

Acute effects of bradykinin on cerebral microvascular permeability in the anaesthetized rat

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1. The permeability response to acutely applied bradykinin and [des-Arg⁹]-bradykinin on single cerebral venular capillaries has been investigated using the low molecular mass fluorescent dyes Lucifer Yellow and Sulforhodamine B with the single vessel occlusion technique.
2. When bradykinin was applied repeatedly for up to 2 h, the permeability increase was small and reversible for concentrations that ranged from 5 nM to 50 μ M.
3. The log EC₅₀ of the permeability response to bradykinin was -5.3 ± 0.15 (log M; mean \pm s.e.m.). This was reduced to -6.37 ± 0.24 with the angiotensin-converting enzyme inhibitor captopril, to -6.33 ± 0.19 with the neutral endopeptidase inhibitor phosphoramidon and to -7.3 ± 0.20 with captopril and phosphoramidon combined.
4. The permeability response to bradykinin was blocked by the bradykinin B₂ receptor antagonist HOE 140, by inhibition of the Ca²⁺-independent phospholipase A₂, by the scavenging of free radicals, or by inhibition of both cyclo-oxygenase and lipoxygenase in combination. Block of Ca²⁺ entry channels with SKF 96365 had no effect on the response.
5. Application of [des-Arg⁹]-bradykinin also increased permeability over the concentration range 5 nM to 50 μ M, with a log EC₅₀ of -5.6 ± 0.37 . This response was not affected by free radical scavenging, but was completely blocked by the histamine H₂ receptor blocker cimetidine.
6. These results imply that the acute permeability response to bradykinin is mediated via the release of arachidonic acid, which is acted on by cyclo-oxygenase and lipoxygenase resulting in the formation of free radicals, and that the response to [des-Arg⁹]-bradykinin is mediated via histamine.

Traumatic brain injury, stroke, and the active phase of multiple sclerosis result in blood–brain barrier disruption, and sometimes lead to a life-threatening cerebral oedema. The mechanisms of this disruption are still unclear but there is evidence that inflammatory mediators are involved, and it has been suggested recently that bradykinin plays an important role in the cerebral oedema that follows stroke and trauma (Narotam *et al.* 1998; and see Raidoo & Bhoola, 1998). Furthermore, treatment with a bradykinin B₂ receptor antagonist following experimental reversible ischaemia reduced the oedema and infarct volume (Relton *et al.* 1997), and patients who were operated on for cerebral arterial stenosis made better progress if preoperative cerebral kinin formation was low (Makevnina *et al.* 1994). The development of non-hydrolysable bradykinin analogues currently under investigation for facilitation of the passage of therapeutic agents into the brain (Jolliet-Riant & Tillement, 1999) additionally highlights the importance of bradykinin in modulating the blood–brain barrier.

Previous experiments on the effects of bradykinin application to the brain have indicated that there is often, but not always, extravasation of marker dyes (e.g. Unterberg *et al.* 1984; Wahl *et al.* 1985). It is possible that this dye leakage is secondary to increased vascular pressure, since hypertension itself can result in blood–brain barrier disruption (Mayhan, 1996). The permeability may be estimated independently of pressure by using single microvessel techniques (Easton & Fraser, 1994), an approach pioneered in the brain by Crone & Olesen (1982) who used changes in the electrical cable properties of 500 μ m lengths of pial venular capillaries as the basis of the measurement. Results from the frog (Olesen & Crone, 1986), and the rat (Butt, 1995), showed that very high concentrations of bradykinin have only a small permeability-increasing effect. This is apparently at variance with the recent findings that bradykinin is important in the development of oedema following experimental cerebral ischaemia in rats (Kamiya *et al.* 1993), but it is possible that the resistance measurements

underestimate the full permeability response due to uneven permeability along the length of the microvessel itself (see Easton *et al.* 1997).

We have used a single pial microvessel occlusion technique that allows measurements of permeability change over small regions of microvessels (*ca* 10 μm in length) in experiments designed to investigate the cerebrovascular permeability effects of bradykinin (which activates B_2 receptors) and its active metabolite [des-Arg⁹]-bradykinin (which activates B_1 receptors; see Hall, 1992) on single pial venular capillaries. We have also investigated the intracellular mechanisms that link the activation of the receptors to the permeability changes. Some of the findings reported here have been previously presented in a preliminary form (Sarker & Fraser, 1994, 1995).

METHODS

The method used in this study, and its theoretical basis, has been described fully elsewhere (Fraser & Dallas, 1993; Easton & Fraser, 1994). Briefly, the microcirculation of the surface of the brain of rats (aged 20–30 days) was exposed by removal of the dura and arachnoid. A low molecular mass fluorescent dye, either Lucifer Yellow (457 Da) or Sulforhodamine B (580 Da), was introduced into single venular capillaries via a bolus injection into the carotid artery. Sulforhodamine B has the advantage of requiring a longer wavelength for excitation and results in a more stable preparation. The fluorescence signal, which has been shown to increase linearly with dye concentration, 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 200–300 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 were 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. The dye concentration in the measured portion will depend on axial flow and diffusion as well as on transmural convection and diffusion. We have shown that when the dye is injected into the arterial blood supplying the pial vasculature the trapped erythrocytes prevent dye diffusion from the open end of the occluded vessel and the axial dye concentration gradient is flat (Easton *et al.* 1997). Under these circumstances axial diffusion and convection have no effect on the dye concentration in the lumen (Fraser & Dallas, 1993). As in this preparation there is no continuous barrier to free diffusion of dye from the surface of the vessel (Butt *et al.* 1990), the abluminal dye concentration can be kept at undetectably low levels by maintaining a flow of superfusate of about 1–2 ml min^{-1} . Previous experiments showed that there was no difference in the rate of fall of luminal dye concentration even when the superfusate flow was stopped (Easton, 1993). 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 the rate of fall of the intravascular concentration of a small polar molecule is independent of the hydrostatic pressure (Fraser & Dallas, 1993), the concentration of dye in the vessel 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 C_t and C_0 are the concentrations at times t and 0, and $k = 4P/d$, where P is the permeability and d the diameter of the vessel.

