Permeabilization in a cerebral endothelial barrier model by pertussis toxin involves the PKC effector pathway and is abolished by elevated levels of cAMP

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Accepted 21 January 2003
Journal of Cell Science 116, 1837-1846 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00378

Summary

Respiratory tract infections caused by *Bordetella pertussis* are occasionally accompanied by severe neurologic disorders and encephalopathies. For these sequelae to occur the integrity of cerebral barriers needs to be compromised. The influence of pertussis toxin, a decisive virulence factor in the pathogenesis of pertussis disease, on barrier integrity was investigated in model systems for blood-liquor (epithelial) and blood-brain (endothelial) barriers. While pertussis toxin did not influence the barrier function in *Plexus chorioideus* model systems, the integrity of cerebral endothelial monolayers was severely compromised. Cellular intoxication by pertussis toxin proceeds via ADP-ribosylation of α-G$_i$ proteins, which not only interferes with the homeostatic inhibitory regulation of adenylate cyclase stimulation but also results in a modulation of the membrane receptor coupling. Increasing intra-endothelial cAMP levels by employing cholera toxin or forskolin even inhibited the pertussis toxin-induced permeabilization of endothelial barriers. Therefore, pertussis-toxin-induced permeabilization has to be mediated via a cAMP-independent pathway. To investigate potential signalling pathways we employed several well established cellular drugs activating or inhibiting central effectors of signal transduction pathways, such as phosphatidylinositol 3-kinase, adenylate cyclase, phospholipase C, myosin light chain kinase and protein kinase C. Only inhibitors and activators of protein kinase C and phosphatidylinositol 3-kinase affected the pertussis toxin-induced permeability. In summary, we conclude that permeabilization of cerebral endothelial monolayers by pertussis toxin does not depend on elevated cAMP levels and proceeds via the phosphokinase C pathway.

Key words: Cerebral endothelial barriers, Pertussis toxin, Transient permeabilization, PKC, cAMP

Introduction

The secreted pertussis toxin (PT) is a decisive virulence factor of *Bordetella pertussis,* the causative agent of the childhood disease whooping cough. PT is organized according to the A-B structural principle which is typical for numerous bacterial toxins (Krueger and Barbieri, 1995; Tamura et al., 1983). The A-protomer consists of a single polypeptide (S1) that mediates ADP-ribosylation of the α-subunit of several heterotrimeric inhibitory G proteins. This modification blocks the inhibitory effect of G$_i$ proteins on adenylate cyclase and in this way not only interferes with the homeostatic inhibitory regulation of adenylate cyclase stimulation but also results in a modulation of G-protein-mediated signal transduction, a central step in cellular communication (Katada and Ui, 1982a; Katada and Ui, 1982b). However, intoxication by PT alone exhibits only minor changes in basal cAMP levels (Weiss and Hewlett, 1986; Glineur and Locht, 1994). The B-oligomer mediates binding and uptake of PT by target cells and the translocation of the S1 subunit. PT recognizes sialoglycoproteins on many if not all types of cells (el Bayâ et al., 1995; Armstrong et al., 1994; Brennan et al., 1988). Additionally, Hausmann and Burns (Hausmann and Burns, 1993) reported binding of PT to glycolipids harbouring terminal sialic acids. However, quartz crystal microbalance measurements investigating PT interactions with gangliosides did not support direct binding to glycolipids (Janshoff et al., 1997).

Although the structure-function of PT has been thoroughly investigated, and the toxin is frequently applied in biochemical and pharmacological studies, its role in the onset of systemic disease is still not completely understood. Especially, whether PT might be instrumental in the development of neurological complications that are occasionally observed as a sequelae of pertussis disease has not been elucidated. In the pathogenesis of pertussis-related neurologic disorders an important step might affect the integrity of cerebral barriers represented either by the *Plexus chorioideum* epithelium or the cerebral capillary endothelium. The presence of tight junctions in both barriers severely limits the passage of even small solutes from blood into the brain and the central nervous system (Franke et al., 1999; Wegener and Galla, 1996). Previous studies by Amiel (Amiel, 1976) already showed an increase in cerebral vascular permeability of mice 24 hours after i.v. injection of killed pertussis organisms. Furthermore, PT is frequently used in immunological studies to enhance the onset of autoimmune

disease in experimental animals (Munoz, 1985). Experimental autoimmune encephalomyelitis (EAE), an accepted model for multiple sclerosis, can be induced in genetically susceptible laboratory animals by a single injection of a central nervous system tissue homogenate or purified myelin antigens. The mechanism by which PT might enhance the development of EAE has not been elucidated but it appears to involve an increase of the vascular permeability of the blood-brain-barrier (Linthicum et al., 1982; Yong et al., 1993; Ben-Nun et al., 1997).

To investigate the influence of PT on cerebral barriers we employed tissue culture systems to model cerebral barriers using epithelial *Plexus chorioideus*-derived as well as cerebral endothelial monolayers. Although in both cell types α-G_i proteins were nearly completely ADP-ribosylated by PT, in epithelial monolayers the barrier function was not affected. In contrast, in endothelial monolayers PT substantially enhanced their permeability for the marker protein horse radish peroxidase (HRP). In parallel the transendothelial resistance decreased. Raising cAMP levels with cholera toxin (CT) as well as forskolin proved to be inhibitory for the PT-induced permeabilization. Thus, in cerebral endothelial cells PT appears to act via a different signalling pathway. Activating or inhibiting central effectors with various well established cellular drugs indicated protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3-kinase) to be involved in the PT-mediated permeabilization of endothelial monolayers. Recent studies already indicated PKC as a downstream enzyme of PI3-kinase and a connection between PT-sensitive G proteins and PI 3-kinase activity has been suggested (Vanhaesebroeck et al., 1997; Takeda et al., 1999). This study shows that the PT-induced permeability of cerebral endothelial barriers is mediated through the PKC and PI3-kinase pathways and is abrogated by high cAMP levels. Furthermore, this study implies a potential mechanism for the onset of neurological disorders associated with pertussis disease due to the effect of PT on the integrity of the blood-brain-barrier.

