, inset) provides a qualitative analysis that shows the distribution of green and red pixels as well as the colocalized ones (white) displayed in panel D. The significant colocalization of CaR and TRPC1 is consistent with previous reports showing that these proteins localize to caveolin-rich plasma membrane domains (33, 34).

Once we established that HEK-CaR cells express TRPC1, we proceeded to examine the hypothesis that this cation channel mediates the CaR-mediated transient \([Ca^{2+}]\) oscillations. HEK-CaR cells were transfected with siRNA targeted against TRPC1 to examine changes in \([Ca^{2+}]\) after stimulation of the CaR as described under “Experimental Procedures.” siRNAs Targeting Different Regions of TRPC1 Prevent Transient \([Ca^{2+}]\) Oscillations in Response to L-Phe—As illustrated in Fig. 2A, intracellular \([Ca^{2+}]\) imaging revealed that addition of 5 mM L-Phe to HEK-CaR cells stimulated striking transient \([Ca^{2+}]\) oscillations and that each \([Ca^{2+}]\) peak returned to baseline value. In contrast, an increase in the \([Ca^{2+}]\) from 1.5 to 5 mM induced a rapid elevation in \([Ca^{2+}]\), to a new baseline level followed by oscillatory \([Ca^{2+}]\), fluctuations. These sinusoidal \([Ca^{2+}]\) oscillations ceased when \([Ca^{2+}]\) was returned to 1.5 mM. Addition of L-Phe or \([Ca^{2+}]\) to wild-type HEK-293 cells did not induce any detectable change in \([Ca^{2+}]\) (Fig. 2B), demonstrating that the \([Ca^{2+}]\) oscillations observed in HEK-CaR in response to L-Phe and \([Ca^{2+}]\) stimulation were associated to the expression of the CaR. In some occasions, we observed in less than 3% of non-stimulated HEK-293 or HEK-CaR cells an upward drift in baseline \([Ca^{2+}]\), that never exceeds 5% of the initial measurement over 600 s of recording (data not shown).
Transfection of HEK-CaR cells with non-targeted siRNA did not affect the production of \([Ca^{2+}]\), Oscillations induced by L-Phe or [Ca^{2+}]_i (Fig. 2C). However, the transient, but not sinusoidal, [Ca^{2+}]_i, oscillations were selectively inhibited in HEK-CaR cells transfected with a pool of TRPC1 siRNA (Fig. 2D). Analysis of the ratio between the amplitude of the [Ca^{2+}]_i oscillations in cells transfected with the different TRPC1 siRNAs and non-targeted siRNA indicates that TRPC1 knockdown did not significantly affect the CaR-mediated response to extracellular Ca^{2+} (pool TRPC1 siRNA/non-targeted siRNA = 1.00 ± 0.16, mean ± S.E.; TRPC1 siRNA 1/non-targeted siRNA = 1.07 ± 0.18; TRPC1 siRNA 2/non-targeted siRNA = 0.87 ± 0.16; see “Experimental Procedures” for further details).

To exclude the possibility that the inhibitory effect on L-Phe-induced [Ca^{2+}]_i, oscillations by the pool of TRPC1 siRNA was due to off-target effects (35), we performed additional experiments using two individual siRNA that targeted different coding regions of TRPC1. We found that the individual siRNAs prevented the production of [Ca^{2+}]_i oscillations in response to L-Phe as effectively as the pool of TRPC1 siRNA (Fig. 2, E and F). [Ca^{2+}]_i mobilization in response to carbachol stimulation, which acts through muscarinic receptors endogenously expressed in HEK-293 cells, was not affected by TRPC1 siRNA (data not shown).

The siRNA directed against TRPC1 reduced the L-Phe-elicited [Ca^{2+}]_i oscillations in a dose-dependent manner, i.e. the number of cells responding to L-Phe stimulation decreased as a function of higher concentrations of TRPC1-targeted siRNA (Fig. 2G). As illustrated in Fig. 2, H and I, a significant reduction of TRPC1 protein expression, but not of \(\alpha\)-tubulin or actin, was detected in HEK-CaR cells treated either with the pool or with the individual TRPC1 siRNA, indicating that its down-regulation was specific. Non-targeting siRNA neither affects the number of cells responding to L-Phe stimulation nor decreases TRPC1 protein expression levels in HEK-CaR cells (Fig. 2 H and I). Together, these results indicate that TRPC1 siRNA produced an on-target effect and that this effect, i.e. TRPC1 expression down-regulation, inhibits the generation of transient [Ca^{2+}]_i oscillations mediated by the CaR in response to L-Phe stimulation.

