Am J Physiol Cell Physiol
280: C1233–C1241, 2001.

Pertussis toxin directly activates endothelial cell p42/p44 MAP kinases via a novel signaling pathway

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Received 6 October 2000; accepted in final form 15 December 2000

Garcia, Joe G. N., Peiyi Wang, Feng Liu, Marc B. Hershenson, Talaibek Borbiev, and Alexander D. Verin. Pertussis toxin directly activates endothelial cell p42/p44 MAP kinases via a novel signaling pathway. *Am J Physiol Cell Physiol* 280: C1233–C1241, 2001.—*Bordetella pertussis* generates a bacterial toxin utilized in signal transduction investigation because of its ability to ADP ribosylate specific G proteins. We previously noted that pertussis toxin (PTX) directly activates endothelial cells, resulting in disruption of monolayer integrity and intercellular gap formation via a signaling pathway that involves protein kinase C (PKC). We studied the effect of PTX on the activity of the 42- and 44-kDa extracellular signal-regulated kinases (ERK), members of a kinase family known to be activated by PKC. PTX caused a rapid time-dependent increase in bovine pulmonary artery endothelial cell ERK activity that was significantly attenuated by *1*) pharmacological inhibition of MEK, the upstream ERK activating kinase, *2*) an MEK dominant-negative construct, and *3*) PKC inhibition with bisindolylmaleimide. There was little evidence for the involvement of either Gβγ-subunits, Ras GTPases, Raf-1, $p60^{src}$, or phosphatidylinositol 3′-kinases in PTX-mediated ERK activation. Both the purified β-oligomer binding subunit of the PTX holotoxin and a PTX holotoxin mutant genetically engineered to eliminate intrinsic ADP ribosyltransferase activity completely reproduced PTX effects on ERK activation, suggesting that PTX-induced ERK activation involves a novel PKC-dependent signaling mechanism that is independent of either Ras or Raf-1 activities and does not require G protein ADP ribosylation.

signal transduction; endothelium; bacterial toxin; adenosine 5′-diphosphate ribosylation; extracellular signal-regulated kinases; β-oligomer; Raf-1 activation; p21 Ras activity

PERTUSSIS TOXIN (PTX) is a product of *Bordetella pertussis* infection and is a widely used tool for examination of cellular signaling pathways. The watershed discovery was that pertussis toxin (PTX) exerted its effect as a modulatory virulence factor by ADP ribosylation of guanine nucleotide-binding G proteins, key components of stimulus/coupling signal transduction. The pertussis holotoxin comprises an ADP-ribosyltransferase fragment (S_1) whose targets include the α-subunit of G_i and G_o subclasses of heterotrimeric G proteins and a β-oligomer containing several binding subunits (S_2–S_6) (24, 40, 41). ADP ribosylation of Gα uncouples the G protein from its receptor in a way that disruption of a signaling pathway by PTX is presumptive evidence of a G protein-regulated pathway. Direct effects of PTX on cellular function, i.e., in the absence of agonist/ligand stimulation, have also been noted in numerous cell systems (7, 27, 36, 46–48, 52). This has generally been perceived as evidence for tonic regulation by a PTX-sensitive G protein; however, PTX directly elicits several second messenger cascades capable of evoking specific biochemical and physiological responses such as Ca^{2+} mobilization and cAMP and diacylglycerol synthesis (46–48, 52). For example, PTX was noted to directly increase lung weight gain in isolated lung preparations, consistent with lung cell activation (8, 49), although the exact mechanism or target of the edemagenic response was not identified. We previously noted PTX to be a potent direct stimulus for endothelial paracellular gap formation and increases in macromolecular permeability across confluent endothelial cell monolayers in vitro (37, 38). In these studies, neither increases in cytosolic Ca^{2+} nor increases in myosin light chain phosphorylation were noted, unique findings compared with other models of endothelial cell permeability (15). However, there was strong evidence that PTX-mediated endothelial cell activation was dependent on protein kinase C (PKC) activity, because PKC inhibition attenuated the extent of PTX-induced endothelial cell barrier dysfunction (38). The exact PKC permeability targets responsible for PTX-mediated endothelial cell gap formation and permeability have not yet been defined; however, signaling pathways frequently involved in cellular activation such as phosphatidylinositol-specific phospholipase C or phospholipase D do not appear to participate in PTX-induced endothelial cell activation (16–18). In fact, our prior results suggested a novel PKC-dependent model of endothelial cell permeability that is

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independent of contractile protein rearrangement driven by a myosin motor.

The mitogen-activated protein (MAP) kinase family of serine/threonine protein kinases includes several potential participants in PTX-induced endothelial cell activation, since several members of this family are known to be activated by PKC. This MAP kinase family includes three subgroups [extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38] that are structurally related, yet exhibit distinct substrate specificity and biological effects. ERK was the first MAP kinase to be discovered and participates in cell proliferation, contraction, apoptosis, and a number of other important cellular responses (19). Increased ERK activation can follow increases in either p21 Ras GTPase activity or PKC activity, both of which result in Raf-1 kinase-mediated autophosphorylation and, subsequently, increased activity of the dual-specificity kinase MEK, the direct upstream activator of p42/p44 ERK. The mechanism by which G proteins activate p42/p44 MAP kinases is poorly understood but has been attributed to βγ-subunit involvement as well as α-subunit-associated coupling (35). In this study, we have examined whether p42/p44 MAP kinase activity is involved in endothelial cell activation produced by the important G protein modulator PTX. Our results indicate PTX to be a robust stimulus for activation of p42 and p44 ERK1 and ERK2 activation via a novel signaling pathway that does not involve p21 Ras GTPases, Raf-1, or G protein βγ-subunits. In contrast, PKC was critical to the MEK-dependent, PTX-mediated ERK activation. Both an S_1 mutant devoid of ADP ribosyltransferase activity and the purified β-oligomer of the PTX holotoxin directly produced ERK activation. These studies strongly suggest the involvement of ERK signaling pathways in endothelial cell activation evoked by PTX.