Animal preparation

The experiments were performed on Wistar rats of either sex and were carried out within guidelines directed by The Animals (Scientific Procedures) Act 1986. The animals were anaesthetized by an intraperitoneal injection of 60 mg (kg body weight)⁻¹ sodium pentobarbitone diluted in water (25% w/v), supplemented (10% of the original dose) as necessary, as assessed by the foot pinch-withdrawal reflex. At the end of the experiment the animal was killed with an overdose of 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 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 lamboid sutures, was thinned with a dental drill. A metal ring (internal diameter, 7 mm; external diameter, 13 mm) was glued onto the cranium surrounding the thinned bone with cyanoacrylate adhesive. The lumen of the ring was filled with artificial cerebrospinal fluid (ACSF; for composition see below), whilst the adhesive set. The thinned cranial surface within the ring was constantly superfused by a pump at a rate of 1–2 ml min^{-1} with ACSF 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, which in these young rats leaves the microvessels free from a continuous layer of overlying tissue (Butt *et al.* 1990). The rat was then placed on the modified stage of a microscope (ACM, Zeiss Oberkochen) and the exposed cerebral surface illuminated with a 50 W mercury vapour lamp through a $\times 20$ water-immersion objective lens (Cooke; NA, 0.5).

Artificial cerebrospinal fluid and drug application

The cerebral surface was superfused with ACSF at a rate of 0.5–1 ml min^{-1} , warmed to 37 ± 1 °C and buffered to pH 7.40. The ACSF contained (mM): NaCl, 110.5; KCl, 4.7; CaCl₂, 2.5; KH₂PO₄, 1.1; MgSO₄·7H₂O, 1.25; NaHCO₃, 25; and HEPES, 15. All chemicals were obtained from BDH (Poole, UK) except MgSO₄·7H₂O (Fisons, Loughborough, UK) and HEPES (Sigma). The bradykinin B_1 receptor agonist [des-Arg⁹]-bradykinin, the B_2 agonist bradykinin, and the B_1 antagonist [des-Arg⁹-Leu⁸]-bradykinin were generously provided by Dr Martin Perkins of the Novartis Institute for Medical Science. The B_2 antagonist HOE 140 was generously provided by Dr Klaus J. Wirth of Hoechst. Possible second messengers were investigated by using the cation channel blocker SKF 96365 (Merritt *et al.* 1990; generously supplied by SmithKline Beecham Pharmaceuticals), the phospholipase A₂ (PLA₂) inhibitors aristolochic acid and palmitoyl trifluoromethyl ketone (Calbiochem), the H₂ receptor antagonist cimetidine (SmithKline Beecham), the free radical scavengers butylated-hydroxytoluene (BHT) and superoxide dismutase (SOD) with catalase, the cyclo-oxygenase and lipoxygenase blockers indomethacin and nordihydroguaiaretic acid (NDGA), the neutral endopeptidase (NEP) inhibitor phosphoramidon, the H₂ receptor agonist dimaprit, and the angiotensin-converting enzyme (ACE) inhibitor captopril (all

purchased from Sigma). Bradykinin, [des-Arg⁹]-bradykinin and other substances were applied abuminally into the pool of ACSF over the exposed cerebral surface, and the concentrations given are the final concentration in the pool.

Protocols

Permeability measurements were carried out on venular capillaries between 10 and 18 μm in diameter in the following manner. The selected dye-containing vessel was occluded, and once a stable level of fluorescence was obtained (after about 30 s) bradykinin or [des-Arg⁹]-bradykinin, or kinin mixed with other drugs, 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 were washed off the brain surface. The order of application of the kinin–drug mixtures was randomized in any series, except with a slowly reversible drug, such as indomethacin, when this was applied towards the end of an experiment. 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 concentrations of the drug in pseudo-random order to a single vessel, and at least

four vessels from four rats⁹ were used for each dose–response curve. The actions of inhibitors and free radical scavengers were investigated by co-application with the stated concentration of bradykinin or [des-Arg⁹]-bradykinin, and within any series these too were paired experiments.

Statistics

Unless otherwise stated, the results are expressed as the mean ± s.e.m., and the significance of any changes was assessed by a non-parametric one-way analysis of variance test (Kruskal–Wallis) with Dunn’s multiple comparison test. Regression lines and sigmoidal dose–response curves were fitted using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

The present experiments were carried out on pial venular capillaries of at least 200 μm in length, which had negligible or low initial permeabilities, that is from below the limit of

