The Calcitonin Receptor Stimulates Shc Tyrosine Phosphorylation and Erk1/2 Activation

IN Volvement of Gs, Protein Kinase C, and Calcium*

(Received for publication, January 12, 1998, and in revised form, May 18, 1998)

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While it is well established that adenylyl cyclase and phospholipase C-β are two proximal signal effectors for the calcitonin receptor, the more distal signaling pathways are less well characterized. G protein-coupled receptors can activate Erk1/2 by Gs-, Gi-, or Gq-dependent mechanisms, and Erk1/2 can couple to all three of these G proteins, the ability of calcitonin to activate Erk1/2 was investigated. Calcitonin induced time- and concentration-dependent increases in Shc tyrosine phosphorylation, Shc-Grb2 association, and Erk1/2 phosphorylation. Our results suggest that the calcitonin receptor induces Shc phosphorylation and Erk1/2 activation in a HEK 293 cell line that stably expresses the rabbit calcitonin receptor C1a isoform. Pertussis toxin, which inactivates Gs, and calphostin C, a protein kinase C inhibitor, each partially inhibited calcitonin-induced Shc tyrosine phosphorylation, Shc-Grb2 association, and Erk1/2 phosphorylation. In contrast, neither forskolin nor H89, a protein kinase A inhibitor, had a significant effect on basal or calcitonin-stimulated Erk1/2 phosphorylation. Our results suggest that the calcitonin receptor induces Shc phosphorylation and Erk1/2 activation in HEK293 cells by parallel Gs- and PKC-dependent mechanisms. The calcitonin-induced elevation of cytosolic free Ca²⁺ was required for Erk1/2 phosphorylation, since preventing any change in cytosolic free Ca²⁺ by chelating both cytosolic and extracellular Ca²⁺ abolished the response. However, the change in Ca²⁺ that is induced by calcitonin is not sufficient to account for the calcitonin-induced Erk1/2 phosphorylation, since treatment with 100 nM ionomycin or 10 µM thapsigargin, each of which induced elevations of Ca²⁺ comparable to those induced by calcitonin, induced significantly less Erk1/2 phosphorylation than that induced by calcitonin. Erk1/2 may have important roles as downstream effectors mediating cellular responses to calcitonin stimulation.

Calcitonin (CT)³ is a polypeptide hormone that regulates calcium homeostasis by inhibiting osteoclastic bone resorption and enhancing renal calcium excretion (1). High affinity CT binding has also been demonstrated in other tissues, including the central nervous system, pituitary gland, and gastrointestinal tract. Because of its marked hypocalcemic effect, CT has been widely used for treating diseases that are characterized by elevated osteoclast activity, such as hypercalcemia of malignancy, osteoporosis, and Paget’s disease (1). The CT receptor (CTR) couples to multiple heterotrimeric G proteins, leading to the activation of the proximal effectors adenylyl cyclase and phospholipase Cβ (2–4). Recently, the CTR-dependent activation of phospholipase D has also been reported (5). CT-induced activation of adenylyl cyclase and phospholipase Cβ appears to elicit different components of a cell’s response to CT. For example, in isolated osteoclasts, cAMP-dependent mechanisms lead to reduced cell motility while protein kinase C-dependent events are apparently involved in the CT-induced retraction of the cell (6, 7). In contrast to the well-documented activation of adenylyl cyclase and phospholipase Cβ following stimulation of the CTR, less progress has been made in characterizing the more distal signaling pathways elicited by CT (8).

Mitogen-activated protein kinases (MAPKs) are a group of serine/threonine protein kinases, including Erk1/2, JNK, and SAPK, that lie at the end of parallel protein kinase cascades. MAPKs are activated in response to the stimulation of several distinct classes of cell-surface receptors, including receptor tyrosine kinases and G protein-coupled receptors (GPCRs), and play important roles in integrating the effects of extracellular signals on multiple cellular functions, including differentiation, proliferation, and transformation (9, 10). Erk1/2 (also denoted as p42/44 MAPK) were the first to be identified and are the best characterized. Their activation by several GPCR ligands has recently been well documented (11). Depending on the receptors and the cell types, several distinct signal transduction pathways leading from GPCRs to Erk1/2 have been demonstrated. The pertussis toxin-sensitive Gq-coupled receptors G2A-

