The Ca²⁺-sensing Receptor Activates Cytosolic Phospholipase A₂ via a G₁α-dependent ERK-independent Pathway*

The Ca²⁺-sensing receptor (CaR) stimulates a number of phospholipase activities, but the specific phospholipases and the mechanisms by which the CaR activates them are not defined. We investigated regulation of phospholipase A₂ (PLA₂) by the Ca²⁺-sensing receptor (CaR) in human embryonic kidney 293 cells that express either the wild-type receptor or a nonfunctional mutant (R796W) CaR. The PLA₂ activity was attributable to cytosolic PLA₂ (cPLA₂) based on its inhibition by arachidonyl trifluoromethyl ketone, lack of inhibition by bromoeno lactone, and enhancement of the CaR-stimulated cPLA₂ activity by coexpression of a cDNA encoding the 85-kDa human cPLA₂. No CaR-stimulated cPLA₂ activity was found in the cells that expressed the mutant CaR. Pertussis toxin treatment had a minimal effect on CaR-stimulated arachidonic acid release and the CaR-stimulated rise in intracellular Ca²⁺ (Ca²⁺i), whereas inhibition of phospholipase C (PLC) with U73122 completely inhibited CaR-stimulated PLC and cPLA₂ activities. CaR-stimulated PLC activity was inhibited by expression of RGS4, an RGS (Regulator of G protein Signaling) protein that inhibits Gαi activity. CaR-stimulated cPLA₂ activity was inhibited 80% by chelation of extracellular Ca²⁺ and depletion of intracellular Ca²⁺ with EGTA and inhibited 90% by treatment with W7, a calmodulin inhibitor, or with KN-93, an inhibitor of Ca²⁺-calmodulin-dependent protein kinases. Chemical inhibitors of the ERK activator, MEK, and a dominant negative MEK, MEK97R, had no effect on CaR-stimulated cPLA₂ activity but inhibited CaR-stimulated ERK activity. These results demonstrate that the CaR activates cPLA₂ via a Gαq, PLC, Ca²⁺-CaM, and calmodulin-dependent protein kinase-dependent pathway that is independent the ERK pathway.

The extracellular Ca²⁺-sensing receptor (CaR) is a G protein-coupled receptor that is expressed in the parathyroid and kidney and senses extracellular Ca²⁺ in the millimolar range. This receptor acts through at least two G proteins, Gq and Gαq, to regulate multiple intracellular enzymes that control production of second messengers including cAMP, inositol trisphosphate (IP₃), diacylglycerol (DAG), intracellular Ca²⁺ (Ca²⁺i), and arachidonic acid (AA) metabolites (1). In the parathyroid, the CaR inhibits parathyroid hormone production and secretion in response to elevated extracellular Ca²⁺ (Ca²⁺i) levels, and in the kidney, activation of the CaR inhibits Na⁺KCl cotransporter activity, Ca²⁺ resorption, and the action of vasopressin leading to a Na⁺, Cl⁻, Ca²⁺, and H₂O diuresis. CaR-stimulated production of AA metabolites, possibly products of 12- and 15-lipoxygenase, contributes to inhibition of parathyroid hormone secretion in parathyroid cells (2–4). In cells from the thick ascending limb of Henle, activation of phospholipase A₂ (PLA₂) by the CaR results in the production of 20-hydroxyicosatetraenoic acid, a cytochrome P450 metabolite, that inhibits the apical 70-picosiemens potassium channel activity that would reduce Na⁺KCl transporter activity (5). In these tissues and others including the brain, pancreas, stomach, colon, and skin, the CaR may also sense Ca²⁺ extrusion by adjacent cells and function in cell-cell communication (6–8).

