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Amino acids and Ca^{2+} stimulate different patterns of Ca^{2+} oscillations through the Ca^{2+}-sensing receptor

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Young, Steven H., and Enrique Rozengurt. Amino acids and Ca^{2+} stimulate different patterns of Ca^{2+} oscillations through the Ca^{2+}-sensing receptor. *Am J Physiol Cell Physiol* 282: C1414–C1422, 2002. First published February 6, 2002; 10.1152/ajpcell.00432.2001.—We determined the effect of aromatic amino acid stimulation of the human extracellular Ca^{2+}-sensing receptor (CaR) on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in single HEK-293 cells. Addition of L-phenylalanine or L-tryptophan (at 5 mM) induced $[Ca^{2+}]_i$ oscillations from a resting state that was quiescent at 1.8 mM extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$). Each $[Ca^{2+}]_i$ peak returned to baseline values, and the average oscillation frequency was ~1 min^{-1} at 37°C. Oscillations were not induced or sustained if the $[Ca^{2+}]_e$ was reduced to 0.5 mM, even in the continued presence of amino acid. Average oscillation frequency in response to an increase in $[Ca^{2+}]_e$ (from 1.8 to 2.5–5 mM) was much higher (~4 min^{-1}) than that induced by aromatic amino acids. Oscillations in response to $[Ca^{2+}]_e$ were sinusoidal whereas those induced by amino acids were transient. Thus both amino acids and Ca^{2+}, acting through the same CaR, produce oscillatory increases in $[Ca^{2+}]_i$, but the resultant oscillation pattern and frequency allow the cell to discriminate which agonist is bound to the receptor.

sinusoidal calcium oscillations; baseline calcium oscillations; allosteric; G protein-coupled receptors

THE EXTRACELLULAR CA^{2+}-SENSING RECEPTOR (CaR) is a member of the superfamily of heptahelical G protein-coupled receptors that was originally cloned from parathyroid chief cells (for review, see Ref. 5). Inactivating and activating mutations of the CaR in humans (15) and genetic disruption of the CaR gene in mice (16) 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 extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) by regulating parathyroid hormone secretion (5). Subsequent studies demonstrating that the CaR is also expressed in many other tissues and cells including kidney (29), keratinocytes (27), gastrointestinal cells (6, 7), and nerve terminals suggest that the CaR could play additional, as yet less well-defined, physiological roles in the regulation of secretion, gene expression, cell proliferation, and apoptosis (5). Thus the mechanisms that regulate CaR function are attracting major interest. A recent study of CaR activation in single cells has shown that intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) oscillates upon stimulation of CaR by increases in $[Ca^{2+}]_e$ (4). It is increasingly recognized that the pattern and frequency of $[Ca^{2+}]_i$ oscillations play a key role in signal transduction, regulating Ca^{2+}- and calmodulin-dependent protein kinase II (30), protein kinase C (PKC) (26), mitochondrial metabolism (14), and nuclear transcriptional activity leading to differential gene expression (10, 17, 21, 34).

In addition to its role as a sensor of $[Ca^{2+}]_e$, Conigrave et. al. [8; reviewed by Kobilka (20)] have recently demonstrated that CaR activity is also regulated by aromatic amino acids which, like extracellular Ca^{2+}, stimulate increases in $[Ca^{2+}]_i$. These elegant findings indicated that the CaR is an allosteric protein that recognizes and responds to two different agonists, namely, Ca^{2+} and aromatic amino acids. Because the frequency of $[Ca^{2+}]_i$ oscillations plays a key role in signal transduction, an allosteric interaction of $[Ca^{2+}]_e$ and amino acids on the production of $[Ca^{2+}]_i$ oscillations by the CaR could provide a novel mechanism that could distinguish as well as integrate the effect of these agonists in different cell types that express the CaR. However, the effects of amino acids on CaR-mediated regulation of $[Ca^{2+}]_i$ in single cells have not been investigated, and thus it is not known whether extracellular aromatic amino acids, like extraellular Ca^{2+}, also induce $[Ca^{2+}]_i$ oscillations in CaR-expressing cells and/or modulate the pattern and frequency of $[Ca^{2+}]_i$ oscillations initiated by an increase in $[Ca^{2+}]_e$.

In the present study, we demonstrate, for the first time, that single HEK-293 cells transfected with the human CaR respond to the addition of the aromatic amino acids L-tryptophan and L-phenylalanine with striking and lasting $[Ca^{2+}]_i$ oscillations. As recently reported (4), these cells also respond to increases in $[Ca^{2+}]_e$ with $[Ca^{2+}]_i$ oscillations. However, the average frequency of the oscillations induced by extracellular

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Ca^{2+} (~4 min^{-1}) was much higher than that produced by amino acid stimulation (~1 min^{-1}). Furthermore, $[Ca^{2+}]_i$ oscillations in response to $[Ca^{2+}]_e$ were of sinusoidal pattern, whereas those induced by aromatic amino acids were of transient type. Thus the pattern and frequency of the $[Ca^{2+}]_i$ oscillation-induced signals which agonist (Ca^{2+} or aromatic amino acid) is predominantly bound to the CaR.

METHODS

Materials. Fura 2-AM was obtained from Molecular Probes (Eugene, OR); Hanks' balanced salt solution (HBSS), Opti-MEM I, and Lipofectin were from Invitrogen (Carlsbad, CA). Amino acids were of the highest grade commercially available. 2-Aminoethoxydiphenylborane (2-APB), Ro-31-8220, and phorbol 12,13-dibutyrate were from Calbiochem (San Diego, CA).

Cell culture. HEK-293 cells, maintained as previously described (25), were plated onto clean 18-mm-diameter glass coverslips resting in the bottom of 60-mm plastic culture dishes and were grown in high-glucose Dulbecco's modified Eagle's medium (Sigma, catalog no. D5796) supplemented with 10% fetal bovine serum containing penicillin (10 U/ml), streptomycin (10 μg/ml), and amphotericin B (25 ng/ml). Cells were maintained in a humidified incubator under 10% CO_2-90% air at 37°C.