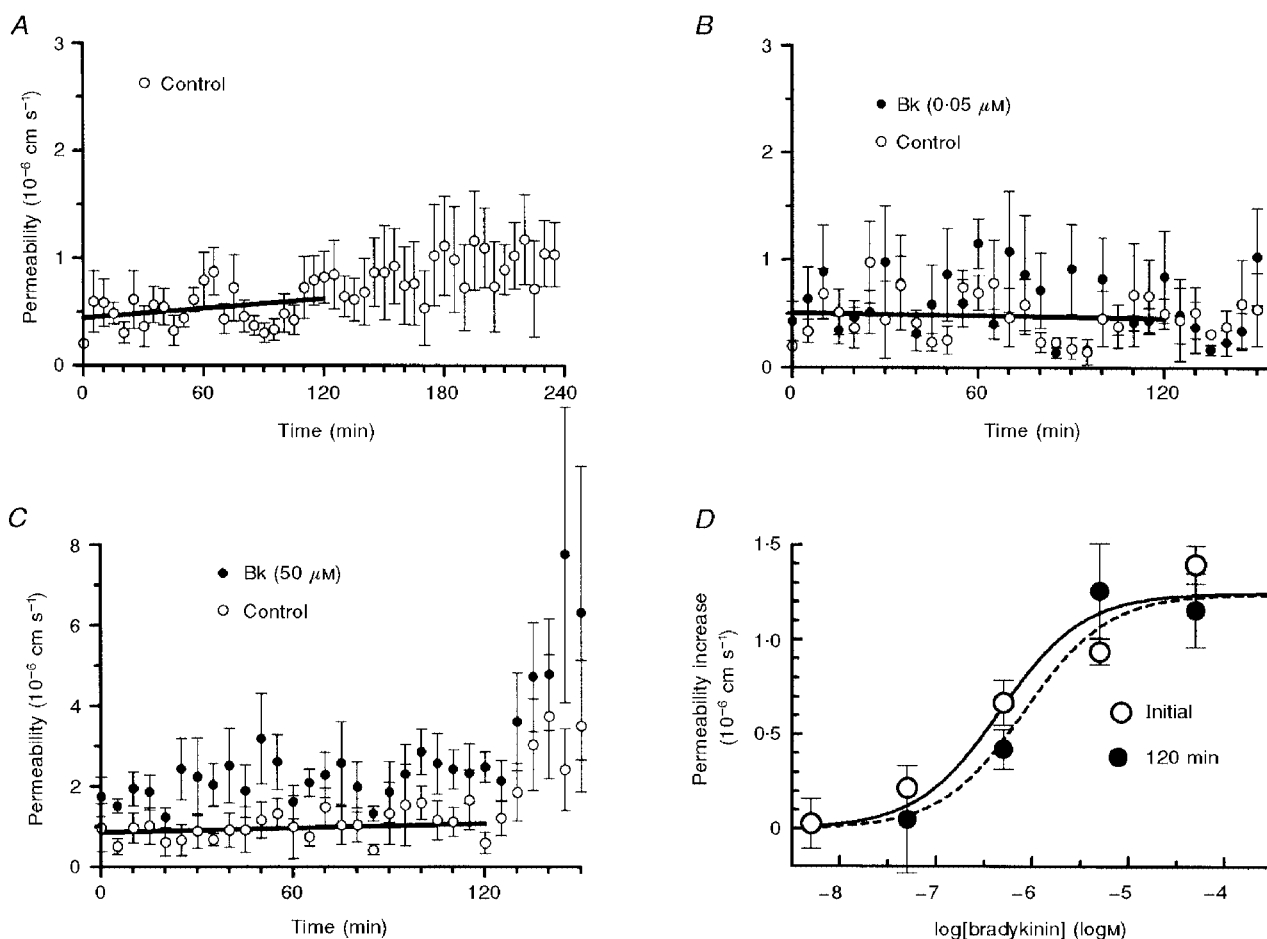


Figure 1. The stability of the permeability response to acute topical application of bradykinin

A, permeability measured with no addition of bradykinin at 5 min intervals in ten venular capillaries to assess the stability of the preparation. *B* and *C*, examples of the permeability response to bradykinin application to the brain surface at concentrations of 0.05 and 50 μM, respectively, at 5 min intervals. The permeability was measured immediately before, and from 30 to 60 s during, each application. Each concentration was repeated on four microvessels. The regression lines through the control values, shown for the first 2 h, do not increase significantly. *D*, dose–response curve for the permeability increase due to bradykinin application, estimated from initial values and those obtained 2 h later.

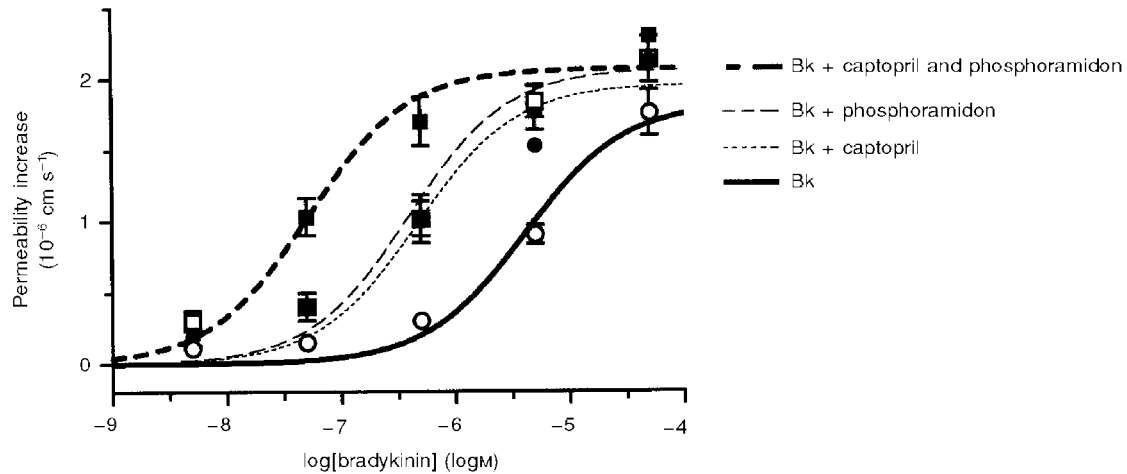


Figure 2. The effect of inhibiting bradykinin-destroying enzymes on the permeability response to bradykinin

Dose–response curves for bradykinin (Bk; 5 nM to 50 μ M) when applied to the brain surface for 30 to 60 s, alone (○) or in combination with the neutral endopeptidase inhibitor phosphoramidon (□; 200 μ M), the angiotensin-converting enzyme inhibitor captopril (●; 1 μ M) or with both inhibitors in combination (■). Four vessels were used for each dose–response curve.

detection (nominally $0.2 \times 10^{-6} \text{ cm s}^{-1}$; Easton *et al.* 1997) up to $2.0 \times 10^{-6} \text{ cm s}^{-1}$. A control series of experiments was carried out in which permeability measurements were made at 5 min intervals on ten venular capillaries from ten rats. There was a very slow spontaneous permeability increase at a rate of $0.09 \times 10^{-6} \pm 0.06 \times 10^{-6} \text{ cm s}^{-1} \text{ h}^{-1}$ over the first 2 h (see Fig. 1A), which is in contrast with our previous observation on spontaneous permeability increase (Easton *et al.* 1997), and can be ascribed to improvements in the technique. The effect of repeated bradykinin application was studied by applying it to the cerebral surface for 30–60 s during an occlusion that was released as soon as the new rate of decrease in fluorescence signal was established. The bradykinin was then washed off and a new pair of

measurements (control followed by bradykinin) were made 5 min later. This was continued for 150 min (i.e. 30 repetitions of the same concentration), in each of four vessels (from 4 rats) for each concentration of bradykinin from 5 nM to 50 μ M. The responses were stable for the first 2 h, and the permeability value returned to the baseline level before a subsequent bradykinin application (Fig. 1B and C). The baseline permeability changed little during this time (rates of change were $-0.23 \times 10^{-6} \pm 0.08 \times 10^{-6}$ and $0.11 \times 10^{-6} \pm 0.11 \times 10^{-6} \text{ cm s}^{-1} \text{ h}^{-1}$ for the 50 nM and 50 μ M applications, respectively), although it did change rapidly after this time with the highest concentration of bradykinin. The response to bradykinin also increased dramatically after 2 h, and for these reasons all the