Phospholipase A₂, the rate-limiting enzyme in AA metabolism, hydrolyzes cellular phospholipids to form lysophospholipids that lead to the production of platelet-activating factor and liberation of polyunsaturated fatty acids including AA that are the precursors for prostaglandins, thromboxanes, leukotrienes, and a variety of other metabolites (eicosanoids) (1, 9). Eight different groups of PLA₂ enzyme have been described. The majority of these enzymes are extracellular and not hormonally regulated, whereas two groups, group IV and group VI, are intracellular and are subject to regulation by extracellular signals. The hormone-regulated enzymes are the 85-kDa Ca²⁺-sensitive cytosolic PLA₂ (cPLA₂, a group IV enzyme) and the 80–88-kDa Ca²⁺-insensitive PLA₂ (iPLA₂, a group VI enzyme). Both enzymes are subject to activation by G protein-dependent signaling systems (10, 11).

cPLA₂ is expressed in most tissues and is activated by many G protein-coupled receptors including those for angiotensin II, ATP, bradykinin, endothelin, lysophosphatidic acid, and thrombin (1). The AA products of cPLA₂ appear to be involved primarily in signaling functions. cPLA₂ activity is inhibited by the substrate analogue arachidonyl trifluoromethyl ketone (AACOCF₃) but not bromoeno lactone (BEL) (12). The precise mechanism of activation of cPLA₂ is variable and depends on the receptor. Activation of cPLA₂ requires a rise in Ca²⁺, which leads to translocation of the enzyme from the cytosol to the membranous fraction where it becomes active in hydrolyzing phospholipids that are present in the plasma membrane and in the membrane of the endoplasmic reticulum.

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‡ The abbreviations used are: CaR, extracellular Ca²⁺-sensing receptor; AACOCF₃, arachidonyl trifluoromethyl ketone; BEL, bromoeno lactone; Ca²⁺i, intracellular Ca²⁺; Ca²⁺o, extracellular Ca²⁺; CaM, calmodulin; CaMK, calcium, calmodulin-dependent protein kinase; ERK, extracellular signal-regulated kinase; HA, influenza hemagglutinin antigen; HEK-293 cells, human embryonic kidney 293 cells; IP₃, inositol 1,4,5 trisphosphate; LPS, lipopolysaccharide; MEK, mitogen-activated protein kinase kinase-ERK kinase; PLA₂, phospholipase A₂; cPLA₂, cytosolic PLA₂; PKC, protein kinase C; iPLA₂, Ca²⁺-insensitive PLA₂; PMA, phorbol myristate acetate; RGS protein, Regulator of G protein Signaling protein; bp, base pairs; DAG, diacylglycerol; MDCK, Madin–Darby canine kidney cells; BAPTA, 1,2-bis(2-aminoethoxy)ethane-N,N,N',N'-tetraacetic acid.

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13941
plasma membrane. In different cell types, pathways that involve pertussis toxin-sensitive or -insensitive G proteins, phospholipase C (PLC), protein kinase C (PKC), MAP kinases (extracellular signal-regulated kinases, ERK), calmodulin (CaM), and calmodulin-dependent protein kinase (CaMK) have been described (13). cPLA2 is a substrate for ERKs in many cell types, and phosphorylation of cPLA2 by ERK appears to be required for activation of the enzyme (14).

iPLA2 also appears to be ubiquitously expressed and is activated by receptors for ATP, α2 agonists, vasopressin, and Fas (15–17). The proposed functions of iPLA2 include cellular lipid remodeling, generation of substrates for leukotriene biosynthesis, and generation of second messengers that regulate ion channel activity (11, 18). iPLA2 activity is inhibited by both AAOCCF₃ and BEL (12). iPLA2 is activated by Ca²⁺ store depletion and PKC and is inactivated by association with calmodulin (16).

The form of PLₐα th at is activated by the CaR and the mechanism by which the CaR stimulates PLₐα activity have not been defined in any cell type. To determine which PLₐα, cPLA2 or iPLA2, is activated by the CaR and which protein, second messenger, and kinase pathway(5) are involved, we expressed the CaR in HEK-293 cells and defined the early components of the signaling pathway that lead to activation of PLₐα. We find that the CaR activates cPLA2 via a Gα₅, PLC, Ca²⁺/CaM, and CaMK-dependent but ERK-independent signaling pathway.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were purchased from the Sigma or Fisher unless specified otherwise. Fura-2-AM was obtained from Molecular Probes (Eugene, OR). PMA was supplied by LC Laboratories (Woburn, MA). Antibodies to myo-PLC were purchased from Biomol (Plymouth Meeting, PA). PD89059, U0124, W7, KN-93, and AAOCCF₃ were obtained from Calbiochem-Novabiochem. G418 sulfate was supplied by Life Technologies, Inc. The myo-[2-³H]inositol (10–25 Ci/mmol) and [5,6,8,9,11,12,14,15-³H]IAA (60–100 Ci/mmol) were purchased from NEN Life Sciences. SuperSignal West Pico chemiluminescent substrate was obtained from Pierce. AG1-X8 anion resin (200–400 mesh, formate form) was purchased from Bio-Rad.