Transient transfection. The human CaR cDNA cloned in the pCR3.1 expression vector was kindly provided by Dr. Allen Spiegel. Plasmid DNA (5 μg) was used in each 60-mm culture dish. DNA was diluted into serum-reduced medium (Opti-MEM I), mixed with diluted Lipofectin, and allowed to incubate at room temperature for 1 h. The resulting solution was diluted to a final volume of 2.5 ml with Opti-MEM I and was added to the cells. After 4–6 h, 2.5 ml of Opti-MEM I supplemented with 20% fetal bovine serum were added to the dish. Cells were used 1, 2, and 3 days after transfection. As a check for transfection efficiency, cotransfections were performed with the addition of the expression vector pDsRed1-N1 (Invitrogen), which produces a red fluorescent protein. More than 80% of cells that expressed the red protein also responded to an increase in $[Ca^{2+}]_e$ or to amino acid stimulation. Less than 6% of physiologically responsive cells lacked the red protein.

Solutions. Physiological saline consisted of HBSS without phenol red, supplemented with 0.5 mM $CaCl_2$ and 20 mM HEPES buffer, pH 7.4. Final concentrations (in mM) were 138 NaCl, 4 $NaHCO_3$, 0.3 Na_2HPO_4, 5 KCl, 0.3 KH_2PO_4, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.4 $MgSO_4$, 5.6 D-glucose, and 20 HEPES. Solutions of Ca^{2+} concentrations <1.3 mM were made by starting with Ca^{2+}- and Mg^{2+}-free HBSS to which the appropriate divalent cations were added.

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 45–60 min 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 (Zeiss TV 100; Carl Zeiss, Thornwood, NY) to which was attached a digital imaging system (Attofluor; Atto Instruments, Rockville, MD) and associated software (RatioVision). Ratio images (340 nm divided by 380 nm) were obtained about 1 s^{-1}, and the average ratio values from small regions (10 μm^2) 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.

RESULTS

Aromatic amino acids produce $[Ca^{2+}]_i$ oscillations through the CaR in HEK-293 cells. To examine the effect of aromatic amino acids on $[Ca^{2+}]_i$ in single cells, HEK-293 cells transiently transfected with the human CaR cDNA were loaded with the fluorescent Ca^{2+} indicator fura 2-AM and incubated in the presence of either 1.8, 1.3, or 0.5 mM $[Ca^{2+}]_e$. Intracellular Ca^{2+} imaging revealed that most transfected cells exhibit a stable $[Ca^{2+}]_i$, although a small proportion of cells (7% at 1.8 mM Ca^{2+}, n = 940 cells; 1% at 1.3 mM Ca^{2+}, n = 1,263 cells) showed spontaneous oscillatory activity.

Addition of 5 mM L-tryptophan to the perfusion saline bathing CaR-transfected cells stimulated striking oscillations in $[Ca^{2+}]_i$ that continued for hundreds of seconds (Fig. 1*A*). In cells exposed to 5 mM L-tryptophan, each $[Ca^{2+}]_i$ peak returned to baseline value, and the mean frequency was 1.3 ± 0.1 min^{-1} (n = 10 cells). Baseline $[Ca^{2+}]_i$ spiking (referred to as transient oscillations) is a well-defined oscillatory pattern that has been described for many other agonists in a variety of cell types (2). We verified that addition of 5 mM L-tryptophan to nontransfected HEK-293 cells did not induce any detectable change in $[Ca^{2+}]_i$ in any of the cells analyzed (n = 401).

Induction and maintenance of amino acid-induced $[Ca^{2+}]_i$ oscillations were dependent on the continued presence of an aromatic amino acid. Thus $[Ca^{2+}]_i$ oscillations were completely stopped upon removal of L-tryptophan (Fig. 1*B*) but were reinitiated by the introduction of an aromatic amino acid to the bathing solution (5 mM L-phenylalanine in Fig. 1*B*). The average frequency of the baseline $[Ca^{2+}]_i$ peaks in cells stimulated with 5 mM L-phenylalanine was 1.2 ± 0.1 min^{-1} (n = 14), which is not significantly different from that evoked by 5 mM L-tryptophan. In contrast, exposure of the cells to 5 mM L-proline produced small to nonexistent shifts in $[Ca^{2+}]_i$ and did not elicit any detectable $[Ca^{2+}]_i$ oscillations (Fig. 1, *B* and *C*). The lack of response to 5 mM L-proline was not due to desensitization of CaR responsiveness; when L-proline was applied before any other treatment, it did not produce $[Ca^{2+}]_i$ oscillations (Fig. 1*C*). We verified that subsequent addition of 5 mM L-phenylalanine to cells unresponsive to 5 mM L-proline initiated robust oscillatory changes in $[Ca^{2+}]_i$. These results indicate that aromatic amino acids induce striking and lasting transient oscillations in HEK-293 cells.

Amino acid-induced $[Ca^{2+}]_i$ oscillations are dependent on $[Ca^{2+}]_e$. Amino acid-induced $[Ca^{2+}]_i$ oscillations via the CaR depended on a threshold $[Ca^{2+}]_e$. Thus addition of 5 mM L-tryptophan did not produce any detectable $[Ca^{2+}]_i$ oscillations when the cells were exposed to a saline solution containing 0.5 mM $[Ca^{2+}]_e$, but a robust $[Ca^{2+}]_i$ response was initiated when the amino acid was present in a solution containing 1.8 mM $[Ca^{2+}]_e$ (Fig. 2*A*). Not only the induction but also the maintenance of amino acid-induced $[Ca^{2+}]_i$ oscilla-