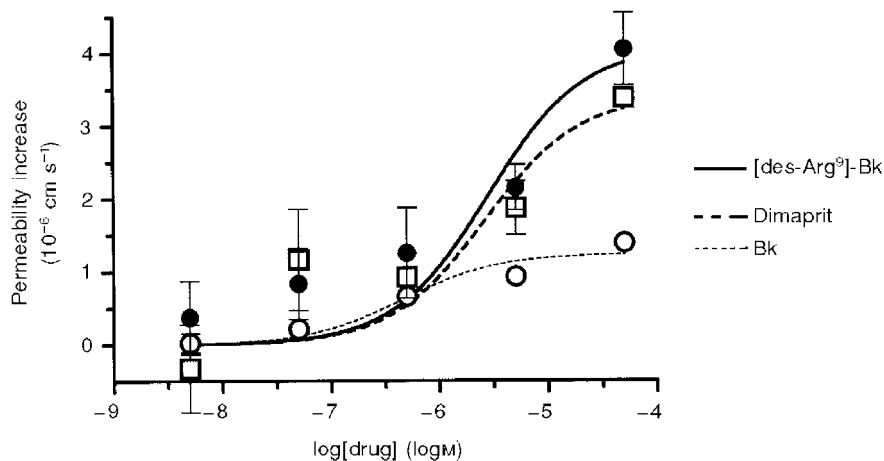


Figure 3. Permeability dose–response curve for [des-Arg⁹]-bradykinin compared with those for bradykinin and dimaprit

The bradykinin B₁ receptor agonist [des-Arg⁹]-bradykinin (●) was applied to the brain surface over a range of concentrations (from 5 nM to 50 μ M) and the permeability response measured in eight vessels. The dose–response curves for bradykinin (○) and dimaprit (□; histamine H₂ receptor agonist) are shown for comparison, the data being taken from Fig. 1D and Sarker *et al.* (1998), respectively.

subsequent experiments were carried out prior to this. The permeability response to bradykinin was concentration dependent and the dose-response curves obtained were unchanged over the 2 h (see Fig. 1D). The estimated maximal permeability increase was initially $1.25 \times 10^{-6} \pm 0.13 \times 10^{-6} \text{ cm s}^{-1}$ with a log median effective concentration ($\log EC_{50}$; units, $\log M$) of -6.34 ± 0.243 , and this did not change significantly after 2 h ($1.24 \times 10^{-6} \pm 0.106 \times 10^{-6} \text{ cm s}^{-1}$ and -6.08 ± 0.188 , respectively).

Bradykinin-destroying enzymes

The $\log EC_{50}$ value for the permeability response to bradykinin in the above experiments was much higher than would be predicted from the K_D for bradykinin on the B_2 receptor (see Hall, 1992). We investigated the possibility that bradykinin-destroying enzymes are present on the cerebral microvessels (Brecher *et al.* 1981; Brownson *et al.* 1994) by constructing dose-response curves for bradykinin alone, and in the presence of the ACE inhibitor captopril or the NEP inhibitor phosphoramidon, or both enzyme inhibitors together (Fig. 2). The $\log EC_{50}$ of the response to bradykinin was -5.30 ± 0.15 (rather higher than in the earlier series of experiments) and this decreased to -6.37 ± 0.24 with captopril, to -6.33 ± 0.19 with phosphoramidon, and to -7.3 ± 0.20 with captopril and phosphoramidon combined. One-way analysis of variance showed that this decrease was significant when captopril and phosphoramidon were applied separately ($P < 0.05$), and when they were applied together ($P < 0.001$), as assessed by Dunn's correction. There was no change in the estimated maximum permeability increase with any of the enzyme inhibitors, and the apparent increase in the maximum response when compared with that in Fig. 1D (i.e. $1.82 \times 10^{-6} \pm 0.13 \times 10^{-6}$ vs. $1.25 \times 10^{-6} \pm 0.29 \times 10^{-6} \text{ cm s}^{-1}$) was also not significant.

Bradykinin and [des-Arg⁹]-bradykinin receptor antagonists

Bradykinin is rapidly converted to [des-Arg⁹]-bradykinin by the action of kininase I (see Bhoola *et al.* 1992), and when [des-Arg⁹]-bradykinin was applied in a similar way to bradykinin there was also a permeability increase. The dose-response curve is illustrated in Fig. 3; the estimated $\log EC_{50}$ of -5.6 ± 0.37 was similar to that of bradykinin, although the maximal permeability increase of $4.1 \times 10^{-6} \pm 0.82 \times 10^{-6} \text{ cm s}^{-1}$ was higher.

It is possible that all the permeability response is mediated via the B_1 receptor. However, when the specific subtype receptor inhibitors ([des-Arg⁹-Leu⁸]-bradykinin for the B_1 receptor and HOE 140 for the B_2 receptor) were co-applied with bradykinin and [des-Arg⁹]-bradykinin, there was no evidence of such cross-reactivity (see Fig. 4). The permeability increase in response to bradykinin was abolished by HOE 140 (from $1.6 \times 10^{-6} \pm 0.42 \times 10^{-6}$ to $0.02 \times 10^{-6} \pm 0.21 \times 10^{-6} \text{ cm s}^{-1}$), and was not significantly affected by B_1 blockade ($1.0 \times 10^{-6} \pm 0.28 \times 10^{-6} \text{ cm s}^{-1}$). Blocking the B_1 receptor with [des-Arg⁹-Leu⁸]-bradykinin

($0.5 \mu M$) abolished the response to $5 \mu M$ [des-Arg⁹]-bradykinin ($1.2 \times 10^{-6} \pm 0.38 \times 10^{-6}$ to $0.0 \times 10^{-6} \pm 0.14 \times 10^{-6} \text{ cm s}^{-1}$). B_2 receptor blockade with HOE 140 did not affect the response to [des-Arg⁹]-bradykinin ([des-Arg⁹]-bradykinin with HOE 140: $1.1 \times 10^{-6} \pm 0.17 \times 10^{-6} \text{ cm s}^{-1}$).

Second messengers that mediate the permeability response to bradykinin and [des-Arg⁹]-bradykinin

Raised cytosolic Ca^{2+} in the endothelium has been implicated as having an important role in the rapid permeability response to a variety of agonists (see Crone, 1986), and we have found previously that the permeability response to histamine in pial venular capillaries was attenuated by the Ca^{2+} channel blocker SKF 96365 (Sarker *et al.* 1998). Co-application of SKF 96365 ($10 \mu M$) with bradykinin ($5 \mu M$), however, did not alter the permeability response ($2.3 \times 10^{-6} \pm 0.12 \times 10^{-6} \text{ cm s}^{-1}$ with bradykinin alone and $2.5 \times 10^{-6} \pm 0.05 \times 10^{-6} \text{ cm s}^{-1}$ with SKF 96365 in 4 vessels; see Fig. 5). On the other hand, the response to [des-Arg⁹]-bradykinin ($5 \mu M$) was much reduced by SKF 96365 ([des-Arg⁹]-bradykinin alone, $3.8 \times 10^{-6} \pm 0.12 \times 10^{-6} \text{ cm s}^{-1}$; with SKF 96365, $0.89 \times 10^{-6} \pm 0.10 \times 10^{-6} \text{ cm s}^{-1}$ in 4 vessels).

