Potentiation by cholera toxin of bradykinin-induced inositol phosphate production in the osteoblast-like cell line MC3T3-E1

Yoshiko BANNO,* Takayuki SAKAI,† Takahiko KUMADA‡ and Yoshinori NOZAWA*§

Departments of *Biochemistry, †Oral and Maxillo-Facial Surgery and ‡Otorhinolaryngology, Gifu University School of Medicine, Tsukasamachi-40, Gifu 500, Japan

Cells of the osteoblastic cell line MC3T3-E1 were shown to contain at least three phosphatidylinositol-specific phospholipase C (PI-PLC) isoenzymes (PLC-β, PLC-γ and PLC-δ) by Western blotting analysis with various anti-PLC antibodies. Stimulation of inositol phosphate production in MC3T3-E1 cells by bradykinin (BK) occurred via a GTP-binding protein. Inositol phosphate formation on stimulation by BK was not affected by pretreatment with pertussis toxin, whereas it was potentiated by cholera toxin pretreatment. Elevation of cellular cyclic AMP levels by brief pretreatment with dibutyryl cyclic AMP or forskolin failed to enhance the BK-mediated generation of inositol phosphates, but long-term preincubation with these agents partially mimicked the action of the cholera toxin. Cholera toxin also caused an increase in BK receptor number. Cycloheximide, a protein biosynthesis inhibitor, prevented the potentiating actions of the cholera toxin and the cyclic AMP-elevating agents on BK-induced inositol phosphate production, and also inhibited the increase in BK receptor number. The specific binding of [^{3}H]BK to the whole MC3T3-E1 cells in the presence or absence of cholera toxin was completely inhibited by the B2 BK receptor antagonist D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]BK, but not by the B1 BK receptor agonist des-Arg^9-BK. These data suggest that the activation of PI-PLC induced by cholera toxin in BK-stimulated MC3T3-E1 cells was caused by an enhancement of the synthesis of BK receptor protein(s), at least part of which was mediated by a sustained increase in the intracellular level of cyclic AMP.

INTRODUCTION

It is known that inflammation-induced bone resorption is a consequence of interactions between cells in the inflammatory infiltrate and cells on the bone surface. Prostaglandins and tumour necrosis factor α are mediators that are known to be capable of stimulating bone resorption *in vitro* [1,2] and *in vivo* [3,4]. It has been found that bradykinin (BK), generated from high-molecular-mass kininogen by activated plasma kallikrein, also stimulates bone resorption *in vitro* [5]. BK-induced bone resorption is considered to be mediated via endogenous prostaglandin formation as a result of activation of phospholipase A_2 [6,7]. Moreover, BK was reported to induce a rapid increase in inositol 1,4,5-trisphosphate levels, with a subsequent increase in the cytosolic free Ca^{2+} concentration, in the osteoblastic cell line MC3T3-E1 [8,9]. Although MC3T3-E1 cells contain the B2 type of BK receptor [7], the mechanism of BK-induced inositol phosphate production has not been fully elucidated.

Mammalian tissues have been shown to contain several forms of phosphatidylinositol-specific phospholipase C (PI-PLC) [10]. The regulation of these activities varies: PLC-γ is phosphorylated on tyrosine residues by growth factors [11–14], and PLC-β is stimulated via a pertussis toxin-insensitive GTP-binding protein (termed G_q) [15,16]. GTP-binding proteins have been assumed to be involved in the coupling of the BK receptor to PI-PLC in various cells [17–20]. BK stimulates inositol phosphate production by mechanisms involving both pertussis toxin-sensitive and -insensitive GTP-binding proteins, probably via different subtypes of the BK receptor [17–20]. It has been shown that, in osteoblast-like MC3T3-E1 cells, PI-PLC activation by parathyroid hormone [21], thrombin [22] and prostaglandin E_2 [23] is mediated through different types of GTP-binding protein. Inositol phosphate production is pertussis toxin-insensitive with thrombin [22], but pertussis toxin-sensitive (via G_i2) with prostaglandin E_2 [23].

Earlier studies have demonstrated that cholera toxin affects signal transduction in various cell types. Pre-exposure of cells to cholera toxin caused inhibition of PI turnover, which was partly due to a reduction in the hormone receptor number [24–26]. On the other hand, it has been shown that cholera toxin potentiates hormone-stimulated PI turnover: the toxin increased Ca^{2+} influx, which in turn enhanced PI-PLC activity [27]. The action of cholera toxin was also reported to involve protein biosynthesis; elevations in cyclic AMP concentration caused by cholera toxin promoted transcription of mRNA encoding a protein that stimulates arachidonic acid metabolite release [28]. In addition, epidermal growth factor-stimulated inositol phosphate formation was potentiated by cholera toxin pretreatment, but this potentiating action was inhibited by cycloheximide [29]. In the present study we show that BK-induced inositol phosphate formation is markedly enhanced by pre-exposure to cholera toxin in osteoblast-like MC3T3-E1 cells, and that this potentiating effect of cholera toxin is abolished by the addition of cycloheximide.