Materials and Methods

Pertussis toxin, cellular drugs and reagents

Pertussis toxin was obtained as a generous gift from Aventis Pasteur (Lyon, France). Secondary antibodies either coupled to alkaline phosphatase, or labeled with fluorescein-isothiocyanat were purchased from Miles Scientific (Naperville, IL) or Nordic Immunology (Tilburg, The Netherlands), respectively. Sialidase from *Clostridium perfringens* and ABTS was obtained from Roche (Mannheim, Germany). N-octylglycosid and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma (St Louis, MO). Prestained molecular mass standards were purchased from BioRad Laboratories (Richmond, CA). ^{32}P-NAD (1000 Ci/mmol) was obtained from Amersham/Buchler (Braunschweig, Germany). Media and solutions used in cell culture systems were obtained from PAA Laboratories GmbH (Cölbe, Germany) and Biochrom KG (Berlin, Germany). Collagen G was purchased from Biochrom (Berlin, Germany). Dispase II from *Bacillus polymyxa* and collagenase/dispase from *Achromobacter iophagus/Bacillus polymyxa* was from Roche (Mannheim, Germany). BAPTA/AM, calcium ionophore A23187, cyclosporin A, okadaic acid and anti-CD62E were purchased from Calbiochem-Novabiochem Corporation (Bad Soden, Germany). Forskolin, phorbol 12-myristate 13-acetate (PMA), U73122, LY294002, 1,2-dioctanoyl-sn-glycerol, H-7, W-7, H-89, ML-7, ML-9, amiodarone, staurosporine, mastoparan, bradykinin, thapsigargin, wortmannin, dibutyryladenosine 3′, 5′-cyclic monophosphate (dbcAMP), horse radish peroxidase (HRP), anti-vinculin, anti-vimentin, anti-α-, β-catenin, anti-α-tubulin, and FITC-phalloidin were obtained from Sigma (St Louis, MO). Anti-zonula occludens-1 protein (ZO-1) was purchased from Nordic immunology (Tilburg, The Netherlands).

Cell lines and tissue culture

Experiments were performed on brain capillary endothelial cells (BCECs) isolated from pig brains in primary and secondary culture. Cells were cultured at 37°C in a humidified atmosphere in DMEM medium with 10% CO_2 supplemented with 10% fetal calf serum, 1 mM L-glutamine, 100 units penicillin/ml and 100 μg streptomycin/ml. SCP, ESP and SP-R cell lines derived from sheep *Plexus chorioideus* were obtained as a kind gift from R. Riebe (BFA für Viruskrankheiten der Tiere, Insel Riems, Germany) and were cultured on laminin-coated Transwell-clear filters (Corning Costar, Bodenheim, Germany).

Isolation of porcine brain capillary endothelial cells (BCECs)

Brain capillary endothelial cells were isolated from pig brains essentially as described by Mischek et al. (Mischek et al., 1989) with minor modifications (Tewes et al., 1997). Pig brains were obtained fresh from a slaughterhouse and transported on ice cold Dulbecco's PBS containing penicillin and streptomycin. Cerebra were cleared of meninges and homogenized mechanically. The brain homogenate was digested enzymatically using 1% (w/v) dispase II from *Achromobacter iophagus/Bacillus polymyxa* in BCEC preparation medium (Medium 199 Earle supplemented with 0.7 mM L-glutamine, 100 μg/ml gentamycin, 100 U/ml penicillin and 100 μg/ml streptomycin; 100 ml medium/brain). After a 2.5 hour incubation at 37°C with continuos stirring, brain capillaries were separated from myelin and cell debris by dextrane density centrifugation. For this, aliquots of the suspension (100 ml) were mixed with 230 ml 15% (w/v) dextrane solution and centrifuged at 5800 ***g*** for 10 minutes at 4°C. Each pellet was resuspended in plating medium (BCEC preparation medium containing 10% v/v ox serum) and triturated using a glass pipette. A second enzymatic digestion with 0.1% (w/v) collagenase/dispase II for 60 minutes at 37°C was performed to remove the capillary basement membrane. The suspension was centrifuged at 100 ***g*** for 10 minutes and the pellet was resuspended in plating medium (10 ml/brain). The endothelial cells released were further purified using a discontinous percoll density gradient. Endothelial cells were collected from the percoll gradient with a pipette, resuspended in plating medium and seeded on collagen G-coated culture flasks at a density of approx. 2×10^5 cells/cm^2. Twenty-four hours after initial plating the cells were washed with Dulbecco's PBS and supplied with BCEC culture medium (BCEC plating medium without gentamycin).

Preparation of cell extracts

Extracts of BCECs were prepared according to Brennan et al. (Brennan et al., 1988). Briefly, cells were grown to confluency, scraped from the tissue culture flasks, centrifuged (200 ***g***, 5 minutes, 4°C) and washed with phosphate buffered saline (PBS). After extraction with 0.2 M n-octylglucoside containing 2 mM phenylmethylsulfonyl fluoride (PMSF) and 50 mM K_3PO_4 with shaking at 4°C for 30 minutes, the extracts were centrifuged at 15,000 ***g*** for 20 minutes in an Eppendorf centrifuge (Model 5403) and the supernatant was collected. For measuring the uptake of PT cells were preincubated in culture with pertussis toxin for various intervals. In some experiments extracted proteins were treated with 40 μg sialidase from *Clostridium perfringens*/ml 50 mM sodium acetate pH 5.0 for 2 hours at 37°C in order to remove terminal sialic acid residues.

In vitro ADP-ribosylation assay

The ADP-ribosylation assay was performed essentially as described by Xu and Barbieri (Xu and Barbieri, 1995) and more recently by el Bayâ et al. (el Bayâ et al., 1995) with minor modifications. PT uptake and intoxication of various cells were investigated by an indirect ADP-ribosylation assay. If PT were routed to its target α-G_i proteins resulting in successful ADP-ribosylation during the incubation period, the target proteins would have been blocked for further PT-mediated modification. Thus, incorporation of ^{32}P-ADP ribose in vitro serves as a measure of the residual α-G_i subunits available compared with controls without prior PT incubation ('subtractive modification'). Cells were extracted as described above and incubated for 2 hours at 20°C with 100 ng activated PT (activated by preincubation for 1 hour at 20°C with 50 mM dithiothreitol in 100 mM Tris-HCl, pH 8.2) and 1 μCi ^{32}P-NAD in 100 mM Tris-HCl pH 8.2, 25 mM dithiothreitol and 2 mM ATP in a final volume of 10 μl. Sample buffer was added (final concentration: 5% glycerol, 0.75% SDS, 2% β-mercaptoethanol, 16 mM Tris-HCl, pH 6.8) and the mixture was heated at 95°C for 10 minutes. After separation by SDS-PAGE (15% polyacrylamide), labeled proteins were visualized and quantified using a bioimager (Phosphoimager Fuji BAS 1000). All assays were performed in at least four independent experiments.

SDS-PAGE and western blotting

Cell extracts were separated by SDS-PAGE with 10% polyacrylamide in buffer containing 5% glycerol, 0.75% SDS, 2% β-mercaptoethanol, 16 mM Tris-HCl (pH 6.8). After protein transfer to nitrocellulose membranes by electroblotting the blots were blocked in 5% BSA/PBS, washed with 0.05% Tween 20/PBS and incubated with the monoclonal antibody 6FX1 (1:100 dilution of a hybridoma supernatant), washed again, and then incubated with goat anti-mouse antibodies coupled to alkaline phosphatase [1:7500; 30 minutes at room temperature (RT)]. Bound antibodies were visualized by enzyme reaction using nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl-phosphate-p-toluidine (BCIP) as substrate.

