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The role of guanylyl cyclases in the permeability response to inflammatory mediators in pial venular capillaries in the rat

M. H. Sarker and P. A. Fraser

Centre for Cardiovascular Biology and Medicine, King's College London, New Hunt's House, Guy's Campus, London SE1 1UL, UK

Inflammatory mediators have a role in the formation of cerebral oedema and there is evidence that cGMP is an important signal in vascular permeability increase. We have investigated the role and the source of cGMP in mediating the permeability response to acutely applied bradykinin and the histamine H_2 agonist dimaprit on single cerebral venular capillaries, by using the single vessel occlusion technique. We found that 8-bromo-cGMP applied acutely resulted in a small and reversible permeability increase with a log EC_{50} −7.2 ± 0.15 M. KT 5823, the inhibitor of cGMP-dependent protein kinase, abolished the permeability responses to both bradykinin and dimaprit, while zaprinast, an inhibitor of type 5 phosphodiesterase, potentiated the response to bradykinin. On the other hand, L-NMMA blocked the response to dimaprit, but not that to bradykinin. Inhibitors of soluble guanylyl cyclase, LY 85353 and methylene blue, also inhibited the permeability response to dimaprit, but not bradykinin. The permeability responses to the natriuretic peptides ANP and CNP were of similar magnitude to that of bradykinin with log EC_{50} −10.0 ± 0.33 M and −8.7 ± 0.23 M, respectively. The natriuretic peptide receptor antagonist HS-142-1 blocked permeability responses to bradykinin as well as to ANP, and leukotriene D_4 blocked the responses to CNP and bradykinin, but not to dimaprit. In conclusion, the histamine H_2 receptor appears to signal via cGMP that is generated by a NO and soluble guanylyl cyclase, while bradykinin B_2 receptor also signals via cGMP but through particulate guanylyl cyclase.

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Corresponding author P. A. Fraser: Centre for Cardiovascular Biology and Medicine, King's College London, New Hunt's House, Guy's Campus, London SE1 1UL, UK. Email: paul.fraser@kcl.ac.uk

The normally tight blood–brain barrier formed by cerebral endothelium can be modified by inflammatory mediators, and there is evidence that these have a role in the cerebral oedema that can develop following trauma or cerebral ischaemia (Abbott, 2000). Two of these mediators, histamine and bradykinin, have been shown to produce a rapid, small and reversible permeability increase of similar magnitude when briefly applied directly to the pial microvessels. The permeability-increasing effects of histamine are mediated via the H_2 receptor (Sarker *et al.* 1998) and those of bradykinin via the B_2 receptor (Sarker *et al.* 2000). Interestingly, H_1 receptor activation results in a decrease in permeability (Sarker *et al.* 1998), while B_1 receptor activation results in an increase in permeability (Sarker *et al.* 2000), but mediated via histamine release. It appears that different signal transduction mechanisms are used by these two mediators. Thus, the acute response to histamine was inhibited by the calcium-entry blocker SKF 96365, which had no effect on the response to bradykinin. It is possible that calcium entry can occur across the cell membrane through sites of lipid peroxidation (Graier *et al.* 1998). Although Ca^{2+} influx has long been associated with an endothelial permeability increase, there is considerable evidence suggesting that cyclic nucleotides have an important role in regulating permeability changes: increased cAMP is associated with decreased permeability whereas raised cGMP increases permeability *in vivo* (Michel & Curry, 1999).

Both bradykinin and histamine can raise cGMP in endothelial cells in culture via a calcium–calmodulin-dependent activation of endothelial nitric oxide synthase (eNOS) to release nitric oxide, which in turn activates soluble guanylyl cyclase (Furchgott & Vanhoutte, 1989), and this pathway has also been shown to be important for the permeability increase in mesenteric microvessels (He *et al.* 1998). Cytosolic cGMP also increases when particulate guanylyl cyclase is activated by atrial, brain and C-type natriuretic peptides (ANP, BNP and CNP, respectively), for which cerebral microvessels have receptors (Vigne & Frelin, 1992).

The aim of this study was to investigate the role of the guanylyl cyclases in mediating permeability changes in response to activating the histamine H_2 and the bradykinin B_2 receptors. Some of the data have been previously presented in a preliminary form (Sarker & Fraser, 1998).

METHODS

The method used in this study, and its theoretical basis, have been described fully (Fraser & Dallas, 1993; Easton & Fraser, 1994). Briefly, the microcirculation of the surface of the brain of rats (aged 20–30 days) was exposed by removing the dura and arachnoid. A low molecular weight fluorescent dye, sulforhodamine B (580 Da), was introduced into the cerebral microcirculation via a bolus injection into the carotid artery, and viewed through a Zeiss ACM fluorescence microscope under 525 to 535 nm illumination. The fluorescent signal, which has been shown to be linearly related to the dye concentration (Fraser & Dallas, 1990), was captured through a microscope, an image-intensifier camera, and analysed through a video-densitometer. Permeability was measured from the rate of loss of dye trapped in a single pial venular capillary by a glass-occluding probe. The fluorescence measurements were made from a small segment 200 to 300 μm from the open end of the occluded vessel, but not so close to the occluding probe that the vessel diameter was distorted. If the vessel was leaky, a transmural hydrostatic pressure gradient would drive fluid across the wall, which would be replaced by fresh dye-free fluid entering the open end of the occluded portion. Dye concentration in the measured portion depends on axial flow and diffusion as well as transmural convection and diffusion. Permeability was calculated from the rate of decrease in fluorescence under the measured portion of the occluded segment. The diameter of the vessels did not change during the occlusion (Easton & Fraser, 1994). As rate of fall of intravascular concentration of a small polar molecule is independent of the hydrostatic pressure (Fraser & Dallas, 1993), the concentration of dye early in the occlusion, before axial volume flux distorts the uniform axial concentration of dye in the region of measurement, will be $C_t = C_0\, e^{-kt}$, where $k = 4P/d$ and P is the permeability and d the diameter of the vessel.

Animal preparation

The experiments were performed on Wistar rats of either sex aged between 20 and 30 days and were within guidelines directed by the Animals (Scientific Procedures) Act, 1986. The animals were anaesthetized by an intraperitoneal injection of 60 mg kg^{-1} body weight sodium pentobarbitone diluted in water (25% w/v), and maintained by supplementary injection of 10% of the original dose when necessary. At the end of the experiment the animal was killed by administering an overdose of the anaesthetic. Once anaesthetized, a thermal probe was inserted rectally, and the animal was kept on a heating blanket connected via the probe to a feedback circuit (CFP 8105 Harvard Instruments) to maintain the animal body temperature at 37 ± 1 °C. The trachea and the right common carotid artery were cannulated routinely, the arterial cannula being placed orthograde and filled with heparinized saline (100 U ml^{-1}) to prevent clot formation. A section of the frontoparietal bones on the left side, between coronal and lambdoid sutures, was thinned with a dental drill. A metal ring (internal diameter 7 mm and outside diameter 13 mm) was glued onto the cranium surrounding the thinned bone with cyanoacrylate adhesive. The thinned cranial surface within the ring was constantly superfused by a pump at the rate of 1–2 ml min^{-1} with artificial cerebrospinal fluid (CSF) warmed to 37 ± 0.3 °C and delivered through a fine plastic tube to ensure that a layer of fluid was present at all times. The thinned bone within the ring was then removed carefully avoiding damage to the meninges and cerebral surface. Pial microvessels were exposed by cutting away the overlying meninges. The rat was then placed on the modified stage of a microscope (ACM, Zeiss Oberkocken) and the exposed cerebral surface illuminated with a 100 W xenon discharge lamp through a × 20 water-immersion objective lens (Cooke, N.A. 0.5).