MATERIALS AND METHODS

Materials

Myo-[2-^{3}H]Inositol (19.1 Ci/mmol), [^{32}P]NAD$^+$ (800 Ci/mmol), 2,3-prolyl-[3,4-^{3}H(n)]BK (102 Ci/mmol) and [^{3}H]phosphatidylinositol 4,5-bisphosphate ([^{3}H]PIP_2) (8.8 Ci/mmol) were pur-

Abbreviations used: BK, bradykinin; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; IBMX, 3-isobutyl-1-methylxanthine; PI-PLC, phosphatidylinositol-specific phospholipase C; PE, phosphatidylethanolamine; G_i and G_s, G-proteins that mediate inhibition and stimulation respectively of adenylate cyclase; G_q, pertussis toxin-insensitive GTP-binding protein(s) coupled to phosphoinositide breakdown; GTP[S], guanosine 5′-[γ-thio]triphosphate; GDP[S], guanosine 5′-[β-thio]diphosphate; α-MEM, α-minimal essential medium.

§ To whom correspondence should be addressed.

chased from Du Pont/New England Nuclear. Pertussis and cholera toxins were purchased from Funakoshi (Tokyo, Japan). β-Escin was from Nacalai Tesque Co. (Kyoto, Japan). BK, the BK analogues D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]BK and des-Arg^9-BK, and cycloheximide were from Sigma. Guanosine 5′-[γ-thio)-triphosphate (GTP[S]) and guanosine 5′-[β-thio]diphosphate (GDP[S]) were from Boehringer Mannheim. Thapsigargin was a gift from Dr. J. W. Putney (National Institutes of Health, Research Triangle Park, NC, U.S.A.). Other reagents used were of the highest grade available.

Cell culture

MC3T3-E1 cells, a cloned murine calvarial cell line, were kindly supplied by Dr. H. Kodama (Ohou University, Japan). The cells were maintained in α-minimal essential medium (α-MEM) containing 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 units/ml) at 37 °C in air/CO_2 (19:1). Cells were subcultured every 3 days as previously described [30,31].

Measurement of inositol phosphates

MC3T3-E1 cells were plated at a density of 4×10^4 cells/dish in 6-well (35 mm-diam.) dishes. At near-confluence the growth medium was changed to inositol-depleted α-MEM (diluted 10-fold with inositol-free medium), and then *myo*-[2-^{3}H]inositol (0.75 μCi/ml) was added and cells were cultured for 36 h. Stimulation experiments were initiated by replacing the culture medium with 0.6 ml of the reaction medium (HBS; 10 mM Hepes, pH 7.4, 145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$ and 5 mM glucose) containing 10 mM LiCl. The reaction was terminated by adding 10% (v/v) perchloric acid to give a final concentration of 3% and then neutralized by adding KOH; inositol phosphates were extracted as described by Bone et al. [32]. The inositol phosphates were separated by anion-exchange chromatography over Dowex AG 1-X8 columns (200–400 mesh, formate form, Bio-Rad) using a stepwise gradient of ammonium formate as described [33].

Cell permeabilization

MC3T3-E1 cells prelabelled with *myo*-[2-^{3}H]inositol were collected and suspended in a solution consisting of 20 mM Hepes, pH 7.3, 10 μg/ml leupeptin, 74.1 mM KCl, 2 mM $MgCl_2$, 4.5 mM MgATP, 2 mM EGTA, 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and 10 mM phosphocreatine. Permeabilization was achieved by incubating the cell suspension at 25 °C in 20 μM β-escin in the above solution for 20 min [34]. The extent of permeabilization was monitored by the Trypan Blue exclusion test; almost all cells (> 95%) were permeable to Trypan Blue.

Treatment of whole cells and membranes with pertussis toxin or cholera toxin

Pertussis toxin or cholera toxin (0.5–2 nM) was added to *myo*-[2-^{3}H]inositol-labelled MC3T3-E1 monolayers 12–24 h before exposure to BK. MC3T3-E1 cells, either untreated or pretreated with the toxins, were washed twice with HBS. Cells were then scraped from the dish with a rubber policeman and homogenized in a solution consisting of 20 mM Tris/HCl, pH 7.4, 10 μg/ml leupeptin, 1 mM phenylmethanesulphonyl fluoride and 1 mM EDTA. The membrane fraction obtained by centrifugation at 15000 *g* for 30 min was resuspended in homogenizing buffer. ADP-ribosylation assays were performed by the methods of Okajima et al. [35] for pertussis toxin and Lotersztajn et al. [36] for cholera toxin. Pertussis toxin and cholera toxin were incubated with 0.2 M dithiothreitol at 30 °C for 15 min to release the enzymically active fragment [35]. ADP-ribosylation with pertussis toxin was assayed by incubating membranes (100 μg of protein) with the preactivated pertussis toxin (4 μg/ml) in 0.1 ml of ADP-ribosylation reaction mixture, consisting of 40 mM potassium phosphate buffer (pH 7.5), 0.4 mM ATP, 0.4 mM GTP, 10 mM thymidine, 10 mM nicotinamide, 2.5 mM $MgCl_2$ and 25 μM [^{32}P]NAD^+ (10 μCi). Cholera toxin-catalysed [^{32}P]-ADP-ribosylation was carried out by incubating membranes (150 μg of protein) with the preactivated cholera toxin (20 μg/ml) in the same medium as used for pertussis toxin treatment, except that 2 mM dithiothreitol was added and higher concentrations of ATP (1 mM) and thymidine (0.1 M) were used. After 1 h of incubation with either toxin at 30 °C, the reactions were terminated by adding 1.2 ml of ice-cold 15% trichloroacetic acid. The precipitated proteins were resuspended in 50 μl of Laemmli sample buffer [37] and electrophoresed in SDS/10%-polyacrylamide gels. Autoradiography was performed by exposing the dried gel to Kodak X-Omat AR film for 24 h at −80 °C.