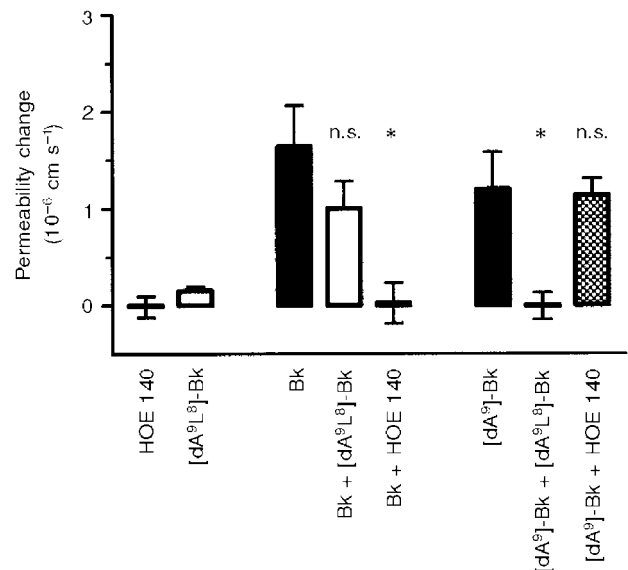


Figure 4. The effect of bradykinin receptor antagonists on the permeability responses to bradykinin and [des-Arg⁹]-bradykinin

The permeability-increasing effects of $5 \mu M$ bradykinin and $5 \mu M$ [des-Arg⁹]-bradykinin ([dA⁹]-Bk) were tested against the bradykinin B_1 and B_2 receptor antagonists [des-Arg⁹-Leu⁸]-bradykinin ([dA⁹L⁸]-Bk; $0.5 \mu M$) and HOE 140 ($0.5 \mu M$), respectively. The bradykinin-induced permeability increase was completely blocked by HOE 140 ($*P < 0.05$, Kruskal-Wallis test, Dunn's correction), but not by the B_1 receptor antagonist [des-Arg⁹-Leu⁸]-bradykinin. Similarly, [des-Arg⁹-Leu⁸]-bradykinin blocked the effect of [des-Arg⁹]-bradykinin ($*P < 0.05$), but HOE 140 had no effect. [des-Arg⁹-Leu⁸]-Bradykinin and HOE 140 alone had no effect on permeability. These paired experiments were carried out on two groups of four vessels.

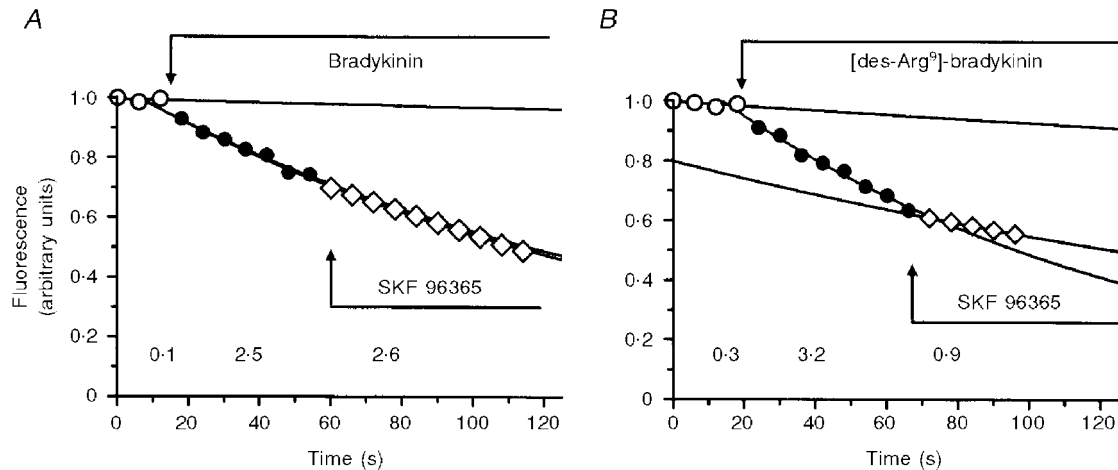


Figure 5. Differences in the effects of Ca^{2+} channel block on the permeability responses to bradykinin and [des-Arg⁹]-bradykinin

A and B, representative occlusion experiment carried out on single venular capillaries with bradykinin ($5 \mu\text{M}$; A) and [des-Arg⁹]-bradykinin ($5 \mu\text{M}$; B) applied to the brain surface. Permeability (as measured from the rate of fall of fluorescence signal) increased with both agonists. The Ca^{2+} channel blocker SKF 96365 ($10 \mu\text{M}$) had no effect on the increase induced by bradykinin, while it reduced that to [des-Arg⁹]-bradykinin. The permeability values (shown below the relevant portions of the traces and expressed as $10^{-6} \text{ cm s}^{-1}$) were calculated from the rate constants of mono-exponentials fitted to the fluorescence signals from the occluded microvessel segments.

Free radicals

Free radicals released as a consequence of arachidonic acid metabolism have been shown to increase the permeability of these vessels (Easton & Fraser, 1998), and the possibility that they are involved in the bradykinin-mediated increase in permeability was tested by applying known scavengers.