Artificial cerebrospinal fluid and drug application

The cerebral surface was superfused with an artificial cerebrospinal fluid (CSF) at the rate of 0.5 to 1 ml min^{-1}, warmed so that it arrived at the brain's surface at 37 ± 1 °C and buffered to pH 7.40. The CSF contained (mM): NaCl (110.5), KCl (4.7), $CaCl_2$ (2.5), KH_2PO_4 (1.1), $MgSO_4.7H_2O$ (1.25), $NaHCO_3$ (25) and Hepes (*N*-[2-hydroxyethyl]piperazine-*N*´-[2-ethanesulphonic acid]). All chemicals were obtained from BDH, Poole, except $MgSO_4.7H_2O$ from Fisons, Loughborough, and Hepes from Sigma. HS-142-1 (Imura *et al.* 1992) was a kind gift from Dr Y. Matsuda (Kyowa Hakko Kogyo, Tokyo, Japan), leukotriene D_4 was purchased from Affinity Research (Exeter, England). KT 5823, the inhibitor of cGMP-dependent protein kinase (PKG; Nazario *et al.* 1995), the soluble guanylyl cyclase inhibitor LY 85383 (Schmidt *et al.* 1985) and the type 5 phosphodiesterase (PDE_5) inhibitor zaprinast (Frossard *et al.* 1981) were purchased from CN Biosciences (Nottingham, England). Bradykinin, the histamine H_2-specific agonist dimaprit, *N*-monomethyl-L-arginine (L-NMMA), D-NMMA, super oxide dismutase, catalase, methylene blue, atrial natriuretic peptide and C-type natriuretic peptide were all purchased from Sigma (Poole, England).

A static pool of artificial CSF formed over the exposed pial surface when the delivering pump was switched off. The volume of the static pool was estimated by pipette as being approximately 100 μl. All the drugs were applied abluminally into this pool, and the doses are expressed as the final concentration, unless otherwise specified.

Protocols

Permeability measurements were carried out on venular capillaries between 10 and 18 μm diameter in the following manner. There was sufficient autofluorescence in the fluorescein filter set for the cerebral surface to be viewed directly under the microscope. The long, straight arteries and arterioles were easily distinguished from the venular capillaries, which were shorter and more tortuous. A venular capillary with an unbranching section of at least 200 μm was selected and the glass occluding probe positioned at one end of the vessel. A bolus of fluorescent dye-containing solution was injected through the carotid arterial cannula, and during its transit through the microcirculation the arterioles filled rapidly, followed by a later venular filling. The occluding probe was lowered on to the venular capillary, thereby forming a segment containing dye, while dye was cleared from the rest of the microcirculation, and once a stable level of fluorescence was obtained (after about 30 s) the drug, or mixture of drugs in question, was applied to the brain surface while the occlusion was maintained. Once the video-densitometer trace had stabilized (between 30 and 60 s) the occlusion was released and the drugs washed off the brain surface. The order of application of the drug mixtures was randomized in any series. All the experiments, unless otherwise stated, were completed within 2 h of the first measurement. Dose–response curves were constructed by applying all the different doses of the drug in pseudo-random order to a single vessel, and at least four vessels from four rats were used for each dose–response curve.

Statistics

Unless otherwise stated, the results are expressed as the mean ± S.E.M., and the significance of any changes assessed by using a non-parametric one-way analysis of variance test (Kruskal–Wallis) with Dunn's multiple comparison test, or the

Mann–Whitney test where appropriate. Regression lines and sigmoidal dose–response curves were fitted using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, CA, USA.

RESULTS

This study was designed to explore the signal transduction pathways linked to acute permeability responses to histamine and bradykinin. Previous experiments have demonstrated that this preparation is stable for the first 2 h following exposure, and acute application of histamine or bradykinin produced reproducible, transiently increased permeability responses without affecting the resting permeability (Sarker *et al.* 2000).

Permeability response to 8-bromo-cGMP

Previously experiments have shown that raising intra-endothelial cGMP resulted in increased permeability of venular microvessels, but often decreased permeability in cultured endothelium (Draijer *et al.* 1995; He *et al.* 1998). Which of these responses occurs in pial venular microvessels was assessed by applying the cell permeant analogue of cGMP, 8-bromo-cGMP, from 0.5 nM to 0.5 mM, to the abluminal surface. There was a dose-dependent permeability increase, with the estimated maximal increase being $2.0 \pm 0.16 \times 10^{-6}$ cm s^{-1} (mean ± S.E.M.), and the log median effective dose (logEC_{50}) −7.2 ± 0.15 M (Fig. 1).

Requirement of cGMP for acute permeability responses to histamine or bradykinin

The importance of cGMP in the development of a rapid permeability increase was also investigated by using the PKG inhibitor, KT 5823, in conjunction with the histamine H_2 and bradykinin B_2 receptor agonists, dimaprit and bradykinin. KT 5823 (5 μM) alone had negligible effect on permeability ($0.3 \pm 0.27 \times 10^{-6}$ cm s^{-1}, $n = 11$), but the permeability response to bradykinin (5 μM bradykinin alone, $1.3 \pm 0.15 \times 10^{-6}$ cm s^{-1}, $n = 6$) was very much reduced when co-applied with KT 5823 ($0.1 \pm 0.18 \times 10^{-6}$ cm s^{-1}, $n = 6$). KT 5823 similarly blocked the response to dimaprit (50 μM dimaprit alone, $1.6 \pm 0.44 \times 10^{-6}$ cm s^{-1}, $n = 5$; with KT 5823, $0.0 \pm 0.31 \times 10^{-6}$ cm s^{-1}, $n = 4$).

Conversely, a sub-threshold concentration of bradykinin (50 pM) that resulted in no net change in permeability ($-0.1 \pm 0.16 \times 10^{-6}$ cm s^{-1}, $n = 10$) produced a significant increase when co-applied with the PDE_5 inhibitor zaprinast (50 pM + 50 μM zaprinast, $1.4 \pm 0.26 \times 10^{-6}$ cm s^{-1}, $n = 4$). Zaprinast alone produced little change in permeability ($0.2 \pm 0.13 \times 10^{-6}$ cm s^{-1}, $n = 4$). These results are illustrated in Fig. 2.

The role of NO in the permeability response to histamine and bradykinin

The permeability responses to both histamine and bradykinin require the presence of extracellular Ca^{2+} (Sarker *et al.* 2000) and a rise in intra-endothelial [Ca^{2+}] can activate endothelial nitric oxide synthase (eNOS) to form nitric oxide (NO), which will, in turn, activate soluble guanylyl cyclase (sGC) to form cGMP from GTP. When the NOS inhibitor L-NMMA (50 μM) was applied to the brain surface a small, significant increase in permeability resulted (Fig. 3; $0.3 \pm 0.04 \times 10^{-6}$ cm s^{-1}, $P < 0.05$). L-NMMA blocked the response to 50 μM dimaprit (dimaprit alone 1.2 ± 0.25, with L-NMMA, $0.3 \pm 0.20 \times 10^{-6}$ cm s^{-1}), but had no effect on the permeability response to 5 μM bradykinin (bradykinin alone, 1.7 ± 0.23; with L-NMMA, $1.4 \pm 0.36 \times 10^{-6}$ cm s^{-1}). This inhibition was partially reversed by the addition of 50 μM L-arginine ($0.8 \pm 0.16 \times 10^{-6}$ cm s^{-1}). D-NMMA (50 μM), a biologically inactive isomer of L-NMMA, had no effect on the dimaprit-mediated increase in permeability.