Assay of PI-PLC activity

MC3T3-E1 cells treated or not with cholera toxin for 13 h were lysed by sonication. PI-PLC activity was measured using [^{3}H]PIP_2 as substrate, as described previously [38]. The reaction mixture (50 μl) contained 20 mM Tris/maleate buffer, pH 6.8, 80 mM KCl, [^{3}H]PIP_2 (20000 d.p.m.)/PIP_2 (100 μM)/phosphatidylethanolamine (PE) (500 μM), 0.1% (w/v) deoxycholate, 1.66 mM $CaCl_2$, 2 mM EGTA and cell lysate. The reaction was terminated by addition of 0.25 ml of chloroform/methanol/conc. HCl (100:100:0.6, by vol.) and 0.1 ml of 5 mM EGTA/1 M HCl.

Protein was measured using the Bio-Rad protein assay with γ-globulin as standard.

Immunoprecipitation and immunoblotting of PI-PLC isoenzymes

MC3T3-E1 cells were suspended in buffer (0.5% Triton X-100, 0.5% sodium cholate, 10 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol and 20 mM Tris/HCl, pH 7.4) and centrifuged at 15000 *g* for 30 min to obtain supernatants. The supernatants were incubated for 12 h with antibodies against PLC-β, PLC-γ and PLC-δ, as described previously [39]. Monoclonal antibodies against PLC-γ and PLC-δ were kindly supplied by Dr. S. G. Rhee (National Institutes of Health, Bethesda, MD, U.S.A.), and polyclonal anti-PLC-β antibody was prepared as described previously [40].

[^{3}H]BK binding studies

Binding studies were performed with confluent MC3T3-E1 cells in 12-well dishes, with or without cholera toxin treatment, in α-MEM containing 2% calf serum for 13 h prior to binding experiments, as described previously [20]. Growth medium was removed and 1 ml of binding buffer (20 mM Hepes, pH 7.3, 17 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.63 mM $CaCl_2$, 0.21 mM $MgSO_4$, 0.34 mM Na_2HPO_4, 110 mM *N*-methylglucamine, 0.1% (w/v) BSA and 2 mM bacitracin) was added to each well. Plates were then equilibrated on ice for 10 min, after which the binding buffer was removed and replaced with 0.3 ml of binding buffer containing the appropriate concentration of [^{3}H]BK with or without unlabelled BK and BK analogues. After 4 h, binding buffer was removed and cells were rinsed with

4 × 1 ml of binding buffer at 4 °C. Cells were then suspended in 0.3 ml of 2% Na_2CO_3/1 M NaOH; 20 μl of each sample was removed for protein assay, and total activity per well was assessed by scintillation counting.

RESULTS

BK-induced inositol phosphate formation, and effects of GTP[S] and NaF

BK stimulated MC3T3-E1 cells to generate [^{3}H]inositol phosphates in a time-dependent manner. A rapid rise in the sum of inositol polyphosphates [inositol bisphosphates (IP_2) plus inositol trisphosphates (IP_3)] was observed, with a peak at 30 s after addition of BK; the peak was at 15 s for IP_3 and at 30 s for IP_2. In contrast, there was a slower progressive accumulation of inositol monophosphates (IP_1).

To examine whether BK-stimulated inositol phosphate generation was via GTP-binding protein(s), β-escin-permeabilized MC3T3-E1 cells were challenged with BK (100 μM) in the presence or absence of a submaximal concentration of GTP[S] (10 μM). In the absence of BK, GTP[S] promoted a time-dependent production of [^{3}H]inositol polyphosphates (IP_2 plus IP_3) within 20 min, with a subsequent plateau that was 4.5-fold greater than the basal value. The generation of inositol polyphosphates was then examined with a range of GTP[S] concentrations (5–1000 μM). The maximal enhancing effect was obtained at 100 μM. There was no significant increase in [^{3}H]IP_1 on addition of 100 μM GTP[S]. On the other hand, BK induced a transient small increase in inositol polyphosphate production in permeabilized cells. However, the BK-induced generation of inositol polyphosphates was greatly potentiated by the addition of 10 μM GTP[S]. This potentiating effect of GTP[S] was completely lost in the presence of 1 mM GDP[S].

We also examined the effect of NaF, a non-specific activator of GTP-binding proteins [36], on inositol phosphate production by intact MC3T3-E1 cells. Progressive increases in inositol phosphate production stimulated by NaF (30 mM) were observed with increasing time of incubation. After a 10 min incubation, 13-, 36- and 5-fold increases were observed in IP_1, IP_2 and IP_3 respectively. In cells pretreated for 5 min with a submaximal concentration of NaF (15 mM), addition of BK produced an additive effect on inositol phosphate production. However, NaF at the maximal concentration (30 mM) prevented the synergistic effect by BK. These results suggest that the BK receptor is linked to PLC via a GTP-binding protein in MC3T3-E1 cells.

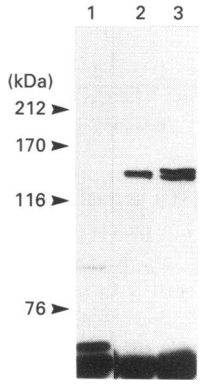

Figure 1 Immunoblotting with anti-PLC antibodies of MC3T3-E1 cells

The cells were lysed and immunoprecipitated with the antibodies raised against PLC-δ (lane 1), PLC-γ (lane 2) and PLC-β (lane 3). The immunoprecipitated proteins were subjected to SDS/PAGE, then transferred to a nitrocellulose membrane and probed with the mixed antibodies against PLC-β, PLC-γ and PLC-δ. Molecular mass standards (from top to bottom) used were myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa) and transferrin (76 kDa).