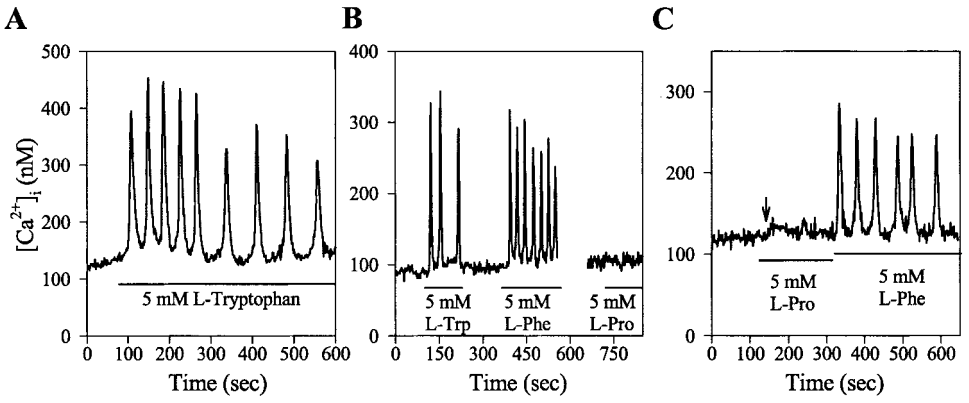

Fig. 1. Aromatic amino acids stimulate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) oscillations. *A*: upon perfusion with 5 mM L-tryptophan (L-Trp), $[Ca^{2+}]_i$ exhibited a small increase followed by a series of transient peaks of $[Ca^{2+}]_i$ that persisted for hundreds of seconds. Solution changes and timings are marked by horizontal bars. *B*: both L-tryptophan and L-phenylalanine (L-Phe) could produce oscillations of $[Ca^{2+}]_i$ in the same cell. Oscillations persisted only in the continued presence of amino acid. L-Proline (L-Pro; 5 mM) produced little to no increase in $[Ca^{2+}]_i$ with no oscillations. *C*: in some cells, 5 mM L-proline produced a small increase in $[Ca^{2+}]_i$ (arrow) but did not produce oscillations.

tions required a threshold $[Ca^{2+}]_e$. As shown in Fig. 2*B*, $[Ca^{2+}]_i$ oscillations induced by 5 mM L-tryptophan in the presence of 1.8 mM $[Ca^{2+}]_e$ were halted when the $[Ca^{2+}]_e$ was reduced to 0.5 mM, even in the continued presence of the amino acid.

Amino acid-induced transient oscillations persisted in the presence of 10 μM nifedipine, an inhibitor of L-type Ca^{2+} channels (Fig. 2*C*), but were completely blocked by addition of 2 μM thapsigargin (Fig. 2*D*), which rapidly depletes intracellular Ca^{2+} stores (32). These results indicate that $[Ca^{2+}]_i$ oscillations induced by aromatic amino acids depend on intracellular Ca^{2+} pools but do not require Ca^{2+} influx through L-type Ca^{2+} channels.

Stimulation of the CaR by external Ca^{2+} produces sinusoidal oscillations of a higher frequency than those produced by amino acids. Having established that aromatic amino acids induce transient $[Ca^{2+}]_i$ oscillations through the CaR in HEK-293 cells, our next step was to compare the pattern and frequency of these responses with those stimulated by an increase $[Ca^{2+}]_e$. As shown in Fig. 3, an increase in $[Ca^{2+}]_e$ in the absence of any exogenously added amino acid initiated $[Ca^{2+}]_i$ oscillations in HEK-293 cells transiently transfected with the CaR and perfused at 37°C. Specifically, an increase in the $[Ca^{2+}]_e$ from 1.8 to 3.0 mM produced a rapid elevation in $[Ca^{2+}]_i$ followed by striking oscillatory fluctuations in $[Ca^{2+}]_i$ that did not return to baseline values, a well-defined pattern known as sinusoidal oscillations (2, 3). The average frequency of these Ca^{2+}-induced sinusoidal oscillations mediated by the CaR was 3.8 ± 0.2 min^{-1} (n = 13). Similar results were obtained when the $[Ca^{2+}]_e$ was raised from 1.8 to 4.0 mM, producing a similar mean frequency of 3.9 ± 0.3 min^{-1} (n = 8; Fig. 3). $[Ca^{2+}]_i$ oscillations induced by increasing $[Ca^{2+}]_e$ to either 3.0 or 4.0 mM were halted when the $[Ca^{2+}]_e$ was returned to 1.8 mM (Fig. 3). An increase in $[Ca^{2+}]_e$ did not induce any $[Ca^{2+}]_i$ oscillations in nontransfected HEK-293 cells (n = 684).

The salient feature of the results presented in Figs. 1–3 is that the sinusoidal oscillations induced by an increase in $[Ca^{2+}]_e$ clearly differ from the transient oscillations induced by exposure to aromatic amino acids in the presence of a threshold $[Ca^{2+}]_e$. Most notably, the frequency of oscillations induced by aromatic amino acids is much lower than those stimulated by an increase in $[Ca^{2+}]_e$. As summarized in Fig. 4, the mean frequency of the $[Ca^{2+}]_i$ peaks was 1.3 ± 0.1 or 1.2 ± 0.1 min^{-1} in cells exposed to 5 mM L-tryptophan or 5 mM L-phenylalanine, respectively. Stimulation with 0.5 mM L-phenylalanine also induced transient oscillations, but the frequency was lower (0.85 ± 0.1 min^{-1}, n = 6, P = 0.06) than that produced by 5 mM L-phenylalanine. Stimulation with 10 mM L-phenylalanine produced oscillations with a slightly higher frequency (1.6 ± 0.1 min^{-1}, n = 21, P = 0.12) than that produced by 5 mM L-phenylalanine. In contrast, the mean frequency of the oscillations induced by increasing the $[Ca^{2+}]_e$ to either 3.0 or 4.0 mM (from 1.8 mM) was ~3.8 min^{-1}, and increasing the $[Ca^{2+}]_e$ to 5.0 mM produced oscillations at ~ 3.4 min^{-1} (3.4 ± 0.2 min^{-1}, n = 9). Reduction of the $[Ca^{2+}]_e$ increase to 2.5 mM produced oscillations of a lower frequency (2.2 ± 0.2 min^{-1}, n = 9) but still not in the frequency range of oscillations produced by 0.5–10 mM amino acid (P = 0.007 vs. 10 mM L-phenylalanine). These results indicate that the CaR mediates $[Ca^{2+}]_i$ oscillations in response to either $[Ca^{2+}]_e$ or L-amino acids, but the pattern and frequency of the $[Ca^{2+}]_i$ oscillation induced (i.e., sinusoidal or transient) discriminate which agonist is predominantly bound to the receptor.