The role of soluble guanylyl cyclase

The observation that blocking nitric oxide synthesis has no effect on the permeability response to bradykinin raises the question of the source of the necessary cGMP. This was examined initially by using the inhibitors of sGC, LY 83583 (5 μM) and methylene blue (5 μM). Both inhibitors completely blocked the permeability increase brought about by dimaprit (50 μM dimaprit alone, $1.4 \pm 0.52 \times 10^{-6}$ cm s^{-1}, dimaprit with LY83583, $0.1 \pm 0.13 \times 10^{-6}$ cm s^{-1}, and dimaprit with methylene blue, $-0.1 \pm 0.20 \times 10^{-6}$ cm s^{-1}), but had no effect on the response to 5 μM bradykinin (bradykinin alone, $1.3 \pm 0.15 \times 10^{-6}$ cm s^{-1}; bradykinin with LY83583, $1.5 \pm 0.36 \times 10^{-6}$ cm s^{-1}, bradykinin with methylene blue, $1.3 \pm 0.25 \times 10^{-6}$ cm s^{-1}, Fig. 4*A*).

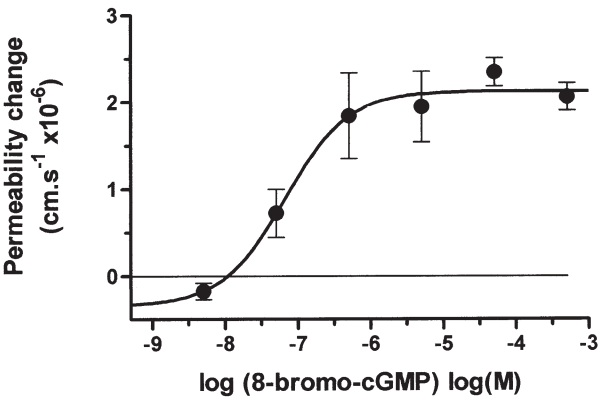


Figure 1. Permeability response to applied 8-bromo-cGMP

Permeability of single venular capillaries was measured before and during the application of 8-bromo-cGMP to the brain surface, and the change in permeability plotted against the dose. Each of four microvessels (diameters 10 to 16 μm) from four rats received a range of concentrations (from 5 nM to 500 μM). Analysis of variance showed that there was a significant dose dependent permeability increase ($P < 0.01$).

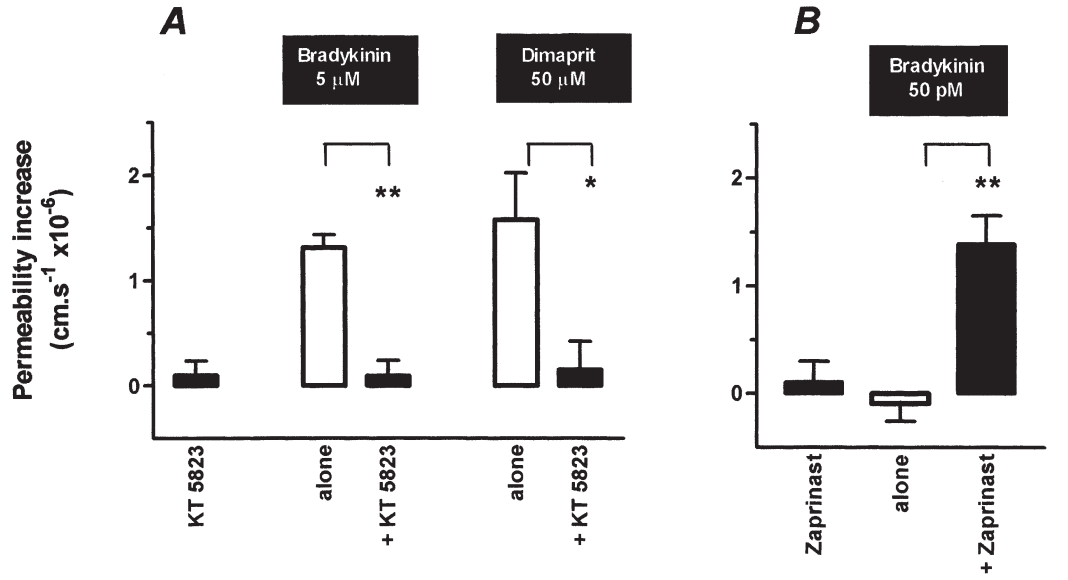


Figure 2. The effect of interfering with cGMP metabolism on the permeability response to bradykinin and dimaprit

A, the PKG inhibitor KT 5823 (0.5 μM) alone had no effect on permeability (11 vessels, five rats), but when co-applied with bradykinin (5 μM) or dimaprit (50 μM) it blocked the permeability responses (Mann–Whitney; $^{**}P < 0.01$, $^{*}P < 0.05$, five vessels from four rats). *B*, a sub-threshold concentration of bradykinin produced a maximal response when co-applied with the PDE_5 inhibitor, zaprinast (50 μM; $^{**}P < 0.01$; four vessels from four rats).

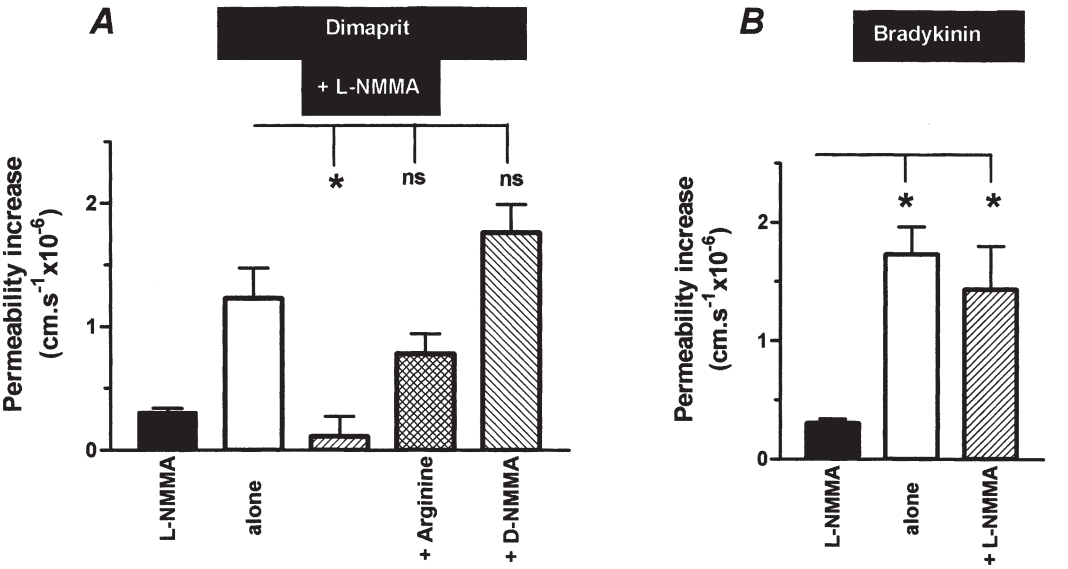


Figure 3. The role of nitric oxide in the permeability response to dimaprit and bradykinin

A and *B*, L-NMMA (50 μM) blocked the response to dimaprit (50 μM; $^{*}P < 0.05$, Kruskal–Wallis, Dunn's multiple comparison test), but had no effect on the permeability response to bradykinin (5 μM). L-NMMA by itself produced a small permeability increase. D-NMMA (50 μM) had no effect on dimaprit-mediated increase in permeability. These experiments were performed on five venular capillaries (diameter range 10 to 16 μm) from five rats.