Table 1 Effects of pertussis toxin and cholera toxin on BK-induced inositol phosphate formation

MC3T3-E1 cells were prelabelled with *myo*-[2-^{3}H]inositol (0.7 μCi/ml) for 36 h and then pretreated with pertussis toxin (2 nM) or cholera toxin (0.5 nM) for 24 h. The cells were then exposed to BK (100 nM) for 30 s. The reactions were terminated by adding 10% perchloric acid. The released inositol phosphates were separated by anion-exchange column chromatography. Results are given as percentages of basal value (without BK) and are expressed as means ± S.E.M. for triplicate determinations from two representative experiments.

	Inositol phosphates produced (% of basal)		
Addition	IP_1	IP_2	IP_3
Untreated	174 ± 22	324 ± 17	244 ± 15
Pertussis toxin	196 ± 32	353 ± 56	290 ± 16
Cholera toxin	621 ± 86	893 ± 46	586 ± 12

Identification of PI-PLC isoenzymes

MC3T3-E1 cell lysates were analysed by immunoblotting with antibodies directed against PI-PLC isoenzymes (PLC-β, PLC-γ and PLC-δ). The anti-PLC-β antibody reacted with two polypeptides of molecular masses 150 kDa and 140 kDa (Figure 1, lane 3), and the anti-PLC-γ antibody reacted with a polypeptide of 145 kDa (lane 2). Moreover, a small amount of a 85 kDa polypeptide was stained by the anti-PLC-δ antibody (lane 1). These results show that MC3T3-E1 cells contain at least three PI-PLC isoenzymes: PLC-β, PLC-γ and PLC-δ.

Effects of pertussis toxin and cholera toxin on BK-stimulated inositol phosphate production and ADP-ribosylation

Pretreatment of the cells with pertussis toxin (2 nM, 13 h) did not affect BK-induced inositol phosphate production (Table 1). However, surprisingly, preincubation of MC3T3-E1 cells with cholera toxin (0.5 nM, 13 h) was found to potentiate the BK-induced generation of inositol phosphates. Identical pretreatment of cells with cholera toxin did not affect inositol phosphate accumulation induced by 30 mM NaF (results not shown).

To characterize the ADP-ribosylating substrates of pertussis toxin and cholera toxin, MC3T3-E1 cells were incubated in the presence or absence of the toxins and then the membrane fractions were assayed for ADP-ribosylation of any remaining unribosylated GTP-binding proteins in the presence of [^{32}P]-NAD^+ (Figure 2). Membranes of control MC3T3-E1 cells contained a substrate for pertussis toxin of 41 kDa (α-subunit of G_i) which was completely lost after pretreatment with pertussis toxin for 13 h (Figure 2a, lane 2). ADP-ribosylation of a major 45 kDa protein by cholera toxin was evident on SDS/PAGE of control membranes (Figure 2b, lane 1). When the cells were pretreated with cholera toxin at 0.5 nM for 13 h, the quantity of radiolabelled 45 kDa protein in the membrane fraction was diminished (Figure 2b, lane 2). However, after short-term exposure to cholera toxin (10 nM for 3 h), 20% of the protein was ADP-ribosylated.

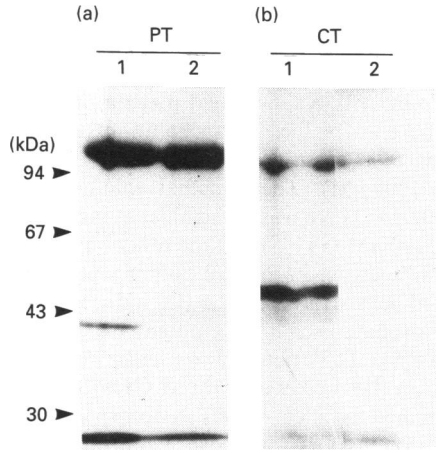

Figure 2 ADP-ribosylation of MC3T3-E1 cell membrane proteins

Cells were pretreated with pertussis toxin (2 nM) and cholera toxin (0.5 nM) for 13 h (lanes 2). Control (lanes 1) and treated samples were then assayed for ADP-ribosylation of the remaining available substrates in the presence of [^{32}P]NAD$^+$ and pertussis toxin (PT) or cholera toxin (CT) as described in the Materials and methods section. Molecular mass standards (from top to bottom) used were phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa).

Mechanism of cholera toxin enhancement of inositol phosphate production in BK-stimulated cells

The enhancement of inositol phosphate accumulation by pretreatment with cholera toxin (0.5 nM, 13 h) was dependent on the BK concentration when measured after 30 s of stimulation (Figure 3). Cholera toxin augmented the maximal BK-induced production, but the toxin alone was unable to stimulate inositol phosphate formation. Inositol phosphate production was potentiated in BK-stimulated MC3T3-E1 cells in a cholera-toxin-concentration-dependent manner over the range 5 pM–1 nM, with a maximum at 50 pM. There are several proposed mechanisms for the potentiating action of cholera toxin: an increase in Ca^{2+} influx [27], an elevation of cyclic AMP levels, and the stimulation of the synthesis of a specific protein(s) [28,29]. Therefore we examined which of these mechanisms is involved in the potentiation by cholera toxin of BK-stimulated inositol phosphate production in MC3T3-E1 cells.