PKC inhibition and 2-APB differentially affect $[Ca^{2+}]_i$ oscillations induced by an increase in $[Ca^{2+}]_e$ or by aromatic amino acid. Previous studies in which HEK-293 cells were transfected with the CaR showed that activation of PKC markedly inhibited $[Ca^{2+}]_e$-elicited increases in $[Ca^{2+}]_i$ via phosphorylation of

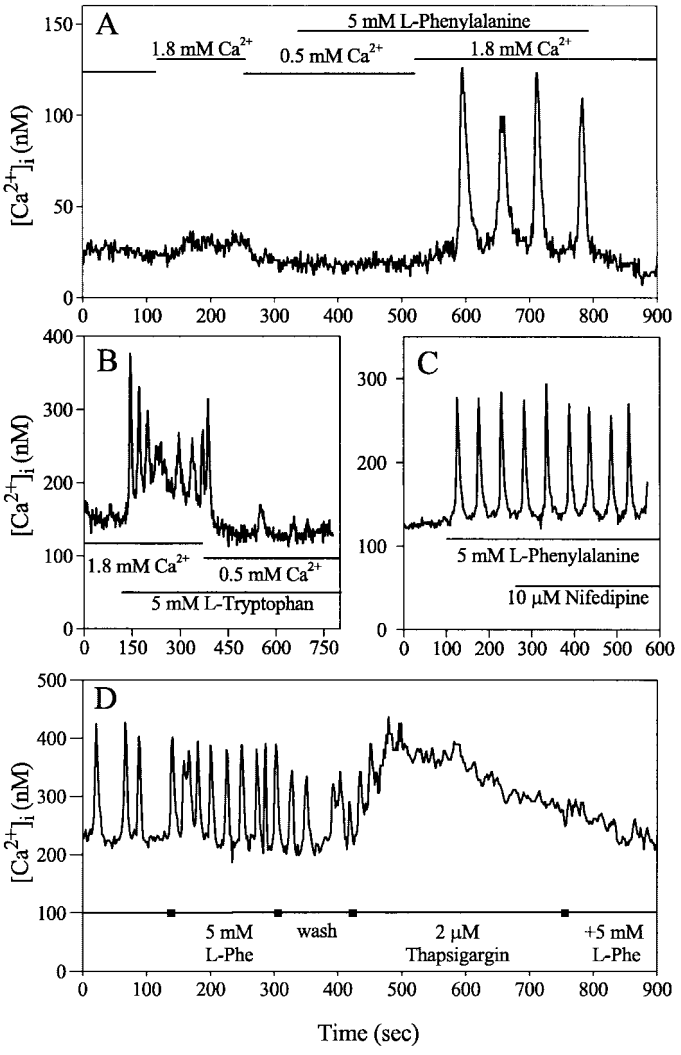

Fig. 2. *A*: initiation of amino acid-induced $[Ca^{2+}]_i$ oscillation is dependent on external Ca^{2+}. L-Phenylalanine (5 mM) did not produce oscillations in a solution containing 0.5 mM Ca^{2+}. Oscillations began when the external Ca^{2+} concentration ($[Ca^{2+}]_e$) was increased to 1.8 mM. Upon removal of the L-phenylalanine, oscillations ceased. *B*: maintenance of amino acid-induced $[Ca^{2+}]_i$ oscillations is dependent on $[Ca^{2+}]_e$. Starting $[Ca^{2+}]_e$ was 1.8 mM. When $[Ca^{2+}]_e$ was lowered, oscillations ceased, even in the continued presence of amino acid. *C*: amino acid-induced $[Ca^{2+}]_i$ oscillations were not eliminated by L-type Ca^{2+} channel blocker nifedipine (10 μM). $[Ca^{2+}]_e$ was 1.8 mM throughout experiment. *D*: treatment with thapsigargin (2 μM) blocked $[Ca^{2+}]_i$ oscillations.

threonine 888 in the CaR (1), indicating that PKC negatively modulates the coupling of the CaR to intracellular signaling systems. Because the inhibitory effect of PKC on CaR signaling could provide a mechanism leading to the generation of sinusoidal $[Ca^{2+}]_i$ oscillations (3, 33), we examined whether exposure to a selective PKC inhibitor reduces or eliminates $[Ca^{2+}]_e$-elicited oscillatory changes in $[Ca^{2+}]_i$. Pretreatment with the PKC inhibitor Ro-31-8220 (1.25 μM; 1 h) significantly reduced the percentage of cells that exhibited oscillations in response to an increase in $[Ca^{2+}]_e$ (from 1.3 to 3.0 mM) from 42% (control cells, $n = 60$) to 8% (Ro-31-8220-treated cells, $n = 59$). This treatment

