Cereport™ (RMP-7) Increases the Permeability of Human Brain Microvascular Endothelial Cell Monolayers[1]

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Purpose. To study Cereport (RMP-7, bradykinin B2 agonist) effects on human brain microvascular endothelial cell (HBMEC) monolayer permeability.

Methods. HBMEC grown on transwell membranes were exposed to Cereport. The monolayer permeability was determined with [^{14}C]-inulin (MW. 5,200) and [^{3}H]-dextran (MW. 70,000).

Results. Cereport increased the HBMEC permeability to [^{14}C]-inulin, but not to [^{3}H]-dextran. The effect was transient, maximal at 15 min (i.e., 79.3% increase), and polarized to the basolateral membrane. An inverted U, dose-response curve was observed with active concentrations of Cereport from 0.01 to 0.5 nmol/L, the plateau maximal effect between 0.5 and 10 nmol/L, and loss of activity at the highest concentration, i.e., 20 nmol/L. Cyclic AMP-specific phosphodiesterase 3 (PDE3) inhibitor rolipram (10 μmol/L) abolished Cereport effects, while cGMP-specific PDE5 inhibitor, zaniprast (50 μmol/L) enhanced by 31% ($p < 0.05$) the effect of 0.1 nmol/L Cereport. Unlabeled Cereport displaced [^{125}I]-bradykinin and/or [^{125}I]-Cereport from the basolateral side. There was no specific Cereport binding to the apical side.

Conclusions. Cereport exerts specific time, dose and size dependent actions on HMBEC monolayer that are restricted to the basolateral membrane. Its effects can be further modulated through changes in cAMP and cGMP second messenger systems.

KEY WORDS: Cereport; bradykinin B2 receptor; human; brain endothelium; permeability.

INTRODUCTION

Cereport (RMP-7), a synthetic bradykinin B2 agonist, has been used as a central nervous system (CNS) delivery agent (1,2) and an effective permeabilizer of the vascular barriers of the CNS and eye in different animal models (1,3–10), and more recently in patients with brain gliomas (11). When compared to bradykinin, Cereport is more stable in biological fluids and has an extended plasma half-life (5,12–14). Cereport acts on brain vasculature via B2 receptors and opens the blood-brain barrier (BBB) and more permeable blood-brain tumor barrier (BBTB) (4,7,11,15). Its effects *in vivo* are rapid, transient and self-regulated (4,5).

It is not clear whether Cereport alters CNS vascular permeability by enhancing cerebral blood flow and producing vasodilation with secondary plasma leakage, as opposed to primarily affecting the tightness of brain endothelium via an endothelial specific mechanism (15,16). It remains still unresolved whether its permeabilizing effect is chiefly mediated through changes in paracellular endothelial transport, and if so is the site of its action localized to the luminal side, abluminal side and/or both sides of the brain vessel wall. Studies demonstrating opening of the BBB and/or BBTB after systemic administration of Cereport and/or bradykinin would argue for the luminal site of action (7,16–18), while increased BBB permeability after bradykinin cortical superfusion (16) and/or intracerebral injections (19) may indirectly suggest the presence of bradykinin receptors at the abluminal side (reviewed in 16).

Bradykinin B2 receptors have also been shown in human cerebral vessels (20). Limited information, however, is available on Cereport vascular permeabilizing effects in human brain. The present study used our human brain microvascular endothelial cell (HBMEC) transport BBB model (21–23) to characterize in greater detail Cereport effects on transporting properties of brain endothelia, and to determine whether modulations of major second messenger systems, i.e., cyclic AMP and cyclic GMP, may affect Cereport permeabilizing endothelial effects.

MATERIALS AND METHODS

Materials

Cereport was supplied by Alkermes, Cambridge MA. [^{14}C]-inulin (specific activity, SA = 2.1 mCi/g), [^{125}I]-bradykinin (SA = 2,200 Ci/mmole) both from NEN DuPont (Boston, MA), [^{3}H]-dextran (SA = 322 mCi/g) and [^{14}H]-cereport (SA = 55 mCi/mmol; custom made) from Amersham (Arlington Heights, IL) were used throughout the study. Fetal bovine serum was obtained from Hyclone (Logan, UT), and fibronectin purchased from Sigma (St. Louis, MO). Hanks balanced salt solution, (HBSS), and RPMI medium 1640 were from Irvine Scientific (Irvine, CA).

