Calcium-sensing receptor activation induces intracellular calcium oscillations

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Breitwieser, Gerda E., and Lucio Gama. Calcium-sensing receptor activation induces intracellular calcium oscillations. Am J Physiol Cell Physiol 280: C1412–C1421, 2001.—Parathyroid hormone secretion is exquisitely sensitive to small changes in serum Ca2+ concentration, and these responses are transduced via the Ca2+-sensing receptor (CaR). We utilized heterologous expression in HEK-293 cells to determine the effects of small, physiologically relevant perturbations in extracellular Ca2+ on CaR signaling via phosphatidylinositol-phospholipase C, using changes in fura 2 fluorescence to quantify Parathyroid hormone secretion is exquisitely sensitive to small

intracellular Ca2+ responses to acute extracellular Ca2+ perturbations but had no effect on thapsigargin-sensitive Ca2+ stores. Modest, physiologically relevant increases in extracellular Ca2+ concentration (0.5 mM increments) caused sustained (30–40 min) low-frequency oscillations of intracellular Ca2+ (~45 s peak to peak interval). Oscillations were eliminated by 1 μM thapsigargin but were insensitive to protein kinase inhibitors (staurosporine, KN-93, or bisindolylmaleimide I). Staurosporine did increase the fraction of cells oscillating at a given extracellular Ca2+ concentration. Serum Ca2+ concentrations thus chronically regulate cells expressing CaR, and small perturbations in extracellular Ca2+ alter both resting intracellular Ca2+ as well as Ca2+ dynamics.

fura 2; intracellular calcium; calcium oscillations; thapsigargin-sensitive calcium stores; HEK-293 cells; protein kinase inhibitors

Methods

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THE CALCIUM-SENSING RECEPTOR (CaR) was hypothesized to exist based on the sensitivity of parathyroid hormone secretion to serum calcium (5, 24). Expression cloning utilizing RNA isolated from parathyroid cells resulted in identification of the CaR (6), which is activated by extracellular Ca2+ in the physiological range (0.5–5 mM). A unique feature of CaR is the steep cooperativity of Ca2+-dependent activation, which is observed both in vivo (26) and when CaR is heterologously expressed (6, 15). In the parathyroid, CaR is chronically exposed to serum Ca2+ concentrations that are within the steeply cooperative range of its dose-response relation (EC50 of 2–3 mM, and Hill coefficient n ~ 2–3), providing tight control over parathyroid hormone secretion. Although these qualitative details of CaR-mediated modulation of parathyroid hormone secretion are well characterized, the kinetics of single-cell responses to tonic, physiological changes in extracellular Ca2+ have not been studied.

CaR couples changes in extracellular Ca2+ to a variety of intracellular responses, including activation of phospholipases, generation of inositol trisphosphate (IP3) and diacylglycerol, increases in intracellular Ca2+, changes in protein phosphorylation, activation of ion channels, regulation of hormone secretion, and modulation of gene expression (32). Chronic activation of CaR resulting from constant exposure to serum Ca2+ would necessarily contribute to regulation of cellular functions through many of these pathways. Under conditions of constant agonist exposure, however, many G protein-coupled receptors undergo desensitization and, ultimately, downregulation of receptor and/or signal transduction pathway protein expression (7, 13). A key question with respect to CaR function is therefore whether CaR desensitizes at a fixed extracellular Ca2+ concentration and responds only to acute changes in serum Ca2+ or whether CaR is chronically activated under physiological conditions.

We have utilized heterologous expression of human CaR in HEK-293 cells to address two fundamental questions with respect to the in vivo contributions of CaR to parathyroid cell function, namely, How do cells expressing CaR adapt to chronic exposure to the concentrations of extracellular Ca2+ that are present in normal serum? and What are the kinetic features of CaR-mediated changes in intracellular Ca2+ in response to small, acute perturbations of extracellular Ca2+? We find that cells expressing CaR are uniquely sensitive to alterations in extracellular Ca2+ and that small perturbations in extracellular Ca2+ induce low-frequency intracellular Ca2+ oscillations. These results have implications for the mechanism(s) by which CaR activation regulates parathyroid hormone secretion as well as diverse cellular signal transduction pathways in many other cell types.