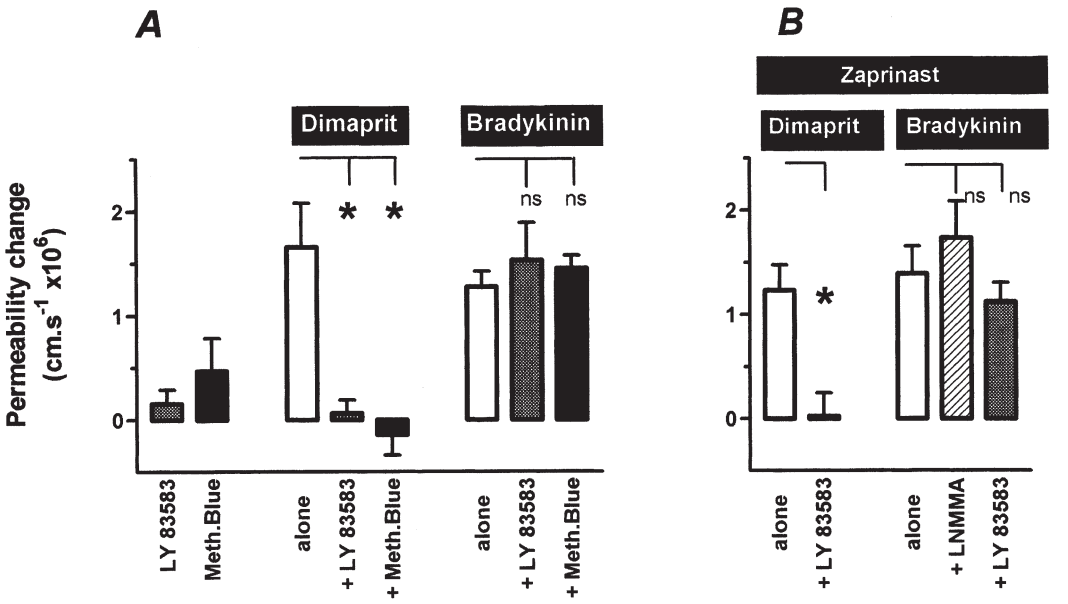


Figure 4. The role of soluble guanylyl cyclase in the permeability response to bradykinin and dimaprit

A, bradykinin (5 μM) and dimaprit (50 μM) were co-applied to the brain surface with the sGC inhibitors LY 83583 (5 μM) or methylene blue (5 μM), neither of which produced a significant permeability increase). The response to dimaprit was abolished, but neither inhibitor affected the bradykinin-induced permeability increase. *B*, the zaprinast (50 μM) potentiation of the response to bradykinin (50 pM) was unaffected by either LY 83583 or L-NMMA (50 μM), and did not protect the response to dimaprit from LY 83583. These experiments were performed on 12 venular capillaries (diameter range, 10 to 18 μm) from eight rats (*$P < 0.05$, Kruskal–Wallis, Dunn's multiple comparison test).

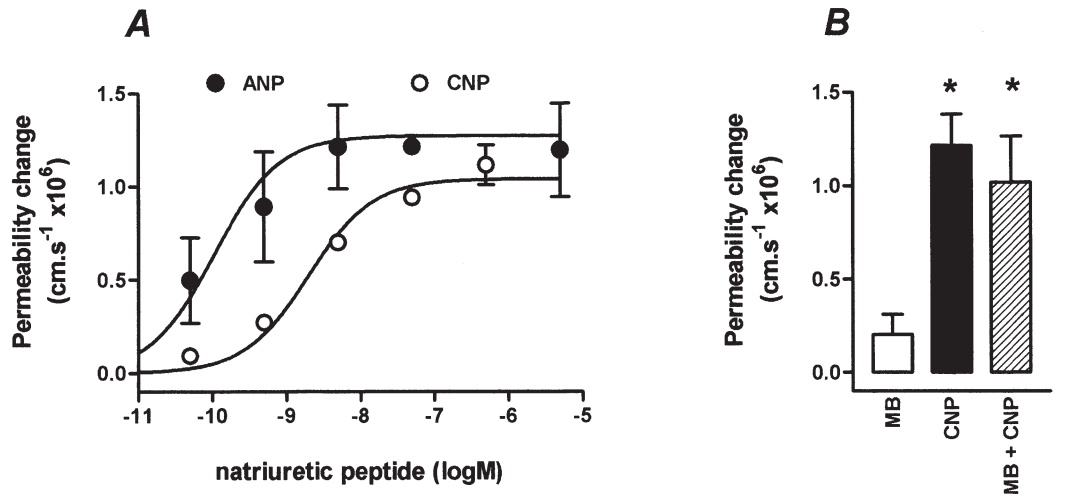
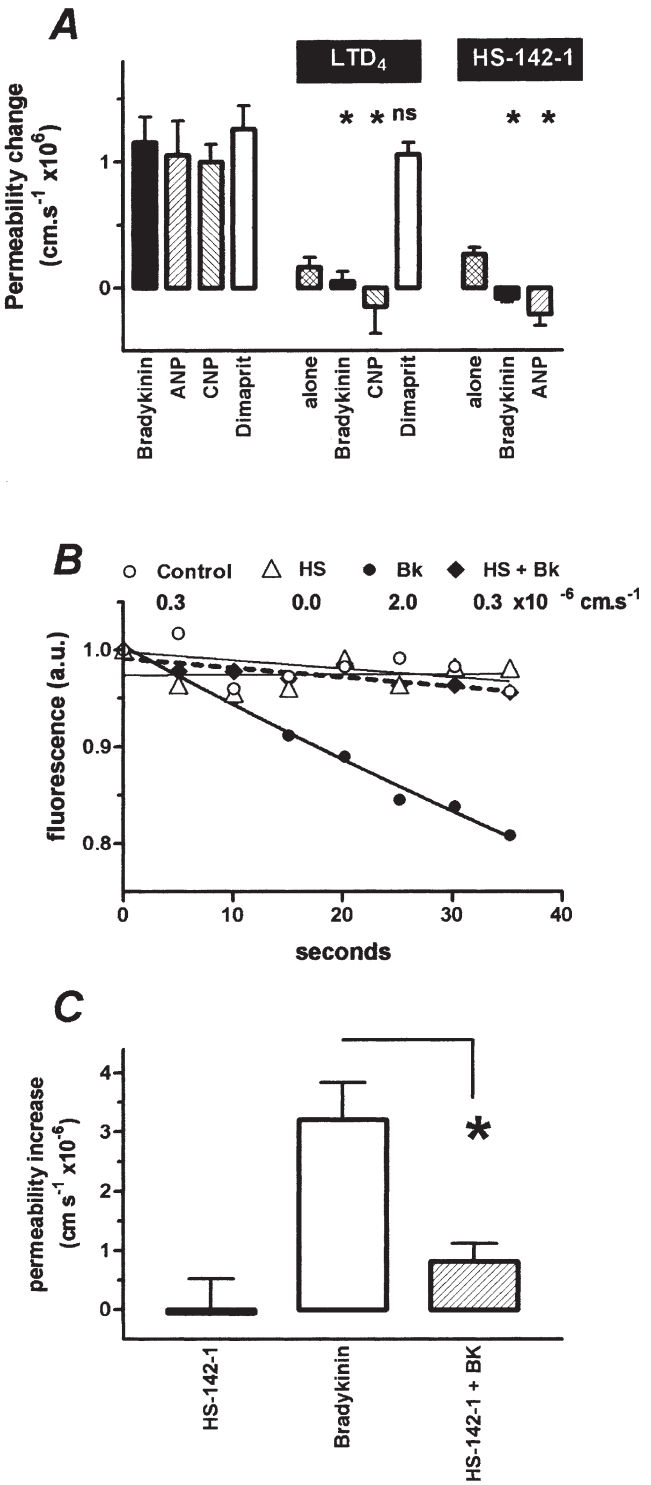