Effect of Ca^{2+} increase

It has been reported that cholera toxin causes an increase in cytoplasmic Ca^{2+} levels by stimulating Ca^{2+} influx into RBL cells [27]. In a previous report we have demonstrated a BK-induced Ca^{2+} influx into MC3T3-E1 cells [9]. Moreover, in the present study we observed that the Ca^{2+} influx was also enhanced by pretreatment with cholera toxin (results not shown). Therefore, in order to examine whether the enhancement by cholera toxin of BK-induced Ca^{2+} influx caused the increase in inositol phosphate production, the cells were depleted of extracellular Ca^{2+} by chelation with EGTA (3 mM) and were examined for BK-stimulated phosphoinositide hydrolysis. The presence or absence of extracellular Ca^{2+} had no effect on BK-induced inositol phosphate production in cholera toxin-treated cells. We further examined the effect of the intracellular Ca^{2+} concentration on BK-induced inositol phosphate production by using the intracellular Ca^{2+}-elevating agents ionomycin and thapsigargin [41]. Neither of these agents affected phosphoinositide breakdown induced by BK. These observations thus suggest that the rise in intracellular Ca^{2+} is not sufficient to explain the potentiating effects of cholera toxin.

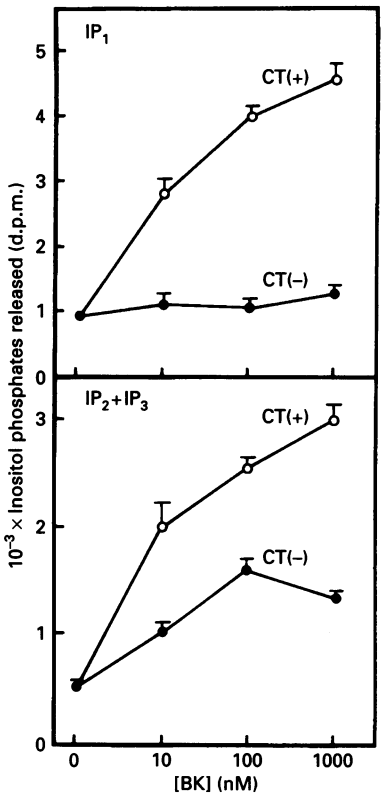

Figure 3 Enhancement by cholera toxin of inositol phosphate production: effect of BK concentration

[^{3}H]Inositol-labelled cells pretreated with cholera toxin (0.5 nM, 13 h) (○), or not treated (●), were incubated with various concentrations of BK for 30 s. Inositol phosphates (IP_1 and IP_2+IP_3) were separated on Dowex AG 1-X8 columns. Each point represents the means ± S.E.M. of 5–6 values.

Effect of cyclic AMP increase

Cholera toxin is known to elevate cellular cyclic AMP levels through activation of G_s in numerous cell types [42]. A question now arises as to whether the elevation of cyclic AMP levels induced by cholera toxin is an obligatory step in the initiation of the enhanced generation of inositol phosphates by PI-PLC stimulation. To this end, MC3T3-E1 cells were incubated with dibutyryl cyclic AMP (1 mM, 30 min) or with forskolin (100 μM, 45 min) plus 3-isobutyl-l-methylxanthine (IBMX), a phosphodiesterase inhibitor (150 μM). We have previously observed that pretreatment with forskolin for 30 min augmented the cyclic AMP level by 40-fold in MC3T3-E1 cells [31]. As shown in Table 2 (Expt. I), production of inositol phosphates in the unstimulated cells (basal level) was unchanged by short term-incubation with dibutyryl cyclic AMP (30 min) or forskolin plus IBMX (45 min). Furthermore, BK-stimulated inositol phosphate production was not affected when the intracellular cyclic AMP level was elevated to a maximum by pretreatment with dibutyryl cyclic AMP or forskolin. On the other hand, when the cells were exposed to these cyclic AMP elevating agents for 13 h, as indicated in Table 2 (Expt. II), the BK-stimulated inositol phosphate production was increased. Cholera holotoxin (40 pM) increased the formation of IP_1 and inositol polyphosphates (IP_2 plus IP_3) by 4-

Table 2 Effects of cholera toxin, dibutyryl cyclic AMP and forskolin on BK-induced inositol phosphate formation

MC3T3-E1 cells were labelled with *myo*-[2-^{3}H]inositol (0.7 μCi/ml) for 36 h and then treated with cholera holotoxin (40 pM), A subunit (0.5 nM) or B subunit (0.5 nM) for 13 h (Expt. II), or with dibutyryl cyclic AMP (1 mM) for 30 min (Expt. I) or 13 h (Expt. II), or with forskolin (100 μM) plus IBMX (150 μM) for 45 min (Expt. I) or 13 h (Expt. II) Cells were then exposed to BK (100 nM) for 30 s. Results are given as percentages of basal value (without BK) and are expressed as mean ± S.E.M. from six experiments