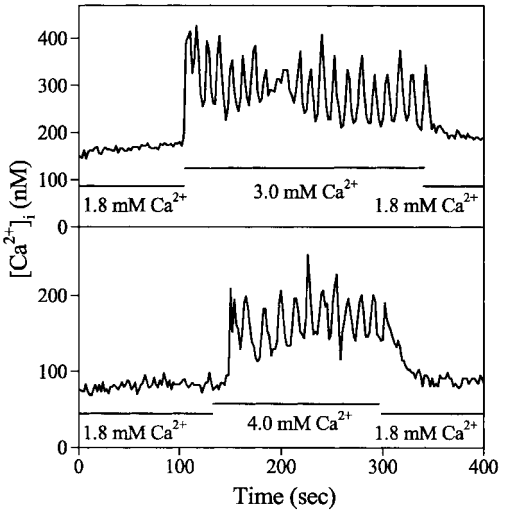

Fig. 3. Activation of the Ca^{2+}-sensing receptor (CaR) by increases in $[Ca^{2+}]_e$ in HEK-293 cells transfected with CaR produced oscillations in $[Ca^{2+}]_i$. *Top*: as $[Ca^{2+}]_e$ was raised from a resting level of 1.8 mM to 3.0 mM, $[Ca^{2+}]_i$ oscillations were induced. Oscillations ceased when $[Ca^{2+}]_e$ was returned to 1.8 mM. *Bottom*: oscillations induced by 4.0 mM $[Ca^{2+}]_e$.

did not block overall $[Ca^{2+}]_i$ responsiveness; the percentage of nonresponding cells remained similar (15% control, 17% Ro-31-8220). The majority of cells after Ro-31-8220 treatment exhibited a transient, nonoscillatory increase in $[Ca^{2+}]_i$ (Fig. 5*A*). These results suggest that PKC plays an important role in the generation of $[Ca^{2+}]_e$-elicited $[Ca^{2+}]_i$ oscillations. In contrast, Ro-31-8220 not only reduced the percentage of oscillatory responses to 5 mM L-phenylalanine (control 24%, $n = 41$ cells; Ro-31-8220 5%, $n = 62$ cells), but it almost completely inhibited all $[Ca^{2+}]_i$ responses (90% unresponsive; Fig. 6).

Recent studies have indicated that 2-APB, a membrane-permeant inhibitor of the D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] receptor (23, 24), is a selective inhibitor of capacitive Ca^{2+} influx (22). To examine the role of 2-APB-sensitive Ca^{2+} entry in

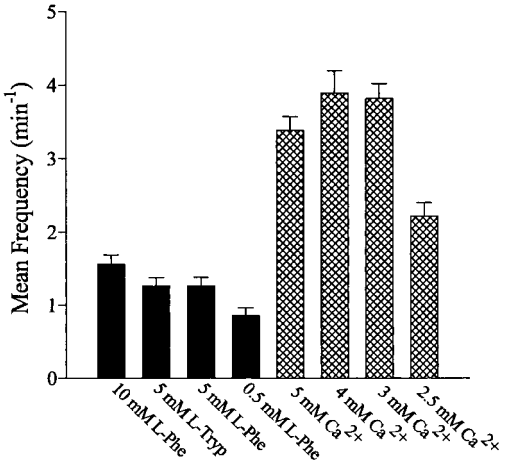

Fig. 4. Comparison of the mean frequency of amino acid-induced $[Ca^{2+}]_i$ oscillations with the mean frequency of Ca^{2+}-induced $[Ca^{2+}]_i$ oscillations. Data are presented as means ± SE.

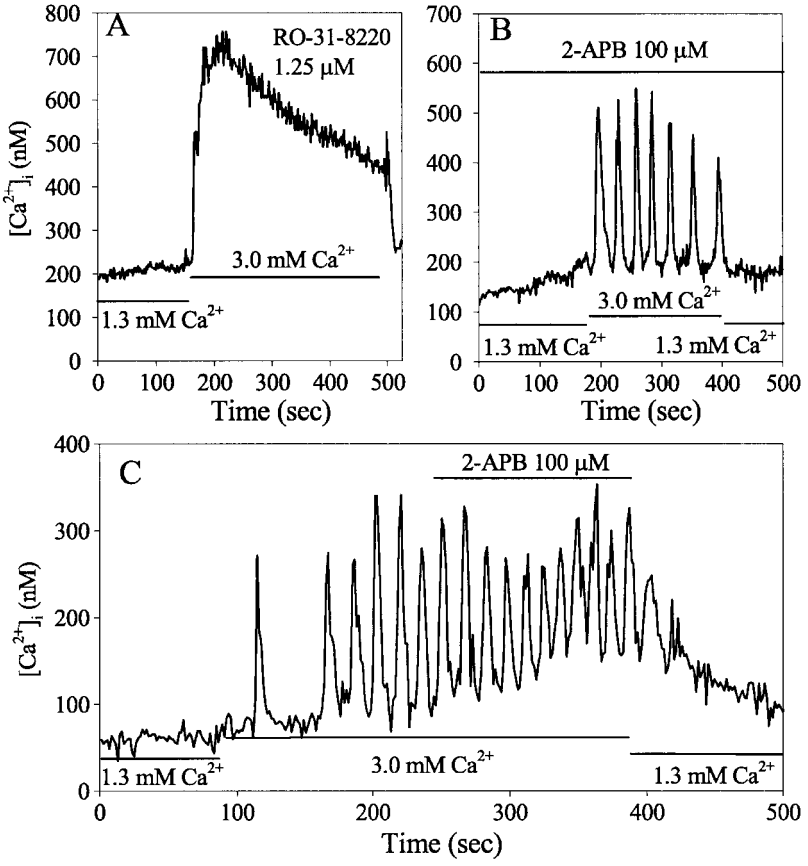

Fig. 5. Effect of Ro-31-8220 or 2-aminoethoxydiphenylborane (2-APB) on $[Ca^{2+}]_e$-induced $[Ca^{2+}]_i$ oscillations. *A*: pretreatment with the protein kinase C (PKC) inhibitor Ro-31-8220 at 1.25 μM for 1 h did not prevent a $[Ca^{2+}]_i$ response to an increase in $[Ca^{2+}]_e$ but converted oscillatory behavior of most cells to that of a slow declining transient. *B*: pretreatment of a cell with 100 μM 2-APB before the increase in $[Ca^{2+}]_i$ did not prevent oscillatory behavior. *C*: addition of 100 μM 2-APB to a cell that was already stimulated to produce $[Ca^{2+}]_i$ oscillations upon an increase in $[Ca^{2+}]_e$ from 1.3 to 3.0 mM did not affect the oscillations.