* This work was supported in part by National Institutes of Health Grant DE-04724 to R. B.) and Medical Research Council of Canada (MRC) Grant MT-10854 (to S. J. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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³ The abbreviations used are: CT, calcitonin; AR, adrenergic receptor; BPAT-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′′-tetraacetic acid tetra(acetoxymethyl) ester; [Ca²⁺]ᵢ, cytosolic free calcium concentration; CTR, calcitonin receptor; DME, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; GPCR, G protein-coupled receptor; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PTX, pertussis toxin; PMA, phorbol 12-myristate 13-acetate; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein.
adrenergic receptor (AR) and M2A-muscarinic acetylcholine receptor activate Erk1/2 by a mechanism that involves the sequential tyrosine phosphorylation of the adaptor protein Shc, association of phosphorylated Shc with the Grb2-moSOS complex, and activation of Ras (12). The same pathway is used by the receptors for EGF and other growth factors. The Gs-coupled receptors α1AR and M1A-muscarinic acetylcholine receptor can activate Erk1/2 via a PKC-dependent but Ras-independent pathway in some cells (12) and via a Ras-dependent pathway in others (13, 14). cAMP, which antagonizes Erk1/2 activation in some cells (15–18), activates Erk1/2 in PC12 cells (19) and in COS-7 cells (20).

Many GPCRs couple to more than one type of heterotrimeric G protein. For example, the thrombin and lysophosphatidic acid (LPA) receptors couple to Gs, Gq, and the thyrotropin receptor couples to Gs, Gi, and Gq (25). Interestingly, the LPA receptor-dependent activation of Erk1/2 is virtually abolished by pertussis toxin, indicating that activation is primarily via Gi, with little contribution from Gq or G12/13 (24). The PTH/PTHrP receptor also couples to at least two different types of heterotrimeric G proteins, Gi and Gq (25).

Recent reports indicate that the PTH/PTHrP receptor can regulate Erk1/2 activity by different pathways, with the specific mechanism and resulting effect on Erk1/2 activity depending on the cellular context (15, 26). These observations suggest that the mechanisms by which individual receptors regulate Erk1/2 activity are highly receptor- and cell type-specific.

Since the CTR is capable of coupling to Gs, Gi, and Go, we examined the ability of CT to regulate Erk1/2 activity. We report here that calcitonin induced Shc tyrosine phosphorylation, Shc-Grb2 association, and Erk1/2 activation in HeK 293 cells that stably express the rabbit osteoclast CTR C1a isoform. Additive effects of pertussis toxin and PKC inhibition suggest that both Gs-dependent and pertussis toxin-insensitive PKC-dependent mechanisms contribute to the CTR-induced activation of Erk1/2. Erk1/2 are therefore downstream effectors of the CTR which may mediate certain cellular responses to CT.

**EXPERIMENTAL PROCEDURES**

**Materials**—Salmon calcitonin was purchased from Peninsula Laboratories, Inc. (Belmont, CA). Antibodies against total Erk1/2 and phosphorylated Erk1/2 were purchased from New England Biolabs, Inc. (Beverly, MA). The polyclonal antibody used for Shc immunoprecipitation, the monoclonal anti-Shc antibody plus 50 μg/ml of monoclonal anti-Shc antibody (1:1000 dilution) to detect the presence of co-precipitated Grb2. The CTR which may mediate certain cellular responses to CT.

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Calcitonin Induces Transient and Concentration-dependent Activation of Erk1/2—To examine the effect of CT on Erk1/2 phosphorylation, C1a-HEK cells were treated with 1 nM CT for increasing periods of time, then lysed and analyzed for phosphorylated Erk1/2 as described under “Experimental Procedures.” CT induced rapid phosphorylation of Erk1/2, which was apparent as early as 30 s, reached a maximum at 5 min, and decreased to a low level by 60 min (Fig. 1A). Plateau phases of the responses were quantified as the value of [Ca\(^{2+}\)] at 485 nm measured at low and saturating concentrations of Ca\(^{2+}\), respectively, and \(\beta\) was the ratio of the fluorescence at 485 nm measured at low and saturating Ca\(^{2+}\) concentrations (29).

Peak Ca\(^{2+}\) responses were quantified as the maximum elevation of [Ca\(^{2+}\)], above basal levels induced by the test substance. Plateau phases of coupling to Gs, Gi, and Gq, we investigated which pathways are involved in CT-induced Erk1/2 activation. Although cAMP generally attenuates growth factor-induced Erk1/2 activation (16–18), the activation of Erk1/2 by cAMP has been reported (19, 20). We therefore examined whether cAMP participated in CT-induced Erk1/2 activation (Fig. 3). Serum-starved C1a-HEK cells were treated with 200 \(µ\)M forskolin for 25 min alone or followed by 1 nM CT for an additional 5 min. Forskolin induced only a slight increase in Erk1/2 phosphorylation (lane 3) and had no apparent effect on CT-induced Erk1/2 phosphorylation (lane 4). Furthermore, the protein kinase A-specific inhibitor H89 (10 \(µ\)M) had little effect on CT-stimulated Erk1/2 phosphorylation (lane 6). Taken together, these data suggest that the cAMP/PKA pathway does not contribute significantly to CT-induced Erk1/2 activation in HEK-293 cells.