Sources and Construction of cDNAs—The cDNAs encoding human wild-type and nonfunctional mutant CaR (R796W) in pcDNA3 were generous gifts from Drs. E. M. Brown, S. C. Hebert, and M. Bai in the Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, and Vanderbilt University School of Medicine. To add the HA epitope tag to the C terminus of CaR, we synthesized a 57-bp oligonucleotide coding for the last 5 amino acids of CaR, followed by 9 amino acids of the HA epitope, a stop codon, an Xbal restriction site, and a 3-bp tail (5’ GCC TCT AGA GTA AGC GTA GTC GAC GTC GTA TGG GTA ATT CAC TAC GAT TTC 3’). The cDNA was amplified by polymerase chain reaction using an oligonucleotide that is 5’ to a unique BamHI site in the C-terminal region of receptor (5’ ACC TTT ACC TGT CCC CTG AA 3’) and the 57-bp oligonucleotide coding for C terminus of receptor and the HA epitope. The polymerase chain reaction products were digested with BamHI and XbaI, purified, and ligated into the expression vector (pcDNA3) that contained the remain-
Inhibitor of iPLA2 (1, 12). The CaR was activated with neomycin, both ligands for the CaR, stimulated [3H]AA release (IC50 for neomycin was 0.15 mM. To determine which form of PLA2, cPLA2, and we measured [3H]AA release (19). Fig. 3A shows that RGS4, an RGS protein that interacts preferentially with members of the Goq family, accelerates their GTPase activity, and reduces their activation (20, 27). Fig. 4C shows that RGS4 eliminated CaR-stimulated PLC-β activity. These results indicate that the CaR acts through a pertussis toxin-insensitive, Goq-dependent pathway to activate PLC, the products of which stimulate cPLA2.

Role of Ca2+-PLC-β produces both DAG, which activates PKC, and IP3, which raises Ca2+ intracellular stores. A rise in Ca2+ could activate cPLA2 by mechanisms involving calmodulin, Ca2+-calmodulin-dependent protein kinase, Ca2+-dependent tyrosine kinases, PKC in cooperation with DAG, or the ERK pathway. To document and characterize the CaR-stimulated rise in Ca2+, in the cells that express the CaR, we measured Ca2+ in cells that express the wild-type and mutant CaR (CaR R796W) using Fura-2 fluorescence (Fig. 5). In the cells that express the wild-type CaR, activation of the CaR with 4 mM extracellular Ca2+ leads to a rapid rise in Ca2+, followed by a slow fall to a plateau level above base line. This type of Ca2+ signal has two components, release of Ca2+ from intracellular stores and Ca2+ entry across the plasma membrane. Subsequent stimulation of purinergic receptors in these cells with ATP (100 μM) also leads to a rapid rise in Ca2+, and a plateau phase. Cells that express the CaR R796W do not respond to 4 mM extracellular Ca2+ but do respond to ATP. Fig. 5B shows that the effect of pertussis toxin on the CaR-stimulated Ca2+ signal, like its effect on cPLA2 and PLC activity, is minimal. Incubation of the cells with EGTA to chelate Ca2+ and deplete Ca2+ (Fig. 5C) reduced CaR-stimulated [3H]AA release by 80% when the CaR was activated with neomycin, indicating that Ca2+ and a rise in Ca2+ are required for activation of cPLA2 by the CaR (28). In studies similar to those shown in Fig. 5, A and B, preincubation of cells with 2 mM EGTA for 20 min in nominally Ca2+-free medium prevented bradykinin and ATP-stimulated rises in

**RESULTS**

Expression of CaR in Mammalian Cells—To study regulation of PLA2 by the CaR, we expressed an HA-tagged wild-type and an HA-tagged nonfunctional mutant receptor (R796W) in HEK-293 cells (23). The expressed proteins were detected by immunoblotting with the monoclonal antibody 12CA5 that recognizes the HA tag on the receptors.