$[Ca^{2+}]_i$ oscillations elicited by $[Ca^{2+}]_e$ or amino acid stimulation of the CaR, we applied 2-APB either before or during stimulation. In control cells, an increase in $[Ca^{2+}]_e$ from 1.3 to 3.0 mM produced oscillatory behavior in 42% of the cells (n = 41). As shown in Fig. 5, *B* and *C*, addition of 100 μM 2-APB did not block $[Ca^{2+}]_e$-induced $[Ca^{2+}]_i$ oscillations in HEK-293 cells transfected with the CaR either when 2-APB was applied 4 min before stimulation or during the oscillatory activity. Specifically, after treatment with 100 μM 2-APB for 4 min, the percentage of oscillatory cells was similar to that for control cells (45%; n = 95), and the mean oscillation frequency was not significantly altered whether 2-APB was applied before (P = 0.33) or during (P = 0.18) oscillatory responses. These results are in striking contrast to the effect of 2-APB on the $[Ca^{2+}]_i$ responses elicited by stimulation with 5 mM L-phenylalanine (Fig. 6). Addition of 100 μM 2-APB completely blocked all responses of $[Ca^{2+}]_i$ to 5 mM L-phenylalanine (0% responding; n = 60).

L-Phenylalanine increases the sensitivity of the CaR to small changes in $[Ca^{2+}]_e$. Whereas certain cells that express CaR, typically in the gastrointestinal tract, are exposed to large changes in the concentration of aromatic amino acids and/or $[Ca^{2+}]_e$, many other cells in internal organs and tissues (e.g., parathyroid and kidney cells) are bathed by the interstitial milieu in which the concentrations of these agonists are maintained in a narrow range. We therefore examined whether aromatic amino acids, at concentrations present in plasma

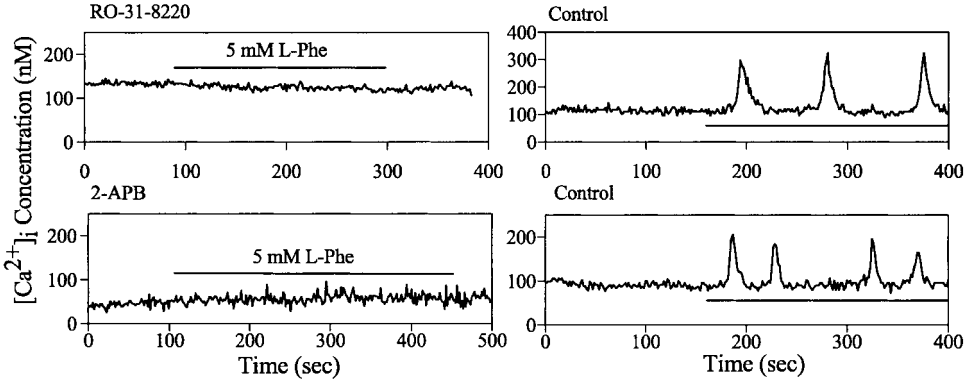

Fig. 6. L-Phenylalanine-induced oscillations were blocked by treatment with either Ro-31-8220 or 2-APB. *Top*: after treatment with 1.25 μM Ro-31-8220 for 1 h, $[Ca^{2+}]_i$ responses were severely reduced with 90% of cells showing no response to 5 mM L-phenylalanine. *Bottom*: after treatment with 100 μM 2-APB for 120 s, cells no longer responded with increases in $[Ca^{2+}]_i$ after stimulation with 5 mM L-phenylalanine.

(~0.1 mM), can modulate the frequency of $[Ca^{2+}]_i$ oscillations induced by an increase in $[Ca^{2+}]_e$ in a physiological range (1.3–2.3 mM).

As shown in Fig. 7, an increase in $[Ca^{2+}]_e$ from 1.3 to 2.3 mM in the absence of any exogenously added amino acid did not initiate any detectable $[Ca^{2+}]_i$ oscillations in HEK-293 cells transfected with the human CaR (although some cells do exhibit a small transient increase in $[Ca^{2+}]_i$; Fig. 7, *D–G*). Similarly, addition of a mixture of aromatic amino acids at the concentrations found in fasting plasma (0.1 mM L-phenylalanine + 0.1 mM L-tryptophan) did not induce any oscillatory changes in $[Ca^{2+}]_i$ in cells exposed to a solution containing 1.3 mM $[Ca^{2+}]_e$. In contrast, striking sinusoidal oscillations were produced when the $[Ca^{2+}]_e$ was increased from 1.3 to 2.3 mM in the presence of physiological concentrations of aromatic amino acids (0.1 mM L-phenylalanine + 0.1 mM L-tryptophan). These $[Ca^{2+}]_i$ oscillations were halted when the $[Ca^{2+}]_e$ was returned to 1.3 mM (Fig. 7, *A–C*). These results demonstrate that aromatic amino acids, at physiological concentrations, dramatically increase the ability of the CaR to mediate sinusoidal $[Ca^{2+}]_i$ oscillations in response to an increase in $[Ca^{2+}]_e$.