Isolation and Characterization of HBMEC

Human brain capillaries were isolated from small fragments of cerebral cortex obtained from surgical resections from 3 adults with seizure disorder, as recently described (21,24). Portions with no pathologic lesions were used. Cell viability was greater than 95% as judged by trypan blue exclusion test. Human brain microvessels were plated on collagen coated support and resulting HBMEC were cultured and characterized as previously described (24). The HBMEC were positive for endothelial cell markers (24) and expressed gamma glutamyl transpeptidase, indicating their brain origin. Detailed procedures regarding characterization of HBMEC were as we previously reported (21,24).

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ABBREVIATIONS: receptor mediated permeabilizer-7; RMP-7; Blood-brain barrier, BBB; blood-brain-tumor barrier, BBTB; human brain microvascular endothelial cells, HBMEC; hanks balanced salt solution, HBSS; disintegration per minute, d. p.m.; %RD, percent radioactive dose.

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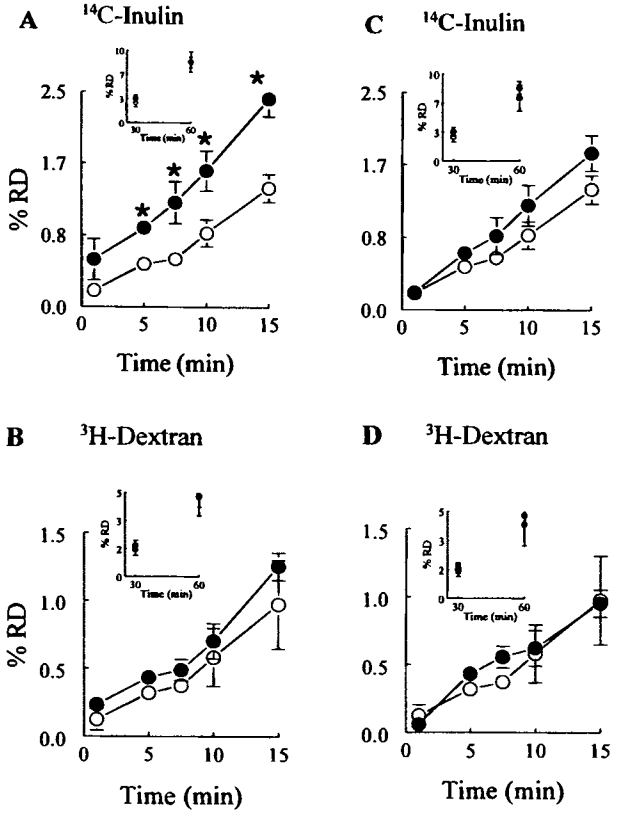

Fig. 1. HBMEC monolayer permeability to [^{14}C]-inulin and [^{3}H]-dextran in the presence (●) or absence (○) of 10 nmol/L Cereport added to either bottom chamber (A, B) or upper chamber (C, D). Tracers were introduced to the upper chamber simultaneously with Cereport. Major graphs—transport of tracers over 15 min; insets—transport of tracers at 30 and 60 min. Each point is the mean ± SE, from 3 different human monolayers. Three separate wells were used for each time point, and each well was sampled at the specific time point. The protocol was repeated 3 times. * $p < 0.05$, for Cereport and vehicle, by Student's t-test.

In Vitro Model of the Endothelial Barrier

Using the HBMEC, the endothelial barrier model was constructed in inserts with collagen-fibronectin coated polycarbonate in Transwell membrane filters as we reported (22). The average electrical resistance of the monolayers was between 120 and 180 Ω cm^2. As reported, the presence of astrocytes did not positively affect the tightness of the HBMEC monolayer in this model (23) and therefore they were not used in present studies.