METHODS

Cell culture and transfection. HEK-293 cells (American Type Culture Collection, Rockville, MD) were grown in high-
RESULTS

Contribution(s) of CaR expression to resting intracellular Ca\(^{2+}\) concentration. Activation of CaR is steeply dependent on extracellular Ca\(^{2+}\). Figure IA illustrates a typical experiment in which extracellular Ca\(^{2+}\) is progressively increased from a baseline value of 0.5 to 30 mM, and intracellular Ca\(^{2+}\) is monitored with fura 2 fluorescence. Data compiled from 20 cells in this representative experiment were fitted with the Hill equation (Fig. 1B) to obtain the EC\(_{50}\) for extracellular Ca\(^{2+}\) (3.4 ± 0.4 mM) and the Hill coefficient (1.8 ± 0.4), as we have previously reported (15). These results suggest that exposure of cells expressing CaR to normal serum-containing media (Ca\(^{2+}\) of ~1.8–2 mM) should partially activate the receptor.

The steady-state contributions of CaR activation on intracellular Ca\(^{2+}\) in the presence of physiological levels of extracellular Ca\(^{2+}\) were investigated with a pretreatment protocol. HEK-293 cells expressing CaR were loaded with fura 2-AM at various extracellular Ca\(^{2+}\) concentrations (0.5, 2, or 3 mM), and the Ca\(^{2+}\) imaging experiments were then begun in the same concentration of extracellular Ca\(^{2+}\). Cells were therefore maintained in a fixed concentration of extracellular Ca\(^{2+}\) throughout loading and initiation of the experiment (30–40 min). Intracellular Ca\(^{2+}\) was determined at the start of the experiment (within the first minute of recording). The averaged results of this type of experiment are illustrated in Fig. 2A. Culture, loading, and initiation of the experiment in elevated extracellular Ca\(^{2+}\) (>0.5 mM) significantly increased both intracellular Ca\(^{2+}\) and the range of intracellular Ca\(^{2+}\)
Fig. 2. Steady-state intracellular Ca$^{2+}$ in cells expressing CaR. A: HEK-293 cells transiently expressing CaR-GFP were loaded with fura 2-AM in various Ca$^{2+}$ concentrations (0.5, 2, and 3 mM), and experiments were begun in the same Ca$^{2+}$ concentration. Intracellular Ca$^{2+}$ concentrations were measured during the first minute of recording. Data represent average of 60 cells/condition from at least 3 independent transfections. Significance was determined relative to 0.5 mM at $P<0.01$ (*) or 2 mM at $P<0.01$ (**). B: untransfected HEK-293 cells were subjected to the same protocol as in A, with significance determined relative to 0.5 mM at $P<0.01$ (*). C: 20 cells were exposed to 2 mM extracellular Ca$^{2+}$, then to 0.5 mM, and then returned to 2 mM. Average intracellular Ca$^{2+}$ at steady state in each condition was measured. Significance is relative to 2 mM at $P<0.01$ (*). Data are representative of 3 independent experiments. D: protocol similar to that of C performed in untransfected HEK-293 cells.

Concentrations observed (Fig. 2A, 2 mM Ca$^{2+}$ > 0.5 mM Ca$^{2+}$; 3 mM Ca$^{2+}$ > 2 mM Ca$^{2+}$). Similar experiments were performed on control HEK-293 cells (Fig. 2B); no systematic effects on intracellular Ca$^{2+}$ were observed, i.e., intracellular Ca$^{2+}$ concentration after 2 mM preincubation was approximately equal to that after 3 mM preincubation (although both had an intracellular Ca$^{2+}$ concentration higher than that observed after preincubation in 0.5 mM Ca$^{2+}$). Acute CaR-mediated changes in intracellular Ca$^{2+}$ were assessed by measuring the response to a decrease in extracellular Ca$^{2+}$ from 2 to 0.5 mM, followed by a return to 2 mM extracellular Ca$^{2+}$. The averaged responses of 20 cells from a single experiment are illustrated in Fig. 2C (data representative of 3 independent experiments). There was a statistically significant decrease in intracellular Ca$^{2+}$ in 0.5 mM extracellular Ca$^{2+}$, with full recovery on return to 2 mM Ca$^{2+}$. Similar changes in intracellular Ca$^{2+}$ were not observed in untransfected HEK-293 cells (Fig. 2D). Thus, by several criteria, cells expressing CaR are sensitive to steady-state perturbations of extracellular Ca$^{2+}$ over the range of 0.5–3 mM and respond with changes in the steady-state concentration of intracellular Ca$^{2+}$.