A series of experiments was carried out on four vessels from four rats. Figure 6A shows that co-application of bradykinin with a combination of SOD (100 U ml^{-1}) and catalase (100 U ml^{-1}) was required to block the permeability response to bradykinin (bradykinin alone, $1.52 \times 10^{-6} \pm 0.26 \times 10^{-6} \text{ cm s}^{-1}$; with SOD and catalase, $-0.07 \times 10^{-6} \pm$

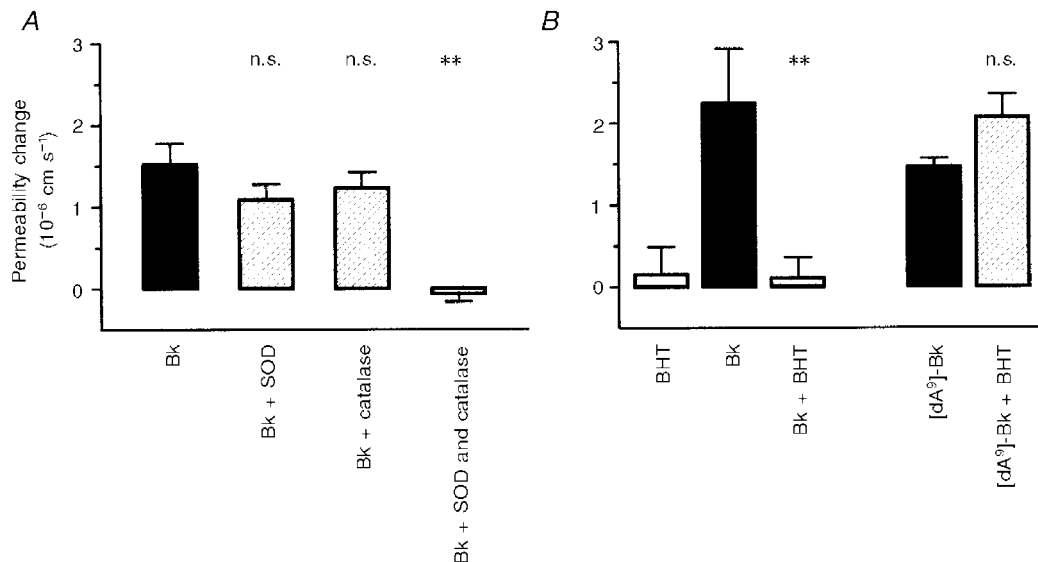


Figure 6. The effects of antioxidants on the permeability response to kinins

A, superoxide dismutase (SOD) and catalase (each 100 U ml^{-1}) applied separately to the brain surface had little effect on the permeability response to $5 \mu\text{M}$ bradykinin, but completely blocked it when used in combination (** $P < 0.01$, Kruskal-Wallis test, Dunn's correction). B, the lipid peroxidation chain blocker butylated-hydroxytoluene (BHT; $1 \mu\text{M}$) also inhibited the permeability response to bradykinin ($5 \mu\text{M}$; ** $P < 0.01$), but had no effect on that to [des-Arg⁹]-bradykinin. Each of the two groups of experiments was carried out on four vessels.

$0.09 \times 10^{-6} \text{ cm s}^{-1}$). In a further series of four experiments BHT ($1 \mu\text{M}$), a lipid peroxidation chain blocker, also prevented the response to $5 \mu\text{M}$ bradykinin, but not that to $5 \mu\text{M}$ [des-Arg⁹]-bradykinin (bradykinin alone, $2.2 \times 10^{-6} \pm 0.67 \times 10^{-6} \text{ cm s}^{-1}$; with BHT, $0.11 \times 10^{-6} \pm 0.26 \times 10^{-6} \text{ cm s}^{-1}$; [des-Arg⁹]-bradykinin alone, $1.47 \times 10^{-6} \pm 0.10 \times 10^{-6} \text{ cm s}^{-1}$; with BHT, $2.07 \times 10^{-6} \pm 0.28 \times 10^{-6} \text{ cm s}^{-1}$; Fig. 6*B*).

The possibility that arachidonic acid is the source for the free radicals was investigated by co-applying bradykinin with the Ca²⁺-dependent PLA₂ inhibitor aristolochic acid (Rosenthal *et al.* 1989) and the Ca²⁺-independent PLA₂ inhibitor palmitoyl trifluoromethyl ketone (PaF₃MeK; Liu, 1999). Figure 7*A* shows that PaF₃MeK ($50 \mu\text{M}$) completely blocked the bradykinin-mediated permeability response (bradykinin alone $1.65 \times 10^{-6} \pm 0.30 \times 10^{-6} \text{ cm s}^{-1}$, and with PaF₃MeK $-0.27 \times 10^{-6} \pm 0.23 \times 10^{-6} \text{ cm s}^{-1}$), whereas aristolochic acid had no effect on the response (bradykinin with aristolochic acid, $1.46 \times 10^{-6} \pm 0.42 \times 10^{-6} \text{ cm s}^{-1}$). The permeability increase in response to dimaprit, a histamine H₂ agonist previously shown to act via SKF 96365-sensitive Ca²⁺ entry (Sarker *et al.* 1998), was unaffected by PaF₃MeK.

Free radicals are formed during the metabolism of arachidonic acid via either cyclo-oxygenase or lipoxygenase, so inhibition of both these enzymes should abolish the

permeability response to bradykinin. This was tested by co-applying bradykinin with indomethacin and NDGA, inhibitors of cyclo-oxygenases and lipoxygenases, respectively, separately and in combination. Figure 7*B* shows that only combined application of the inhibitors prevented the permeability increase ($5 \mu\text{M}$ bradykinin alone, $1.64 \times 10^{-6} \pm 0.33 \times 10^{-6} \text{ cm s}^{-1}$; with $1 \mu\text{M}$ indomethacin, $1.52 \times 10^{-6} \pm 0.21 \times 10^{-6} \text{ cm s}^{-1}$; with $1 \mu\text{M}$ NDGA, $1.96 \times 10^{-6} \pm 0.19 \times 10^{-6} \text{ cm s}^{-1}$; with both, $0.10 \times 10^{-6} \pm 0.14 \times 10^{-6} \text{ cm s}^{-1}$). These results indicate that bradykinin stimulates the production of arachidonic acid and that free radicals are generated via its metabolism through cyclo-oxygenase and lipoxygenase pathways.

There is evidence that kinins are able to release histamine from mast cells (see Hall, 1992), which raises the possibility that histamine has a role in the permeability response. This was examined by blocking the H₂ receptor (responsible for cerebrovascular permeability increase; Sarker *et al.* 1998) during bradykinin and [des-Arg⁹]-bradykinin application. Figure 8 shows that 2 nM cimetidine blocked the response to $5 \mu\text{M}$ [des-Arg⁹]-bradykinin ([des-Arg⁹]-bradykinin alone, $1.15 \times 10^{-6} \pm 0.22 \times 10^{-6} \text{ cm s}^{-1}$; with cimetidine, $0.03 \times 10^{-6} \pm 0.03 \times 10^{-6} \text{ cm s}^{-1}$), but had no effect on that to $5 \mu\text{M}$ bradykinin (bradykinin alone, $1.75 \times 10^{-6} \pm 0.31 \times 10^{-6} \text{ cm s}^{-1}$; with cimetidine, $1.59 \times 10^{-6} \pm 0.46 \times 10^{-6} \text{ cm s}^{-1}$).