DISCUSSION

Our results demonstrate, for the first time, that single HEK-293 cells transfected with the human CaR

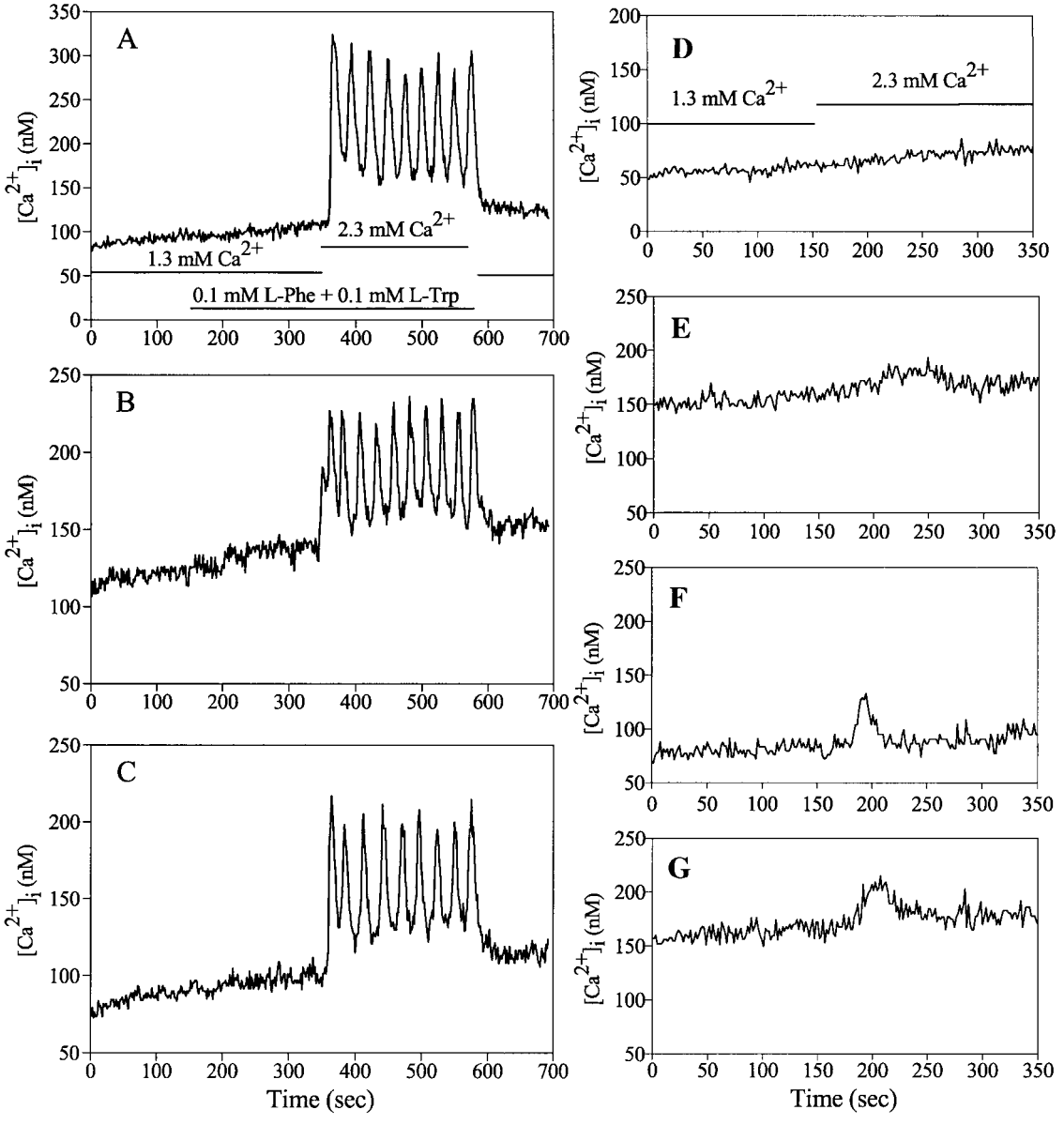

Fig. 7. Amino acids potentiated the induction of $[Ca^{2+}]_i$ oscillations. *A–C*: addition of a mixture of 0.1 mM L-phenylalanine + 0.1 mM L-tryptophan to a resting $[Ca^{2+}]_e$ of 1.3 mM did not produce $[Ca^{2+}]_i$ oscillations, but a subsequent increase to 2.3 mM Ca^{2+} produced oscillatory behavior. *D–G*: increasing $[Ca^{2+}]_i$ from 1.3 to 2.3 mM without amino acids did not produce oscillations. Responses of $[Ca^{2+}]_i$ to the 1 mM $[Ca^{2+}]_e$ increase varied from no effect (*D*), to small, steady shifts (*E*), to single small transient increases (*F* and *G*).

and incubated in the presence of a threshold $[Ca^{2+}]_e$ respond to the addition of the aromatic amino acids L-tryptophan and L-phenylalanine, at millimolar concentrations, with striking and lasting $[Ca^{2+}]_i$ oscillations that occurred as discrete transients arising from a baseline level. Transient oscillations is a well-defined oscillatory pattern that has been described for many other agonists in a variety of cell types (2). Aromatic amino acids are known to reach high concentrations in the lumen of the gastrointestinal tract during the digestion of ingested protein. It is conceivable that amino acid-induced Ca^{2+} oscillations through the CaR, which is expressed in cells of the gastrointestinal tract including the G cells and parietal cells of the stomach (6, 28) and endocrine cells of the small intestine (7), provide a mechanism by which the release of amino acids from ingested protein regulates gastrointestinal function. Amino acid-induced $[Ca^{2+}]_i$ oscillatory changes in gut endocrine cells could trigger the release of gastrointestinal peptides that regulate multiple functions including secretion, motility, and cell proliferation (9).

Our results also demonstrate that HEK-293 cells transfected with the CaR respond to increases in $[Ca^{2+}]_e$ with $[Ca^{2+}]_i$ oscillations, in agreement with a recent study (4). A salient feature of our results is that the average frequency of the oscillations induced by an increase in $[Ca^{2+}]_e$ ($\sim$4 min^{-1}) was much higher than that produced by aromatic amino acid stimulation ($\sim$1 min^{-1}) and that the pattern was sinusoidal rather than transient. Because external Ca^{2+} is both an agonist and the permeant species of any Ca^{2+} influx, oscillations induced with increased $[Ca^{2+}]_e$ could reflect not only an increase in agonist concentration but also an increase in driving force for Ca^{2+} influx with a resultant increase in influx that might influence oscillation frequency.