The phosphorylation and activation of Erk1/2 is catalyzed by ERK kinases 1 and 2 (MEK1/2) (9). To further confirm that Erk1/2 phosphorylation correlates with activity, we examined the effect of the MEK-specific inhibitor PD 98059 on Erk activity and phosphorylation. As expected, pretreatment of the cells with 50 \(µ\)M PD 98059 for 30 min almost completely abolished both the phosphorylation (Fig. 2A) and activation (Fig. 2B) of Erk1/2 induced by CT or by EGF.

The Cyclic AMP-Protein Kinase A Pathway Has Little Effect on Erk1/2 Phosphorylation—GPCR may regulate Erk1/2 activity via one or more of several different pathways involving G\(_q\)-, G\(_i\)-, and G\(_s\)-dependent mechanisms, depending on the receptors and the cellular context (11). Since the CTR is capable of coupling to G\(_o\), G\(_i\), and G\(_s\), we investigated which pathways are involved in CT-induced Erk1/2 activation. Although cAMP generally attenuates growth factor-induced Erk1/2 activation (16–18), the activation of Erk1/2 by cAMP has been reported (19, 20). We therefore examined whether cAMP participated in CT-induced Erk1/2 activation (Fig. 3). Serum-starved C1a-HEK cells were treated with 200 \(µ\)M forskolin for 25 min alone or followed by 1 nM CT for an additional 5 min. Forskolin induced only a slight increase in Erk1/2 phosphorylation (lane 3) and had no apparent effect on CT-induced Erk1/2 phosphorylation (lane 4). Furthermore, the protein kinase A-specific inhibitor H89 (10 \(µ\)M) had little effect on CT-stimulated Erk1/2 phosphorylation (lane 6). Taken together, these data suggest that the cAMP/PKA pathway does not contribute significantly to CT-induced Erk1/2 activation in HEK-293 cells.

**RESULTS**

Calcitonin induces transient and concentration-dependent Erk1/2 phosphorylation. Subconfluent C1a-HEK cells were incubated in DMEM containing 0.5% serum for 18 h and subsequently treated (A) with 1 nM CT for the indicated times or (B) with the indicated concentrations of CT or 10 ng/ml EGF for 5 min. Cells were lysed in modified RIPA and 20 \(µ\)g of total cell lysates were processed as described under “Experimental Procedures” for immunoblotting with anti-P-Erk antibody to determine the phosphorylation state of Erk1/2 (upper panel) or anti-Erk antibody to determine the amount of Erk1/2 in the samples (lower panel).

FIG. 1. Calcitonin induces transient and concentration-dependent Erk1/2 phosphorylation. Subconfluent C1a-HEK cells were incubated in DMEM containing 0.5% serum for 18 h and subsequently treated (A) with 1 nM CT for the indicated times or (B) with the indicated concentrations of CT or 10 ng/ml EGF for 5 min. Cells were lysed in modified RIPA and 20 \(µ\)g of total cell lysates were processed as described under “Experimental Procedures.” The enzymatic activity was determined from the relationship \([\text{Ca}^{2+}] = K_d (R - R_{\text{max}})/(R_{\text{max}} - R)\), where \(K_d\) (the dissociation constant for the Indo-1-Ca\(^{2+}\) complex) was 250 nM, \(R_{\text{max}}\) and \(R_{\text{max}}\) were the values of \(R\) at low and saturating concentrations of Ca\(^{2+}\), respectively, and \(\beta\) was the ratio of the fluorescence at 485 nm measured at low and saturating Ca\(^{2+}\) concentrations (29).

Peak Ca\(^{2+}\) responses were quantified as the maximum elevation of \([\text{Ca}^{2+}]\), above basal levels induced by the test substance. Plateau phases of coupling to Gs, Gi, and Gq, we investigated which pathways are involved in CT-induced Erk1/2 activation. Although cAMP generally attenuates growth factor-induced Erk1/2 activation (16–18), the activation of Erk1/2 by cAMP has been reported (19, 20). We therefore examined whether cAMP participated in CT-induced Erk1/2 activation (Fig. 3). Serum-starved C1a-HEK cells were treated with 200 \(µ\)M forskolin for 25 min alone or followed by 1 nM CT for an additional 5 min. Forskolin induced only a slight increase in Erk1/2 phosphorylation (lane 3) and had no apparent effect on CT-induced Erk1/2 phosphorylation (lane 4). Furthermore, the protein kinase A-specific inhibitor H89 (10 \(µ\)M) had little effect on CT-stimulated Erk1/2 phosphorylation (lane 6). Taken together, these data suggest that the cAMP/PKA pathway does not contribute significantly to CT-induced Erk1/2 activation in HEK-293 cells.