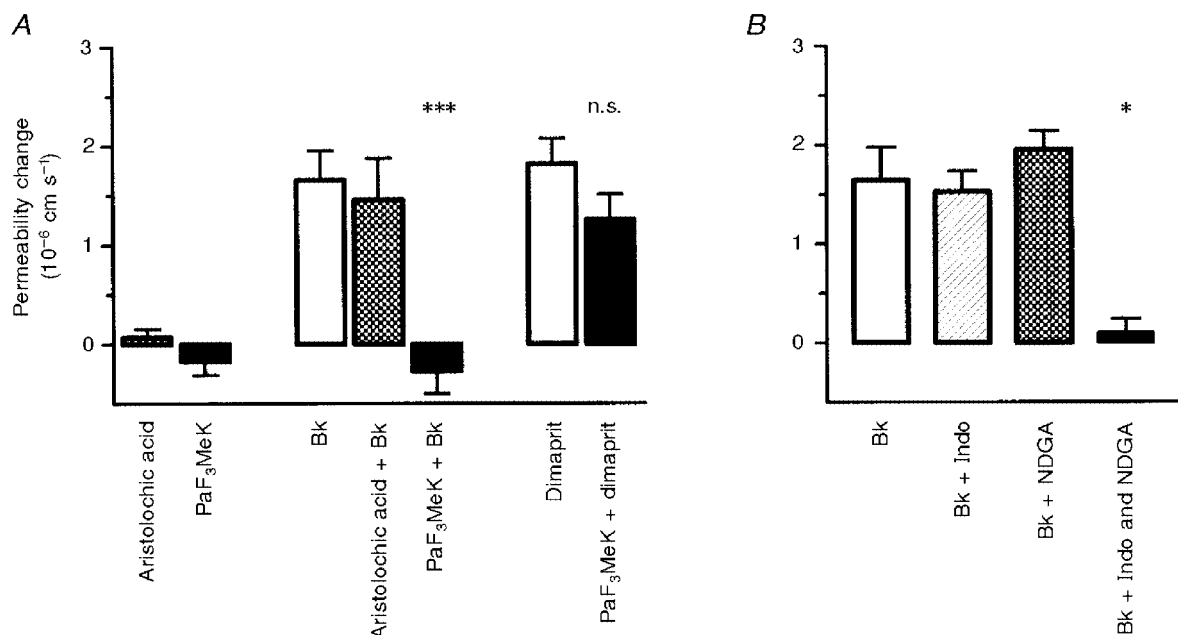


Figure 7. Arachidonic acid mediates the bradykinin-induced permeability increase

The idea that arachidonic acid release following acute bradykinin ($5 \mu\text{M}$) application leads to free radical generation was tested by co-application of PLA₂ antagonists, and blockers of lipoxygenase and cyclo-oxygenase. *A*, the antagonist of the Ca²⁺-dependent PLA₂ (aristolochic acid; $5 \mu\text{M}$) had no effect, but that of the Ca²⁺-independent PLA₂ (palmitoyl trifluoromethyl ketone, PaF₃MeK; $50 \mu\text{M}$) completely blocked the permeability increase. PaF₃MeK did not affect the response to the histamine H₂ agonist dimaprit ($50 \mu\text{M}$) ($***P < 0.001$, Kruskal-Wallis test, Dunn's correction). *B*, the permeability response to bradykinin was also abolished by a combination of indomethacin (Indo) and nordihydroguaiaretic acid (NDGA) ($1 \mu\text{M}$ of each), but not when they were applied separately ($*P < 0.05$, Kruskal-Wallis test, Dunn's correction). The two groups of experiments were carried out on five vessels each.

DISCUSSION

The present study shows that brief applications of bradykinin that last no longer than a minute can increase cerebrovascular permeability to a limited extent (about half of that to histamine; Sarker *et al.* 1998), but only at relatively high concentrations. Longer lasting applications can give rise to complicated interactions with the release of other inflammatory mediators. For example, a 10 min application resulted in an irreversible permeability increase due to the release of interleukin-1 β (Hu & Fraser, 1997). Applications for 30–60 s at 5 min intervals, on the other hand, produced fully reversible responses which were stable for 2 h even with a bradykinin concentration as high as 50 μM .

This lack of sensitivity (high EC_{50}) can be explained by the activity of the bradykinin-destroying enzymes ACE and NEP, which are widely distributed on microvessels and in many tissues including the brain (Bhoola *et al.* 1992). We found that inhibition of either of these enzymes shifted the dose–response curve to the left by an order of magnitude, and when both were blocked the EC_{50} was much closer to the reported K_D of bradykinin on the B_2 receptor (see Hall, 1992). A similar enhancement by a combination of captopril and phosphoramidon on the effect of bradykinin on permeability was also observed in hamster cheek pouch (Yong *et al.* 1992). It is possible that the kinin-destroying enzymes ACE and NEP have a physiological role in protecting the blood–brain barrier from circulating bradykinin. It has been reported that the expression of these enzymes can vary between individual animals

(Winkler *et al.* 1998) and this may be at the root of the differences in the observed $\log \text{EC}_{50}$ for the two series of experiments shown in Figs 1 and 2.

A relatively stable, low-level disruption of the blood–brain barrier may account for the observations of workers who found that bradykinin was unable to open the barrier to proteins or other macromolecules (Gabbiani *et al.* 1970; Saria *et al.* 1983; Unterberg *et al.* 1984). The values obtained in the present series of experiments are similar to those obtained with previous single vessel studies. Thus bradykinin (5 μM) acutely applied to frog pial microvessels reduced the specific resistance of the vessel wall from 1500 to 280 $\Omega \text{ cm}^2$ (equivalent to a Lucifer Yellow permeability of $1 \times 10^{-6} \text{ cm s}^{-1}$; Olesen & Crone, 1986), but no lower. Likewise, the rat pial microvascular specific resistance fell to a mean of about 550 $\Omega \text{ cm}^2$ (*ca* $0.5 \times 10^{-6} \text{ cm s}^{-1}$) even after 30 min superfusion with 100 μM bradykinin (Butt, 1995).