METHODS

Reagents. Bovine pulmonary artery endothelial cell cultures were maintained in DMEM (GIBCO, Chagrin Falls, OH) supplemented with 20% (vol/vol) colostrum-free bovine serum (Irvine Scientific, Santa Ana, CA), 15 μg/ml endothelial cell growth supplement (Collaborative Research, Bedford, MA), 1% antibiotic and antimycotic solution (10,000 U/ml penicillin, 10 μg/ml streptomycin, and 25 μg/ml amphotericin B; K. C. Biologicals, Lenexa, KS), and 0.1 mM nonessential amino acids (GIBCO). Unless specified, reagents were obtained from Sigma Chemical (St. Louis, MO). Phosphate-buffered saline (PBS) and Hanks' balanced salt solution without phenol red were purchased from GIBCO (Grand Island, NY). Polyacrylamide gradient 4–15% ready-to-use gels were purchased from Bio-Rad (Hercules, CA). The MEK inhibitor PD-98059 was purchased from Calbiochem (La Jolla, CA). Endotoxin-free pertussis holotoxin and β-oligomer were purchased from List Biological Laboratories (Campbell, CA). The S_1 mutant, β-adrenergic receptor kinase (βARK) minigene, hemagglutinin (HA)-tagged ERK2, and MEK constructs were kindly provided by Drs. Rappuoli (Sienna, Italy), W. J. Koch (Duke University, Durham, NC), R. Pestell (Albert Einstein College of Medicine, Bronx, NY), and M. Rosner (University of Chicago, Chicago, IL), respectively.

Bovine pulmonary artery endothelial cell cultures. Endothelial cells were obtained from American Type Tissue Culture Collection (CCL 209; Rockville, MD) at 16 passages, utilized at *passages 19–24*, and cultured in complete media (15, 17). The endothelial cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2-95% air and grew to contact-inhibited monolayers with typical cobblestone morphology. Cells from each primary flask were detached with 0.05% trypsin, resuspended in fresh culture medium, and passaged into 30- or 60-mm dishes for MAP kinase activity, Raf-1 kinase activity, and p21 Ras activity determination.

ERK activation assays. Endothelial cell monolayers in 35-mm dishes (100% confluence) were either serum-starved by incubation with DMEM for 20 h or challenged in complete media and treated with either vehicle or PTX (Calbiochem, CA) for specified periods of time. The cells were lysed with 150 μl of boiling lysis buffer containing 10 mM Tris·HCl, pH 7.4, 1% SDS, and 1 mM sodium orthovanadate, heated to boiling for 5 min, and centrifuged for 5 min. The protein concentration of the resulting supernatant was determined using BCA (bicinchoninic acid) Protein Assay Reagent (Pierce). ERK activity of samples was assessed by either Western blotting with specific phospho-MAPK (MAP kinase) antibody (New England BioLab, Beverly, MA) or an in-gel MAP kinase assay. To perform the in-gel MAP kinase assay, we separated MAP kinases from other proteins by SDS-PAGE (33) with the use of 12.5% polyacrylamide gel containing 0.5 mg/ml myelin basic protein (MBP) (Sigma). After electrophoresis, the gel was washed with two changes of 100 ml of 20% isopropanol in 50 mM Tris·HCl, pH 8.0, for 2 h to remove SDS and then incubated with 250 ml of *buffer A* (50 mM Tris·HCl, pH 8.0, and 5 mM 2-mercaptoethanol) for 1 h with continuous agitation. To denature the proteins, we incubated the gel with 100 ml of 6 M guanidine-HCl in *buffer A* for 1 h with two exchanges. The proteins in the gel were then renatured by five changes of *buffer A* containing 0.04% Tween 20 at 4°C with continuous agitation for 16 h. To assess phosphorylation of MBP, we preincubated the gel with 25 ml of kinase buffer [40 mM HEPES-NaOH, pH 8.0, 2 mM dithiothreitol (DTT), 0.1 mM EGTA, 0.1 mM sodium orthovanadate, and 10 mM $MgCl_2$] for 30 min at 25°C and then with 10 ml of kinase buffer containing 25 μM ATP and 50 μCi of [γ-^{32}P]ATP for 1 h with continuous agitation. The reaction was stopped by washing the gel extensively with 5% TCA containing 1% sodium pyrophosphate with continuous agitation until all free radioactivity was liberated. The gel was then dried and exposed to X-Omat film (Kodak).

Raf-1 activity assay. Raf-1 kinase activity was assessed by using a commercially available assay kit (Upstate Biotechnology, Lake Placid, NY). Confluent endothelia were treated with 1 μg/ml PTX or the same volume of PBS as vehicle control for 5 min after 18 h of serum starvation. As a positive control, cells were treated with phorbol 12-myristate 13-acetate (PMA; 100 nM) or DMSO as vehicle control (5 min). Cells were lysed at the end of the incubation period, and Raf-1 kinase was immunoprecipitated with 4 μg of anti-human c-Raf kinase carboxy terminus at 4°C for 2 h. This was followed by gentle agitation with 100 μl of PBS-prewashed protein G-Sepharose slurry (containing 30% protein G-Sepharose 4 Fast Flow; Amersham Pharmacia Biotech, Piscataway, NJ) for 2 h at 4°C. Immunoprecipitated active Raf was used to phosphorylate and activate GST-MAPKK (glutathione-*S*-transferase-MAPK kinase), which in turn phosphorylates p42 GST-MAPK, resulting in phosphorylation of MBP in the presence of [γ-^{32}P]ATP. The radiolabeled substrates were allowed to bind to P81 phosphocellulose

paper, and the radioactivity was measured in a scintillation counter.