**An Antibody Directed against the Pore Region of TRPC1 Blocks the Transient [Ca^{2+}]_i Oscillations in Response to L-Phe**—As an independent test of the hypothesis that TRPC1 mediates the transient [Ca^{2+}]_i oscillations elicited by L-Phe-stimulation of the CaR, we used an antibody that interferes in vivo with the activity of this channel by binding the peptide sequence QLYDKYTSKQD73 located in the pore region of the human TRPC1 (36). Exposure of HEK-CaR cells to the anti-TRPC1 antibody for 30 min inhibited L-Phe-elicited transient [Ca^{2+}]_i oscillations (Fig. 3B) in a dose-dependent manner (Fig. 3C). Importantly, the anti-TRPC1 antibody did not interfere with the [Ca^{2+}]_i-stimulated sinusoidal [Ca^{2+}]_i oscillations (Fig. 3B). The transient [Ca^{2+}]_i oscillations inhibition mediated by the anti-TRPC1 antibody was abolished by preabsorbing the antibody during 1 h with 15 \(\mu\)g/ml of the immunizing peptide (Fig. 3C). No inhibition of sinusoidal or transient [Ca^{2+}]_i oscillations was observed when the cells were...
exposed to an unrelated antibody, e.g. anti-Myc (Fig. 3D). These results further demonstrate that interference with TRPC1 function blocks the transient, but not the sinusoidal, [Ca\(^{2+}\)]\(_i\) oscillations mediated by the CaR. Because TRPC1 can form homo- and heterotetramers (37, 38), further experiments are needed to determine whether other TRPCs also participate in the generation of [Ca\(^{2+}\)]\(_i\) oscillations mediated by the CaR in response to aromatic amino acid stimulation.

Role of PKC\(\alpha\) and Calmodulin in the Generation of Transient [Ca\(^{2+}\)]\(_i\) Oscillations—We next asked how TRPC1 could contribute to the generation of [Ca\(^{2+}\)]\(_i\) oscillations in response to CaR activation rather than produce a persistent increase in Ca\(^{2+}\) influx, its well known role in mediating store-operated Ca\(^{2+}\) entry in response to Ca\(^{2+}\) depletion from intracellular stores (22). In this context, recent studies demonstrating that the activity of TRPC1 is positively regulated by PKC\(\alpha\) and negatively by CaM binding are highly relevant (39, 40). We hypothesized that PKC\(\alpha\)-mediated TRPC1 activation could enhance the upward phase of the [Ca\(^{2+}\)]\(_i\) spike whereas CaM-activated CaM binding mediates the downward phase of each spike. Accordingly, we examined whether PKC and CaM were involved in the production of transient [Ca\(^{2+}\)]\(_i\) oscillations by the CaR.

In agreement with our hypothesis, intracellular Ca\(^{2+}\) imaging revealed that the preferential PKC\(\alpha\) inhibitors Ro-31-7549 and Ro-32-0432 (41) abolished Ca\(^{2+}\) signaling elicited by l-Phe stimulation of the CaR (Fig. 4, B and C). Because the IC\(_{50}\) of these compounds for the PKCs is not sufficient to unambiguously distinguish between the different isoforms (42), we used siRNA-mediated knock down to define the role of PKC\(\alpha\) in CaR-mediated [Ca\(^{2+}\)]\(_i\) oscillations. HEK-CaR cells were transfected with a pool of PKC\(\alpha\) or PKC\(\beta\) siRNA and the changes in [Ca\(^{2+}\)]\(_i\), after l-Phe stimulation examined in single cells. As we previously demonstrated (see Fig. 2C), non-targeted siRNA did not affect the production of transient [Ca\(^{2+}\)]\(_i\) oscillations (Fig. 4D). However, these oscillations were inhibited by the siRNA targeting PKC\(\alpha\) (Fig. 4E) but not PKC\(\beta\)1 (Fig. 4F). This inhibition was dose dependent (Fig. 4G) and concurrent with a marked reduction of PKC\(\alpha\) expression.