Measurement of endothelial barrier function

To determine the permeability of cell monolayers endothelial cells were subcultured on polycarbonate filters (10 mm in diameter, 0.4 μm pore size; Transwell, Costar) mounted in Transwell inserts. For subculture, endothelial cells grown on flasks (75 cm^2) were washed twice with Dulbecco's PBS and incubated with 4 ml of a trypsin (0.25%)/ ethylene diamine tetraacetic acid (EDTA; 0.1%) solution until the cells had detached. The suspension was added to 2 ml ox serum to inactivate trypsin and was subsequently centrifuged at 100 ***g*** for 10 minutes at RT. The pellet was resuspended in culture medium (described above) and seeded onto rat tail collagen coated Transwell filters. 24 hours later, the cell layers were washed with PBS and incubated in serum-free medium (DMEM/Ham's F12 supplemented with 550 nM hydrocortisone, 865 nM insulin, 6.5 nM L-glutamine, 100 units penicillin/ml and 100 μg streptomycin/ml). Confluent monolayers formed within 2-4 days after plating at a density of 2×10^5 cells/cm^2.

For measurements of monolayer permeability for horse radish peroxidase (HRP) a solution of serum-free medium containing HRP (30 μg/ml) was added to the medium in the upper or luminal compartment for 1 hour at day 4 of subculture. The transendothelial HRP transport was then assessed by measuring HRP in the medium of the abluminal compartment.

Application of cellular drugs

For the investigation of effector pathways cellular monolayers subcultured for 4 days were treated with different established pharmacological agents at the concentrations given in Table 1. The drugs were applied either alone or in combination with PT. All reagents were incubated for 4 hours before and for one additional hour throughout the permeability assay (total incubation time 5 hours). All reagents had been evaluated for potentially toxic activities at the concentration applied during the time course of the experiment. The analysis were performed in at least four independent experiments.

Measurements of transendothelial resistance (TER)

The TER was measured using a custom made epithelial tissue voltohmmeter (Evom, World Precision Instruments, Sarasota, FL), where the chambers for measurements are designed specifically for use with Transwell filters. Confluency of the monolayers and barrier properties were documented by measuring transendothelial resistance (200-1000 Ohm cm^2). TER values of cell layers obtained from different BCEC preparations varied from ~200 to ~1000 Ω cm^2 and reached their maxima on day 4 of subculture.

Measurements of horse radish peroxidase (HRP)

HRP concentrations were determined spectrophotometrically by assaying peroxidase activity using the chromogen ABTS [2,2′-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid)] as substrate. For ABTS-buffer preparation 50 mg ABTS were dissolved in 500 ml of 100 mM citric acid, pH 5.0. Immediately before performing the assay 28 μl

Table 1. Activators and inhibitors of different effector pathways used in this study

Effector	Inhibitor*	Activator*	References
Phospholipase C (PLC)	U73122 (4 μM)		Chin and Chueh, 1998
Phosphatidylinositol 3-kinase (PI3-kinase)	Wortmannin (100 nM), LY294002 (10 μM)		Takeda et al., 1999; Scheid and Duronio, 1996
Myosin light chain kinase (MLCK)	ML-7 (1 μM), ML-9 (50 μM)		Garcia et al., 1995; Zhang et al., 1998
Protein kinase A (PKA)	H-89 (1 μM)		Quest, 1996
Protein kinase C (PKC)	Staurosporine (50 nM), H-7 (50 μM)	PMA (500 nM), dioctanoyl-sn-glycerol (100 μg/ml)	Buchan and Martin, 1992; Narayan et al., 1998; Zhang et al., 1998
Adenylat cyclase		CT (1 μg/ml), forskolin (50 μM)	Garcia et al., 1992; Stelzner et al., 1989
Calcium	BAPTA/AM (2 μM)	Bradykinin (20 nM), thapsigargin (20 nM), A23187 (200 nM)	Garcia et al., 1995
Calmodulin	W-7 (20 μM)		Zhang et al., 1998
G protein		Mastoparan (500 nM), amiodarone (12 μM)	Chahdi et al., 1998; Hagelüken et al., 1995
Phosphatases (PP1, PP2A, PP2B)	Cyclosporin A (150 nM), okadaic acid (25 nM)		Diwan et al., 1997; Wu et al., 1998

*The concentrations of the various inhibitors/activators used in this study are shown in brackets.

H_2O_2 were added to 100 ml of ABTS-buffer. Samples containing an unknown HRP concentration (60 μl) were mixed with 200 μl ABTS-buffer and – after 15 minutes of incubation at RT in the dark – the OD was measured at 405 nm. The HRP that had traversed through the monolayer as indicated by the OD values was taken as a measure of the level of relative permeability.

Measurements of cyclic AMP (cAMP)

Intracellular cAMP levels were determined using a cAMP enzyme immunoassay system (Amersham Pharmacia Biotech, Braunschweig, Germany). Endothelial cells were subcultured on 24-well tissue culture plates for 24 hours. After incubation with medium alone or medium containing PT (200 ng/ml or 1000 ng/ml), CT (1 μg/ml) or forskolin (50 μM) for 2 hours, the medium was removed by aspiration and cells were washed three times with Dulbecco's PBS before cell-lysis was performed using the reagent provided with the cAMP enzyme immunoassay system. An aliquot of the supernatant was transfered to the assay-plate. The assay is based on the competition between unlabeled cAMP and a fixed quantity of peroxidase-labeled cAMP for a limited number of binding sites of a cAMP specific antibody

Distribution of cellular components by immunofluorescence

For the localization of cytoskeletal proteins, endothelial cells were seeded on tissue culture chamber slides (Labtek; Nalge Nunc Int., Wiesbaden, Germany) and grown to confluency. After overnight incubation with medium alone or medium containing PT (200 ng/ml), cells were washed with Dulbecco's PBS three times. Cells were fixed with 4% (w/v) paraformaldehyde in water for 15 minutes followed by a 5 minute incubation of 0.2% (v/v) Triton X-100 solution. Actin microfilaments were stained with FITC-phalloidin a specific stain for F-actin. The distribution of vinculin, vimentin, α-tubulin, α- and β-catenin and zonula occludens-1 proteins was visualized by a 60 minutes incubation with specific monoclonal IgG antibodies directed against vinculin (Sigma, Deisenhofen, Germany), vimentin (Sigma, Deisenhofen, Germany), α-tubulin (Sigma, Deisenhofen, Germany), ZO-1 (Chemicon Int., Single Oak Dr, CA), and α- and β-catenin followed by a 60 minute incubation of FITC-labeled secondary antibodies (goat anti-mouse IgG or goat anti-rabbit IgG; Nordic Immunological Antibodies, Tilburg, The Netherlands). The chamber slides were then separated and the cells were mounted in Mowiol. After 24 hours the cytoskeletal proteins were visualized by immunofluorescence microscopy.