Cotransfection with plasmids encoding HA-ERK 2 and the Gβγ-binding domain of βARK1. Endothelial cells grown to 80% confluence in 35-mm dishes were transiently transfected with a plasmid encoding HA-ERK2 (23) and a second plasmid encoding either the G protein βγ-binding domain of βARK1 (22, 29) or a dominant-negative MEK construct (EE-MEK-2E) (57). The βARK minigene plasmid (pRK-βBARK1-495-689) contains the carboxy terminus of the β-adrenergic receptor kinase (the Gβγ-binding domain) and was kindly provided by Dr. Walter J. Koch (Duke University). Cells were incubated with 1 μg of total DNA (1:1 ratio of the DNA of the 2 plasmids) and 10 μl of Lipofectamine (GIBCO) in 1 ml of OPTI-MEM for 6 h. The solution was then replaced with 1 ml of normal growth medium and incubated for 24 h, and the cells were subsequently serum-starved in DMEM for 20 h. The transfected endothelial cell monolayers were then treated with either PTX (1 μg/ml) for 5 min or lysophosphatidic acid (LPA; 1 μM) for 5 min. ERK2 kinase activity was assessed by immunoprecipitation of HA-tagged ERK2, followed by in vitro phosphorylation assay using MBP as substrate. Briefly, after treatment with agonists, the cells were quickly rinsed with PBS and lysed with 150 μl of immunoprecipitation buffer containing 10 mM Tris·HCl, pH 7.4, 1% Triton X-100, 0.5% Nonidet P-40 (NP-40), 150 mM NaCl, 20 mM NaF, 0.2 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, and 1% inhibitor cocktails (Calbiochem) for 30 min at 4°C. The cells were scraped, homogenized by being passed through a 26-gauge syringe three times, and centrifuged for 10 min at 4°C. The soluble cell lysate (100 μl), containing ~100 μg of total protein, was incubated with mouse anti-HA antibody for 1.5 h and then with 15 μl of protein G-Sepharose at 4°C for 1.5 h. The immune complexes were washed three times with immunoprecipitation buffer and three times with kinase buffer containing 10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, and 0.5 mM DTT. The immune complexes were resuspended in 40 μl of kinase buffer with 0.5 mg/ml MBP, 25 μM ATP, and 2.5 μCi of [γ-^{32}P]ATP and incubated at 30°C for 30 min. The reaction was stopped by adding 14 μl of boiling 4× sample buffer. The samples were then boiled for 5 min and centrifuged for 5 min, and 15 μl of supernatant were loaded for SDS-PAGE (33). After electrophoresis, the gel was stained with Coomassie blue R250, destained, dried, and exposed to X-Omat film (Kodak).

Ras activity assay. Endothelial cell monolayers were cultured in 35-mm dishes for 7 days in serum-containing culture medium, serum-starved for 16 h, and radiolabeled with 220 μCi/ml [^{32}P]orthophosphate in DMEM for an additional 4 h to label ATP pools. Cells were challenged with 1 μg of PTX or 100 nM PMA in 1 ml of phosphate-free DMEM for the indicated times. Medium was then removed, and cells were lysed in 500 μl of buffer containing 25 mM Tris, pH 7.5, 150 mM NaCl, 16 mM $MgCl_2$, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml p21^{ras} primary antibody (anti-v-H-ras; Calbiochem). Plates were incubated on ice for 30 min, and then lysates were scraped from dishes and centrifuged for 10 min (16,000 *g* at 4°C). An additional 2 μg of primary antibody were added to each supernatant and incubated on ice for 1 h, and 50 μl of lysis buffer preequilibrated protein G-Sepharose were added to each tube and allowed to incubate for 1 h with gentle mixing at 4°C. Protein G-Sepharose was spun down at 80 *g* for 1 min at 4°C and was washed four times with washing buffer (lysis buffer without proteinase inhibitors and antibody). Immunoprecipitates were resuspended in 20 μl of elution buffer containing 2 mM EDTA, 2 mM DTT, and 0.2% SDS and then boiled for 3 min. Sepharose was pelleted by centrifugation at 16,000 *g* for 10 min at room temperature. Supernatants were collected and counted for radioactivity using a scintillation counter. Equal amounts of radioactivity for each sample were loaded on 20 × 20-cm thin-layer chromatography (TLC) plates (Baker-flex cellulose PEI-F; J. T. Baker, Phillipsburg, NJ) and performed in 0.75 M KH_2PO_4, pH 3.4. The TLC plates were exposed to a phosphorimager plate overnight and were read in a Molecular Dynamics PhosphorImager 445SI. The intensities of separated [^{32}P]GTP and [^{32}P]GDP were quantitated, and the data were expressed as the ratio of [^{32}P]GTP to [^{32}P]GTP and [^{32}P]GDP.

ADP ribosylation of endothelial cell proteins. Bacterial toxin-catalyzed ADP ribosylation of proteins contained within endothelial cell homogenates was measured by incorporation of ^{32}P-labeled NAD (10–20 μCi/ml in ribosylation cocktail; NEN) as we have previously described (16, 18, 37). PTX (final concentration 1 μg/ml) was preactivated with 20 mM DTT. ADP-ribosylated proteins were separated via SDS-PAGE gels (33) and detected by autoradiography.

RESULTS

PTX induces rapid ERK activation in endothelium. We initially assessed whether MAP kinases participate in PTX-induced endothelial cell signal transduction and cellular activation. Figure 1 depicts the rapid increase in p42, p44 ERK activation elicited by PTX as detected by either immunoblotting with an antibody that only recognizes ERKs phosphorylated at Thr-183 and Tyr-185, a requirement for full enzymatic activity (2, 39) (Fig. 1*A*) or an in-gel MAP kinase assay using MBP as substrate (Fig. 1*B*). PTX-induced ERK activation was evident in serum-starved endothelium (maximal at 5 min) as well as in cells challenged in complete medium (maximal at 15 min), with a steady decline to basal or below basal levels thereafter (Fig. 1*C*). Near-maximal stimulation was observed with concentrations as low as 10 ng/ml (Fig. 1*D*).