Protocols to Study HBMEC Monolayer Opening by Cereport

The HBMEC were preincubated for 1 hr in RPMI culture medium with 10% fetal bovine serum at 37 °C. Cereport (0.01 to 20 nmol/L) was added to either upper chamber (apical side) or lower chamber (basolateral side). In all experiments the tracer solution with [^{14}C]-inulin and [^{3}H]-dextran was added to the upper chamber to determine apical-to-basolateral transport. When the effects of rolipram and zaniprast were studied, these specific cAMP and cGMP phosphodiesterase (PDE) inhibitors, respectively, were added to the basolateral side 5 min prior to Cereprot. Cultures were stirred upon addition of Cereport and/or tracers. There was no significant binding of Cereport to fetal bovine serum. In most studies the tracer solution and Cereport were administered simultaneously. In some studies, tracers were infused 5 min before Cereport or 15 min after Cereport (see Results).

All measurements were done in triplicate. [^{3}H] and [^{14}C] radioactivities were determined in a Beckman LS-7500 liquid scintillation spectrometer. The amount of radiolabeled inulin or dextran transported across the HBMEC was expressed as the percent of their respective radioactivity dose (%RD) added to the apical chamber (14):

$$\%RD = d.p.m._{BOTTOM\ CHAMBER}/(d.p.m._{TOP\ CHAMBER} + d.p.m._{BOTTOM\ CHAMBER})$$

[^{125}I]-Bradykinin and [^{125}I]-Cereport Binding to HBMEC

HBMEC monolayers were incubated for 15 minutes with [^{125}I]-bradykinin or [^{125}I]-Cereport from either apical or basolateral side in the presence and absence of ~100 molar excess of unlabeled Cereport at 4°C. After the incubation cells were washed three times in cold HBSS, lifted from the filters by short incubation in cold medium containing EDTA, homogenized and counted in Beckman Gamma 4000 counter.

RESULTS

Figure 1A illustrates transient opening of the HBMEC monolayer to [^{14}C]-inulin by 10 nmol/L Cereport added to the basolateral side; there was no significant effect on simultaneously determined [^{3}H]-dextran transport (Fig. 1B). In contrast, there was no change in HBMEC permeability to either tracer when Cereport was added to the apical side (Fig. 1C and D). In all studies Cereport and tracers were introduced to their respective chambers at the same time, and transport of tracers measured from the apical to basolateral compartment.

The permeability of monolayers to inulin (MW ≤ 5.2 kDa) in the present study of 7.9 cm/sec × 10^{-6} compares well with the permeability to similar molecular weight inert polar molecule, polyethylene glycol (MW 4 kDa) in a previous study (25). Our permeability measurements performed typically for 15 min resulted in ~1.3% of the dose being transported from the apical into the basolateral chamber under control conditions. This value compares well with ~5.5% of the dose found for smaller sucrose molecule (MW 342) transport across bovine endothelial monolayers (26). In these in vitro models, the diffusion across the pores of two different size polar molecules is proportional to the reciprocal values of the square roots of their respective molecular weights, that in case of sucrose vs. inulin is 3.9.

The rationale for selecting 10 nmol/L Cereport in the initial experiments was that this concentration produced maximal increase in the intracellular free calcium in rodent brain endothelial cells (13), and also opened the vascular barriers of the CNS (2) and eye (8–9) *in vivo*. In the present model, the effect of Cereport on inulin permeability was maximal at 15 min. and the permeability was restored to control values at 30 and 60 min.

Next, we studied the effect of different concentrations of Cereport (0.01 to 20 nmol/L) on permeability of HBMEC

monolayers to ^{14}C-inulin (Fig. 2). Cereport and tracer were introduced to the system simultaneously. We selected 15 min as at this time point the opening of the monolayer to Cereport was maximal with 10 nmol/L (Fig. 1A). Cereport added to the basolateral side exhibited the dose-dependent increase in HBMEC permeability to [^{14}C]-inulin between 0.01 and 0.5 nmol/L (Fig. 2A). With further increases in Cereport concentration (from 0.1 to 10 nmol/L) an asymptote was observed, with transport values being similar to those seen with 0.5 nmol/L. However, with this protocol the effect was completely lost with 20 nmol/L Cereport (Fig. 2A), and could not be observed at any other time point within 60 min (not shown). In contrast, none of the studied concentrations exhibited an effect on HBMEC permeability when Cereport was added to the apical side either after 15 min (Fig. 2B) and/or at any other time point within 60 min (not shown).