The finding that [des-Arg⁹]-bradykinin also produced a reversible permeability increase was unexpected, as the bradykinin B_1 receptor is usually expressed only in response to an inflammatory event subsequent to interleukin-1 β release (see Marceau *et al.* 1998). The specific receptor subtype inhibitors for the B_1 and B_2 receptors, [des-Arg⁹-Leu⁸]-bradykinin and HOE 140, respectively, showed no cross-reactivity for the natural agonists, [des-Arg⁹]-bradykinin and bradykinin. As cimetidine blocked the permeability-increasing effect of [des-Arg⁹]-bradykinin, it is likely that the B_1 receptors are not located on the endothelium, but on a histamine-secreting cell. For instance, histaminergic neurones are distributed widely and have nerve endings close to most small blood vessels of the brain (Takagi *et al.* 1986). This idea is supported by the similarity between the dimaprit and [des-Arg⁹]-bradykinin dose–response curves (see Fig. 3), and is consistent with the finding that B_2 , and not B_1 , ligand binding sites were found on isolated rat and pig cerebral microvessels (Homayoun & Harik, 1991).

Signal transduction linked to bradykinin receptor

In the present study we found that the cation channel blocker SKF 96365 failed to inhibit the permeability increase due to bradykinin. This was surprising as in cultured and large vessel endothelium, bradykinin is known to raise $[\text{Ca}^{2+}]_i$ via a phospholipase C pathway. The IP_3 so formed mediates release of Ca^{2+} from Ca^{2+} stores, and their depletion results in the opening of SKF 96365-sensitive Ca^{2+} channels (Schilling *et al.* 1992). Increased $[\text{Ca}^{2+}]_i$ is often linked to a rapid increase in microvascular permeability both *in vivo* (He & Curry, 1993) and in cell culture (Wysolmerski & Lagunoff, 1991), and we have previously shown that the H_2 receptor on these vessels operates via such channels (Sarker *et al.* 1998). The resulting endothelial nitric oxide synthase (eNOS) activation and subsequent stimulation of soluble guanylyl cyclase to form cGMP are thought to be key steps in increasing microvascular

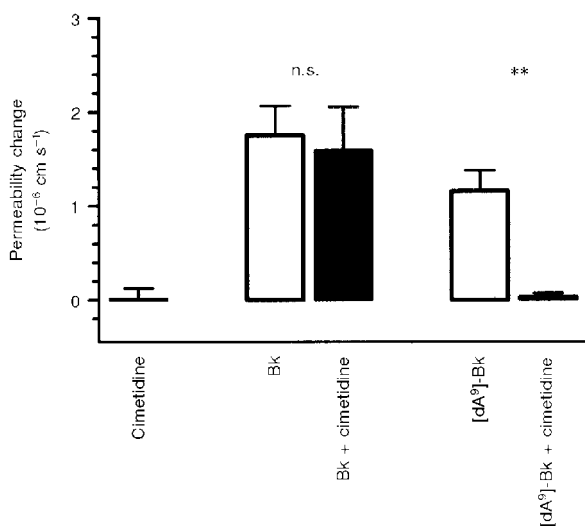


Figure 8. The [des-Arg⁹]-bradykinin-induced permeability increase is blocked by cimetidine

The histamine H_2 receptor antagonist cimetidine (2 nM) blocked the permeability response to [des-Arg⁹]-bradykinin (5 μM), but not that to bradykinin (5 μM ; ** $P < 0.01$, Kruskal-Wallis test, Dunn's correction).

permeability (He *et al.* 1998), and bradykinin has been shown to be a very potent mediator of vascular relaxation mediated by eNOS activation (Cherry *et al.* 1982). In many *in vivo* studies little attempt has been made to separate the effects of vasodilatation from those of permeability (Mayhan, 1992), but where this was done (in the hamster cheek pouch preparation: Félétou *et al.* 1996; and the rat knee: Cambridge & Brain, 1995) bradykinin application resulted in nitric oxide-dependent vasodilatation, but nitric oxide-independent macromolecular extravasation.

There are other instances where the effects of bradykinin have been shown to be mediated via a free radical-generating pathway. Thus, bradykinin-mediated cerebral arteriolar dilatation was blocked by either scavenging free radicals (Navari *et al.* 1979; Kontos *et al.* 1984; Rosenblum, 1987) or by blocking their production by chelating iron or haem with desferrioxamine (Kontos *et al.* 1990).

We found that the permeability response to bradykinin in cerebral microvessels is linked to free radical formation as it was completely blocked by either a combination of SOD and catalase, or by inhibiting lipid peroxidation with BHT. Bradykinin has been shown to release arachidonic acid by activating PLA₂ in cultured endothelium (Briand *et al.* 1996) and the present experiments indicate that bradykinin activates a Ca²⁺-independent PLA₂, one variety of which has recently been shown to be strongly expressed in all regions of rat brain (Molloy *et al.* 1998). The increase in cerebral microvascular permeability induced by direct application of arachidonic acid was blocked by scavenging free radicals (Wei *et al.* 1986) that were formed from both cyclo-oxygenase and lipoxygenase (Easton & Fraser, 1998). Thus the observation that a combination of low concentrations of indomethacin and NDGA was effective in blocking the acute response to bradykinin further supports the idea that the B₂ receptor activates PLA₂. This is of particular interest as arachidonic acid release from endothelium is one of the earliest events in cerebral ischaemia (Bazan & Rodriguez de Turco, 1980). It has been pointed out to us that the relatively low concentrations of indomethacin and NDGA used in the present experiments may allow arachidonic acid to be shunted into a cytochrome P450 or 12 or 15 lipoxygenase pathway, which may be protective of inflammation (Conrad, 1999), and thus may not necessarily reflect a reduction of free radical production. This is an interesting possibility, but as these pathways would still result in free radical formation we think that it is more likely that the direct effect is the inhibition of their generation from the actions of both lipoxygenase and cyclo-oxygenase.

In conclusion we have found that bradykinin and [des-Arg⁹]-bradykinin applied to the brain surface for no more than 60 s results in a small reversible opening of the blood-brain barrier, which is mediated via B₂ and B₁ receptors, respectively. The B₁ receptors do not appear to be located on the endothelium, but on some histamine-

releasing structure, possibly on the histaminergic nerve endings. The B₂ receptor appears to activate a Ca²⁺-independent PLA₂ which releases arachidonic acid, which in turn is the substrate for cyclo-oxygenase and lipoxygenase. The permeability increase results from the free radicals that are formed during the metabolism of arachidonic acid, possibly as a consequence of Ca²⁺ entry through areas of lipid that have undergone peroxidation.

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