Results

Pertussis toxin entry and intoxication of endothelial and epithelial cells

To demonstrate the activity of PT in epithelial and/or endothelial monolayers, its uptake and G protein modification by PT in various cell types were assessed with the indirect ADP-ribosylation assay by using radioactively labeled NAD as substrate (el Bayâ et al., 1995; Xu and Barbieri, 1995). Epithelial cells (ESP, SP-R, SCP and *Plexus chorioideus*) and BCECs were incubated with pertussis toxin for intervals up to 5 hours. At various time points of PT-incubation the membrane proteins were solubilized and subsequently used in an in vitro-ADP ribosylation assay employing ^{32}P-NAD (Materials and Methods). The amount of ^{32}P-ADP ribose transferred after solubilization is equivalent to the residual unmodified α-G_i subunits still available in the cellular extracts after the given time of incubation with PT. The intracellular target proteins were nearly completely ADP-ribosylated in all cell lines and in primary *Plexus chorioideus* as well as BCECs in less than 2 hours with half-lifes (el Bayâ et al., 1999) ranging from about 30 minutes (ESP, SP-R, SCP and *Plexus chorioideus*) to 75 minutes (BCEC; Fig. 1). PT has to be routed by the retrograde transport mechanism to achieve efficient intoxication (el Bayâ et al., 1995; Xu and Barbieri, 1995; el Bayâ et al., 1999). To compare the time course of ADP-ribosylation and to demonstrate the necessity of Golgi transition also in BCECs (el Bayâ et al., 1997), the effect of interference by brefeldin A is shown in Fig. 1.

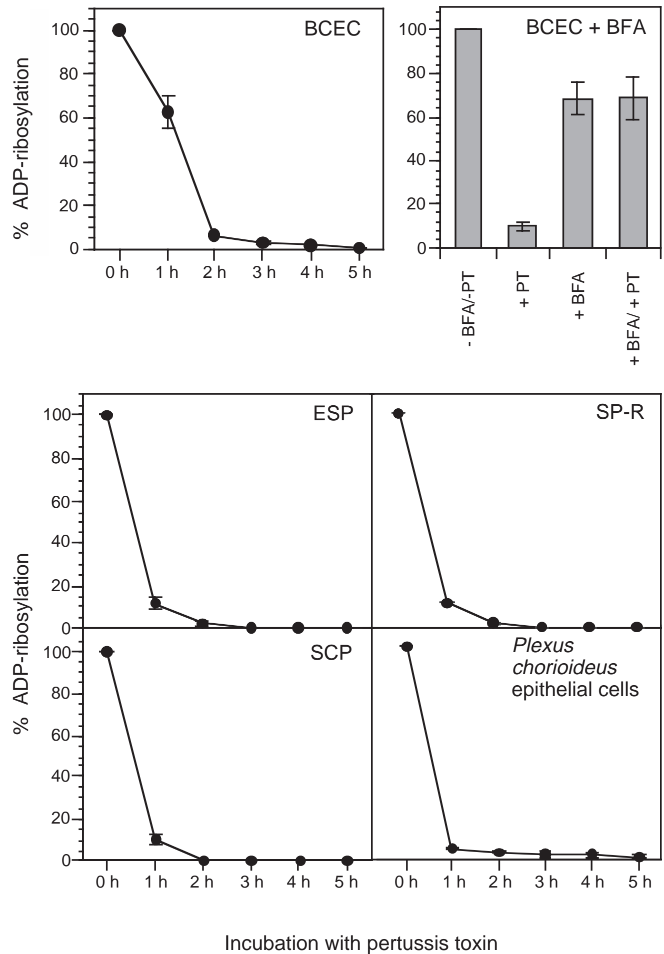

Fig. 1. In vitro ADP-ribosylation of available α-G_i subunits extracted from BCEC, various *Plexus-chorioideus*-derived epithelial cell lines (ESP, SP-R and SCP), and isolated porcine *Plexus chorioideus* epithelial cells (lower panels) after preincubation with PT in culture. Cells at 80% confluency were incubated with 200 ng/ml PT for up to 5 hours and the solubilized membrane proteins were used as substrate in an in vitro ADP-ribosylation assay with 140 ng activated PT. ^{32}P-ADP-ribose labeled target proteins were measured using a Phosphoimager. All assays were performed in four independent experiments. The standard deviations (error bars, n=4) are indicated. The values have been normalized to the amount of protein employed in the assay. The signals obtained in solubilized cells without prior incubation with PT has been set to 100% for each cell type. The residual ADP-ribosylation was measured after pre-incubation with PT for the indicated time. Prior incubation with Brefeldin A (1 μg/ml) for 1 hour inhibits the PT-mediated ADP-ribosylation as shown for BCECs (top right).

Morphological changes of endothelial cells treated with PT

As in some cells, such as CHO cells (Hewlett et al., 1983), PT induces profound changes in cell morphology; therefore, we investigated its effect in sub-confluent and confluent BCEC monolayers. In sub-confluent BCEC monolayers PT was found to cause elongations of the cell body in a time and dose dependent manner (data not shown). BCECs in confluent and non-confluent cultures were treated with PT concentrations ranging from 1 pg/ml to 2 μg/ml. Already after 2.5 hours, PT-induced changes could be detected in sub-confluent BCECs when the cells were incubated with 100 pg/ml. Incubation of BCECs with up to 1 μg PT/ml for several days did not produce obviously detrimental or toxic effects. Concentrations of PT exceeding 2 μg/ml proved to be lethal for BCECs. However, the distribution of cytoskeletal proteins, such as actin and α-tubulin, or of proteins involved in cell-cell contacts, such as vinculin, vimentin, α-catenin, β-catenin and ZO-1, in confluent BCEC monolayers was not obviously rearranged.

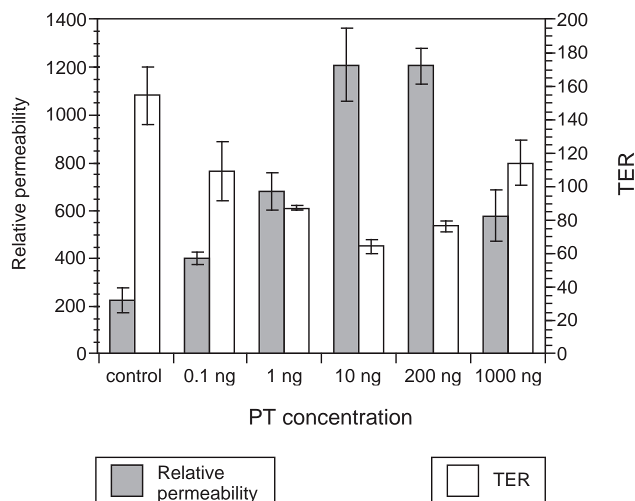

Fig. 2. Transendothelial resistance (TER) and permeability for horse-radish peroxidase (HRP) of BCEC monolayers. TER was measured using a custom-made epithelial tissue voltohmmeter designed for Transwell filter inserts. The transendothelial transport of HRP to the lower compartment was assessed spectrophotometrically. OD values were representative of the traversed HRP and thus for the level of relative permeability. The standard deviations (error bars, n=4) are indicated.