The effect of Cereport was further investigated by using a protocol in which the apical side of the monolayer was exposed to [^{14}C]-inulin 5 min prior to addition of 20 nmol/L Cereport to the basolateral side. Samples were collected between 1 and 15 min after addition of Cereport (Fig. 3A). This was done to help determine if the loss of Cereport's activity at 20 nmol/L might be due to rapid tachyphylaxis induced by the high concentration of the agonist. With this protocol there was a modest 22% increase in monolayer permeability after 10 min of exposure to Cereport, but the effect was lost at 15 min. Addition of Cereport to the apical side with this same inulin protocol had no effect on monolayer permeability (data not shown). To further test the tachyphylaxis hypothesis, 5 nmol/L Cereport was added to the basolateral side of HBMEC 15 min prior to [^{14}C]-inulin and the monolayer permeability studied within next 30 min (Fig. 3B). Although 5 nmol/L Cereport had a significant effect on HBMEC permeability to inulin when introduced into the system simultaneously with the tracer (Fig. 2A), the effect was completely lost when the monolayer was pretreated with Cereport (Fig. 3B). Again, there was no effect when Cereport was added to the apical side (not shown).

Figure 4A shows significant binding of [^{125}I]-bradykinin (trace concentration) to the basolateral side that was displaced by 48% with 100 times molar excess of unlabeled Cereport. Binding of [^{125}I]-bradykinin to the apical side was low and

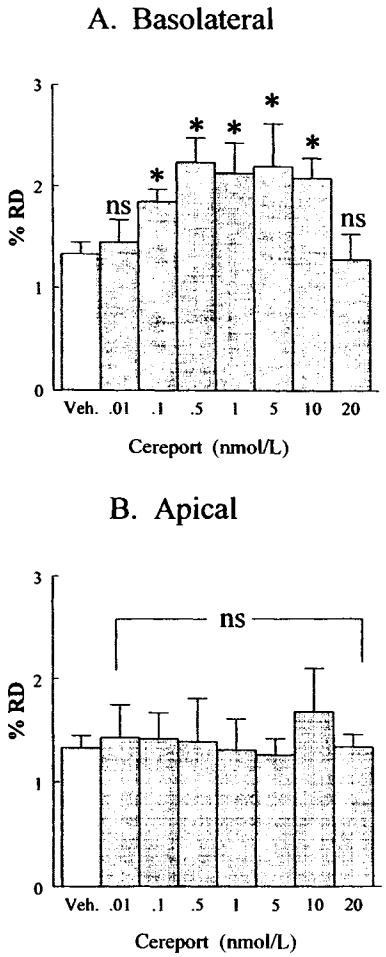

Fig. 2. Effect of different Cereport concentrations on HBMEC permeability to [^{14}C]-inulin. Cereport (0.01–20 nmol/L) was added to the basolateral side (A) or to the apical side (B); [^{14}C]-inulin was introduced to the upper chamber simultaneously with Cereprort. The permeability of the monolayers was examined at 15 min. Each point is the mean ± SE, from 3 different monolayers determined in triplicate. * $p < 0.05$, ^{ns}non-significant for different concentrations of Cereport vs. vehicle (Veh.), by Student's t-Test.

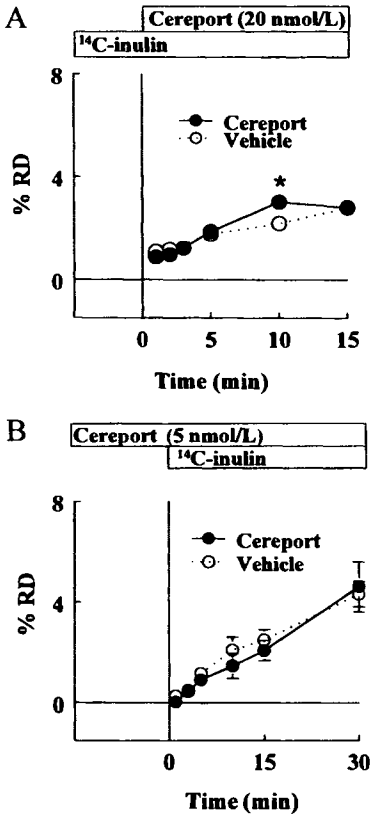

Fig. 3. A. Modest effect of 20 nmol/L Cereport added to the basolateral side of HBMEC on [^{14}C]-inulin transport; [^{14}C]-inulin was introduced 5 min before Cereport to increase the sensitivity of transport measurements. B. Loss of Cereport (5 nmol/L) effect by pre-treatment of HBMEC monolayers with Cereport for 15 min; [^{14}C]-inulin was introduced 15 min after Cereport. Inulin transport was determined from the apical-to-basolateral compartment in the absence (○) and presence (●) of Cereport. Each point is mean ± SE from 3 different monolayers determined in duplicate. * $p < 0.05$, for Cereport and vehicle, by Student's t-Test.