Figure 5. Natriuretic peptides increase permeability independently of soluble guanylyl cyclase

A, the dose–response curve of particulate guanylyl cyclase (pGC) activators ANP (●) and CNP (○). ANP and CNP were applied to the brain surface of pial venular capillaries in a range of concentration (5 pmol l^{-1} to 5 μmol l^{-1}). The full range of concentrations of either ANP or CNP was applied to four microvessels from four rats. Analysis of variance showed that there was a significant dose-dependent permeability increase ($P < 0.05$). *B*, methylene blue (MB; 5 μM) produced a small (but insignificant) permeability increase when applied alone, but did not affect the permeability response to CNP (0.5 μM); results from four vessels from four rats (*$P < 0.05$, Kruskal–Wallis, Dunn's multiple comparison test).

The possibility that the zaprinast-enhanced response to bradykinin (Fig. 2*B*) was due to potentiation of a low-level activation of the eNOS–sGC pathway was examined by co-applying L-NMMA and LY 83583 (5 μM). Neither of these affected the response (permeability increase $1.7 \pm 0.35 \times 10^{-6}$ cm s^{-1} and $1.1 \pm 0.18 \times 10^{-6}$ cm s^{-1}, respectively, Fig. 4*B*).



Particulate guanylyl cyclase

Particulate guanylyl cyclase is a possible alternative source for cGMP, but this is usually activated by a natriuretic peptide receptor, and C-type natriuretic peptide (CNP) has been shown to raise cGMP in rat cerebral microvessels (Kobayashi *et al.* 1994). We found that both atrial natriuretic peptide (ANP) and CNP increased cerebral microvascular permeability in a dose-dependent manner, and the dose–response curves (Fig. 5) give estimated maximal permeability increases for ANP and CNP of $1.2 \pm 0.11 \times 10^{-6}$ cm s^{-1} and $1.0 \pm 0.1 \times 10^{-6}$ cm s^{-1} with $\log EC_{50}$ of -10.0 ± 0.33 M and -8.7 ± 0.23 M, respectively. The permeability response to CNP (0.5 μM) was unaffected when co-applied with methylene blue ($1.2 \pm 0.17 \times 10^{-6}$ cm s^{-1} CNP alone, and $1.0 \pm 0.25 \times 10^{-6}$ cm s^{-1} with methylene blue, 5 μM).

The possibility that bradykinin activates particulate guanylyl cyclase was tested by co-applying bradykinin, natriuretic peptides and dimaprit with leukotriene D_4 (LTD_4, which has been shown to prevent ANP-dependent production of cGMP; Terada *et al.* 1989), and the specific natriuretic peptide antagonist HS-142-1 (Imura *et al.* 1992). Both LTD_4 and HS-142-1 blocked the response to bradykinin and the natriuretic peptides. The permeability changes, expressed as the change from the control level ($n = 4$ to 10 for each group) were: bradykinin (5 μM) alone, 1.2 ± 0.20; co-applied with LTD_4 (0.5 μM), $0.1 \pm 0.08 \times 10^{-6}$ cm s^{-1}; and with HS-142-1(5 μM), $-0.1 \pm 0.01 \times 10^{-6}$ cm s^{-1}; CNP (0.5 μM) alone, $1.0 \pm 0.15 \times 10^{-6}$ cm s^{-1}; with LTD_4, $0.1 \pm 0.22 \times 10^{-6}$ cm s^{-1}; ANP (5 μM) alone, $0.8 \pm 0.08 \times 10^{-6}$ cm s^{-1}, and with HS-142-1, $-0.3 \pm 0.18 \times 10^{-6}$ cm s^{-1}. The response to dimaprit was unaffected by the inhibitory action of LTD_4 on particulate guanylyl cyclase (dimaprit, 50 μM alone, $1.3 \pm 0.18 \times 10^{-6}$ cm s^{-1}, with LTD_4, $1.1 \pm 0.10 \times 10^{-6}$ cm s^{-1}).

Figure 6. Permeability response to bradykinin was blocked by blocking particulate guanylyl cyclase

A, 5 μM bradykinin, 50 μM dimaprit, 0.5 μM CNP and 5 μM ANP all increased permeability significantly. When co-applied with 1 μM LTD_4, the response to bradykinin and CNP was blocked (Kruskal–Wallis, Dunn's multiple comparison test, $^*P < 0.05$), but had no effect on the response to dimaprit. Similarly, the natriuretic peptide receptor blocker, HS-142-1, inhibited the responses to 5 μM bradykinin and 5 μM ANP. *B*, a series of occlusions on a single venular capillary in which bradykinin and the specific natriuretic receptor inhibitor HS-142-1 were applied in the lumen. The permeability coefficients shown were calculated from rate of decrease of fluorescence. *C*, summary of five paired luminal application experiments. The permeabilities measured with bradykinin alone were significantly greater than those with bradykinin combined with HS-142-1.

It is possible to interpret the inhibition of the response to bradykinin by HS-142-1 as evidence that bradykinin causes release of a natriuretic peptide from a nearby tissue (Wennberg *et al.* 1999) similar to the method by which des-arg^9 bradykinin was shown to act via histamine release (Sarker *et al.* 2000). The question whether cells other than endothelial cells were involved was tested by delivering bradykinin (5 μM), and bradykinin with HS-142-1 (5 μM), to the lumen of venular capillaries via intracarotid bolus injections (Fig. 6). The mean permeability increase with bradykinin alone (compared to a saline bolus immediately preceding) was $3.2 \pm 0.62 \times 10^{-6}$ cm s^{-1}, which is considerably higher than maximum obtained for the abluminal application. This is possibly due to a difference in the expression of kinin-destroying enzymes (Sarker *et al.* 2000). The response to bradykinin was reduced in boluses that included HS-142-1 ($0.8 \pm 0.30 \times 10^{-6}$ cm s^{-1}), and applied HS-142-1 alone also resulted in no permeability increase ($-0.1 \pm 0.59 \times 10^{-6}$ cm s^{-1}). These paired experiments were carried out on five vessels in five rats.

We found in a control experiment that there was no effect of HS-142-1 on the relaxation produced by bradykinin in a rat aortic ring preparation, which confirms that the drug does not act by binding to or inactivating bradykinin.

DISCUSSION

The dose–response relationship between 8-bromo-cGMP and permeability is consistent with the association between raised cGMP and increased permeability found in studies in pig coronary, frog mesenteric and rat cerebral microvessels (Yuan *et al.* 1993; He *et al.* 1998; Chi *et al.* 1999). Inhibition of the permeability response to histamine and bradykinin by blocking PKG with KT 5823 suggests that both agonists require cGMP for the permeability response in these microvessels, and thus may involve the activation of vasodilator-stimulated phosphoprotein, similar to that found in cultured cerebral endothelium (Sporbert *et al.* 1999). Potentiation of the sub-threshold concentration of bradykinin by the PDE$_5$ inhibitor zaprinast to a maximal response is consistent with this view. Intriguingly, there is also a suggestion that the lowest 8-bromo-cGMP concentration used here (5 nM) resulted in a permeability decrease (not statistically significant as the baseline permeability was very low), and, if real, some of the processes that lower permeability with raised cGMP in cultured endothelium may operate here (Draijer *et al.* 1995; Holschermann *et al.* 1997).