Changes in permeability and transendothelial resistance

Although in confluent monolayers no obvious changes in the investigated cytoskeletal proteins could be observed the effect of PT on cell morphology in sub-confluent monolayers raised the question whether PT might, nevertheless, affect barrier formation by enhancing the permeability of endothelial cell monolayers. To perform permeability studies BCECs were subcultured on Transwell polycarbonate filters. We measured the transendothelial resistance (TER) to monitor the generation and to assess the quality of the barrier formed by the cell monolayers. The resistance of the BCEC monolayers varied with time in culture, and on Transwell filters reached its maximum usually on day 4. To address the influence of PT on the BCEC monolayers the cells were incubated with different concentrations of PT for 12 hours starting on day 3 of subculture. For these experiments only BCEC monolayers exhibiting a transendothelial resistance at least between 250 and 300 Ω cm^2 were used. Measurements of TER and permeability were always performed in at least four independent experiments. The permeability of monolayers induced by the incubation with PT was assessed by the translocation of the 44 kDa horse radish peroxidase (HRP) protein as an enzymatic marker protein. The permeability of BCEC monolayers started to increase with a PT concentration of 0.1 ng/ml, reaching a plateau at 10-200 ng/ml (Fig. 2). Interestingly, when the concentration of PT was increased further to 1 μg/ml, the TER increased and permeability decreased indicating a reversal of the PT-mediated response. As expected, transendothelial permeability and TER exhibit an inverse relationship (Fig. 2). The PT-induced effect on BCEC monolayers were followed for several hours. The time course of PT-enhanced permeability and the concomitant reduction of TER is shown for a PT concentration of 200 ng/ml (Fig. 3).

To address the question whether the S1-subunit or the B-oligomer of PT is responsible for the PT effect, cell monolayers were incubated with the isolated B-oligomer alone instead of whole PT. No changes in permeability or TER were observed (data not shown). This clearly indicated that the PT effect on cerebral endothelial cells is due to the activity of the S1 subunit.

The PT-mediated increase in permeability is not due to elevated intracellular cAMP levels

Cellular intoxication by PT is thought to proceed via the ADP-ribosylation of heterotrimeric G proteins that interferes with the homeostatic inhibitory regulation of adenylate cyclase, potentially leading to non-physiological levels of intracellular cAMP. In most cells, including the BCECs in this study, however, PT itself only induces an incremental increase in cAMP (e.g. Weiss and Hewlett, 1986; Glineur and Locht, 1994). To investigate whether the observed

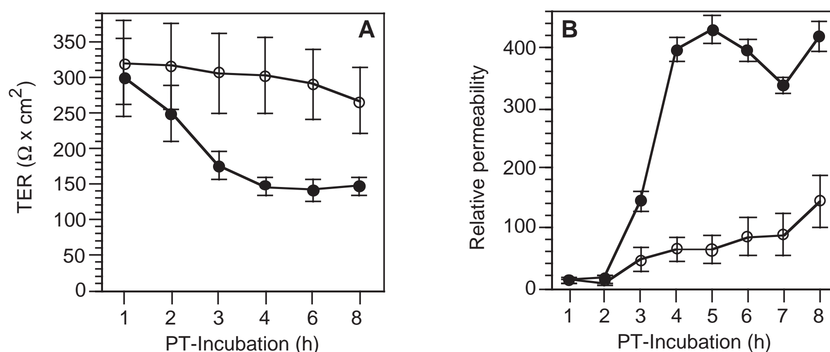

Fig. 3. Time course of PT-enhanced permeability and reduced TER. BCEC monolayers were exposed to medium or medium containing 200 ng/ml PT for the times indicated. TER and permeability for HRP was measured as described in the legend to Fig. 1. The standard deviations (error bars, n=6) are indicated.

increase in permeability might be affected by elevated cAMP levels we employed cholera toxin (CT) and forskolin for the direct stimulation of the adenylate cyclase. Both reagents efficiently raised the level of cAMP in BCECs (Fig. 4). CT and forskolin alone had no effect on the permeability of BCEC monolayers. However, when CT or forskolin were applied in combination with PT, also no increase in permeability was found (Fig. 5). This demonstrates that elevated intracellular cAMP levels completely abolish the PT-induced permeability of BCEC monolayers. Comparing intracellular cAMP levels after treatment with PT (200 ng/ml or 1000 ng/ml), CT (1000 ng/ml) or forskolin (50 μM), by using a cAMP enzyme immunoassay system, showed that even at a concentration of 1000 ng/ml PT induced only a minor increase in cAMP in BCECs. Incidentally, at this PT concentration the permeability induced at lower concentrations was reversed (Fig. 2). This indicates that higher levels of cAMP stabilize the barrier function of BCEC monolayers.

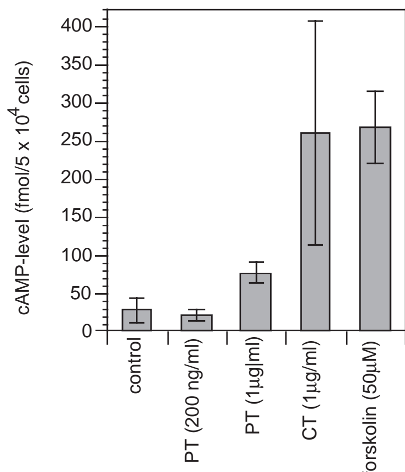

Fig. 4. Measurements of intracellular cAMP levels after treatment with PT, CT or forskolin. BCEC monolayers were incubated with PT (low: 200 ng/ml, high: 1 μg/ml), cholera toxin (CT; 1 μg/ml) or forskolin (50 μM) for 2 hours. Intracellular cAMP levels were determined using a cAMP enzyme immunoassay system (Amersham Pharmacia Biotech, Braunschweig, Germany). The values shown represent the means of analysis performed in triplicate. The standard deviations (error bars, n=3) are indicated.