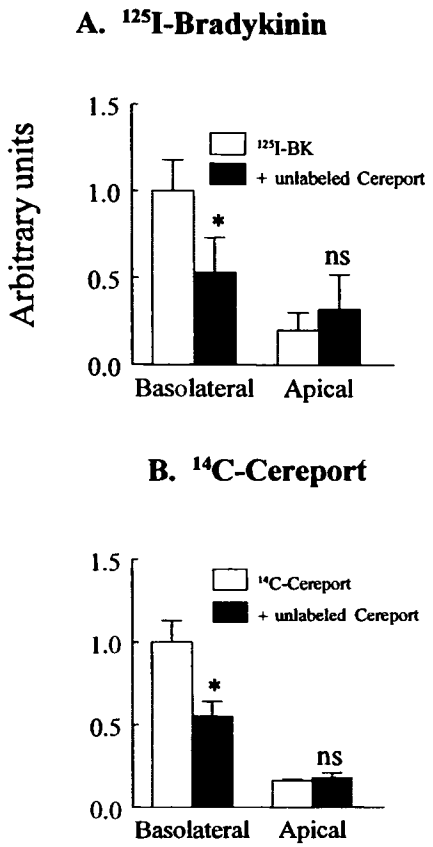

Fig. 4. Binding of [^{125}I]-bradykinin (A) and [^{125}I]-Cereport (B) at trace concentrations to the basolateral and apical side of the HBMEC monolayers in the absence (open bars) and presence of a 100 times molar excess of unlabeled Cereport (black bars). Values are mean ± SE of 3–4 monolayers in triplicates. Control binding in the absence of unlabeled Cereport is arbitrarily set as 1.

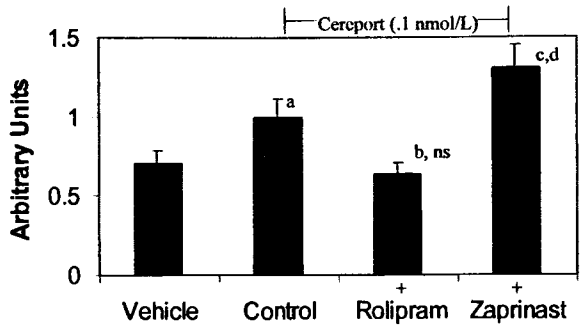

Fig. 5. Effects of cyclic AMP-specific and cyclic GMP-specific PDE inhibitors Rolipram and Zaprinast on Cereport-induced increase in HBMEC monolayer permeability to inulin. HBMEC were exposed to either Rolipram (10 μmol/L) or Zaprinast (50 μmol/L) from the basolateral side 5 min prior to 15 min exposure to Cereport (0.1 nmol/L) from the basolateral side; [^{14}C]-inulin was introduced to the upper chamber simultaneously with Cereport and the permeability examined at 15 min. Each point is the mean ± SE, from 3 different monolayers determined in triplicate $^{a,b,c,d}p < 0.05$, for Cereport vs. vehicle; Rolipram + Cereport vs. Cereport; Zaprinast + Cereport vs. Cereprort; and for Zaprinast + Cereport vs. vehicle, respectively.^{ns}, non-signifcant, Rolipram + Cereport vs. vehicle. Control effect of 0.1 nmol/L Cereport on HBMEC permeability is arbitrarily set as 1.

could not be inhibited by unlabeled Cereport possibly reflecting non-specific binding. Since the amount of protein is very small in individual wells, cells from several wells were collectively assayed for protein content and binding of radiolabeled ligand (e.g., ^{125}I-bradykinin) Tto the basolateral side in the absence of unlabeled bradykinin was 0.033 pmol/mg protein, and was reduced to 0.018 pmol/mg in the presence of 100-molar excess of Cereport. Binding of radiolabeled ligands to the apical side was between 0.013 and 0.018 pmol/mg protein, and could not be displaced by excess of unlabeled either bradykinin or Cereport. The HBMEC cellular uptake of [^{14}C]-inulin was barely above the background level indicating the absence of a non-specific leak into the cells and minimal entrapping of the tracer in the medium between the cells at the sites of junctional complexes (not shown). Similar effects were obtained with [^{14}C]-Cereport (Fig. 4B).