Addition	Inositol phosphates produced (% of basal)		
	IP_1	IP_2	IP_3
Expt. I			
Untreated	144 ± 19	320 ± 16	240 ± 21
Dibutyryl cyclic AMP	179 ± 21	254 ± 12	202 ± 16
Forskolin + IBMX	192 ± 30	302 ± 30	246 ± 13
		(IP_2/IP_3)	
Expt. II			
Untreated	139 ± 15	204 ± 13	
Cholera toxin			
Holotoxin	604 ± 11	556 ± 69	
A subunit	206 ± 18	203 ± 18	
B subunit	189 ± 10	199 ± 10	
Dibutyryl cyclic AMP	472 ± 48	424 ± 56	
Forskolin ± IBMX	222 ± 18	327 ± 31	

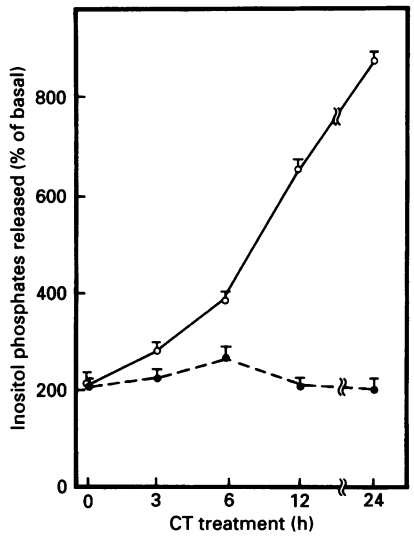

Figure 4 Effect of cycloheximide on the potentiating action of cholera toxin

[^{3}H]Inositol-labelled cells were pretreated with 40 pM cholera toxin in the presence (●) or absence (○) of 10 μg/ml cycloheximide for the indicated times and then stimulated with BK (100 nM for 30 s). Values are expressed as a percentage of the basal inositol polyphosphate production (IP_2 + IP_3; 210 d.p.m.). Data are means ± S.E.M. from three experiments.

and 3-fold respectively, and dibutyryl cyclic AMP (2 mM) gave 3- and 2-fold increases respectively. The increase induced by forskolin plus IBMX was smaller. Cholera toxin A subunit (0.5 nM) or B subunit (0.5 nM) did not affect inositol polyphosphate production. These results indicated that a sustained elevation of cyclic AMP levels was able to augment BK-stimulated PI-PLC activity.

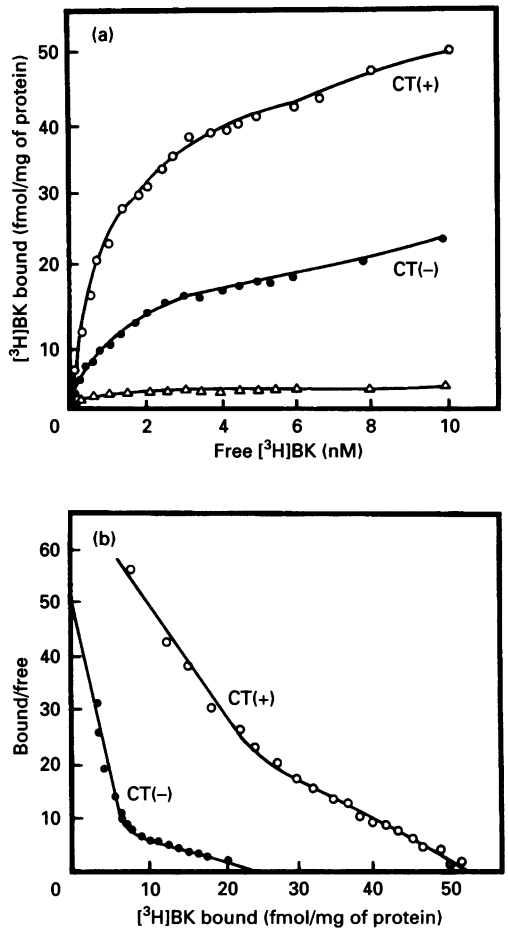

Figure 5 Saturation (a) and Scatchard (b) analysis of [^{3}H]BK binding to MC3T3-E1 cells

MC3T3-E1 cells with (CT+, ○) or without (CT−, ●) cholera toxin treatment (40 pM for 16 h) were used for binding analysis. Background binding (△) was measured in the presence of a 500-fold excess of unlabelled ligand and subtracted from total binding to give specific binding. (**a**) Increasing binding was observed with increasing ligand concentration, measured after 4 h at 4 °C; (**b**) Scatchard transformation of the specific binding data shown in (**a**). Points are means of duplicate determinations from three separate experiments.

Effect of protein synthesis

Previous studies have suggested that a newly synthesized protein might be important in mediating the secretory response to cholera toxin [43,44]. However, such a protein mediator(s) has not been identified. Peterson et al. [28] reported synthesis is required for cholera toxin-induced stimulation of arachidonic acid metabolite release. The potentiating action of cholera toxin on BK-stimulated inositol phosphate production was examined to see whether it was affected by cycloheximide. The cells were pretreated with cholera toxin in the presence or absence of cycloheximide. Cholera toxin increased BK-stimulated inositol phosphate production in time-dependent manner up to 24 h (Figure 4); however, in the presence of cycloheximide (10 μg/ml), this action of cholera toxin was completely inhibited. Administration of actinomycin D (10 μg/ml) also prevented the potentiating effect of cholera toxin. All of the potentiations by various cyclic AMP elevating agents (indicated in Table 2) were completely abolished by simultaneous addition of cycloheximide or actinomycin D. These results suggest a requirement for protein