PTX-mediated ERK activation does not involve ADP ribosylation. We have previously shown that PTX catalyzes the ADP ribosylation of 40-kDa G proteins in human and bovine endothelial cells that are not substrates for ADP ribosylation by other bacterial toxins (18, 37). These 40-kDa proteins have previously been shown to comigrate with a band that is immunoreactive with antibodies directed against a synthetic peptide corresponding to an amino acid sequence common to all known Gα proteins and migrates to the expected position of $G_i\alpha$ (18). Prior studies also indicated that maximal PTX-mediated Gα ADP ribosylation occurs at 1–2 h (16), a time frame that differs markedly from that of the maximal PTX-mediated ERK activation (5–15 min) shown in Fig. 1. To assess the linkage between ADP ribosylation and ERK activation, we next performed experiments to carefully detect the earliest evidence of PTX-mediated ADP ribosylation in endothelium. Lysates were retrieved after several defined periods of PTX exposure (1 μg/ml) to allow endogenous ADP ribosylation, which is assessed by comparison with the magnitude of the subsequent activated

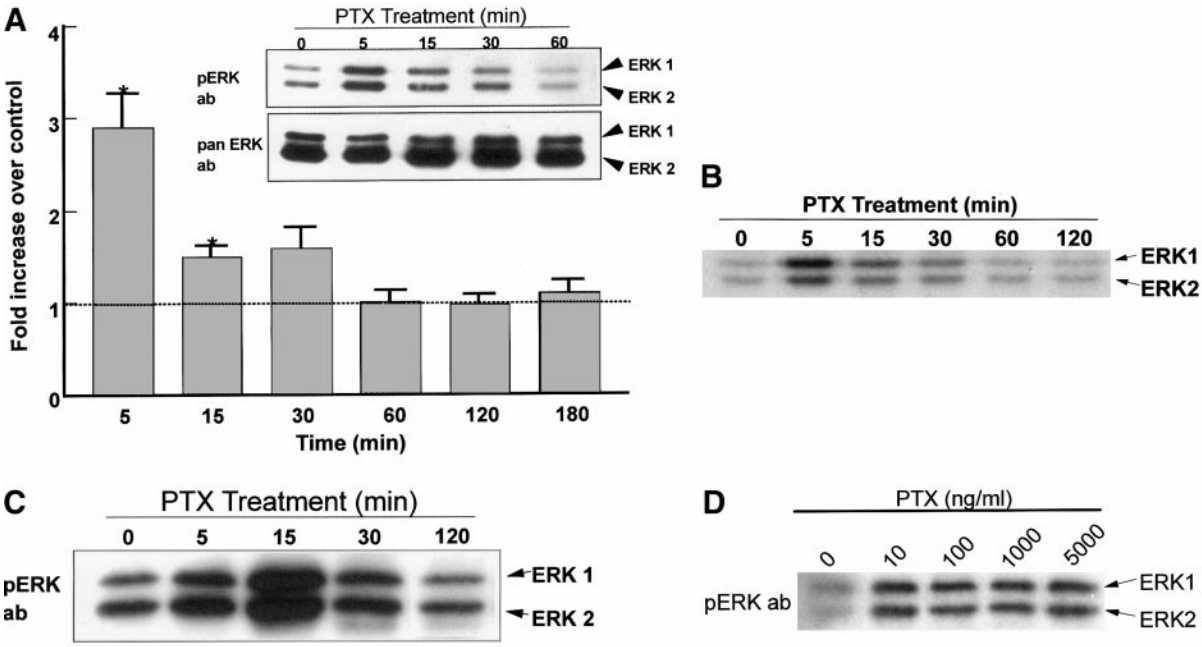

Fig. 1. Effect of pertussis toxin (PTX) on mitogen-activated (MAP) kinase activity in bovine pulmonary artery endothelial cell monolayers. *A*: confluent bovine endothelium from 35-mm dishes were serum-starved for 20 h and then challenged with 1 μg/ml PTX for the indicated periods of time. After treatment, cells were lysed with SDS lysis buffer, scraped off dishes, heated for 5 min, and microcentrifuged. The supernatant was used for Western immunoblotting analysis with phospho-specific extracellular signal-regulated kinase (p-ERK) and pan-ERK antibodies (ab). Plot represents data averaged from 4 independent experiments quantitated by scanning densitometry. $^*P < 0.05$ vs. control. *Inset*: representative Western blot. PTX significantly increased ERK activity in a time-dependent manner. *B*: similar to results in *A*, PTX induced maximal ERK activity as detected by an in-gel assay utilizing myelin basic protein (MBP) as the in-gel substrate. *C*: similar to *A*, confluent bovine endothelium was challenged with 1 μg/ml PTX but without serum starvation, and ERK activity was assessed by p-ERK blotting. *D*: increasing concentrations of PTX were added to confluent serum-starved endothelium and then analyzed by Western blotting with phospho-specific ERK antibodies as described in *A*. Concentrations of PTX as low as 10 ng/ml produce near-maximal ERK activation.

PTX-induced ADP ribosylation assessed in vitro. These studies confirmed that ADP ribosylation evoked by PTX in intact cells begins after 30 min and continues to increase up to 120 min (Fig. 2). Thus PTX-induced ADP ribosyltransferase activity occurs well after the point of maximal ERK activation (5–15 min), suggesting that Gα ADP ribosylation is unlikely to account for PTX-mediated ERK activation in endothelium. Because of the important implication of these findings, two strategies were next used to further confirm the lack of involvement of G protein ADP ribosylation in PTX-induced ERK activation in endothelium. One series of experiments utilized the purified β-oligomer binding subunit, which is the PTX component responsible for cellular binding and facilitating toxin entry into the cell but which is devoid of ADP ribosyltransferase activity (24). Figure 3*A* depicts the time-dependent increase in ERK activation produced by the PTX β-oligomer-binding subunit over control values, which mirrors the holotoxin effects. The β-oligomer preparation is reported by the manufacturer (List Biological Laboratories) as potentially containing up to 0.01% contamination by the PTX holotoxin, resulting in a holotoxin concentration of <0.01 ng/ml. Although we found that this toxin concentration does not affect endothelial cell ERK activity (data not shown), we utilized a second strategy employing a genetically engineered PTX holotoxin S_1 mutant with two site-directed mutations that totally eliminates ADP ribosyltransferase activity (40). Similar to the β-oligomer, the S_1 PTX mutant significantly increased ERK activation in a time-dependent fashion that closely mimicked the native holotoxin (Fig. 3*B*). Together, these results strongly indicate that PTX effects on MAP kinase activity are completely independent of Gα ADP ribosylation and suggest that ligation of a PTX receptor on the cell surface by the PTX binding subunit is sufficient to activate ERK.