Both G\(_{i}\) and PKC Mediate CT-induced Shc Tyrosine Phosphorylation and Erk1/2 Phosphorylation—In HEK 293 cells, both PTX-sensitive G\(_i\)-coupled receptors and PTX-insensitive G\(_q\)-coupled receptors can activate Erk1/2 via the Shc/Grb2/Ras pathway (13) by which receptor tyrosine kinases such as the EGF receptor activate Erk1/2 (30). To determine whether Shc also plays a role in the mechanism of CT-induced Erk1/2 activation, the tyrosine phosphorylation of Shc and its association...
with Grb2 were analyzed. C1a-HEK cells were treated with 1 nM CT for various times and lysed as described under "Experimental Procedures." Shc was immunoprecipitated from 1.5 mg of cell lysates and the immunoprecipitated proteins were analyzed for Shc phosphorylation and Grb2 association as described under "Experimental Procedures." As shown in Fig. 4A, 1 nM CT induced the tyrosine phosphorylation of predominantly the 52-kDa Shc, which reached a maximum level at 5–10 min and remained elevated during the time course examined for up to 120 min. The broad band below 52-kDa Shc was a nonspecific interaction of the immunoprecipitation antibody with the immunoblotting antibodies, since it was present in mock immunoprecipitations performed in the absence of cell lysate (not shown). The amount of immunoprecipitated Shc is shown in the middle panel of Fig. 4A. The induction of Shc tyrosine phosphorylation was also concentration-dependent, with the maximum effect induced by CT concentrations ≥ 1 nM (Fig. 4B). Co-immunoprecipitated Grb2 was readily detectable in the samples in which Shc was phosphorylated. As expected, EGF also induced Shc tyrosine phosphorylation and its association with Grb2 (Fig. 4B), although to a much greater extent than did CT.

Since the CTR can couple to both Gi and Gq, we examined the relative roles of PTX-sensitive and -insensitive Gi proteins in the CT-induced Shc tyrosine phosphorylation and Erk1/2 phosphorylation. C1a-HEK cells were pretreated with 200 ng/ml PTX for 18 h prior to stimulation with 1 nM CT or 10 μM LPA. LPA was used as a positive control, since it was reported to activate Erk1/2 via Gi, coupling (31). Total cell lysates were analyzed for Shc tyrosine phosphorylation, Shc-Grb2 association, and Erk1/2 phosphorylation. As shown in Fig. 5A, PTX partially inhibited CT- and LPA-induced Shc tyrosine phosphorylation and Shc-Grb2 association. PTX completely abolished LPA-stimulated Erk1/2 phosphorylation but only partially inhibited the phosphorylation induced by CT (Fig. 5B).

A peptide derived from the carboxyl terminus of the β-adrenergic receptor kinase 1 (βARK1ct) has been reported to block signaling mediated by the βy subunits of the Gi proteins, including the activation of Erk1/2 (13). To demonstrate further that the CTR-mediated Erk1/2 phosphorylation involves Gi coupling, HEK 293 cells were transiently transfected with CTR-C1a and pβARK1ct or CTR-C1a and pβARK1ct. As shown in Fig. 5C, pβARK1ct reduced the CT-induced Erk1/2 phosphorylation relative to the amount seen in cells transfected with the empty pβARK5 vector, indicating the involvement of βy subunits and supporting the involvement of Gi in the CTR-Erk1/2 coupling mechanism.

Since CT stimulates PKC activity in C1a-HEK cells, we sought to determine the involvement of PKC in the process of CT-dependent Erk1/2 phosphorylation. Subconfluent C1a-HEK cells were incubated overnight with or without PTX, then treated with the PKC-specific inhibitor calphostin C for 30 min prior to the addition of 1 nM CT. The cells were harvested and analyzed for Shc phosphorylation and Shc-Grb2 complex formation (Fig. 6A) and for Erk1/2 phosphorylation (Fig. 6B). Like PTX, calphostin C only partially blocked the CT-induced Shc phosphorylation and Erk1/2 phosphorylation, while the Shc tyrosine phosphorylation and Erk1/2 phosphorylation induced by 100 nM PMA were largely inhibited by the same concentration of calphostin C. In addition, the effects of PTX and calphostin C on Shc and Erk1/2 phosphorylation were additive. The results suggest that signal transduction from CTR to Shc and Erk1/2 involves both Gi-dependent and PKC-dependent mechanisms.