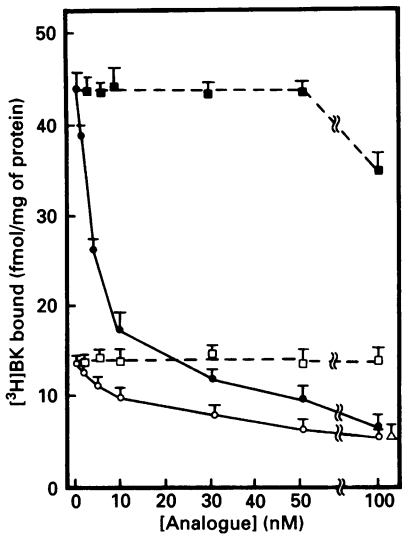

Figure 6 Displacement of [^{3}H]BK binding to MC3T3-E1 cells by BK analogues

Residual binding of [^{3}H]BK was measured at several peptide concentrations after incubation for 4 h at 4 °C of the MC3T3-E1 cells with (■, ●) or without (□, ○) cholera toxin treatment (40 pM, 16 h). Points are means ± S.E.M. of three determinations from two different experiments. □, ■, des-Arg^9-BK; ●, ○, D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]BK; ▲, BK.

synthesis for the potentiating action of cholera toxin on BK-stimulated inositol phosphate production.

Effect of [^{3}H]BK binding

Cholera toxin has been shown to exert an effect by modulating the binding of a hormone to its receptor [24–26]. To see whether the potentiation by cholera toxin of BK-stimulated inositol phosphate production in MC3T3-E1 cells occurs at the level of the BK receptor, we investigated receptor number and affinity in cells treated with cholera toxin (0.5 nM, 13 h) by using [^{3}H]BK. Specific [^{3}H]BK binding to MC3T3-E1 cells treated or not with cholera toxin was saturable (Figure 5a). Scatchard analysis of both saturation curves revealed two components of binding having biphasic Scatchard plots, with mean dissociation constants (K_d) of 0.2 nM (range 0.19–0.23 nM) and 2.3 nM (range 2.3–2.4 nM) (Figure 5b). The K_d was not changed by treatment with cholera toxin, but the toxin caused a 250–300% increase in the maximal binding number ($B_{max.}$) of both sites. For high-affinity sites the $B_{max.}$ values of whole MC3T3-E1 cells untreated or treated with the toxin were 7.7 ± 0.5 and 25.0 ± 3.5 fmol/mg of protein respectively, and for low-affinity sites they were 20.0 ± 3.5 and 56.5 ± 2.3 fmol/mg of protein respectively. Furthermore, the increase in the BK receptor number caused by cholera toxin treatment was completely abolished when cycloheximide (10 μg/ml) was present, suggesting that the amount of the BK receptor protein is increased by cholera toxin.

At least two different BK receptor subtypes have been described, designated B1 and B2 [45]. To examine which type(s) of BK receptor was increased in the cholera toxin-treated MC3T3-E1 cells, we used BK analogues such as the B1-type agonist des-Arg^9-BK and the B2 receptor antagonist D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]BK. As shown in Figure 6, [^{3}H]BK (5 nM) binding to MC3T3-E1 cells treated or not with cholera toxin was inhibited by the B2 receptor antagonist in a dose-dependent manner (range 1–50 nM), while it was not affected by the B1 receptor agonist. These results suggest that MC3T3-E1 cells contain only the B2-type BK receptor, and this receptor subtype was increased by cholera toxin treatment via protein synthesis.

We also compared PI-PLC activities in isolated membranes from MC3T3-E1 cells treated or not with cholera toxin (0.5 nM, 13 h). As measured using [^{3}H]PIP$_2$ as substrate, PI-PLC activities of untreated and treated cells were 208 ± 30 and 235 ± 23 pmol hydrolysed/min per mg of protein respectively. Thus no significant changes in the PI-PLC activity were caused by long-term pretreatment with cholera toxin. Furthermore, the extent of GTP[S]-induced stimulation of PIP$_2$ hydrolysis was not altered in the membrane fraction by cholera toxin treatment.

DISCUSSION

The data presented here demonstrate that the BK receptor is linked to PI-PLC via a pertussis toxin-insensitive GTP-binding protein in osteoblastic MC3T3-E1 cells. NaF caused a much greater increase in inositol phosphate accumulation than did GTP[S] stimulation in permeabilized cells. MC3T3-E1 cells contain three PLC isoenzymes (PLC-β, PLC-γ and PLC-δ), with a large amount of PLC-β, as estimated by Western blotting using anti-PLC antibodies. Furthermore, we found that the $G_L2\alpha$ [46], a member of the G_q class, was detected in the cell membranes by Western blotting using an appropriate antibody (results not shown), thus suggesting that stimulation of MC3T3-E1 cells by BK results in activation of PLC-β1 via G_q. Interestingly, the BK-mediated generation of inositol phosphates was found to be greatly enhanced by cholera toxin pretreatment, although the mechanism(s) by which cholera toxin potentiates hormonal stimulation of PI-PLC activity remains incompletely defined.

It has been reported in RBL cells [27] that cholera toxin enhanced the antigen-stimulated Ca^{2+} influx, which in turn led to activation of PI-PLC, thereby potentiating the production of inositol phosphates. However, our results obtained in the present study showed that intracellular Ca^{2+}-modulating agents such as ionomycin and thapsigargin failed to potentiate BK-induced inositol phosphate production in MC3T3-E1 cells. Furthermore, depletion of extracellular Ca^{2+} by EGTA did not affect the potentiating action of the toxin on PI-PLC activity. These findings indicate that the Ca^{2+} influx was not directly involved in the action of cholera toxin in our cell system.