Independence of ERK activation from Ras and Raf-1 activity. One well-recognized pathway for ERK induction is via G protein βγ-subunit activation of Ras GTPases, which increase Raf-1-mediated phosphorylation of MEK and subsequent ERK activities (22, 50, 55). Although, in general, these responses have been noted in response to ligation of specific G protein-coupled receptors, we next examined whether PTX employs G protein βγ-subunit interaction with p21 Ras to increase ERK activities. Endothelial cell monolayers were cotransfected with plasmids encoding HA-ERK2 and a βARK minigene encoding a peptide that serves as a dominant negative for βγ-subunit activities (21, 29). Epitope-tagged ERK was immunoprecipitated,

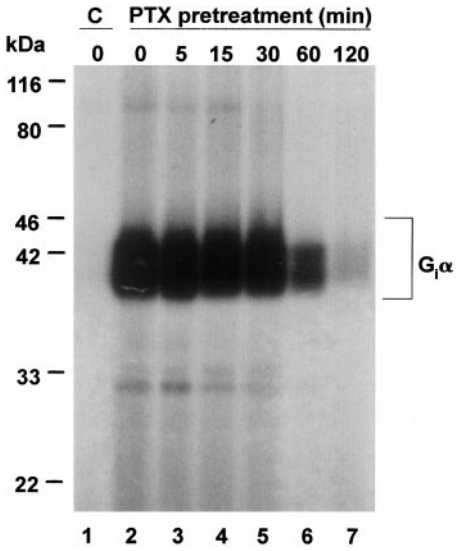

Fig. 2. Time course of PTX-mediated ADP ribosylation of endothelial cell G proteins. Endothelial cell monolayers in 100-mm dishes were incubated with either vehicle (*lanes 1* and *2*) or 1 μg/ml PTX (*lanes 3–7*) for the indicated periods of time, lysed, and homogenized, followed by centrifugation at 100,000 *g*. Pellets were collected, and 60 μg of protein were used for in vitro PTX-catalyzed (1 μg/ml) ADP ribosylation reaction (except for control, *lane 1*). The reactions were carried out in the presence of 5 μCi of ^{32}P-labeled NAD at 30°C for 30 min. Proteins were TCA precipitated and electrophoresed on 15% SDS-PAGE. The resulting gel was dried and exposed to Kodak X-OMAT film for 5 h. *Lanes 1* and *2* were not pretreated with PTX, and PTX was omitted from the in vitro reaction mixture that was loaded in *lane 1* but was present in the reaction mixture loaded in *lane 2*. *Lanes 3–7* represent samples derived from monolayers pretreated with PTX, which results in diminished incorporation of ^{32}P during subsequent in vitro PTX-catalyzed ADP ribosylation. Together, these results indicate that PTX-induced ADP ribosylation begins after 30 min of PTX challenge, a time frame that markedly differs from the time course of ERK activation shown in Fig. 1.

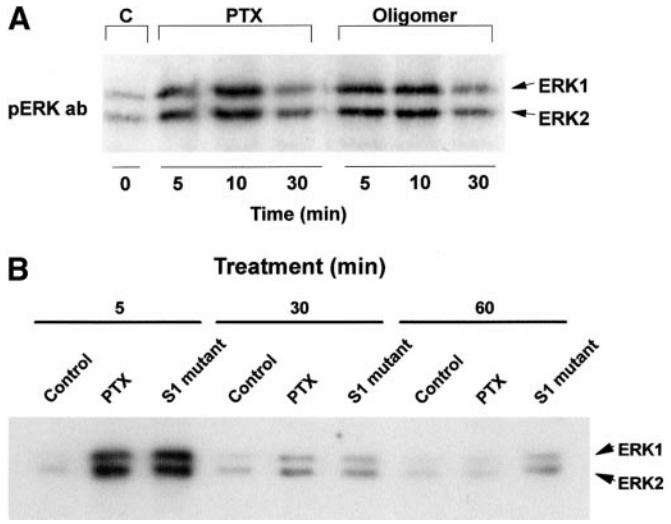

Fig. 3. Effect of PTX, β-oligomer, and the S_1 PTX mutant on endothelial cell MAP kinase activation. *A*: endothelial cell monolayers were treated with 1 μg/ml PTX or 0.734 μg/ml β-oligomer (equimolar amount) for 5, 10, and 30 min. The soluble cell lysates containing comparable total protein were used for Western blotting with phospho-specific ERK1/ERK2 antibodies. *B*: endothelial cell monolayers were treated for 5, 30, or 60 min with PTX (1 μg/ml)or an S_1 PTX mutant (1 μg/ml) that is completely devoid of ADP ribosyltransferase activity. The soluble cell lysates containing comparable total protein were used for Western blotting with phospho-specific ERK1/ERK2 antibodies. These data confirm that ADP ribosyltransferase activity is not required for PTX-induced endothelial cell ERK activation.

and enzymatic activity was assessed by in vitro MBP phosphorylation. These studies demonstrated that βγ inhibition does not significantly alter PTX-induced HA-ERK activity, whereas LPA-stimulated ERK activation was significantly attenuated by βγ inhibition with the βARK minigene (Fig. 4). Consistent with these results, measurements of Ras-associated GTP levels were not increased after PTX (Fig. 5), indicating the absence of Ras activation, although there was substantial evidence of increased Ras GTP content after the PKC-activating phorbol ester (PMA) (Fig. 5 and Table 1). Together, these studies indicate that PTX-mediated ERK activation does not follow a Ras GTPase-dependent pathway.