CT-induced Increase in Cytosolic Calcium Is Required for Erk1/2 Phosphorylation—A recent report suggested that the increase in cytosolic Ca2+ is one of the key mediators of both Gi- and Gq-coupled adrenergic receptor-stimulated Erk1/2 phosphorylation in HEK 293 cells (13). On the other hand, the PTH/PTHR receptor (structurally related to CTR) activated Erk1/2 via a Ca2+-independent mechanism in Chinese hamster ovary cells (26). Since CT increases [Ca2+]i (32), we investigated the role of [Ca2+]i in CT-induced Erk1/2 phosphorylation. [Ca2+]i levels were manipulated using EGTA and BAPTA to chelate extracellular and intracellular Ca2+, respectively, the calcium ionophore ionomycin to induce the elevation of [Ca2+]i, and thapsigargin to deplete the intracellular Ca2+ stores. Changes in [Ca2+]i were monitored by fluorescence spectroscopy as described under "Experimental Procedures."

Consistent with earlier reports (32, 33), 1 nM CT induced a biphasic elevation of [Ca2+]i, which rose within 30 s from basal levels of 134 ± 19 nM to peaks of 319 ± 37 nM above basal levels, followed by a sustained plateau of 57 ± 5 nM above basal levels (n = 7, Fig. 7B). The earlier reports showed that the initial transient results from the release of Ca2+ from intracellular stores and the sustained phase results from the influx of
extracellular Ca$^{2+}$ (33). No change in fluorescence was detected when C1a-HEK cells were treated with vehicle alone (Fig. 7A). When cells were loaded with BAPTA and then suspended in nominally Ca$^{2+}$-free Na$^+$ buffer, the Ca$^{2+}$ response to CT was virtually abolished (Fig. 7E). Similarly, pretreatment of cells with 5 mM EDTA for 30 min markedly decreased the amplitude of both the peak and plateau phases of the [Ca$^{2+}$] elevation induced by CT (Fig. 7F). These treatments abolished the CT-induced Erk1/2 phosphorylation (Fig. 9A, lanes 5 and 6), indicating that an increase in [Ca$^{2+}$]$_i$, is required for the CT-mediated response. In contrast, preincubation of the cells with EDTA for 30 min had little effect on EGF-induced Erk1/2 phosphorylation (Fig. 9A, lanes 7 and 8).

We next examined the relative contributions of the initial Ca$^{2+}$ transient and the sustained Ca$^{2+}$ plateau to CT-stimulated Erk1/2 phosphorylation. Incubation with 5 mM EDTA for 2 min prior to the addition of CT had little effect on the initial transient, but decreased the plateau phase to 12 ± 3 nM above basal levels (n = 7, Fig. 7C). Under these conditions, CT-stimulated Erk1/2 phosphorylation was reduced by more than half (Fig. 9A, compare lanes 2 and 3), suggesting that while the CT-induced release of Ca$^{2+}$ from intracellular stores supports CTR/Erk coupling to some degree, it is not sufficient for the full response. In cells loaded with BAPTA, the initial Ca$^{2+}$ transient was abolished, while the sustained plateau (53 ± 4 nM above basal levels) was the same as cells not loaded with BAPTA (Fig. 7D and B, respectively). Under these conditions, BAPTA had little effect on CT-induced Erk1/2 phosphorylation (Fig. 9A, lane 4). Thus, a small sustained increase in [Ca$^{2+}$]$_i$ is necessary to support the full Erk1/2 response to CT.