**Activation of cPLA2 by the CaR—Extracellular Ca2+ and neomycin, both ligands for the CaR, stimulated [3H]AA release from cells that express the CaR in a dose-dependent manner but not from cells that express the nonfunctional mutant CaR R796W (Fig. 2). The EC50 for Ca2+ was 4.2 μM, and the EC50 for neomycin was 0.15 μM. To determine which form of PLA2, cPLA2 or iPLA2, is activated by the CaR, we treated cells with A22516, an inhibitor of both enzymes, and BEL, a specific inhibitor of iPLA2 (1, 12). The CaR was activated with neomycin to avoid a possible increase in Ca2+-entry as a result in increased extracellular calcium. As shown in Fig. 3A, A22516 inhibited the CaR-stimulated [3H]AA release (IC50 25 μM), but BEL had no effect up to a concentration of 10 μM, a concentration that inhibits iPLA2 in other systems (26). In parallel experiments, 10 μM BEL inhibited LPS-stimulated [3H]AA release in RAW 264.7 cells, an iPLA2-dependent response, by 92% (26). To confirm that the CaR activates cPLA2, we transiently coexpressed cDNAs coding for the CaR and cPLA2, and we measured [3H]AA release (19). Fig. 3B shows that coexpression of increasing amounts of the cPLA2 cDNA with the CaR resulted in increased CaR-stimulated [3H]AA release without an increase in basal [3H]AA release. Inhibitor studies and increasing CaR-stimulated PLA2 activity with increasing levels of expression of cPLA2 demonstrate that the CaR activates cPLA2.

Role of PLC and G Proteins—We tested the role of PLC-β in activation of cPLA2 by first demonstrating inhibition of CaR-stimulated [3H]AA release by U73122, an inhibitor of PLC-β. Pretreatment of cells that express the CaR with U73122 eliminated the CaR-stimulated cPLA2 activity demonstrating that the CaR activates cPLA2 via PLC-β (Fig. 4A). To confirm that U73122 inhibits PLC-β activity, we measured CaR-stimulated IP3 production with and without U73122 (Fig. 4B), and we found that it was completely inhibited by U73122. PLC-β can be activated by pertussis toxin-sensitive (Goq-dependent) or pertussis toxin-insensitive (presumably Goq-dependent) signaling systems. Treatment of the cells with pertussis toxin had a minimal inhibitory effect on [3H]AA release (Fig. 4A), and IP3 production (Fig. 4B) indicating that G proteins of the Goq family have a minimal role in stimulation of cPLA2 and PLC-β by the CaR. To test specifically for a role for a Goq family member in the activation of PLC-β and consequently cPLA2, we transiently expressed the CaR in HEK-293 cells that stably express RGS4, an RGS protein that interacts preferentially with members of the Goq family, accelerates their GTPase activity, and reduces their activation (20, 27). Fig. 4C shows that RGS4 eliminated CaR-stimulated PLC-β activity. These results indicate that the CaR acts through a pertussis toxin-insensitive, Goq-dependent pathway to activate PLC, the products of which stimulate cPLA2.

**Ca2+-Sensing Receptor Activates Cytosolic PLA2**

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**Fig. 1. Stable and transient expression of the CaR in HEK-293 cells.** Membranes from cells that transiently (upper panel) or stably (lower panel) express the HA-tagged wild-type (WT) or mutant (CaR<sup>R796W</sup>, Mut) CaR or G418-resistant control cells (V) were immunoblotted with a monoclonal antibody 12CA5 that recognizes the HA tag on the receptors.