ERK activity, in response to growth factor and tumor promoter stimulation, is known to depend on Src and phosphatidylinositol 3′-kinase (PI 3K) activities (4, 6, 11, 51). However, our experiments with specific Src and PI 3K inhibitors (PP-2 and LY-294002, respectively) do not support involvement of these kinases in PTX-induced ERK activation (data not shown). In addition, phorbol ester- and growth factor-mediated ERK stimulation is dependent on PKC-mediated phosphorylation of Raf-1, a serine/threonine kinase situated upstream to the ERK kinase MEK (5, 12, 30, 31, 45). To explore this potential signal sequence, we pretreated endothelial cell monolayers with the PKC inhibitor bisindolylmaleimide, which significantly reduced PTX-induced ERK activation (Fig. 6). However, kinase activity in Raf-1 immunoprecipitates obtained from PTX-stimulated endothelial cell monolayers and assessed by quantifying MBP phosphorylation was not increased compared with PMA (Fig. 7) and was similar to control values from vehicle-stimulated monolayers, suggesting that, unlike PMA, PTX-induced ERK activation may proceed via a Raf-1-independent pathway. Consistent with this notion, treatment of endothelial cells with forskolin, which decreases ERK activity via cAMP-mediated inactivation of Raf-1 (10, 20, 56), de-

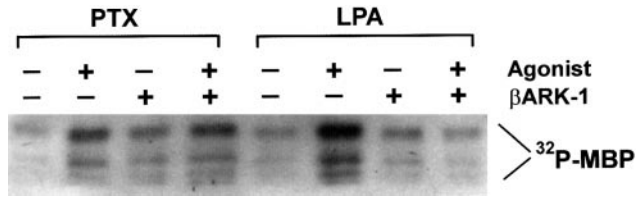

Fig. 4. Effect of G protein βγ-subunit inhibition on PTX-induced ERK activation. Endothelial cells were cotransfected with plasmids encoding hemagglutinin (HA)-tagged ERK 2 and the Gβγ-binding domain of the β-adrenergic receptor kinase (βARK-1). After transfection, the cells were treated with either vehicle, PTX (1 μg/ml), or lysophosphatidic acid (LPA; 1 μM). The cell lysates were immunoprecipitated with HA antibody, and the immune complexes were used for an in vitro kinase assay utilizing MBP as substrate. Phosphorylation of MBP was visualized by gel electrophoresis followed by autoradiography. Unlike LPA, PTX-induced ERK activation does not involve G protein βγ subunit-mediated activation.

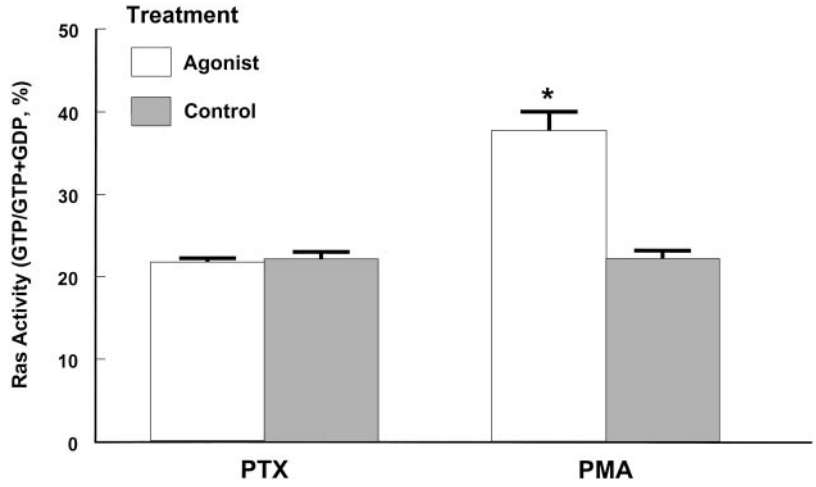

Fig. 5. Effect of PTX on Ras activity. Bovine pulmonary endothelial cells were incubated with either PTX (1 μg/ml agonist) or phorbol 12-myristate 13-acetate (PMA; 100 nM positive control) for 5 min. The cell lysates were subjected to immunoprecipitation with 14 μg of $p21^{ras}$ antibody. Equal amounts of protein were loaded, and the active GTP-bound Ras and the inactive GDP-bound Ras were separated by thin-layer chromatography. Ras activity is presented as the ratio of active Ras to total Ras. Data are means ± SE for 5 independent experiments. Unlike PMA, PTX does not affect Ras activity.

creased the basal level of ERK activity but did not significantly alter PTX-induced ERK activation (data not shown). In contrast to the lack of Raf-1 involvement, PTX-induced MEK activation appears to be essential to subsequent increases in ERK activity, because PD-098059, an inhibitor of the upstream ERK-activating dual kinase MEK (13), abolished PTX-induced ERK activation (Fig. 8*A*). These studies were confirmed by manipulating the activity of ERK in vivo by transiently expressing a dominant-negative MEK construct that is unable to be phosphorylated by substitution of the regulatory serine phosphorylation sites with alanine, thereby inhibiting signaling through the ERK pathway (57). Endothelial cell cotransfection of a plasmid encoding this MEK mutant with HA-ERK2 established a causal relationship between PTX-mediated MEK activation and subsequent ERK activation (Fig. 8*B*).