 Contributions of CaR expression to Ca$^{2+}$ content of thapsigargin-sensitive stores. Expression of CaR has a significant effect on resting intracellular Ca$^{2+}$ concentrations when cells are exposed to extracellular Ca$^{2+}$ concentrations in the physiological range, i.e., 0.5–3 mM (Fig. 2). A second compartment of intracellular Ca$^{2+}$ that might be affected by CaR expression (and chronic activation) is the thapsigargin-sensitive store, which releases Ca$^{2+}$ on CaR activation (32). To assess the content of thapsigargin-sensitive stores after exposure of CaR-expressing HEK-293 cells to 0.5, 2, or 3 mM extracellular Ca$^{2+}$, experiments were begun in the loading Ca$^{2+}$ concentration to establish the resting intracellular Ca$^{2+}$ level, switched to a nominally zero Ca$^{2+}$ bath solution, and then exposed to 1 μM thapsigargin in the continued absence of bath Ca$^{2+}$. Figure 3, A–C, illustrates this type of experiment in HEK-293 cells preincubated in 0.5, 2, and 3 mM Ca$^{2+}$, respectively, and Fig. 3, D–F, illustrates the results obtained with CaR-expressing cells.

To define the differences conferred by CaR expression, the total thapsigargin-releasable Ca$^{2+}$ content was determined by measuring the area under the averaged Ca$^{2+}$ transients of experiments such as those illustrated in Fig. 3. As illustrated in Fig. 4A, there was no difference in the magnitude of the thapsigargin-releasable Ca$^{2+}$ store as a function of extracellular Ca$^{2+}$ concentration for either HEK-293 cells or CaR-expressing cells. There was, however, a significant difference between HEK-293 and CaR-expressing cells with respect to the magnitude of the thapsigargin-releasable Ca$^{2+}$ pool. The horizontal lines in Fig. 4A indicate an estimate of the thapsigargin-releasable Ca$^{2+}$ pool determined by averaging all data obtained, i.e., at 0.5, 2, and 3 mM Ca$^{2+}$, for either HEK-293 cells (11,744 ± 1,362 arbitrary units (AU)) or CaR-expressing cells (9,325 ± 1,148 AU). Expression of CaR reduces the content of the thapsigargin-sensitive Ca$^{2+}$ store by ~20%, but the content of this Ca$^{2+}$ store is not altered by 30–40 min of incubation in extracellular Ca$^{2+}$ concentration in the range from 0.5 to 3 mM.

Close inspection of the traces illustrated in Fig. 3 suggest that, in addition to a significant decrease in the content of thapsigargin-releasable Ca$^{2+}$ stores, expression of CaR also increases the kinetic complexity of the intracellular Ca$^{2+}$ transients. In particular, cells expressing CaR are more sensitive to removal of bath Ca$^{2+}$, e.g., HEK-293 cells (Fig. 3C) are relatively insensitive to switching from 3 mM to zero bath Ca$^{2+}$, whereas CaR-expressing cells (Fig. 3F) exhibit elevated intracellular Ca$^{2+}$ (some cells are undergoing intracellular Ca$^{2+}$ oscillations) in 3 mM Ca$^{2+}$, and this decreases significantly on removal of extracellular Ca$^{2+}$, confirming the results illustrated in Fig. 2. Furthermore, the kinetics of the thapsigargin-induced intracellular Ca$^{2+}$ transient are more variable than those exhibited by HEK-293 cells. To quantify the kinetic diversity, the half times for the rising ($t_R$) and declining ($t_D$) phases of the thapsigargin-induced intracellular Ca$^{2+}$ transient were determined for HEK-293
cells and CaR-expressing cells preincubated in various extracellular Ca\(^{2+}\) concentrations (Fig. 4B). There were no significant differences in half times for the thapsigargin response when the averages of all cells in each condition were used to determine half times (>60 cells/condition).