The foregoing experiments do not, however, address the question of whether the receptor-mediated increase in [Ca$^{2+}$]$_i$ by itself is sufficient to induce the full receptor-mediated Erk1/2 phosphorylation, as suggested by Della Rocca et al. (13). To examine this question, the effect of CT on Erk1/2 phosphorylation was compared with the effect of ionomycin, a Ca$^{2+}$ ionophore, and thapsigargin, an inhibitor of the endoplasmic reticular Ca$^{2+}$-ATPase that induces a transient increase in [Ca$^{2+}$]$_i$ by blocking the re-uptake of Ca$^{2+}$ into intracellular storage sites. The increase in [Ca$^{2+}$]$_i$, induced by 10 nM to 1 µM ionomycin were determined (not shown), and the changes in [Ca$^{2+}$]$_i$, induced by 100 nM ionomycin (peak 480 ± 61 nM and plateaus 114 ± 17 nM above basal levels, n = 5, Fig. 8B) approximated the CT-induced [Ca$^{2+}$]$_i$ changes. However, the Erk1/2 phosphorylation induced by 100 nM ionomycin (Fig. 9C, lane 3) was significantly smaller that induced by 1 nM CT (Fig. 9C, lane 2). (A similar concentration of ionomycin (3 µM) induced Erk1/2 phosphorylation to a similar level as 1 nM CT). Similarly, treatment of the cells with 1 µM thapsigargin caused a biphasic increase in [Ca$^{2+}$]$_i$ that approximated the [Ca$^{2+}$]$_i$ response to CT (peak 357 ± 43 nM and plateaus 74 ± 15 nM above basal levels, n = 5, Fig. 8C), but resulted in a minimal increase in Erk1/2 phosphorylation (Fig. 9C, lane 4). When the cells were treated with 10 µM thapsigargin for 25 min and subsequently treated with 1 nM CT for 5 min, CT did not further increase [Ca$^{2+}$], over the plateau phase induced by thapsigargin (absolute [Ca$^{2+}$], 314 ± 16 nM, n = 3, Fig. 8D), but

FIG. 5. G$_i$ is involved in the process of CT-stimulated Shc tyrosine phosphorylation, Shc-Grb2 association, and Erk1/2 phosphorylation. Subconfluent C1a-HEK cells were incubated in DMEM containing 0.5% serum for 18 h with (+) or without 200 ng/ml PTX. Cells were stimulated with 1 nM CT or 10 µM LPA for 5 min. Cells were lysed and analyzed as described in Figs. 1 and 2. A, Shc tyrosine phosphorylation (upper panel), total Shc (middle panel), and Shc-associated Grb2 (lower panel). B, phospho-Erk1/2 (upper panel) and total Erk1/2 (lower panel). C, HEK 293 cells were transiently co-transfected with pBK-C1a and either pRK5 or pRK-bErk1/2 (of both the peak and plateau phases of the CT-induced release of Ca$^{2+}$ half (Fig. 9A, lanes 2). Similarly, treatment of the cells with 10 nM to 1 µM ionomycin were determined (not shown), and the changes in [Ca$^{2+}$]$_i$, induced by 100 nM ionomycin (peak 480 ± 61 nM and plateaus 114 ± 17 nM above basal levels, n = 5, Fig. 8B) approximated the CT-induced [Ca$^{2+}$]$_i$ changes. However, the Erk1/2 phosphorylation induced by 100 nM ionomycin (Fig. 9C, lane 3) was significantly smaller that induced by 1 nM CT (Fig. 9C, lane 2). (A much higher concentration of ionomycin (3 µM) induced Erk1/2 phosphorylation to a similar level as 1 nM CT). Similarly, treatment of the cells with 1 µM thapsigargin caused a biphasic increase in [Ca$^{2+}$]$_i$ that approximated the [Ca$^{2+}$]$_i$ response to CT (peak 357 ± 43 nM and plateaus 74 ± 15 nM above basal levels, n = 5, Fig. 8C), but resulted in a minimal increase in Erk1/2 phosphorylation (Fig. 9C, lane 4). When the cells were treated with 10 µM thapsigargin for 25 min and subsequently treated with 1 nM CT for 5 min, CT did not further increase [Ca$^{2+}$], over the plateau phase induced by thapsigargin (absolute [Ca$^{2+}$], 314 ± 16 nM, n = 3, Fig. 8D), but
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**Fig. 7.** Ca\(^{2+}\) responses to CT in C1a-HEK cells in the presence of EGTA or BAPTA. Cells were loaded with the Ca\(^{2+}\)-sensitive dye indo-1 and suspended in the indicated buffer in a fluorimetric cuvette at 37 °C with continuous stirring. [Ca\(^{2+}\)]\(_i\) was monitored by fluorescence spectrophotometry as described under "Experimental Procedures." Test substances were added to the cuvette where indicated by arrows. Note the difference in [Ca\(^{2+}\)]\(_i\), scale bars for upper and lower traces. Traces are each representative of the responses of four to seven separate samples. A, cells were suspended in Na\(^{+}\) buffer (1 mM Ca\(^{2+}\)) and challenged with CT vehicle. B, cells were suspended in Na\(^{+}\) buffer (1 mM Ca\(^{2+}\)) and challenged with CT (1 nM). C, cells were suspended in Na\(^{+}\) buffer (1 mM Ca\(^{2+}\), 5 mM EGTA) and, 2 min later, challenged with CT (1 nM). D, cells were loaded with BAPTA (as described under "Experimental Procedures"), and later suspended in nominally Ca\(^{2+}\)-free Na\(^{+}\) buffer and challenged with CT (1 nM). E, cells were loaded with BAPTA and later suspended in nominally Ca\(^{2+}\)-free Na\(^{+}\) buffer and challenged with CT (1 nM). F, cells pretreated with EGTA (5 mM, 30 min) then washed, suspended in Na\(^{+}\) buffer (1 mM Ca\(^{2+}\), 5 mM EGTA), and challenged with CT (1 nM). (Prolonged incubation in the absence of extracellular Ca\(^{2+}\) often increased optical noise seen in traces E and F.)