To examine the role of CaM in the production of transient [Ca\(^{2+}\)]\(_i\) oscillations, HEK-CaR cells were exposed to the CaM antagonists W7 (43) and W-13 (44). These agents did not prevent the rise in [Ca\(^{2+}\)]\(_i\), elicited by l-Phe stimulation of the CaR but impaired the downward phase of the transient [Ca\(^{2+}\)]\(_i\) oscillation, resulting in a sustained, non-oscillatory [Ca\(^{2+}\)]\(_i\) increase (Fig. 5, B and C). To independently corroborate these results, we interfered with the function of endogenous CaM by expressing a DN-CaM form of this protein that has an alanine substitution in each of the four Ca\(^{2+}\)-binding sites (D20A, D56A, D93A, D129A) (30). Transfection of HEK-CaR cells with a vector encoding wild-type CaM did not had any effect in the transient [Ca\(^{2+}\)]\(_i\) oscillations (Fig. 5, D and E). We next asked how TRPC1 could contribute to the generation of [Ca\(^{2+}\)]\(_i\) oscillations in response to CaR activation rather than produce a persistent increase in Ca\(^{2+}\) influx, its well known role in mediating store-operated Ca\(^{2+}\) entry in response to Ca\(^{2+}\) depletion from intracellular stores (22). In this context, recent studies demonstrating that the activity of TRPC1 is positively regulated by PKC\(\alpha\) and negatively by CaM binding are highly relevant (39, 40). We hypothesized that PKC\(\alpha\)-mediated TRPC1 activation could enhance the upward phase of the [Ca\(^{2+}\)]\(_i\) spike whereas CaM-activated CaM binding mediates the downward phase of each spike. Accordingly, we examined whether PKC and CaM were involved in the production of transient [Ca\(^{2+}\)]\(_i\) oscillations by the CaR.

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CaR-mediated Ca\(^{2+}\) Oscillations

**FIGURE 6. Model of CaR-mediated [Ca\(^{2+}\)]\(_{i}\) oscillations.** The activation of the CaR by an elevation in [Ca\(^{2+}\)]\(_{i}\) leads to phospholipase C-mediated production of Ins(1,4,5)P\(_3\) and diacylglycerol. Ins(1,4,5)P\(_3\) binds to its intracellular receptor and triggers the release of Ca\(^{2+}\) from internal stores. The rise in the concentration of free intracellular Ca\(^{2+}\) and diacylglycerol activates PKC. Activated PKC then phosphorylates the CaR, at Thr-688, providing the negative feedback needed to cause sinusoidal Ca\(^{2+}\) oscillations. The binding of aromatic amino acids to the CaR engages a multiprotein complex that includes Rho, filamin-A, and TRPC1. This interaction leads to TRPC1 channel opening and extracellular Ca\(^{2+}\) entry, which is further stimulated by PKCs. As [Ca\(^{2+}\)]\(_{i}\) increases, activated CaM binds the C terminus of TRPC1 and inhibits its activity.

HEK-CaR promoted oscillatory translocations of PKCs from the cytosol to the plasma membrane (18). Furthermore, recent reports indicate that the binding of Ca\(^{2+}\) to the C2 domain of PKCs not only leads to membrane translocation but also promotes a conformational change that enables phosphatidylinositol (4,5)-bisphosphate to access a lysine-rich cluster located in a separated region of the C2 domain and activate the enzyme (45–47). Thus, it is reasonable to assume that the membrane-associated state of PKCs is active, as suggested by our model. As [Ca\(^{2+}\)]\(_{i}\) increases, activated (i.e. Ca\(^{2+}\) bound) CaM binds the C terminus of TRPC1 and inhibits its activity initiating the down ward phase of the spike, a function impaired by CaM antagonists and the DN-CaM. The capacity of the CaR to couple to G\(_{i/o}\)/phospholipase C (18) or to TRPC1, as proposed in Fig. 6, provides a plausible explanation for the remarkable ability of this receptor to mediate sinusoidal and transient patterns of [Ca\(^{2+}\)]\(_{i}\) oscillations in response to different agonists, i.e. Ca\(^{2+}\) and aromatic amino acids.

In conclusion, our studies identify a novel function of TRPC1, namely as a component of a functional signaling complex formed in the presence of the CaR that mediates transient [Ca\(^{2+}\)]\(_{i}\) oscillations in response to aromatic amino acids. In addition to the CaR, the members of the C family of G protein-coupled receptors include the metabotropic glutamate receptors, \(\gamma\)-aminobutyric acid receptors, V2R pheromone receptors, and the taste receptors of the T1R subfamily. In view of the structural and functional similarities of the members of this important receptor family, it will be of interest to determine whether TRPC1 or other members of the TRPC family play a role in the generation of [Ca\(^{2+}\)]\(_{i}\) oscillations initiated by activation of these receptors.

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