Table 1. *Effect of PTX on p21 Ras activation in bovine pulmonary artery endothelial cells*

	p21 Ras Activity, %		
Agent	1 min	5 min	30 min
Control	25	22	19
PTX	25	22	18
PMA	ND	40	ND
FPT	ND	ND	5

Endothelial cells were incubated with either pertussis toxin (PTX; 1 μg/ml) or phorbol 12-myristate 13-acetate (PMA; 100 nM, positive control) for 5 min, and then cell lysates were subjected to immunoprecipitation with 14 μg/ml $p21^{ras}$ antibody. Equal amounts of protein were loaded, and the active GTP-bound Ras and the inactive GDP-bound Ras were separated by thin-layer chromatography. Ras activity is presented as the ratio of active Ras to total Ras. Data are means for 5 independent experiments (in each case, the SE was <2%). Ras activity was determined in ^{32}P-labeled endothelial cell monolayers by thin-layer chromatography (as described in METHODS) and expressed as the percentage of Ras-associated radiolabeled GTP. The control Ras GTP values shown for comparison for 1–30 min ranged from 19 to 28%. There was no significant alteration in Ras activity induced by PTX. In contrast, PMA (100 nM) rapidly increased Ras activity, and the Ras farnesyltransferase inhibitor FPT significantly attenuated Ras GTP levels. ND, not determined.

DISCUSSION

Pertussis infection remains a worldwide health problem affecting target tissues such as the respiratory tract, with many of these pathological features directly attributed to the holotoxin generated during this bacterial infection (24, 41). We have previously noted that PTX elicits substantial endothelial cell activation with increases in paracellular gap formation and loss of semiselective vascular barrier properties (37, 38). Although the exact signaling pathways by which PTX produces endothelial cell activation were previously elusive, we have now identified a major role for the 42- and 44-kDa MAP kinases known as ERK. We monitored ERK activity *1*) by immunoprecipitating the ERK kinase and measuring its activity toward an in vitro substrate, MBP (the most direct measure of its activity); *2*) by Western blotting with antibodies that specifically recognize the phosphorylated and, hence, activated form of the kinase; and *3*) by performing an in-gel assay of MBP phosphorylation. These studies unequivocally demonstrate that PTX is a rapid and potent inducer of ERK activation. We also initiated studies to more precisely define the cellular events evoked by PTX that lead to ERK activation in endothelium. Because disruption of signaling pathway by PTX is often used as evidence that ADP ribosylation of specific heterotrimeric G proteins is involved, our initial experiments assessed this pathway. As noted before, the pertussis holotoxin consists of the A protomer, which contains ADP ribosyltransferase activities (24), whereas the β-oligomer binds the toxin to target cells and increases the efficiency of noncovalently bound S_1 entry and translocation to target sites (24, 53). We utilized commercially available β-oligomer subunits as well as a recombinant S_1 mutant to clarify specific mechanisms by which PTX may increase MAP kinase activities. Interestingly, the S_1 mutant and the catalytically inactive β-oligomer of the toxin completely reproduced the holotoxin effects on ERK activation. In addition, comparison of the temporal sequences of PTX-mediated MAP kinase activities and ADP ribosy-

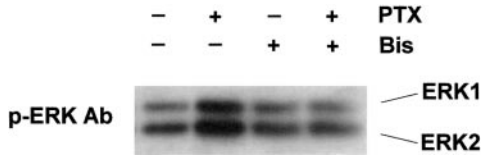

Fig. 6. Effect of protein kinase C (PKC) inhibition on PTX-induced ERK activation. Bovine endothelial cells were preincubated with either vehicle (0.1% DMSO) or the specific PKC inhibitor bisindolylmaleimide (Bis, 1 μM) for 1 h and then challenged with PTX (1 μg/ml) for 5 min. ERK activation was assessed by Western immunoblotting with specific p-ERK antibody. Representative blot (n = 3) demonstrates that PKC inhibition significantly attenuates PTX-induced ERK activation in endothelium.

lation argues effectively that PTX effects on endothelial cell ERK activation are entirely independent of G protein ADP ribosylation. These data are consistent with limited reports suggesting that mere binding of the β-oligomer to eukaryotic cells can alter cellular function independently of ADP ribosylation (27, 46–48, 53). For example, purified β-oligomer induces mitogenic stimulation of human T cells (46, 48), enhances glucose oxidation in adipocytes (53), promotes influx of extracellular Ca^{2+} (38), and promotes leukemic cell adhesion (52). Although the exact identity of these binding sites on endothelium remains unknown, carbohydrate moieties have been speculated to be crucial components of β-oligomer binding sites (24). Given the recent report that PTX appears to induce leukemic cell adhesion via integrin receptor (CD11/CD18) binding (54), it is tempting to speculate that integrin ligation is directly coupled to signaling pathways that can initiate MEK activity as well as other signaling cascades in PTX-challenged endothelium. Further studies are required to evaluate this stimulus/coupling pathway.

We had initially hypothesized that PTX-induced ERK activation might follow a well-recognized activation sequence of G protein βγ-subunit release after

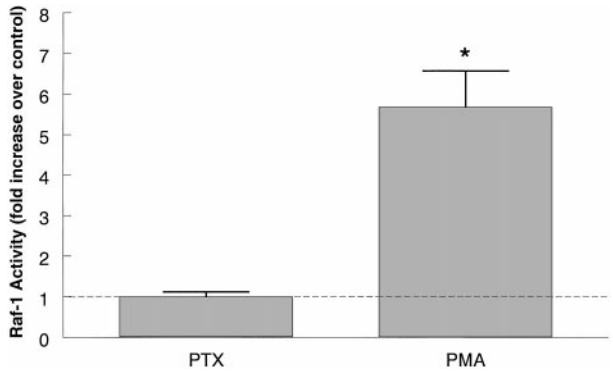

Fig. 7. Effect of PTX on Raf-1 activity. Confluent endothelial cell monolayers were serum-deprived for 18 h and then incubated with either vehicle (PBS) or PTX (1 μg/ml) for 5 min. As a positive control, endothelial cells were incubated with 100 nM PMA or 0.1% DMSO (vehicle) for 5 min. Raf-1 was immunoprecipitated from cell lysates by an antibody directed against the COOH terminus of human c-Raf kinase. Raf-1 activity was measured using a Raf-1 kinase cascade assay as described in METHODS. Data are presented as means ± SE; n = 3. *P < 0.05 vs. basal activity (dotted line). Whereas phorbol ester-stimulated PKC activation produces a >5-fold increase in Raf-1 activity, PTX does not increase Raf-1 activity.