**Fig. 8.** Ca\(^{2+}\) responses to ionomycin or thapsigargin in C1a-HEK cells. Cells were loaded with the Ca\(^{2+}\)-sensitive dye indo-1 and suspended in continuously stirred Na\(^{+}\) buffer (1 mM Ca\(^{2+}\)) in a fluorimetric cuvette at 37 °C. Test substances were added to the cuvette where indicated by arrows. Traces are each representative of the responses of three to five separate samples. A, 1 nM CT. B, 100 nM ionomycin. C, 1 μM thapsigargin (Tg). D, cells were pretreated with 1 μM thapsigargin for 30 min and then challenged with 1 nM CT.

Erk1/2 phosphorylation increased to a level similar to that seen in cells treated with CT alone (Fig. 9C, compare lanes 2 and 5). These results suggest that the small sustained elevation of [Ca\(^{2+}\)]\(_i\), induced by CT or by thapsigargin synergizes with other CT-stimulated signaling events to produce CTR-dependent Erk1/2 activation. To examine whether PKC- and Ca\(^{2+}\)-depend mechanisms are required simultaneously to achieve the full response, the cells were treated with calphostin C and EGTA. As shown in Fig. 9B, the combined treatment reduced Erk1/2 phosphorylation to a minimum level.

**DISCUSSION**

This study demonstrates that CT activates Erk1/2 in HEK 293 cells that express the C1a isoform of the rabbit CTR in a time- and concentration-dependent manner. The CT-induced activation of Erk1/2 is largely independent of cAMP/PKA-dependent pathways. Rather, in these cells the CTR activates the Shc/Grb2/Erk1/2 pathway via both G\(_i\)- and PKC-coupled mechanisms. The CTR-stimulated sustained increase in [Ca\(^{2+}\)]\(_i\) is necessary to support the full CT-dependent Erk1/2 phosphorylation, but the concurrent activity of other signaling events (e.g. PKC activation) is required for the maximal CT-induced response.

Recent results from us and others have shown that signaling via the CTR involves the activation of at least two G proteins, G\(_i\) and G\(_q\) (2–4). In the present study, we demonstrate that the CTR can also activate G\(_i\) since the CTR-stimulated events described here are partly PTX-sensitive. The CTR stimulates prolonged Shc tyrosine phosphorylation, as reported for some other GPCR, such as the thrombin and endothelin receptors, and for the insulin receptor or growth hormone receptor (34–37). This prolonged Shc tyrosine phosphorylation is in contrast to the transient effects observed with the G\(_i\)-coupled α\(_{\text{αα}}\)-AR (31) or the G\(_q\)-coupled α\(_{\text{αβ}}\)-AR (38). On the other hand, the CTR-induced Erk1/2 phosphorylation is transient. While CT induces prolonged Shc tyrosine phosphorylation, it is likely that activated Erk1/2 trigger a negative feedback loop in which MAP kinase phosphatase(s) are activated and in turn dephosphorylate and inactivate Erk1/2, since treatment with the phosphatase inhibitor sodium orthovanadate prolonged CT-stimulated Erk1/2 phosphorylation.\(^2\) The difference in the time course of phosphorylation of the two proteins is the subject of further investigation.

The finding that PTX and calphostin C have additive effects on both Shc and Erk phosphorylation is important for several reasons. It suggests that the G\(_i\)-dependent and PKC-dependent mechanisms that are activated by the CTR each can activate the Shc/Grb2/Erk1/2 pathway independently of the other, and that the G\(_i\)- and PKC-dependent mechanisms converge at or above the level of Shc in HEK 293 cells. Furthermore, it demonstrates for the first time that a single GPCR, the CTR, can activate Erk1/2 through parallel independent signaling pathways in the same cells.