Identification of effector pathways for PT-mediated permeabilization

PT-mediated intoxication of target cells has been thought to involve elevated levels of the second messenger cAMP. However, PT-induced permeabilization of cerebral endothelial barriers has been shown to be reversed by elevated cAMP levels. Therefore, PT activity in cerebral endothelia must involve other messengers and/or signal transduction pathways. To identify potential pathways involved in steps leading to permeabilization we employed various cellular drugs that are well established in their effect on the activation and inhibition of central effectors (Table 1). For this, BCEC monolayers were incubated with medium, medium containing the drug to be tested or PT (200 ng/ml), or medium containing the drug and PT (200 ng/ml) for 4 hours and for 1 additional hour while performing the permeability assay. Most of the cellular drugs

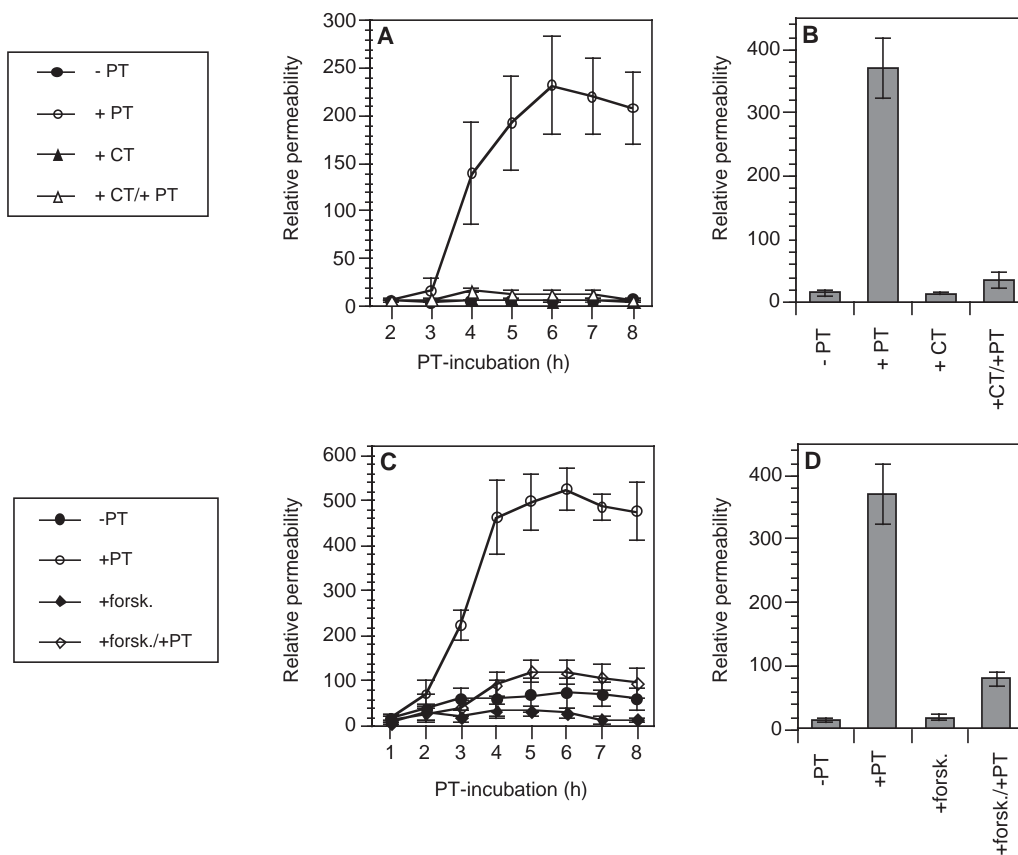

Fig. 5. Influence of elevated cAMP levels on barrier permeability. BCEC monolayers were incubated as indicated with medium or with medium containing PT (200 ng/ml) and/or CT (1 μg/ml) and/or forskolin (50 μM) for 4 hours. The transendothelial HRP transport was assessed by measuring HRP concentrations spectrophotometrically in the lower compartment. OD values were taken as a measure for the traversed HRP indicating the relative permeability. The standard deviations (error bars, n=6) are indicated.

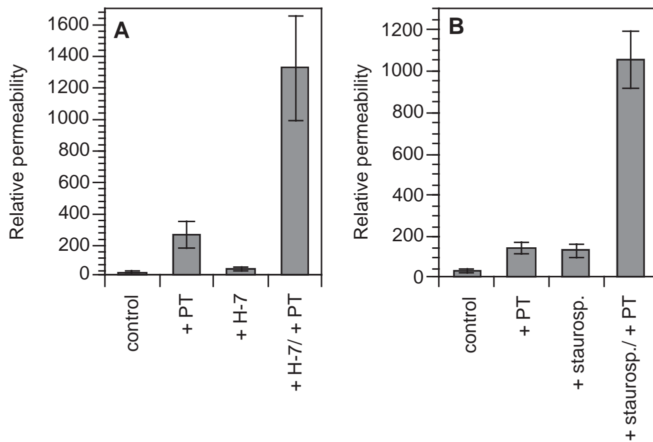

Fig. 6. H-7 and staurosporine enhance the PT-mediated permeabilization of BCECs. BCEC monolayers were exposed to medium, medium containing the drug H-7 (50 µM), staurosporine (50 nM), or PT (200 ng/ml) alone or medium with H-7 in combination with PT for 4 hours (+1 hour for the permeability assay). The transendothelial HRP transport was assessed by measuring HRP concentration spectrophotometrically in the lower compartment. OD values were representative of the traversed HRP as a measure of relative permeability. The standard deviations (error bars, *n*=6) are indicated. (A) Time-course of relative permeability upon incubation with PT and PKC inhibitors H-7 or staurosporine. (B) Effect of PKC inhibitors H-7 and staurosporine on the PT induced permeability after 4 hours of incubation.

investigated had no effect on the PT-induced permeability (data not shown). Interestingly, however, treatment of BCEC monolayers with staurosporine (100 nM) or H-7 (50 µM), which are both used as inhibitors of protein kinase C (PKC), in combination with 200 ng/ml PT enhanced the permeability for HRP substantially (Fig. 6). Control experiments performed with staurosporine or H-7 alone by incubating BCEC monolayers had no effect on permeability. If PKC is crucial in the PT-induced pathway leading to permeabilization this would mean that activation of PKC should significantly reduce the PT-induced permeability increase. This is exactly what we found when we used PMA (500 nM) and dioctanoyl-sn-glycerol (100 µg/ml) as activators of PKC (Fig. 7). Analogous results were obtained by inhibiting PI3-kinase with wortmannin or LY294002 (Fig. 8). Both compounds represent potent and selective inhibitors of PI3-kinase, which enhanced the PT-induced permeability response. Because to our knowledge no biochemical activators of PI3-kinase have been reported the opposite effect could not be investigated. These results strongly suggest that protein kinase C and PI3-kinase are involved in the PT-induced permeability response, where PKC would act as a downstream enzyme of PI3-kinase.