Figure 5 illustrates that pre-exposure of HBMEC to cyclic AMP-specific PDE3 inhibitor rolipram (10 μmol/L) for 5 min abolishes the effect of Cereport (0.1 nmol/L) on HBMEC permeability to inulin determined at 15 min. On the other hand, pre-exposure of HBMEC to cyclic GMP-specific PDE5 inhibitor Zaprinast (50 μmol/L) significantly enhances the effect of Cereport (0.1 nmol/L) on HBMEC permeability to inulin. These PDE inhibitors increase endothelial levels of cAMP and cGMP by preventing their further degradation, respectively. In these studies PDE inhibitors and Cereport were added from the basolateral side. The concentrations of PDE inhibitors in our HBMEC system were selected after pilot studies, since the loss of effects and tachyphylaxis with both rolipram and zaniprast were observed in the present system at higher concentrations of these PDE 3 and PDE5 inhibitors (not shown). On the other hand, changes in monolayer permeability could not be elicited at very low concentrations of these PDE inhibitors (not shown).

DISCUSSION

This study has demonstrated that Cereport directly affects transporting properties of brain endothelia, exerting specific time, dose and size dependent actions that are restricted to the basolateral membrane. Opening of the monolayer was seen only for the smaller molecular weight marker inulin but not for a larger marker dextran (Fig. 1A and B). This supports the hypothesis that increased HBMEC monolayer permeability induced by Cereport is due to an increase in the width of the inter-endothelial tight junctions, i.e., opening of the endothelial "pores" (27,28), rather than due to an enhanced vesicular trans-endothelial transport. The diffusion of tracers through the pores or restricted diffusion is size-dependent, in contrast to vesicular transport that is mediated by a bulk flow transcytosis and is size-independent (27,28).

The present study indicates the polarity of Cereport effect to the basolateral side, since the permeability of the monolayer remains unchanged when Cereport was added to the apical side (Fig. 1C and D and Fig. 2B). *In vivo* studies in rodents were inconclusive with respect whether bradykinin or Cereport actions on the vessel wall are exerted primarily via luminal and/or abluminal B2 receptors (3,4,7,9,15,16). Although B2 receptors have been demonstrated in brain vessels including humans (17,20), their exact endothelial distribution, luminal vs. abluminal by immunolocalization studies is not known. On

the other hand, the electrical BBB properties *in vivo* are much higher than in the present and other *in vitro* endothelial models, and it is possible that several cell receptors or second-messenger systems are miss-sorted *in vitro*, as recently demonstrated for ion channel distribution in intercalated epithelia (29). Thus, it is uncertain to what extent the basolateral endothelial distribution of B2 receptors shown in the present model reflects an *in vivo* situation. Whether the difference in electrical properties of the barriers *in vitro* vs. *in vivo* determines potential differences in their transporting capabilities is also not absolutely clear, as the resistance is not always correlated with transport paracellular properties (30). The polarity for certain proteins in *in vitro* brain endothelial monolayers seems comparable to their *in vivo* distribution, as for example the basolateral localization of the A amino acid transport system and the sodium pump (22,24). Previous work with *in vitro* brain endothelial models indicated that several vasoactive peptides (e.g., vasopressin, atrial natriuretic peptide) have their specific receptors/transporters at the basolateral side, in contrast to insulin and the receptor for advanced glycation end products and the scavenger receptor that have almost all of their binding/transport at the apical side.