**Fig. 2. Concentration dependence of CaR-stimulated PLA2 activity for Ca2+ or neomycin.** HEK-293 cells that stably express the CaR were prelabeled with [3H]AA for 6 h and treated with Ca2+ (0.5–10.0 mM, closed circles) or neomycin (0–2.0 mM, closed squares) at 37°C for 15 min. Cells that stably express the nonfunctional CaR, CaR<sup>R796W</sup>, were treated with Ca2+ (open circles) or neomycin (open squares). [3H]AA release was normalized for [3H]AA incorporated into total cellular lipids. The EC<sub>50</sub> for neomycin was 0.15 mM and the EC<sub>50</sub> for Ca2+ was 4.2 mM.
Ca\(^{2+}\) (data not shown).

Role of ERK—Many receptors act through the ERK pathway to activate cPLA\(_2\) (14). We assessed the role of ERK in the activation of cPLA\(_2\) using chemical inhibitors of MEK, the activator of the ERKs, and a dominant negative form of MEK (MEK\(^{D\Delta 796}\)). Preincubation of the cells with either PD98059 or U0126 had no effect on the ability of the CaR to activate cPLA\(_2\) (Fig. 6A) but inhibited activation of the ERKs by the CaR (Fig. 6B). Treatment of the cells with the inactive U0126 analogue, U0124, did not affect cPLA\(_2\) or ERK activation. Similarly, coexpression of the CaR with the dominant negative MEK, MEK\(^{D\Delta 796}\), and HA-tagged ERK-1 did not inhibit activation of cPLA\(_2\) (Fig. 6C) but resulted in inhibition of ERK activation by the CaR (Fig. 6D). These results indicate that activation of the ERK pathway and presumably phosphorylation of cPLA\(_2\) by p42/44 ERK is not required for activation of cPLA\(_2\) by the CaR.

Role of PKC—The PLC product, DAG, activates PKC in a Ca\(^{2+}\)-dependent manner. To test for a role of the conventional forms of PKC (those that are both DAG- and Ca\(^{2+}\)-dependent), we treated cells with calphostin, a PKC inhibitor, and downregulated PKC by overnight treatment of the cells with 100 nM PMA and then measured CaR-stimulated cPLA\(_2\) activity. As shown in Fig. 7, both calphostin and down-regulation of PKC with PMA pretreatment reduced CaR-stimulated cPLA\(_2\) activity by \(-50\%\) but did not eliminate it. In control experiments, 15 min of exposure of the HEK-293 cells that express the mutant (R796W) CaR to 100 nM PMA prevented a significant stimulation of \(^{3}\)H-AA release to a similar degree as treatment of the cells that were pretreated with 100 ng/ml pertussis toxin (PTx) 100 ng/ml for the 12–15 h before experiments or U73122 for the 30 min before experiments. Cells were treated with 0.5 mM Ca\(^{2+}\) or 5.0 mM Ca\(^{2+}\) for 5 min, and \(^{3}\)HIP\(_3\) production was measured by column chromatography. C, effect of RGS4 on CaR-stimulated PLC activity. HEK-293 cells that stably express RGS4 or G418-resistant control cells (V) were transiently transfected with the CaR (wild-type). Twelve hours after transfection, they were prelabeled with \(^{3}\)Hinositol for 48 h and exposed to 0.5 or 5.0 mM Ca\(^{2+}\) for 5 min, and \(^{3}\)HIP\(_3\) production was separated by column chromatography. Each bar represents the mean of triplicate samples \pm S.D., and this figure is representative of three experiments.

Role of CaM and CaMK—Another mechanism by which a rise in Ca\(^{2+}\), could activate cPLA\(_2\) is via calmodulin (13). To
Ca^2+ Sensing Receptor Activates Cytosolic PLA2

Although different G protein-coupled receptors may act by similar mechanisms such as activation of PLC, raising Ca^{2+}, and activation of PKC, each receptor has unique properties that include its location in the cell, its cycling and processing by the cell, and the set of proteins with which it interacts. These unique characteristics allow it to have discrete functions such as activation of specific effector enzymes. For example, in MDCK cells, \( \alpha_j \)-adrenergic and P2U receptors activate protein kinase C and MAP kinases but activate cPLA2 by different mechanisms (31, 32). Similarly, although the CaR activates PLC, increases Ca^{2+}, and activates PKC and MAP kinases, it activates cPLA2 by mechanisms that are particular to the CaR. To characterize the mechanism by which the CaR activates PLA2 and determine which type of PLA2 was activated by it, we studied activation of PLA2 in HEK-293 cells that stably express the CaR or a nonfunctional mutant form of the receptor, CaR R796W, to control for nonspecific effects of the receptor ligands. We determined that the CaR activated cPLA2 via a novel pathway that involves Goq, PLC, Ca^{2+}, calmodulin, and CaMK. In contrast to many other receptors, activation of the ERKs was not required for CaR-dependent activation of cPLA2.