**Small changes in extracellular Ca\(^{2+}\) induce intracellular Ca\(^{2+}\) oscillations.** While decreases in extracellular Ca\(^{2+}\) over the range from 3 to 0.5 mM cause CaR-mediated changes in the steady-state concentration of intracellular Ca\(^{2+}\) (Figs. 2 and 3), small incremental increases in extracellular Ca\(^{2+}\) (0.5 mM) induce oscillations of intracellular Ca\(^{2+}\). Illustrated in Fig. 5 is a typical experiment, in which HEK-293 cells transiently transfected with CaR were loaded with fura 2-AM and the experiment was begun in 2 mM extracellular Ca\(^{2+}\). Increases (0.5 mM increments) in extracellular Ca\(^{2+}\) from 2 to 3.5 mM initiate oscillatory intracellular Ca\(^{2+}\) changes, which are most pronounced at 3.5 mM extracellular Ca\(^{2+}\). The oscillatory change in intracellular Ca\(^{2+}\) ceased abruptly when extracellular Ca\(^{2+}\) was decreased to 2 mM. At the end of the experiment, the cells were exposed to a concentration of extracellular Ca\(^{2+}\) that maximally activates CaR (20 mM). This concentration of extracellular Ca\(^{2+}\) generated a rapidly activating peak of intracellular Ca\(^{2+}\) (up to 200 nM), which decayed to an elevated plateau for the duration of agonist exposure. This representative experiment illustrates the range of responses to alterations in Ca\(^{2+}\) dynamics.
extracellular Ca\(^{2+}\), from oscillatory changes in intracellular Ca\(^{2+}\) at physiological levels of extracellular Ca\(^{2+}\) to a maximal response that is typical of many G protein-coupled receptors that activate the phosphatidylinositol-phospholipase C (PI-PLC) pathway and increase intracellular Ca\(^{2+}\).

The threshold for CaR-mediated intracellular Ca\(^{2+}\) oscillations. The experiment illustrated in Fig. 5 suggests that the threshold for intracellular Ca\(^{2+}\) oscillations is in the range of 2.5–3 mM extracellular Ca\(^{2+}\). To determine whether this threshold is influenced by the starting extracellular Ca\(^{2+}\) concentration, HEK-293 cells expressing CaR were loaded with fura 2-AM at fixed Ca\(^{2+}\) concentrations (0.5, 2, or 3 mM), and the subsequent experiment was begun in the same extracellular Ca\(^{2+}\) concentration. Extracellular Ca\(^{2+}\) was then increased in 0.5 mM increments. Representative experiments begun in 0.5, 2, or 3 mM extracellular Ca\(^{2+}\) are illustrated in Fig. 6, A, B, and C, respectively. Inspection of the plots reveals that cells do not oscillate in the extracellular Ca\(^{2+}\) concentration in which they have been loaded with fura 2-AM (although there are differences in resting intracellular Ca\(^{2+}\) concentrations, as documented in Figs. 2 and 3). Cells loaded in 2 or 3 mM extracellular Ca\(^{2+}\) begin to oscillate after the first 0.5 mM increment in extracellular Ca\(^{2+}\), with peak numbers of cells oscillating after a 1 mM change from the initial Ca\(^{2+}\) concentration. Cells loaded in 0.5 mM Ca\(^{2+}\) do not oscillate until extracellular Ca\(^{2+}\) reaches 2 mM, with additional recruitment of cells at concentrations up to 5 mM, the highest concentration tested. The extracellular Ca\(^{2+}\) concentration dependence of oscillation threshold, characterized as the percent of cells oscillating in a given condition, was determined for three independent experiments initiated at 0.5, 2, and 3 mM Ca\(^{2+}\), and the results are illustrated in Fig. 6D.

Oscillation frequency is constant. The dependence of the oscillation frequency on extracellular Ca\(^{2+}\), characterized by the peak to peak duration, was analyzed for the experiments in Fig. 6, A–C, plotted in Fig. 6E.
Regardless of the preconditioning extracellular Ca\(^{2+}\), the peak to peak intervals of the oscillations elicited over the range of extracellular Ca\(^{2+}\) concentrations from 2.5 to 5 mM were comparable; the average of all conditions was 46.8 s (indicated by the dashed line in Fig. 6E).

Dependence of oscillations on intracellular Ca\(^{2+}\). Oscillations induced by small changes in extracellular Ca\(^{2+}\) were sustained for periods up to 30 min. An example is illustrated in Fig. 7. In this experiment, the oscillations were initiated by a step change in extracellular Ca\(^{2+}\) from 2 to 3 mM, and intracellular Ca\(^{2+}\) was monitored until oscillations dissipated. The insets in Fig. 7 illustrate the behavior of cells early (from 8 to 16 min) and late (from 33 to 42 min) in the exposure to 3 mM Ca\(^{2+}\). This experiment is representative of 12 experiments [3 independent transient transfections (n = 3 experiments each) plus 3 experiments in cells stably expressing CaR]. In all experiments, oscillatory behavior ceased between 20 and 40 min after initiation by a 1 mM increase in extracellular Ca\(^{2+}\). Despite the cessation of oscillations, the cells exhibited an elevated intracellular Ca\(^{2+}\) in the presence of 3 mM extracellular Ca\(^{2+}\), which decreased on return to 2 mM Ca\(^{2+}\), e.g., Fig. 7.