Discussion

Pertussis toxin and neurologic disorders

Pertussis toxin (PT) is a decisive virulence factor of the causative agent of the childhood disease whooping cough, *Bordetella pertussis*. As the toxin acts by the covalent modification of the α-G$_i$ subunits of heterotrimeric G proteins it is widely applied as a tool in biochemical and pharmacological studies for the investigation of signalling pathways involving heterotrimeric G proteins. However, the role of pertussis toxin in systemic disease and especially in the onset of the occasional severe neurological complications and encephalopathies associated with the infection is still poorly understood. In previous studies it had been speculated that *B. pertussis* might increase cerebral vascular permeability (Amiel, 1976). Additionally, the frequently employed enhancement of the induction of experimental autoimmune encephalomyelitis (EAE) by PT has been suggested to be due to an increase in vascular permeability (Ben-Nun et al., 1997) apparently as a consequence of histamine-sensitization (Yong et al., 1993; Linthicum et al., 1982). Recently, it has been reported that whole-cell but not acellular pertussis vaccine induced convulsive activity in mice. This could be also induced by the administration of active PT and LPS, which is residually present in the whole-cell vaccine but is absent in the acellular vaccine (Donnelly et al., 2001).

Interactions of pertussis toxin with brain barrier cells

To address this issue in more detail we investigated the influence of PT on cells involved in cerebral barriers, such as isolated *Plexus chorioideus*-derived epithelial cells, Plexus chorioideus cell lines, and porcine BCECs.

PT uptake and intoxication efficiency clearly demonstrated that brain-derived epithelial and endothelial cells are target cells for PT. PT uptake is followed by retrograde transport and ADP-ribosylation of α-G$_i$ subunits (e.g. el Bayâ et al., 1997). However, we found that in our model systems the barrier function was compromised only in endothelial cell monolayers. Therefore, in this study we employed tight monolayers of BCECs as an in vitro model system for the blood-brain barrier and focussed on the investigation of the effect of PT on monolayer integrity. Changes in cell

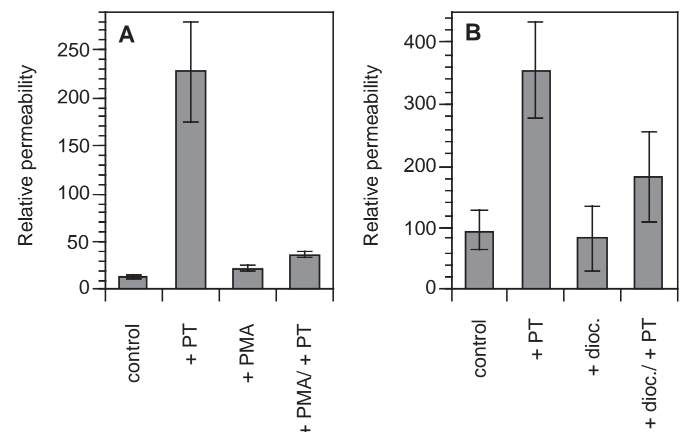

Fig. 7. PKC-activating cellular drugs dioctanoyl-sn-glycerol and PMA reduce the PT-mediated permeabilization of endothelial monolayers. BCEC monolayers were exposed to medium, medium containing the drugs dioctanoyl-sn-glycerol (100 µg/ml) or PMA (500 nM), alone or in combination with PT (200 ng/ml) for 4 hours (+1 hour for the permeability assay). The transendothelial HRP transport was assessed by measuring HRP concentration spectrophotometrically in the lower compartment. OD values indicated the traversed HRP as a measure of relative permeability. The standard deviations (error bars, *n*=6) are indicated.

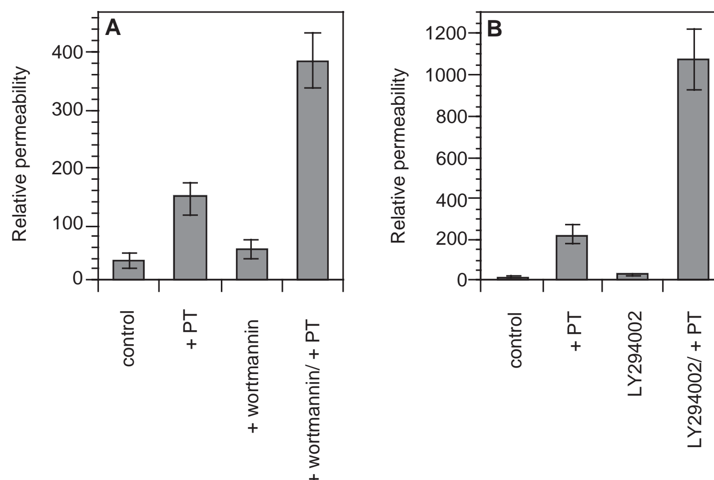

Fig. 8. Effect of the PI3 kinase inhibitors wortmannin and LY294002 on PT-mediated permeabilization of BCEC monolayers. BCEC monolayers were exposed to medium, medium containing wortmannin (100 nM), LY294002 (10 μM) or PT (200 ng/ml) alone or medium with wortmannin or LY294002 in combination with PT for 4 hours (+1 hour for the permeability assay). The transendothelial HRP transport was assessed by measuring HRP concentration spectrophotometrically in the lower compartment. OD values indicated the traversed HRP as a measure of relative permeability. The standard deviations (error bars, n=6) are indicated.

morphology observed in sub-confluent BCECs were not apparent in confluent and tight monolayers. No lethal effect was observed for PT concentrations up to 1000 ng/ml even upon incubation for several days. Changes of cell shape and the maintenance of cell-cell contacts are complex processes where many components of the cytoskeleton, such as microtubules or actin filaments, have to participate. However, in this study no apparent changes in the arrangement and localization of the investigated marker proteins, such as F-actin, α-tubulin, α-catenins, β-catenins, ZO-1, vimentin and vinculin, could be identified by immunofluorescence.

Influence of PT on barrier integrity and the effect of cAMP levels

To further investigate potential consequences of changes in cell morphology we performed permeability assays and measured electrical resistance across endothelial as well as in epithelial cell monolayers. While our results clearly showed that PT had no influence on the performance of *Plexus chorioideus*-derived epithelial cell monolayers we found that PT substantially enhanced the permeability of BCEC monolayers for HRP used as a translocation marker. Using HRP as a marker protein, a contribution to the translocation of HRP by transcytosis cannot be ruled out and previous studies by Karnovsky and co-workers (Reese and Karnovsky, 1967; Karnovsky, 1967) have indeed demonstrated the uptake of HRP in micropinocytotic vesicles. However, HRP-containing vesicles were few in number and appeared not to be involved in peroxidase transport. These early studies already indicated that the main translocation of HRP across endothelial barriers proceeds by way of an intercellular passage. In addition, we have investigated HRP translocation with a stable human brain microvascular endothelial cell line (HBMEC) by electronmicroscopy and rarely found the HRP label intracellularly. However, a contribution of transcytosis to the translocation of HRP cannot be ruled out with certainty and has to be analyzed in more detail in future studies. Our conclusion that intercellular translocation represents the main passageway is further supported by the finding that, in parallel to the translocation of HRP, also the transendothelial resistance (TER) was reduced in a time and dose-dependent manner. Higher concentrations of PT accelerated the effect and maximum effect on TER and permeability was observed after about 5 hours (Fig. 3). However, by raising the PT concentration to 1000 ng/ml the increase in permeability in BCEC monolayers was less pronounced (Fig. 2). This corresponds to the increased cAMP levels generated by this concentration compared with the 200 ng/ml of PT usually employed in the HRP-translocation assays (Fig. 4) and already suggested that the PT-induced permeabilization of BCEC monolayers is not mediated by an increase in cAMP.