Our results confirm the recent report of Della Rocca and colleagues (13) that both G\(_i\)- and G\(_q\)-coupled receptors activate Erk1/2 through the Shc/Grb2 pathway in HEK cells. However, our results differ in some respects. First, inhibition of PKC partially blocks the CTR-induced Erk1/2 phosphorylation, but has no effect on the responses elicited by the G\(_i\)-coupled α\(_{\text{αα}}\)-AR or the G\(_q\)-coupled α\(_{\text{αβ}}\)-AR. This difference may be due to the receptors, the G protein isoforms or other intermediate effec-
A

Erk1/2

untreated

ct

Phospho-Erk1/2

B

Erk1/2

untreated

ct

Phospho-Erk1/2

C

Erk1/2

untreated

ct

Phospho-Erk1/2

Fig. 9. Effects of EGTA, BAPTA, ionomycin, and thapsigargin on CT-induced Erk1/2 phosphorylation. A, subconfluent C1a-HEK cells were treated with 1 nM CT (5 min), 5 mM EGTA (2 min) plus 1 nM CT, 50 μM BAPTA-AM (30 min) plus 1 nM CT, 50 μM BAPTA-AM without extracellular Ca2+ (30 min) plus 1 nM CT, 5 mM EGTA (30 min) plus 1 nM CT, 10 ng/ml EGF, and 5 mM EGTA (30 min) plus 10 ng/ml EGF. B, subconfluent C1a-HEK cells were pretreated with 500 nm calphostin C (30 min), 5 mM EGTA (2 min) or both agents together prior to stimulation with 1 nM CT for 5 min. C, subconfluent C1a-HEK cells were treated with 1 nM CT, 100 mM ionomycin (5 min), 1 μM thapsigargin (30 min), or 1 μM thapsigargin (25 min) plus 1 nM CT (5 min). Cells were analyzed for Erk1/2 phosphorylation as described in the legend to Fig. 1. Phospho-Erk1/2 (upper panel) and total Erk1/2 (lower panel).

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The fact that CT-stimulated Erk1/2 phosphorylation requires sustained [Ca2+]i elevation of 40–50 nM above basal levels is in keeping with the recent report on differential activation of transcription factors by a small Ca2+ plateau versus a large Ca2+ transient in B-lymphocytes (39). In many situations, particularly in cells that express voltage-sensitive Ca2+ channels, an influx of extracellular Ca2+ is both necessary and sufficient for activation of Erk1/2 (40, 41). However, in C1a-HEK cells, the increase in [Ca2+]i, alone is not sufficient to account for the full CT-induced increase in Erk1/2 phosphorylation, since 100 nM ionomycin or 1 μM thapsigargin each induced changes in [Ca2+]i, comparable to those induced by CT, but stimulated little Erk1/2 phosphorylation. Furthermore, although challenging the thapsigargin-treated cells with CT failed to induce any additional change in [Ca2+]i, the level of Erk1/2 activation was much higher in cells treated with both thapsigargin and CT than in cells treated only with thapsigargin. Thus, both the increase in [Ca2+]i, and the activation of other signaling effectors, including PKC, are required for the full CTR-dependent Erk1/2 response.

Although the main focus of investigations of Erk1/2 function has been on their role in mediating the mitogenic effects of diverse ligands for receptor tyrosine kinases and GPCR, these proteins were originally identified as enzymes that phosphorylate microtubule-associated proteins and thereby regulate cytoskeletal stability (42–44). It is now widely established that Erk1/2 are involved in more than just mitogenic signaling (10). The ability of the CTR to activate the Shc to Erk1/2 pathway by two independent mechanisms implies that Erk1/2 may play an important role in mediating the morphological and/or biochemical effects of CT on osteoclast.

The duration of Erk activation has been proposed to determine the cell fate in PC 12 cells (45). Nerve growth factor stimulates sustained Erk activation which leads to the outgrowth of neurites and cessation of cell division, whereas EGF induces transient Erk activation which correlates with cell proliferation. If the model of PC 12 cells can be extended to cells expressing CTR, CT-induced transient Erk1/2 activation may be responsible for the proliferative effect of CT on a human prostate cancer cell line (46). The effect of CT on the proliferation of T47D and MCF-7, two other carcinoma cell lines that express the CTR, is currently under investigation.

In conclusion, we have demonstrated that CT induces Shc tyrosine phosphorylation, Shc-Grb2 association and Erk1/2 activation in C1a-HEK by parallel independent pathways involving G1 and PKC. Both pathways require the elevation of [Ca2+]i, but the change in [Ca2+]i, that occurs in response to treatment with CT cannot by itself induce full Erk1/2 phosphorylation.

Acknowledgments—We thank Dr. Robert J. LeFkowitz for providing the pRK5 and pRK-βARK1ct plasmids and Dr. Hong Sun for helpful discussion.

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