Figure 8A illustrates an experiment designed to determine whether there is a relationship between sustained oscillations and the level of Ca\(^{2+}\) in intracellular stores. In this experiment, cells were exposed twice to 3.5 mM extracellular Ca\(^{2+}\), from a baseline extracellular Ca\(^{2+}\) of 2 mM. Each time, there was an initial peak of intracellular Ca\(^{2+}\) release, followed by oscillations that were sustained for the duration of the exposure to 3.5 mM extracellular Ca\(^{2+}\). The peak to peak intervals of the oscillations during the first and second exposures to 3.5 mM Ca\(^{2+}\) were comparable (Fig. 8B), while the initial peak release of intracellular Ca\(^{2+}\) on agonist addition decreased by 45% and the baseline intracellular Ca\(^{2+}\) in 2 mM extracellular Ca\(^{2+}\) decreased significantly during the experiment (Fig. 8C). Similar behavior was observed in >10 independent experiments.

These results suggest that neither the ability to support sustained intracellular Ca\(^{2+}\) oscillations nor the oscillation frequency is a simple function of the resting level of intracellular Ca\(^{2+}\) or the degree of loading of intracellular Ca\(^{2+}\) stores as inferred from the initial peak response.

Intracellular stores are, however, intimately involved in maintenance of the intracellular Ca\(^{2+}\) oscillations, as indicated by the experiments in Fig. 9. Figure 9A illustrates a control experiment in which...
oscillations were induced by an increase in extracellular Ca$^{2+}$ to 3.5 mM from a baseline Ca$^{2+}$ of 2 mM. Oscillations were sustained until the experiment was ended with exposure of the cells to 10 mM Ca$^{2+}$. A: experiment was begun as in A, but 1 μM thapsigargin was added after 4 min in 3.5 mM Ca$^{2+}$. Experiment was ended by exposure of cells to 10 mM Ca$^{2+}$. Note the reduced response to 10 mM Ca$^{2+}$, indicating compromised thapsigargin-sensitive intracellular stores.

Small step changes in extracellular Ca$^{2+}$ out of intracellular stores. A second widely observed oscillations were initiated by an increase in extracellular Ca$^{2+}$ from 2 to 3.5 mM. Experiment was ended by exposure of the cells to 10 mM Ca$^{2+}$. B: experiment was begun as in A, but 1 μM thapsigargin was added after 4 min in 3.5 mM Ca$^{2+}$. Experiment was ended by exposure of cells to 10 mM Ca$^{2+}$. Note the reduced response to 10 mM Ca$^{2+}$, indicating compromised thapsigargin-sensitive intracellular stores.

**Fig. 9.** Effect of thapsigargin on intracellular Ca$^{2+}$ oscillations. A: control experiment in which intracellular Ca$^{2+}$ oscillations were initiated by an increase in extracellular Ca$^{2+}$ from 2 to 3.5 mM. Oscillations were sustained until the experiment was ended with exposure of the cells to 10 mM Ca$^{2+}$. B: experiment was begun as in A, but 1 μM thapsigargin was added after 4 min in 3.5 mM Ca$^{2+}$. Experiment was ended by exposure of cells to 10 mM Ca$^{2+}$. Note the reduced response to 10 mM Ca$^{2+}$, indicating compromised thapsigargin-sensitive intracellular stores.

**Fig. 9.** Effect of thapsigargin on intracellular Ca$^{2+}$ oscillations. A: control experiment in which intracellular Ca$^{2+}$ oscillations were initiated by an increase in extracellular Ca$^{2+}$ from 2 to 3.5 mM. Oscillations were sustained until the experiment was ended with exposure of the cells to 10 mM Ca$^{2+}$ to elicit a maximal CaR-mediated response. The companion experiment, illustrated in Fig. 9B, begins in the same manner, but after 4 min of oscillations in 3.5 mM Ca$^{2+}$, 1 μM thapsigargin was added to the extracellular solution. There was an immediate increase in intracellular Ca$^{2+}$ as uptake into the thapsigargin-sensitive stores was blocked, followed by a slow decline in intracellular Ca$^{2+}$. Intracellular Ca$^{2+}$ oscillations ceased abruptly on exposure to thapsigargin, before the peak of the thapsigargin response. Thus intracellular Ca$^{2+}$ oscillations are obligatorily linked to the integrity of the thapsigargin-sensitive intracellular Ca$^{2+}$ stores. Small step changes in extracellular Ca$^{2+}$ after the intracellular stores had been compromised by thapsigargin application did not result in the establishment of intracellular Ca$^{2+}$ oscillations but, rather, generated a monotonic increase in intracellular Ca$^{2+}$ consistent with influx of extracellular Ca$^{2+}$ (data not shown).