Activation of B2 receptors by bradykinin (15) or Cereport (4) in *in vivo* animal models resulted in transient increase in vascular CNS permeability. This has been confirmed in the present study (Fig. 1A). Interactions of B2 receptors with their agonists may lead to tachyphylaxis due to receptor desensitization even at relatively low doses of Cereport (5). In the present study, the effect was transient and maximal at 15 min. Moreover, an inverted U, dose-response curve was observed, with active concentrations existing from 0.01 to 0.5 nmol/L, followed by a loss of activity at the highest concentration (i.e., 20 nmol/L). The mechanism of tachyhylaxis observed with Cereport is not clear. Our preliminary studies indicate that modulations of the second messenger cAMP and cGMP systems via inhibition of specific PDE3 and PDE5 involved in their degradation may affect significantly Cereport effects (Fig. 5). Thus, rolipram abolished Cereport effects, while an inhibition of cGMP degradation with zaniprast had the opposite effect, and potentiated Cereport induced increase in HBMEC permeability, similar as in an *in vivo* glioma model (10). Our preliminary data (not shown) indicate that tachyphilaxis in the present HBMEC system may occur with manipulation of both cAMP and cGMP systems, and the effects of these specific PDE inhibitors are lost at increased concentrations. Thus the receptor specificity may be also lost at higher concentrations of Cereport due to activation or tachyphylaxis through these modulatory pathways, and the details of these interactions are currently under study in our laboratory. It is also possible that there is not a true size discrimination in the actions of Cereport, but rather the action of Cereport occur too quickly for appreciable amounts of the larger dextran macromolecule to traverse the barrier. Thus, the window of opportunity may open open and close faster that the larger molecule can diffuse sufficiently to be observed as a difference.

In vivo studies also indicated that higher concentrations of Cereport may hasten receptor desensitization leading to either smaller magnitude of the effect or, after sufficient time, its complete loss (4,5). In this study, 20 nmol/L Cereport resulted in very modest effect on monolayer opening (Fig. 3A), or the effect could not be elicited at all under conditions when lower concentrations of Cereport produced significant effects (Fig. 2A). Pretreatment of HBMEC monolayer with 5 nmol/L Cereport resulted in loss of the effect (Fig. 3B), suggesting tachyphylaxis, possibly due to receptor desensitization (5). Similar, 10 nmol/L Cereport elicited maximal rise in intracellular calcium in rat brain microvascular endothelial cells, while higher concentrations produced significantly lower responses (13).

Studies on rat brain endothelial cells revealed a major class of bradykinin binding sites with $K_D \sim 4$ nmol/L, and a high affinity class with $K_D \sim 0.033$ nmol/L (13). In those studies binding of radiolabeled ligands was displaced by both unlabeled bradykinin or Cereport, but not with B1 agonist desArg^9-bradykinin. The present study confirmed binding of radiolabled bradykinin and Cereport to the basolateral side of HBMEC that was inhibited by a 100-fold excess of unlabeled Cereport (Fig. 4). Binding of both ligands to the apical side of HBMEC was significantly smaller than to the basolateral side and could not be displaced by unlabeled Cereport, possibly reflecting the non-specific binding of the cationic peptides on negatively charged endothelial surface (13). The lack of specific bradykinin and/or Cereport binding to the apical side (Fig. 4) and the inability of Cereport to increase permeability of the HBMEC monolayer when added to the apical side (Fig. 1C and D, Fig. 2B), suggest that the specific binding sites for Cereport in the present model are localized at the basolateral side. The reduced binding detected with 100 molar excess of unlabeled ligand addition to the basolateral side may block the interaction of ligand with its receptors at the basolateral membrane, or produce down-regulation of receptors and rapid taxyphilaxis.

In summary, present studies suggest that Cereport increases vascular permeability by directly acting on brain endothelia to increase its paracellular transporting properties. The responsiveness of human brain microvascular endothelium to Cereport was similar to its previously shown permeabilizing effects on cerebral vasculature in different in vivo animal models (4,5,7,9,12,18). The results suggest that Cereport effects can be modulated through changes in cAMP and cGMP messenger systems that could also be possibly involved in tachyphylaxis observed with Cereprot. This *in vitro* HBMEC monolayer model, although it may not completely reproduce the *in vivo* events, is a useful tool to study the mechanisms underlying Cereport vascular permeabilizing effects, and may be helpful in particular in relating transporting properties to intracellular events and signaling pathways involved in the control of brain inter-endothelial junctions by Cereport and other vasoactive agents.

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