Both cPLA2 and iPLA2 could be activated by G protein-dependent signaling systems, and both enzymes could be activated simultaneously (33). To identify the type of PLA2 that is activated by the CaR, we tested the ability of AACOCF3, an arachidonic acid analogue that inhibits both cPLA2 and iPLA2, and BEL, a "suicide substrate" that is specific for iPLA2 to inhibit CaR-stimulated [3H]AA release from our cells (1). We found that AACOCF3 inhibited all of the CaR-stimulated activity with an IC_{50} of 25 \( \mu \)M, which is comparable to its IC_{50} in other systems (12). BEL, used at a concentration that was considerably higher than that required to inhibit completely iPLA2 in other cell types (10 \( \mu \)M), had no effect on CaR-stimulated [3H]AA release and inhibited LPS-stimulated [3H]AA release from RAW 264.7 cells (12, 34). Additionally, we expressed human cPLA2 with the CaR and demonstrated that CaR-stimulated [3H]AA release increased in parallel with increasing amounts of expressed cPLA2. These results indicate that in our experimental system, CaR-stimulated [3H]AA release is a result of increased cPLA2 activity.

Activation of cPLA2 by the CaR requires stimulation of PLC activity which could occur through members of the Goq family and/or Goq families. Pretreatment of cells with pertussis toxin had a minimal effect on the ability of the CaR to stimulate PLC and consequently cPLA2, through members of the Goq family (10, 13). Pretreatment with W-7, a calmodulin antagonist, and inhibition of its activation of G protein-coupled receptors, the CaR stimulates cPLA2 via a CaM/CaMK-dependent pathway that is independent of the ERK pathway.

**DISCUSSION**

**FIG. 5.** 
**A.** cells expressing either the wild-type or mutant (R796W) CaR were suspended and loaded with Fura-2-AM. The cells were washed and resuspended in medium containing 1.0 mM Ca^{2+}, and then stimulated with Ca^{2+} (4.0 mM). When the tracings returned to base line, ATP (100 \( \mu \)M) was added. **B.** effect of pertussis toxin on the CaR-stimulated Ca^{2+} signal. Cells expressing the CaR were treated with pertussis toxin (PTx, 100 ng/ml) for 12–15 h before the experiments. The CaR response to activation of the CaR was studied as described above. **C.** effect of removal of Ca^{2+}, and Ca^{2+} depletion on CaR-stimulated cPLA2 activity. Cells grown in 24-well trays were washed in buffer and treated with EGTA (0–10 mM) for 30 min before addition of neomycin (500 \( \mu \)M). [3H]AA release was measured over 15 min as described above. The study shown is representative of three similar experiments.