To address the influence of elevated cAMP levels on the PT-mediated permeabilization forskolin and cholera toxin have been used in combination with PT. Both cholera toxin and forskolin induced high levels of intracellular cAMP (Fig. 4). However, both reagents also reduced or even completely abolished the PT-mediated increase in permeability in the BCEC monolayers (Fig. 5). This clearly shows that elevated cAMP levels actually abrogate the PT-mediated reduction of the barrier function. This is in accordance with results reported by Raub (Raub, 1996), who found in bovine endothelial cells co-cultured with rat C6 glioma cells that PT irreversibly obliterates TER.

Potential signalling pathways involved in PT-mediated permeabilization

To identify potential effector pathways we employed a number of established cellular drugs activating or inhibiting central effectors, such as PI3-kinase, adenylate cyclase, PLC, myosin light chain kinase, PKA, PKC, and different phosphatases (PP) such as PP1, PP2A and PP2B (Table 1). BCEC monolayers were incubated either with the respective compounds, with PT, with the drug in combination with PT, or with medium alone. The control experiments excluded the possibility that the drugs themselves had any effects on the integrity of the monolayer. Cellular drugs used for the inhibition of protein kinase C (PKC), such as H-7 and staurosporine, dramatically enhanced the PT effect and, in parallel, activators of PKC, such as PMA and dioctanoyl-sn-glycerol, reduced the PT enhanced permeability of BCEC monolayers. Similar results were obtained using inhibitors selective for PI3-kinase such as wortmannin and LY294002 (Table 1). The PT-mediated permeability of BCEC monolayers increased upon PI3-kinase inhibition. Because no drugs are available that could activate PI3-kinase the effect of PI3 kinase activation could not be examined. All other drugs that would inhibit or activate signal transduction pathways involving effectors distinct from PKC or PI3-kinase had no effect whatsoever on the PT-induced increase in permeability.

The results of these studies strongly suggest that PI3-kinase and PKC are involved in the PT-induced enhancement of the endothelial barrier permeability. Regarding the known signal transduction pathways in eukaryotic cells, PKC is a potential downstream enzyme of PI3-kinase (Duronio et al., 1998). Among the presently known PI3-kinases (PI3Kα, PI3Kβ, PI3Kδ and PI3Kγ) the PI3-kinase isoform γ has been described recently (Stephens et al., 1997). This particular isoform of the PI3-kinases consists of a novel noncatalytic subunit that has been named p101 and was found to be unrelated to p85 (Vanhaesebroeck et al., 1997). G_i-protein (βγ-subunit)-coupled receptors were shown to activate this PI3-kinase isoform (Lopez-Ilasaca et al., 1998; Murga et al., 1998; Vanhaesebroeck et al., 1997). PI3-kinases in turn phosphorylate phosphatidylinositol (PtdIns), PtdIns(4)*P*, and PtdIns(4,5)P_2 to generate PtdIns(3)*P*, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 representing substrates for specific isoforms of PKC (Duronio et al., 1998; Wagey et al., 1998; Ettinger et al., 1996). Recently, it has been shown that PI3-kinase γ products such as PtdIns(3,4,5)P_3 are substrates and activators of a number of isoforms of PKC such as PKC δ, PKC ε, PKC ζ and PKC η (Ettinger et al., 1996; Quest, 1996). The isoforms PKC δ, PKC ε and PKC ζ have been detected in endothelial cells (Wellner et al., 1999) and in the rat brain (Shin et al., 1998). The isoforms PKC δ and PKC ε could be activated by diacylglycerol and phorbolesters as well. Previous studies have shown that activation of MAP kinase as a potentially downstream target of PKC via G_i-coupled receptor, could be blocked by inhibitors of PI3-kinase (wortmannin and LY294002) and PT (Takeda et al., 1999). Among the several cellular phosphorylation systems that potentially regulate cytoskeletal protein phosphorylation, PKC appears as an apparently central regulator (Hazel and Malik, 1996; Stasek et al., 1992). Protein phosphorylation mediated by PKC might also be responsible for the reduced barrier function and altered cell morphology in subconfluent monolayers.

Based on the results described in this study we conclude that PT permeabilizes cerebral endothelial cells via a G protein βγ-subunit-coupled effector involving PI3-kinase and PKC. PLC is not necessary for this pathway because PI3-kinase γ lipid products such as PtdIns(3,4,5)P_3 are direct activators of PKC isoforms. The mechanism by which the inhibition of PKC effects endothelial cells remains to be resolved. The involvement of the PKC isoforms PKC δ and PKC ε in this pathway seems very likely as these isoforms have been detected in endothelial cells and can activated by DAG/PMA as well as PtdIns(3,4,5)P_3. Further studies will identify the specific PKC isoform involved in the pathway of PT-induced barrier dysfunction, and which mechanism is involved in PKC-mediated alteration of cell shape and permeability response.

Potential dual role of PT for blood-brain barrier integrity

This study provides a molecular explanation for the frequently performed enhancement of experimental autoimmune encephalomyelitis (EAE) by the injection of myelin basic protein (MBP) with *B. pertussis* or PT, or with PT alone. While the enzymatic activity of the S1 subunit is clearly needed (Ben-Nun et al., 1997) for the induction of the disease conflicting reports implicate the B-oligomer subunits (Lehmann and Ben-Nun, 1992) as well as the S1 subunit (Robbinson et al., 1996) of PT to be involved in the protective effect of PT against EAE. The dual role of PT observed in vivo might be due to the particular PT concentration available locally as at higher PT concentrations elevated cAMP levels proved to reduce the permeability. Furthermore, as the protective effect of the B-oligomer of PT in EAE might be due to the S2/S3-mediated inhibition of leukocyte adherence to selectins on inflamed endothelial cells (Rozdzinski et al., 1993a), elevated PT concentrations might enhance this effect. Thus, with regard to the induction of encephalopathies as a potential consequence of pertussis infection, which has recently been discussed by Donnelly et al. (Donnelly et al., 2001), PT might exert a dual effect in permeabilizing cerebral endothelial barriers mediated by the activity of the ADP-ribosyltransferase and, by contrast, mediating an anti-inflammatory effect (Rozdzinski et al., 1993b) by competitively blocking leukocyte adherence and recruitment.

This study was funded in part by grants SCHM 770/8-1(2) and GRK 234/2-99 from the Deutsche Forschungsgemeinschaft (DFG). This study is part of the PhD thesis of K.E.B.

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