**Regulation of intracellular Ca$^{2+}$ oscillations.** Intracellular Ca$^{2+}$ oscillations are observed in many cell types, elicited by a wide variety of G protein-coupled receptors that activate the PI-PLC pathway, ultimately causing release of Ca$^{2+}$ from internal stores by activating intracellular IP$_3$ receptors, and often inducing influx of Ca$^{2+}$ from the extracellular medium (16). For CaR-activated oscillations, the data in Fig. 9 indicate an integral contribution of Ca$^{2+}$ cycling into and out of intracellular stores. A second widely observed mechanism regulating G protein-coupled receptor activation is cyclical phosphorylation-dephosphorylation, which has been shown to be responsible for mGluR5-mediated intracellular Ca$^{2+}$ oscillations in neurons (14, 22) and in heterologous expression systems (19). The effects of several protein kinase inhibitors were therefore tested for their ability to regulate CaR-mediated Ca$^{2+}$ oscillations. Figure 10 illustrates the results of typical experiments with staurosporine, a broad-specificity protein kinase inhibitor that was applied at a concentration (100 nM) that should inhibit protein kinases A (IC$_{50}$ ≈ 15 nM), C (IC$_{50}$ ≈ 7 nM), G (IC$_{50}$ ≈ 8.5 nM), myosin light chain kinase (IC$_{50}$ ≈ 1.3 nM), and calmodulin kinase (IC$_{50}$ ≈ 20 nM). In the control experiment, intracellular Ca$^{2+}$ oscillations were elicited by a step change in extracellular Ca$^{2+}$ from 2 to 3.5 mM and were sustained until a maximal response was elicited by 10 mM extracellular Ca$^{2+}$ at the end of the experiment (Fig. 10A). The companion experiment utilized the same 2.0–3.5 mM step in extracellular Ca$^{2+}$ to elicit oscillations, and then 100 nM staurosporine was applied during the oscillations (Fig. 10B). In this and all other experiments of similar protocol, there was no effect on oscillation frequency on addition of staurosporine, although, in some experiments, including that illustrated in Fig. 10B, there was a decrease in the maximum intracellular Ca$^{2+}$ response during oscillations. To determine whether the lack of effect of staurosporine on oscillation frequency was due to slow diffusion of the blocker into the cells, a second protocol was used to assess the effect(s) of the protein kinase inhibitor (Fig. 10C): oscillations were elicited by a step from 2 to 3.5 mM Ca$^{2+}$, followed by return to 2 mM Ca$^{2+}$, application of 100 nM staurosporine for 2 min, and then a second step to 3.5 mM Ca$^{2+}$ in the continued presence of staurosporine. As with the protocol of Fig. 10B, there was no effect of staurosporine on oscillation frequency despite the 2-min preexposure to the blocker. Experiments similar to that illustrated in Fig. 10B were also performed with 1 μM bisindolylmaleimide I and 5 μM KN-93, inhibitors of protein kinase C (IC$_{50}$ ≈ 8–200 nM, depending on isoform) and calmodulin kinase II (IC$_{50}$ ≈ 370 nM), respectively. Although the protein kinase inhibitors did induce a decrease in baseline intracellular Ca$^{2+}$ and the maximal peak Ca$^{2+}$ of the oscillations in some experiments, none of the inhibitors blocked the oscillations or affected the peak to peak interval of the oscillations (Fig. 10D). These results suggest that protein kinases are not required for initiation or maintenance of intracellular Ca$^{2+}$ oscillations elicited by CaR activation.

As a final test for the potential involvement of protein kinases in the establishment or regulation of CaR-mediated intracellular Ca$^{2+}$ oscillations, the ability of staurosporine to alter the threshold for CaR-mediated oscillations was determined. Experiments were performed as in Fig. 6C, with cells exposed to 3 mM extracellular Ca$^{2+}$ during fura 2-AM loading and experiment initiation, with 100 nM staurosporine present throughout the experiment. The percent of oscillating cells was determined over the range of ex-
tracellular Ca\textsuperscript{2+} from 3 to 5 mM. The results are illustrated in Fig. 10E, with the control data (absence of 100 nM staurosporine) redrawn from Fig. 6D. Staurosporine induced an increase in the number of oscillating cells at every extracellular Ca\textsuperscript{2+} concentration, although the oscillation frequency was unaffected (data not shown, but see Fig. 10, A–D). Protein kinase-dependent phosphorylation therefore contributes to setting/resetting the threshold for CaR-mediated intracellular Ca\textsuperscript{2+} oscillations.