Test for a role for calmodulin in CaR-dependent activation of cPLA2, we pretreated cells with W-7, a calmodulin antagonist that competitively inhibits interaction of Ca^{2+}-calmodulin with its target proteins. Pretreatment of the cells with W-7 for 45 min before activation of the CaR eliminated ~90% of the CaR-stimulated cPLA2 activity (IC_{50}, 7 \( \mu \)M), indicating that activation of calmodulin is an essential step in activation of cPLA2 by the CaR (Fig. 5A). The effects of calmodulin could be mediated by Ca^{2+}, calmodulin-dependent protein kinase (CaMK), or other proteins. To test for a role for CaMK, we pretreated cells with KN-93, a specific inhibitor of the CaMK enzymes at the concentrations used (29, 30). We found that over a concentration range up to 25 \( \mu \)M, KN-93 inhibited ~90% of the CaR-stimulated cPLA2 activity with an IC_{50} of ~6.3 \( \mu \)M (Fig. 5B). These results demonstrate that in contrast to other G protein-coupled receptors, the CaR stimulates cPLA2 activity via a CaM/CaMK-dependent pathway that is independent of the ERK pathway.
buffer intracellular Ca\(^{2+}\) with BAPTA but surprisingly found that BAPTA treatment resulted in a slight increase in [\(^{3}\text{H}\)]AA release. This result can be rationalized by the recent finding that BAPTA may lower Ca\(^{2+}\) to the point that Ca\(^{2+}\) influx is stimulated (35, 36). However, by adding EGTA to the medium for 30 min, we were able to inhibit Ca-stimulated cPLA\(_2\) activity by ~80%, demonstrating dependence on intracellular [Ca\(^{2+}\)]. Treatment of the cells for this period with 5–10 mM EGTA presum-ably also reduced, but did not completely deplete, the Ca\(^{2+}\) in the intracellular stores so that the CaR-stimulated rise in Ca\(^{2+}\) due to release of the intracellular stores was reduced. Consequently, we cannot determine whether the 20% of CaR-stimulated cPLA\(_2\) activity that remained was due to incomplete inhibition of the Ca\(^{2+}\) signal or a Ca\(^{2+}\)-independent mechanism.

In many cell types, cPLA\(_2\) is activated by a p42/44 ERK-dependent pathway. Phosphorylation of cPLA\(_2\) by ERK in vitro at Ser-505 results in a change in its mobility in gels (retardation) and increased activity, whereas phosphorylation by PKA or PKC does not result in a mobility shift or increased activity. Similarly, in studies of cPLA\(_2\) from intact cells, p42/44 ERK reduces the mobility of cPLA\(_2\) in gels and increases its activity. Mutation of Ser-505 results in an enzyme that is not activated by and that does not undergo a mobility shift in response to PKC or CaMK (13, 14, 31, 32, 37).

However, some receptors may activate cPLA\(_2\) by mechanisms that are independent of, or only partially dependent on, the ERK pathway (32, 38, 39). P\(_{2X}\) receptors in MDCK-D\(_1\) cells activate cPLA\(_2\) by PKC- and ERK-dependent pathways (32). In MDCK cells, bradykinin stimulates cPLA\(_2\) activity by a mechanism that is independent of both PKC\(_\alpha\) and ERK but that is dependent on tyrosine phosphorylation (39). In renal proximal tubule cells and breast carcinoma cells, ERK can be activated by PLA\(_2\)-dependent AA metabolites rather than ERK-activating PLA\(_2\), reversing the conventional relationship (40, 41).

Our results indicate that the CaR activates cPLA\(_2\) by a pathway that is independent of ERK activation. Inhibition of CaR stimulated ERK activation by three methods; the two
cPLA2 activity at 25 μM KN-93. The IC50 of KN-93 for purified CaMKII is 370 nM, and it is specific up to ~30 μM (30). In PC12 cells with a 3-day incubation, the IC50 value for KN-93-dependent inhibition of tyrosine hydroxylase activity was ~2 μM, and pretreatment of KCl or acetyl choline-stimulated intact PC12 cells with 10 μM KN-93 for 1 h resulted in a 60–80% inhibition of tyrosine hydroxylase activity (30). Consequently, although we cannot exclude the possibility that some other CaM-dependent process participates in activation of cPLA2 by the CaR, we think that it is most likely that the CaR acts through CaM to activate one of the isoforms of CaMK which then activates cPLA2. At this point, we cannot determine which CaMK isoform is involved or if CaMK acts via a direct or indirect mechanism.

Our studies demonstrate that the CaR activates cPLA2 by a novel pathway in which Ca2+, CaM, and CaMK are of principal importance, and ERKs are not involved. CaM is involved in activation of cPLA2 by other G protein-coupled receptors, but CaMK activates the ERKs which then activate cPLA2. Clearly, receptor-dependent mechanisms that do not involve the ERKs can activate cPLA2. The different mechanisms used by various receptors to activate cPLA2 may reflect selective cellular localization of signaling proteins with a particular receptor.

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Ca\textsuperscript{2+}-Sensing Receptor Activates Cytosolic PLA\textsubscript{2}