Both histamine and bradykinin are potent mediators of NO release from endothelial cells (Cherry *et al.* 1982; Lantoine *et al.* 1998), and a phospholipase C, nitric oxide, soluble guanylyl cyclase pathway was shown to mediate the permeability response to histamine in isolated, perfused porcine coronary venules (Yuan *et al.* 1993). The permeability response to bradykinin is not related to this pathway so clearly. In a number of *in vivo* microvascular experiments, it was shown that nitric oxide formation was associated with permeability reduction (e.g. Arnhold *et al.* 1999), but some do indicate a link between bradykinin-mediated permeability increase and nitric oxide production (e.g. Ramirez *et al.* 1995), while other experiments showed that there was no link between nitric oxide and the permeability-increasing effects of bradykinin (Cambridge & Brain, 1995; Félétou *et al.* 1996).

Soluble guanylyl cyclase has been shown to be the source of cGMP following histamine or bradykinin application in the microcirculation of the pig heart (Yuan *et al.* 1993), brain tumour (Nakano *et al.* 1996) and rat mesentery (He *et al.* 1998), and since this enzyme is activated by NO, inhibiting eNOS would be expected to block the permeability response. Such a mechanism appears to operate in this preparation when the histamine H_2 receptor was stimulated. Acute bradykinin application, however, produced NO-independent permeability increases. This is surprising, as a rise in $[Ca^{2+}]_i$ is normally sufficient to activate eNOS (e.g. Weih *et al.* 1998), and we have found that bradykinin application results in such a $[Ca^{2+}]_i$ increase (Sarker & Fraser, 1999), and that the presence of extracellular Ca^{2+} is necessary for the bradykinin-stimulated permeability increase (Sarker *et al.* 2000). In these vessels, however, both the bradykinin-mediated $[Ca^{2+}]_i$ and permeability increases depend on free radicals, probably formed from the interaction of arachidonic acid released by B_2 receptor activation of the Ca^{2+}-independent phospholipase A_2 with either cyclooxygenase or lipoxygenase (Sarker *et al.* 2000). Similarly, it appears that free radical generation is necessary to induce the acute increase in permeability, independently from any contribution from nitric oxide. This is consistent with the ability of free radicals generated by arachidonic acid to increase permeability (Easton & Fraser, 1998).

Both LY 85385 and methylene blue inhibited dimaprit-mediated responses whilst not affecting those mediated by bradykinin or the natriuretic peptides, but there has been some concern as to the specificity of these compounds for soluble guanylyl cyclase as sometimes they have been shown to reduce responses to natriuretic peptides (Ijioma *et al.* 1995; Kook *et al.* 1999). There are, however, a number of instances where it has been found that LY 85385 and methylene blue clearly inhibit the soluble, but not particulate, guanylyl cyclase (Martin *et al.* 1988; Kawada *et al.* 1994; Ali *et al.* 2000). Furthermore, recent work has shown that an alternative and more specific inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Garthwaite *et al.* 1995), can reduce responses to natriuretic peptides in the coronary circulation (Brunner & Wolkart, 2001), and it is possible that some of the natriuretic peptide signalling involves soluble guanylyl cyclase in some tissues. There are further complications, since it has

been shown that LY 85385 and methylene blue are able to inhibit NOS derived from brain tissue directly (Luo *et al.* 1995), albeit only at higher concentrations than in the present experiments, and it has also been shown that free radicals are generated during the cellular metabolism of LY 85385 and methylene blue, and that these radicals inhibit soluble guanylyl cyclase (Wei *et al.* 1996). In this preparation, however, free radical generation increases permeability (Easton & Fraser, 1998; Sarker *et al.* 2000), whereas the soluble guanylyl cyclase inhibitors reduce it.

Particulate guanylyl cyclase

ANP and CNP have been shown to activate the NPR-A and NPR-B receptors (respectively) which are linked across the cell membrane to kinase homology domain and guanylyl cyclase domains, the whole forming particulate guanylyl cyclase (Potter & Hunter, 2001). These natriuretic peptides also have high affinities for the NPR-C clearance receptor, which has been associated with inhibition of adenylyl cyclase (Hempel *et al.* 1998). Natriuretic peptides have been shown to increase the permeability of cerebral and peripheral microvessels (Huxley *et al.* 1987; Vigne & Frelin, 1992) and to increase cGMP levels in brain microvessels (Grammas *et al.* 1991). In the present series of experiments, both ANP and CNP increased permeability in a dose-dependent manner (Fig. 5).

We found that the permeability response to bradykinin was inhibited by the specific natriuretic receptor antagonist HS-142-1, and by LTD_4 (Fig. 6). The finding that HS-142-1 was effective in blocking the permeability response when co-applied with bradykinin in the vessel lumen indicates that no other cell type is involved in these responses, in contrast with our previous observations with $desArg^9$-bradykinin (Sarker *et al.* 2000). There are indications from the literature that bradykinin can increase cGMP formation via particulate guanylyl cyclase directly, in some circumstances (Leibmann *et al.* 1989), and Wennberg *et al.* (1999) showed that HS-142-1 application further inhibited the relaxation of coronary arteries to bradykinin following eNOS blockade. The mechanism by which this occurs is not clear, but it is possible to speculate that bradykinin causes some substance to be released from endothelial cells which then activates the natriuretic receptor. For example CNP has been suggested as a candidate for endothelium-dependent hyperpolarizing

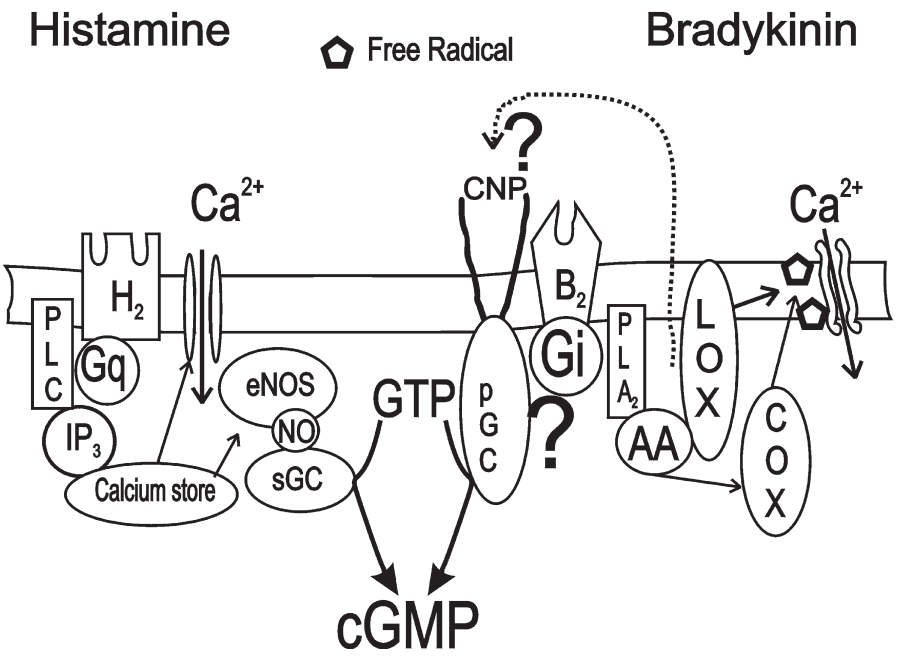


Figure 7. Signalling pathways for the histamine H_2 and the bradykinin B_2 receptors on pial venular endothelium