Whereas the CaR expressed in cells facing the lumen of the gastrointestinal tract are exposed to large changes in the concentration of aromatic amino acids and/or $[Ca^{2+}]_e$ and consequently can function in the two extreme oscillatory modes outlined above, many other cells in internal organs and tissues (e.g., parathyroid and kidney cells) are bathed by the interstitial milieu in which $[Ca^{2+}]_e$ and the concentration of aromatic amino acids are maintained in a narrower range. In this context, our results demonstrate that exposure to aromatic amino acids, at concentrations found in fasting plasma, dramatically increase the generation of $[Ca^{2+}]_i$ oscillations by the CaR in response to a relatively small increase in the $[Ca^{2+}]_e$. Thus we conclude that the allosteric potentiation by physiological concentrations of aromatic amino acids of the response of the CaR to $[Ca^{2+}]_e$, recently reported by Conigrave et al. [8; reviewed by Kobilka (20)] in HEK-293 cell populations, is manifested in single HEK-293 cells by a dramatic increase in the ability of the CaR to produce sinusoidal $[Ca^{2+}]_i$ oscillations.

The spatial organization, amplitude, and frequency of the changes in $[Ca^{2+}]_i$ have been the subject of intense interest. Studies with many other receptors demonstrated the induction of either sinusoidal or transient patterns of $[Ca^{2+}]_i$ oscillations in different cell types and also in individual cells responding to different agonists (2). Even a single agonist can induce different oscillatory patterns of $[Ca^{2+}]_i$ by stimulating closely related receptor subtypes. For example, studies with the metabotropic glutamate receptors, which are structurally related to the CaR (5), showed that glutamate can induce different oscillatory patterns of $[Ca^{2+}]_i$ by stimulating the different receptor subtypes mGlu1 and mGlu 5 (18, 19). In contrast to these systems, the work presented here demonstrates that two different classes of agonists can induce $[Ca^{2+}]_i$ oscillations of different type and frequency through a single receptor, the CaR. Our results indicate that the CaR can function in two different modes, triggering high-frequency sinusoidal oscillations in response to an increase in $[Ca^{2+}]_e$ or low-frequency transient oscillations in response to aromatic amino acids in the presence of a threshold $[Ca^{2+}]_e$. To our knowledge, this is the first time that sinusoidal and transient patterns of $[Ca^{2+}]_i$ oscillations have been shown to be induced by different agonists (i.e., Ca^{2+} and aromatic amino acids) acting through the same receptor.

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 broadly based on negative feedback effects of PKC on the production of $Ins(1,4,5)P_3$ or on the regulatory properties of $[Ca^{2+}]_i$ on the $Ins(1,4,5)P_3$ receptor (2, 31, 33). In an attempt to further distinguish the patterns seen by activation of the CaR by two different agonists, we examined the effect of agents that have been shown to modulate the production of $[Ca^{2+}]_i$ oscillations in a variety of model systems. In our experiments, treatment with the PKC inhibitor Ro-31-8220 significantly reduced oscillatory responses induced by increases in $[Ca^{2+}]_e$ but did not influence overall responsiveness to $[Ca^{2+}]_e$. In the presence of this inhibitor, most cells responded to an increase in $[Ca^{2+}]_e$ by a transient increase in $[Ca^{2+}]_i$ rather than by $[Ca^{2+}]_i$ oscillations. These findings suggest that PKC plays a critical role in the generation of $[Ca^{2+}]_e$-evoked $[Ca^{2+}]_i$ oscillations via the CaR. In contrast, exposure to Ro-31-8220 had a more profound effect on $[Ca^{2+}]_i$ changes induced by 5 mM L-phenylalanine, resulting in almost complete inhibition of response. Furthermore, treatment with 100 μM 2-APB, a membrane-permeant inhibitor of the $Ins(1,4,5)P_3$ receptor (23, 24) as well as an inhibitor of extracellular Ca^{2+} influx (22), did not block $[Ca^{2+}]_e$-induced oscillatory responses or affect mean oscillation frequency. In contrast, 2-APB completely prevented all $[Ca^{2+}]_i$ responses to L-phenylalanine. These results indicate that $[Ca^{2+}]_e$-elicited and amino acid-induced changes in $[Ca^{2+}]_i$ can be further distinguished by their differential sensitivity to Ro-31-8220 and 2-APB. It is tempting to speculate that the CaR mediates sinusoidal or transient patterns of $[Ca^{2+}]_i$ oscillations in response to different agonists through different mechanisms, a proposition that warrants further experimental work. In this context, there is increasing recognition that different agonists induce receptors to

adopt different conformational states (11). For example, the recent work by Kobilka and colleagues (12, 13) has shown that the β_2-adrenergic GPCR can exhibit multiple conformational states dependent on the agonist. Thus 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 downstream signaling pathways.

Oscillatory changes in $[Ca^{2+}]_i$ in response to receptor stimulation is a fundamental mechanism of cell signaling in both nonexcitable and excitable cells that can protect cells from the cytotoxic effects of prolonged increases in $[Ca^{2+}]_i$. It is increasingly recognized that the pattern and frequency of $[Ca^{2+}]_i$ oscillations encode for differential regulation of biological responses including selective gene expression (10, 17, 21, 34). For example, low-frequency $[Ca^{2+}]_i$ oscillations activate the transcription factor nuclear factor (NF)-κB, whereas higher frequencies lead to stimulation of the transcription factor NF-AT (10). Thus our results demonstrating that the CaR mediates oscillations of different type and frequency in response to an increase in $[Ca^{2+}]_e$ in the absence of amino acids, to aromatic L-amino acids in the presence of a threshold $[Ca^{2+}]_e$, or to a relatively small increase in $[Ca^{2+}]_e$ in the presence of aromatic amino acids at concentrations found in fasting plasma are of potential physiological importance because they identify a novel mechanism for encoding different biological responses via a single class of receptor, namely, the CaR.

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