The potentiating effect of cholera toxin on the PI-PLC pathway might be explained by the action of the stimulatory GTP-binding protein G_s, which activates adenylate cyclase to increase intracellular cyclic AMP levels [42]. One can speculate that the increase in the concentration of cyclic AMP caused by treatment with cholera toxin is somehow implicated in the enhancement of BK-mediated phosphoinositide hydrolysis. On the other hand, many studies have indicated that cholera toxin exerts a negative feedback effect on receptor-activated signal transduction, probably via phosphorylation by protein kinase A. For example, in Jurkat T lymphocytes [47], skeletal myoblasts [24] and glomerulosa cells [25], cholera toxin inhibits agonist-induced phosphoinositide breakdown. In these cells the cholera toxin treatment caused a decrease in the numbers of vasopressin and angiotensin II receptors [24,25]. The inhibitory mechanism of cholera toxin was proposed to be due to desensitization or uncoupling of receptors by phosphorylation. Cholera toxin also inhibited the angiotensin II receptor-mediated stimulation of PI-PLC by a dual mechanism in vascular smooth muscle cells: a decrease in the receptor number by a cyclic AMP-independent mechanism, probably receptor down-regulation, and inhibition of GTP[S]-

stimulated inositol phosphate production via phosphorylation by elevating cyclic AMP-dependent kinase activity [26]. Furthermore, Olashaw et al. [48] have shown that brief exposure of cells to cholera toxin or forskolin reduced inositol phosphate formation induced by the GTP-binding protein activator aluminium fluoride, but had no effect on platelet-derived growth factor-mediated inositol phosphate production. These authors demonstrated also serine phosphorylation of PLC-γ resulting from cyclic AMP accumulation caused by cholera toxin.

The results presented here are distinct from those in previous studies demonstrating inhibitory actions of cyclic AMP-elevating agents on phosphoinositide hydrolysis [24–26,48]. Such a discrepancy between previous work and our results may be due to a difference in receptor type. Our data obtained from the experiments with dibutyryl cyclic AMP and forskolin showed that a large but transient (30–45 min) increase in the cellular levels of cyclic AMP is not sufficient to cause augmentation of inositol phosphate production by BK receptor activation in MC3T3-E1 cells. In contrast to the brief preincubation with dibutyryl cyclic AMP and forskolin, the long-term (13 h) incubation partially mimicked the augmentative effect of cholera toxin on BK-induced inositol phosphate production (Table 2). This result indicates that the cyclic AMP concentration needs to remain elevated for several hours before it exerts a potentiating effect. Thus the potentiating effect of cholera toxin is caused via elevation of cyclic AMP, but is unlikely to be due to phosphorylation of the BK receptor. Of great interest is the fact that all of the potentiating effects induced by various agents were completely abolished by addition of cycloheximide or actinomycin D. It can thus be concluded that the potentiating effect of cholera toxin on BK receptor-linked PI-PLC activation in MC3T3-E1 cells requires induction of the synthesis of certain protein(s) synthesis. Several lines of evidence have been presented to demonstrate that the effects of cholera toxin are blocked by cycloheximide and actinomycin D: potentiation of epidermal growth factor receptor modulation by platelet-derived growth factor [49], enhancement of epidermal growth factor-stimulated inositol phosphate formation in BALB/c/3T3 cells [29], and an increase in arachidonic acid release in Chinese hamster ovary cells [28] all occurred with the toxin and were blocked by cycloheximide. Furthermore it has been reported that cholera toxin increases the synthesis of a 133 kDa cell surface glycoprotein or of a 200 kDa membrane glycoprotein in Chinese hamster ovary cells [44]. Our preliminary data with [^{35}S]-methionine labelling showed that, in MC3T3-E1 cells pretreated with cholera toxin for 13 h, membrane-associated proteins of 85 kDa, 60 kDa and 40 kDa were more abundant than in membranes from untreated cells (results not shown). We also found that cholera toxin increased total [^{3}H]BK-binding activity in MC3T3-E1 cells. The increased [^{3}H]BK binding was inhibited by B2 receptor antagonist, but not by a B1 receptor agonist. It is thus tempting to speculate that cholera toxin may modulate the receptor number at the gene level, associated with the sustained elevation of cellular cyclic AMP, since MC3T3-E1 cells are required to be exposed to cholera toxin or dibutyryl cyclic AMP for several hours before the potentiating effect is observed. From these results it is conceivable that cholera toxin induces an increase in BK receptor numbers, which in turn potentiates BK-induced inositol phosphate production in MC3T3-E1 cells.

Some other possibilities could also be considered. It has been reported that ADP-ribosylation by cholera toxin of a G_s-like GTP-binding protein potentiates IgE-mediated secretion from RBL cells by a largely cyclic AMP-independent route [50]. Furthermore, a recent study showed an enhancement of PIP_2 hydrolysis by BK stimulation in NG 108-15 cells treated with dibutyryl cyclic AMP, which was interpreted to be due to an increased $G_q\alpha$ level [51]. Thus the possibility of an increase in the levels of a GTP-binding protein or an interaction of a GTP-binding protein and PLC cannot be completely excluded in our system.

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