**DISCUSSION**

This report delineates the complex intracellular Ca\textsuperscript{2+} responses resulting from activation of CaR by extracellular Ca\textsuperscript{2+}. Cells expressing CaR exhibit both modulation of resting intracellular Ca\textsuperscript{2+} concentrations at extracellular Ca\textsuperscript{2+} concentrations below the EC\textsubscript{50} for CaR activation and a decrease in net thapsigargin-sensitive Ca\textsuperscript{2+} stores. Alterations in the resting intracellular Ca\textsuperscript{2+} concentration may generate low-level activation of Ca\textsuperscript{2+}-sensitive signaling pathways, in particular those with a high affinity for Ca\textsuperscript{2+}. In addition, the low level of CaR activation under these conditions may “sensitize” cells expressing CaR to activation of other G protein-coupled receptors that also utilize the PI-PLC pathway for cellular signaling. This sensitizing effect has recently been seen in monocyes coactivated by extracellular Ca\textsuperscript{2+} and the chemokine MCP-1 (25).

Increasing extracellular Ca\textsuperscript{2+} by as little as 0.5 mM in the range around the EC\textsubscript{50} for CaR activation generates low-frequency (40- to 50-s peak to peak interval) intracellular Ca\textsuperscript{2+} oscillations. The oscillation interval is independent of the extracellular Ca\textsuperscript{2+} concentration, although the number of cells responding to the perturbation of extracellular Ca\textsuperscript{2+} peaks at ~1 mM above the ambient extracellular Ca\textsuperscript{2+}. Cells expressing CaR acclimate to the ambient concentration of extracellular Ca\textsuperscript{2+} with a cessation of oscillations after 30–40 min (Fig. 7). The threshold for initiation of intracellular Ca\textsuperscript{2+} oscillations is thus reset by the concentration of extracellular Ca\textsuperscript{2+} to which the cells are chronically exposed. This “resetting” of the threshold for initiation of oscillations is not due to alterations in the Ca\textsuperscript{2+} content of intracellular stores (Fig. 4). In parathyroid cells in vivo, increases in extracellular Ca\textsuperscript{2+} >1.8 mM induce oscillations with an interval of 42 s (21). Our results suggest that this oscillatory behavior (oscillation interval, threshold) can be conferred on a heterologous cell type by expression of CaR and therefore must be inherent in either CaR or highly conserved elements of the PI-PLC signaling pathway.

Many G protein-coupled receptors that activate the PI-PLC pathway generate intracellular Ca\textsuperscript{2+} oscillations over a limited agonist concentration range. Oscillation frequencies range from very rapid “pacemaker” activity (1 Hz) that signifies elemental Ca\textsuperscript{2+} release events (4) to intermediate (3–4 min\textsuperscript{-1}) (2), to slow (<1 min\textsuperscript{-1}) (12). Agonist concentration-dependent alterations in oscillation frequency have also been noted (10, 12, 23, 27, 28). While we observed relatively slow intracellular Ca\textsuperscript{2+} oscillations, there was no systematic increase in oscillation frequency over the limited range of extracellular Ca\textsuperscript{2+} (agonist) concentrations.
tested. It should be noted that CaR is unique in that its agonist is Ca\(^{2+}\); therefore, increases in extracellular Ca\(^{2+}\) mediate receptor activation and increase the driving force for Ca\(^{2+}\) influx through a variety of permeability pathways. This may limit the range of agonist concentrations that can be explored, since increasing extracellular Ca\(^{2+}\) from 2 to 5 mM is sufficient to induce a peak Ca\(^{2+}\) response, which decays to a sustained plateau, largely eliminating intracellular Ca\(^{2+}\) oscillations. Nevertheless, small increases in extracellular Ca\(^{2+}\) “recruit” cells to oscillate, and the net effect at the level of the parathyroid may be to increase the fraction of cells in which parathyroid hormone secretion is modulated.