The activated H_2 receptor probably signals via Gαq to phospholipase C (PLC), and the formed IP_3 releases Ca^{2+} from calcium stores. This Ca^{2+} is able to activate eNOS to produce nitric oxide, which in turn activates soluble guanylyl cyclase (sGC), and the cGMP so formed activates PKG. The depletion of the calcium stores opens a Ca^{2+} entrance pathway. The B_2 receptor on these cells appears to lack a connection with Gαq, and probably signals via Gαi to a calcium-independent phospholipase A_2 (PLA_2), and possibly to particulate guanylyl cyclase (pGC). Alternatively CNP could be released from the endothelium and activate pGC via the natriuretic peptide receptor. The arachidonic acid forms free radicals from cyclooxygenase (COX) and lipoxygenase (LOX), and the consequent lipid peroxidation increases the plasma membrane permeability to Ca^{2+}. This Ca^{2+} is, with activated PKG, required for increased permeability.

factor (Wennberg *et al.* 1999), but Barton *et al.* (1998) have shown that this is unlikely, and citrulline has been found to cause a HS-142-1-susceptible cGMP formation in endothelial cells (Ruiz & Tejerina, 1998).

There is a possible alternative pathway that is suggested by recent findings. Adrenomedullin has been shown to activate particulate guanylyl cyclase via Gi-protein (Ali *et al.* 2000), and the bradykinin B_2 receptor can signal via Gi-protein in cultured brain endothelium (Fabian *et al.* 1998). So it is possible that bradykinin in pial venular endothelium stimulates particulate guanylyl cyclase via Gi-protein. Such a mechanism would explain the inhibitory effect of LTD_4 on bradykinin, since it can induce the translocation of Gi-protein from the plasma membrane to the cytoskeleton within 15 s (Adolfsson *et al.* 1996). If this were the mechanism by which bradykinin acts, or if there were any other intracellular activation of particulate guanylyl cyclase, then there are implications for the way in which HS-142-1 inhibits. HS-142-1 is a 3 kDa polysaccharide of bacterial origin, and is unlikely to enter the cell membrane, but may act as a false agonist to keep the particulate guanylyl cyclase complex in an inactive state.

In conclusion, we have found that cGMP is a necessary requirement for permeability increases consequent to acute stimulation of the histamine H_2 and the bradykinin B_2 receptors, and that in these pial venular capillaries the two receptors activate soluble and particulate guanylyl cyclase, respectively, for its production. A scheme for these signalling pathways, based on cited references and on the work in our laboratory (Easton & Fraser, 1998; Sarker *et al.* 1998; Sarker *et al.* 2000), is presented in Fig. 7.

REFERENCES

Abbott, N. J. (2000). Inflammatory mediators and modulation of blood-brain barrier permeability. *Cellular and Molecular Neurobiology* **20**, 131–147

Adolfsson, J. L., Ohd, J. F. & Sjolander, A. (1996). Leukotriene D4-induced activation and translocation of the G-protein alpha i3-subunit in human epithelial cells. *Biochemical and Biophysical Research Communications* **226**, 413–419.

Ali, N., Yousufzai, S. Y. & Abdel-Latif, A. A. (2000). Activation of particulate guanylate cyclase by adrenomedullin in cultured SV-40 transformed cat iris sphincter smooth muscle (SV-CISM-2) cells. *Cell Signalling* **12**, 491–498.

Arnhold, S., Antoine, D., Blaser, H., Bloch, W., Andressen, C. & Addicks, K. (1999). Nitric oxide decreases microvascular permeability in bradykinin stimulated and nonstimulated conditions. *Journal of Cardiovascular Pharmacology* **33**, 938–947.

Barton, M., Beny, J. L., D'Uscio, L. V., Wyss, T., Noll, G. & Luscher T. F. (1998). Endothelium-independent relaxation and hyperpolarization to C-type natriuretic peptide in porcine coronary arteries. *Journal of Cardiovascular Pharmacology* **31**, 377–383.

Brunner, F. & Wolkart, G. (2001). Endothelial NO/cGMP system contributes to natriuretic peptide-mediated coronary and peripheral vasodilation. *Microvascular Research* **61**, 102–110.

Cambridge, H. & Brain, S.D. (1995). Mechanism of bradykinin-induced plasma extravasation in the rat knee joint. *British Journal of Pharmacology* **115**, 641–647.

Cherry, P. D., Furchgott, R. F., Zawadzki, J. V. & Jothianandan, D. (1982). Role of endothelial cells in relaxation of isolated arteries by bradykinin. *Proceedings of the National Academy of Sciences of the USA* **79**, 2106–2110.

Chi, O. Z., Liu, X. & Weiss, H. R. (1999). Effects of cyclic GMP on microvascular permeability of the cerebral cortex. *Microvascular Research* **58**, 35–40.

Draijer, R., Atsma, D. E., van der Laarse, A. & van Hinsbergh, V. W. (1995). cGMP and nitric oxide modulate thrombin-induced endothelial permeability. Regulation via different pathways in human aortic and umbilical vein endothelial cells. *Circulation Research* **76**,199–208.

Easton, A. S. & Fraser P. A. (1994). Variable restriction of albumin diffusion across inflamed cerebral microvessels. *Journal of Physiology* **475**, 147–157.

Easton, A. S. & Fraser P. A. (1998). Arachidonic acid increases cerebral microvascular permeability by free radicals in single pial microvessels of the anaesthetized rat. *Journal of Physiology* **507**, 541–547.

Fabian, G., Szabo, C. A., Bozo, B., Greenwood, J., Adamson, P., Deli, M. A., Joo, F., Krizbai, I. A. & Szucs, M. (1998). Expression of G-protein subtypes in cultured cerebral endothelial cells. *Neurochemistry International* **33**, 179–185.

Félétou, M., Bonnardel, E. & Canet, E. (1996). Bradykinin and changes in microvascular permeability in the hamster cheek pouch: role of nitric oxide. *British Journal of Pharmacology* **118**, 1371–1376.

Fraser, P. A. & Dallas, A. D. (1990). Measurement of filtration coefficient in single cerebral microvessels of the frog. *Journal of Physiology* **423**, 343–361.

Fraser, P. A. & Dallas, A. D. (1993). Permeability of disrupted cerebral microvessels in the frog. *Journal of Physiology* **461**, 619–632.

Frossard, N., Landry, Y., Pauli, G. & Ruckstuhl, M. (1981). Effects of cyclic AMP- and cyclic GMP- phosphodiesterase inhibitors on immunological release of histamine and on lung contraction. *British Journal of Pharmacology* **73**, 933–938.

Furchgott, R. F. & Vanhoutte, P. M. (1989). Endothelium-derived relaxing and contracting factors. *FASEB Journal* **3**, 2007–2018.

Garthwaite, J., Southam, E., Boulton, C. L., Nielsen, E. B., Schmidt, K. & Mayer, B.(1995). Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. *Molecular Pharmacology* **48**, 184–188.

Graier, W. F., Hoebel, B. G., Paltauf-Doburzynska, J. & Kostner G. M. (1998). Effects of superoxide anions on endothelial Ca2+ signaling pathways. *Arteriosclerosis Thrombosis Vascular Biology* **18**, 1470–1479.

Grammas, P., Giacomelli, F., Bessert, D. & Wiener, J. (1991). Angiotensin II and atrial natriuretic factor receptor interactions at the blood-brain barrier. *Brain Research* **562**, 93–97.