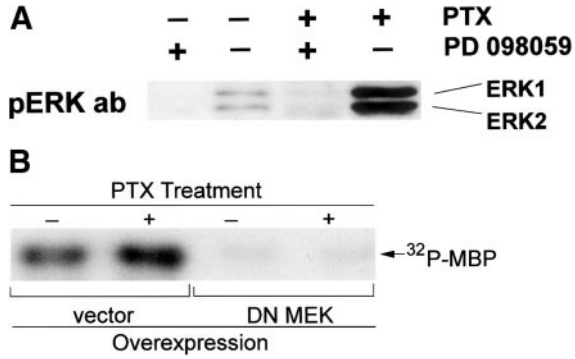

Fig. 8. Effect of MEK (the MAP kinase kinase) inhibition on PTX-induced ERK activation. *A*: confluent endothelial cells (n = 3) pretreated with either vehicle (0.1% DMSO) or PD-098059 (50 μM) for 30 min were stimulated with PTX (1 μg/ml) for 5 min. ERK activity was determined as described in METHODS. Specific inhibition of MEK by PD-098059 significantly attenuated the basal level of ERK phosphorylation and completely abolished PTX-induced ERK activation. *B*: endothelial cells were cotransfected with HA-ERK (vector) and a construct encoding an MEK dominant-negative protein (DN MEK). The PTX-stimulated ERK activity in HA-ERK immunoprecipitates is completely abolished by MEK inhibition (n = 3).

receptor occupancy, followed by Ras- and Raf-1-dependent pathways leading to the sequential activation of MEK and ERK. We cotransfected HA-ERK2 with the βARK minigene encoding a peptide that functions as a dominant negative for βγ-mediated activation sequences. The βARK minigene totally abolished receptor-mediated ERK activation elicited by LPA, an important platelet-derived phospholipid growth factor, but did not attenuate MAP kinase activity after PTX, suggesting that PTX does not stimulate ERK via a pathway involving G protein subunit dissociation. Consistent with the results obtained with the βARK minigene, the PTX-induced ERK response appears to be Ras independent, as convincingly demonstrated by direct measurements of Ras activity.

It was not unexpected that PTX-induced ERK activity is dependent on the activation of the dual-specificity kinase MEK. Growth factors induce ERK activation when activated Raf kinase phosphorylates two regulatory serine residues (Ser-218, Ser-222) on MEK1, which facilitates MEK-mediated phosphorylation of the regulatory threonine (Thr-183) and tyrosine (Tyr-185) residues on ERK2. ERK phosphorylation at these sites increases ERK catalytic activity, whereas removal of either phosphate eliminates this activity (2). PTX-mediated ERK activation was abolished by the synthetic MEK inhibitor PD-98059 as well as by expression of a MEK dominant-negative construct. These results are entirely consistent with the notion that MEK1 participates in the signaling pathway utilized by PTX to completely produce ERK activation in cultured bovine endothelium.

The mechanism by which MEK is activated independently of Ras and Raf-1 after PTX challenge is not clear but appears to require PKC involvement as described in other cell types (9, 25, 32, 34, 42, 43). Ras- and Raf-1-independent ERK activation has been reported (44), and although not addressed in our study, our data

appear to suggest a role for B-Raf or other Raf-like molecules including MEKK2, MEKK3, and the p21-activated kinase (PAK) in the MEK-dependent endothelial cell ERK activation as noted in nonoverlapping studies (3, 14). We had previously noted in bovine endothelium that PTX produces substantial PKC activation and translocation to the plasma membrane in a temporal sequence compatible with a role for PKC in PTX-induced endothelial cell barrier dysfunction (38). The exact mechanism by which PTX accomplishes PKC activation as well as the exact PKC isoforms involved in this response is also unclear. In addition to conventional PKC isotypes, PKC δ- and ζ-isoforms have been suggested to participate in MAP kinase regulation in specific cell systems (9, 34, 42). Recently, we described ERK activation in response to the PKC-activating phorbol esters, which proceeded in a Ras- and Raf-1-dependent fashion. While this certainly suggests PKC isotype-specific activation after PTX and phorbol esters, further work is needed to fully understand the complex regulatory mechanism that involves ERK activation.

In summary, we have explored early signaling events involved in PTX-induced endothelial cell activation and have identified a signaling cascade involving MEK and PKC in the enhancement of ERK MAP kinase activities. PTX-induced ERK activation was completely independent of Ras or Raf-1 activities and does not depend on Gα ADP ribosylation. The physiological importance of PTX-mediated ERK activation in human disease is unknown but is under study. However, ERK has been noted to regulate the stability of the endothelial cell-cell junctions and force development (26, 28), suggesting that PTX may utilize ERK-modified cytoskeletal targets (1) in a manner relevant to human lung epithelial or endothelial cell barrier dysfunction. Our results, which demonstrate rapid ERK activation after cellular interaction with PTX, provide a provocative and potentially important mechanism by which PTX may promulgate the inflammatory response to this bacterial infection, resulting in significant increases in mucosal and vascular permeability.

We gratefully acknowledge the contributions of Drs. Joel Moss (National Institutes of Health Pulmonary and Critical Care Branch), Rino Rappuoli (Sienna, Italy), W. J. Koch (Duke University, Durham, NC), R. Pestell (Albert Einstein College of Medicine, Bronx, New York), and M. Rosner (University of Chicago, Chicago, Illinois) for generously providing reagents for this study, Lakshmi Natarajan and Anila Ricks-Cord for superb technical assistance, and Ellen G. Reather for expert manuscript preparation.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-50533 and HL-58064 and by the Dr. David Marine Endowment (J. G. N. Garcia).

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