A number of mechanisms have been proposed for initiation and maintenance of intracellular Ca\(^{2+}\) oscillations, most requiring cyclical alterations in the state of the Ca\(^{2+}\) release system (16), generally involving fluctuations in either the cellular levels of IP\(_3\) or the activity of IP\(_3\) receptors in the endoplasmic reticulum. Thapsigargin rapidly disrupts CaR-initiated intracellular Ca\(^{2+}\) oscillations, implying that intracellular stores are intimately involved in their initiation and maintenance. In systems in which the intracellular Ca\(^{2+}\) oscillations require plasma membrane level Ca\(^{2+}\) influx, thapsigargin has no effect (30). We cannot exclude a contribution of Ca\(^{2+}\) influx, since we cannot eliminate Ca\(^{2+}\) from the extracellular solution and maintain CaR activation. We attempted to address the question of Ca\(^{2+}\) influx during oscillations by utilizing Mn\(^{2+}\) uptake and quench (29), but found that this approach was not feasible since Mn\(^{2+}\) acted as an agonist for CaR in the range required for quench of fluorescence (data not shown). With respect to receptors having the closest homology with CaR, namely group I metabotropic glutamate receptors, mGluR5-induced intracellular Ca\(^{2+}\) oscillations have been noted both in vivo (14, 22) and in vitro (19) and result from the cyclical protein kinase C-mediated phosphorylation and dephosphorylation of the receptor itself, at a threonine residue within the carboxy terminus (19). Sequence alignment of CaR with mGluR5 suggests that the critical threonine of mGluR5 is present in CaR, although it is not part of a consensus sequence for protein kinase C phosphorylation in CaR (1, 8). Furthermore, we have generated truncations of the carboxy terminus in which all of the consensus sites for protein kinases A and C phosphorylation were removed (15). These truncated forms of human CaR were also observed to oscillate (data not shown).

In this report, we have tested the effects of a variety of protein kinase inhibitors, with a particular focus on those that are regulated by Ca\(^{2+}\). Neither staurosporine, bisindolylmaleimide I, nor KN-93 altered the frequency of oscillations elicited by extracellular Ca\(^{2+}\). Protein kinase C-mediated phosphorylation of CaR causes a reduction in Ca\(^{2+}\) affinity of the receptor (1, 8, 26). For the cholecystokinin receptor, a similar protein kinase C-mediated decrease in agonist affinity of the cholecystokinin S receptor is responsible for the cessation of intracellular Ca\(^{2+}\) oscillations (31). It is therefore possible that protein kinase C-mediated phosphorylation of CaR at low agonist concentrations is responsible for the shift in threshold for initiation of oscillations observed in the “Ca\(^{2+}\) pretreatment” protocol, since staurosporine, a broad-specificity protein kinase inhibitor, increased the number of cells oscillating at each extracellular Ca\(^{2+}\) concentration (Fig. 10E). Further work on protein kinase C-specific site mutants of CaR will be needed to explicitly address this possibility.

Parathyroid hormone secretion is inversely controlled by extracellular Ca\(^{2+}\), i.e., increases in extracellular Ca\(^{2+}\) result in decreases in parathyroid hormone secretion (32). Despite intensive efforts, a fundamental question with regard to CaR regulation of parathyroid hormone secretion remains the locus of the inversion signal, given the observation that CaR activation mediates an increase in intracellular Ca\(^{2+}\). A confocal study that followed subcellular changes in intracellular Ca\(^{2+}\) on a step change in extracellular Ca\(^{2+}\) from 0.5 to 2 mM noted significant time-dependent inhomogeneities in intracellular Ca\(^{2+}\) (9). In this study, we have shown that global intracellular Ca\(^{2+}\) also exhibits oscillations over this concentration range. It is possible that the global and local oscillatory changes in intracellular Ca\(^{2+}\) contribute to the inversion of the secretory response. At a minimum, any model for CaR-mediated regulation of parathyroid hormone secretion must incorporate intracellular Ca\(^{2+}\) oscillations.

CaR contributes to the regulation of diverse physiological functions, and there may be unique implications of CaR-mediated intracellular Ca\(^{2+}\) oscillations that depend on cell type. It is interesting to note, however, that intracellular Ca\(^{2+}\) oscillations have been recognized recently as an extremely potent and specific means of increasing gene expression (11, 17, 20). While direct contributions of CaR activation to changes in gene expression have not been explicitly determined in most systems, there is evidence in the Caco-2 intestinal epithelial cell line for rapid, CaR-mediated increases in c-myc expression (18). In addition, CaR activation potently mediates keratinocyte differentiation (3). Our results suggest that another important locus for CaR-mediated signaling that should be explored is the dynamic regulation of gene expression via intracellular Ca\(^{2+}\) oscillations.

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