He, P., Zeng, M. & Curry, F. E. (1998). cGMP modulates basal and activated microvessel permeability independently of $[Ca^{2+}]_i$. *American Journal of Physiology* **274**, H1865–i1874

Hempel, A., Noll, T., Bach, C., Piper, H. M., Willenbrock, R., Hohnel, K., Haller, H. & Luft F. C. (1998). Atrial natriuretic peptide clearance receptor participates in modulating endothelial permeability. *American Journal of Physiology* **275**, H1818–1825

Holschermann, H., Noll, T., Hempel, A. & Piper, H. M. (1997). Dual role of cGMP in modulation of macromolecule permeability of aortic endothelial cells. *American Journal of Physiology* **272**, H91–98.

Huxley, V. H., Tucker, V. L., Verburg, K. M. & Freeman, R. H. (1987). Increased capillary hydraulic conductivity induced by atrial natriuretic peptide. *Circulation Research* **60**, 304–307.

Ijioma, S. C, Challiss, R. A. & Boyle, J. P. (1995). Comparative effects of activation of soluble and particulate guanylyl cyclase on cyclic GMP elevation and relaxation of bovine tracheal smooth muscle. *British Journal of Pharmacology* **115**, 723–732.

Imura, R., Sano, T., Goto, J., Yamada, K. & Matsuda, Y. (1992). Inhibition by HS-142-1, a novel nonpeptide atrial natriuretic peptide antagonist of microbial origin, of atrial natriuretic peptide-induced relaxation of isolated rabbit aorta through the blockade of guanylyl cyclase-linked receptors. *Molecular Pharmacology* **42**, 982–990.

Kawada, T., Ishibashi, T., Sasage, H., Kato, K. & Imai, S. (1994). Modification by LY 83583 and methylene blue of relaxation induced by nitric oxide, glyceryl trinitrate, sodium nitroprusside and atriopeptin in aortae of the rat, guinea-pig and rabbit. *General Pharmacology* **25**, 1361–1371.

Kobayashi, H., Ueno, S., Tsutsui, M., Okazaki, M., Uezono, Y., Yanagihara, N., Yuhi, T. & Izumi, F. (1994). C-type natriuretic peptide increases cyclic GMP in rat cerebral microvessels in primary culture. *Brain Research* **648**, 324–326.

Kook, H., Rhee, J. H., Lee, S. E., Kang, S. Y., Chung, S. S., Cho, K. W. & Baik, Y. H. (1999). Activation of particulate guanylyl cyclase by Vibrio vulnificus hemolysin. *European Journal of Pharmacology* **365**, 267–272.

Lantoine, F., Iouzalen, L., Devynck, M.A., Millanvoye-Van Brussel, E. & David-Dufilho, M. (1998). Nitric oxide production in human endothelial cells stimulated by histamine requires Ca^{2+} influx. *Biochemical Journal* **330**, 695–699.

Liebmann, C., Roemer, W. & Reissmann, S. (1989). Bradykinin action in the rat duodenum: Ca^{2+}-dependent effects of bradykinin on the activity of particulate guanylate cyclase. *Biomedica Biochimica Acta* **48**, 597–600.

Luo, D., Das, S. & Vincent, S. R. (1995). Effects of methylene blue and LY83583 on neuronal nitric oxide synthase and NADPH-diaphorase. *European Journal of Pharmacology* **290**, 247–251.

Martin, W., White, D. G. & Henderson, A. H. (1988). Endothelium-derived relaxing factor and atriopeptin II elevate cyclic GMP levels in pig aortic endothelial cells. *British Journal of Pharmacology* **93**, 229–239

Michel, C. C. & Curry, F. E. (1999). Microvascular permeability. *Physiological Reviews* **79**, 703–761.

Nakano, S., Matsukado, K. & Black, K. L. (1996). Increased brain tumor microvessel permeability after intracarotid bradykinin infusion is mediated by nitric oxide. *Cancer Research* **56**, 4027–4031.

Nazario, B., Hu, R. M., Pedram, A., Prins, B. & Levin, E. R. (1995). Atrial and brain natriuretic peptides stimulate the production and secretion of C-type natriuretic peptide from bovine aortic endothelial cells. *Journal for Clinical Investigation* **95**, 1151–1157.

Potter, L. R. & Hunter, T. (2001). Guanylyl cyclase-linked natriuretic peptide receptors: structure and regulation. *Journal of Biological Chemistry* **276**, 6057–6060.

Ramirez, M. M., Quardt, S. M., Kim, D., Oshiro, H., Minnicozzi, M. & Duran, W. N. (1995). Platelet activating factor modulates microvascular permeability through nitric oxide synthesis. *Microvascular Research* **50**, 223–234.

Ruiz, E. & Tejerina, T. (1998). Relaxant effects of L-citrulline in rabbit vascular smooth muscle. *British Journal of Pharmacology* **125**, 186–192.

Sarker, M. H., Easton, A. S. & Fraser, P. A. (1998). Regulation of cerebral microvascular permeability by histamine in the anaesthetized rat. *Journal of Physiology* **507**, 909–918.

Sarker, M. H. & Fraser, P. A. (1998). Evidence that bradykinin increases cerebral microvascular permeability via cGMP generated independently from soluble guanylate cyclase in the anaesthetized rat. *Journal of Physiology* **506.P**, 22*P*.

Sarker, M. H. & Fraser, P. A. (1999). Bradykinin induced calcium increase depends on free radical formation in pial venular capillaries. *Journal of Vascular Research* **36**, 327.

Sarker, M. H., Hu, D.-E. & Fraser, P. A. (2000). Acute effects of bradykinin on cerebral microvascular permeability in the anaesthetized rat. *Journal of Physiology* **528**, 177–187.

Schmidt, M. J., Sawyer, B. D., Truex, L. L., Marshall, W. S. & Fleisch, J. H. (1985). LY83583: an agent that lowers intracellular levels of cyclic guanosine 3′,5′-monophosphate. *Journal of Pharmacology and Experimental Therapeutics* **232**, 764–769.

Sporbert, A., Mertsch, K., Smolenski, A., Haseloff, R. F., Schonfelder, G., Paul, M., Ruth, P., Walter, U. & Blasig, I. E. (1999). Phosphorylation of vasodilator-stimulated phosphoprotein: a consequence of nitric oxide- and cGMP-mediated signal transduction in brain capillary endothelial cells and astrocytes. *Brain Research. Molecular Brain Research* **67**, 258–266.

Terada, Y., Tomita, K., Yoshiyama, N., Shiigai, T. & Marumo, F. (1989). Leukotriene D4 inhibits atrial natriuretic factor-dependent cGMP production in rat glomerulus. *American Journal of Physiology* **256**, F95–99.

Vigne, P. & Frelin, C. (1992). C-type natriuretic peptide is a potent activator of guanylate cyclase in endothelial cells from brain microvessels. *Biochemical Biophysical Research Communications* **183**, 640–644.

Wei, E. P., Kontos, H. A. & Beckman, J. S. (1996). Mechanisms of cerebral vasodilation by superoxide, hydrogen peroxide, and peroxynitrite. *American Journal of Physiology* **271**, H1262–1266.

Weih, M. K., Weikert, S., Freyer, D. & Dirnagl, U. (1998). Chemiluminescence detection of nitric oxide production from rat cerebral cortical endothelial cells in culture. *Brain Research. Brain Research Protocols* **2**, 175–182.

Wennberg, P.W., Miller, V. M., Rabelink, T. & Burnett, J. C., Jr (1999). Further attenuation of endothelium-dependent relaxation imparted by natriuretic peptide receptor antagonism. *American Journal of Physiology* **277**, H1618–1621.

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