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SUBSTANCE P: IMMUNOHISTOCHEMICAL LOCALIZATION AND EFFECT UPON CAT PIAL ARTERIES *IN VITRO* AND *IN SITU*

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SUMMARY

1. Nerve fibres containing substance P immunoreactivity were present in the adventitia and the adventitia-media border of all cat cerebral arteries which were examined. Substance P immunoreactivity was most abundant in cerebral arteries from the rostral portion of the circle of Willis.

2. Substance P effected a dose-dependent relaxation of feline middle cerebral arteries which had been contracted with prostaglandin $F_{2\alpha}$. The maximum relaxation ($1{\cdot}6 \pm 0{\cdot}3$ mN) was achieved with substance P at a concentration of 10^{-6} M.

3. In cats anaesthetized with α-chloralose, the perivascular microinjection of substance P effected dose-dependent increases in arteriolar calibre. The maximum increase in calibre ($19 \pm 3\%$) was observed following the injection of 10^{-6} M-substance P.

INTRODUCTION

The innervation of cerebrovascular smooth muscle by sympathetic and parasympathetic nerve fibres has been characterized in considerable detail (see Edvinsson & Mackenzie, 1976). More recently, fibres containing vasoactive intestinal polypeptide (VIP) has been demonstrated around cerebral blood vessels using immunohistochemical techniques (Larsson, Edvinsson, Fahrenkrug, Håkanson, Owman, Schaffalitzky de Muckadell & Sundler, 1976; Edvinsson, Fahrenkrug, Hanko, Owman, Sundler & Uddman, 1980). VIP is a potent dilator of cerebral arteries *in vitro* (Larsson *et al.* 1976) and of cerebral arterioles *in situ* (McCulloch & Edvinsson, 1980). The systemic administration of VIP can elicit marked elevations in the level of cerebral tissue perfusion (McCulloch & Edvinsson, 1980).

The possibility that cerebral blood vessels may additionally be innervated by other vasoactive peptides was suggested by Chan-Paley (1977) who demonstrated immunoreactivity towards substance P and neurotensin in the cerebral blood vessels in the ventral medulla of the rat. In the present study, in cats, the distribution of substance P immunoreactivity in cerebral arteries in the forebrain has been examined. Moreover, in view of the potency of substance P as a dilator in the coronary, pulmonary and other peripheral circulations (von Euler & Gaddum, 1931; Maxwell, 1968; Hallberg & Pernow, 1975), the effects of substance P upon feline cerebrovascular smooth muscle have been examined *in vitro* and *in situ*.

METHODS

Immunohistochemistry. Pial vessels at the base of the brain were collected from five adult cats. The specimens were immersed in a fixative solution (4% formaldehyde in 0·1 M-sodium phosphate buffer, pH 7·2) for 24 h at 4 °C and subsequently rinsed in Tyrode solution containing 5% sucrose at 4 °C for 24–48 h. The vessel segments were frozen on dry ice, sectioned in a cryostat at −20 °C and processed for the immunohistochemical demonstration of substance P (SP). Some of the vessels and cortical pial membranes were stretched on clean microscope slides and fixed for 18 h in a picric acid–formaldehyde mixture (Stefanini, DeMartino & Zamboni, 1967) at 4 °C. Before the immunohistochemical processing, the sections were dehydrated, cleaned in xylol and rehydrated (Costa, Buffa, Furness & Solcia, 1980).

The antiserum (code SP-8), raised in rabbits against synthetic substance P (Beckman, Switzerland), has been used in a previous immunohistochemical study (Alumets, Falkmer, Håkanson, Ljungberg, Sundler & Tibblin, 1980). The antiserum did not cross-react with any other neuronal peptides such as vasoactive intestinal polypeptide, somatostatin or enkephalin. Despite some chemical similarities in the amino acid sequence, the antiserum did not cross-react with bombesin at the immunohistochemical level. Cross-reactivity of the SP antiserum with unknown peptides containing the immunoreactive amino acid sequence cannot be excluded. It would, therefore, be appropriate to use the expression SP-like immunoreactivity for the immunoreactive product. For brevity, nerve fibres displaying substance P-like immunoreactivity are referred to in the text as SP-immunoreactive fibres or substance P fibres.

The substance P antiserum was used in a dilution of 1:80. The site of the antigen–antibody reaction was revealed by fluorescein isothiocyanate-labelled goat antirabbit IgG (Coons, Leduc & Conolly, 1955) used in a dilution of 1:20. Sections to be used as controls were incubated with antiserum and inactivated by the addition of excess antigen (100 μg synthetic substance P per ml diluted antiserum). Immunofluorescence was examined in a Leitz Orthoplan microscope equipped with an epi-illumination system.

Response of isolated pial arterial segments. Seven adult cats of either sex, weighing 2–4 kg, were exsanguinated under pentobarbitone anaesthesia (nembutal 30 mg/kg, I.P.). The brains were removed, the middle cerebral arteries dissected free and placed in a cold Krebs–Ringer solution aerated with 5% CO_2 in O_2. The composition of the buffer solution was (mM): NaCl 119, KCl 4·6, $CaCl_2$ 1·5, $MgCl_2$ 1·2, $NaHCO_3$ 20, NaH_2PO_4 1·2 and glucose 11·0, pH 7·4.

Vessel segments, 3–5 mm long, were mounted between two L-shaped metal prongs in a 5 ml mantled organ bath at 37·5 °C for recording of circular vasomotor activity (Edvinsson, Nielsen & Owman, 1974). Isometric tension was measured with Grass force-displacement transducers and recorded on a Grass Polygraph. The segments (four studied simultaneously) were given a passive load of 4 mN, allowed to attain a steady level of tension and to stabilize for 90 min before testing. When relaxation of the vessels was being examined, the vascular segments were given an active tone by means of the addition of prostaglandin $F_{2\alpha}$ (2.5×10^{-6} M) (Amoglandin, Astra, Sweden). This resulted in a contraction which was stable for at least 30 min.

Vasomotor responses of pial arterioles in situ. These investigations were performed on seven cats weighing 2–4 kg. The animals were anaesthetized initially with a mixture of alfaxolone (6·75 mg/kg) and alfadolone acetate (6·75 mg/kg), administered intravenously. The cats were subsequently intubated and connected to an intermittent positive-pressure ventilation system delivering room air in an open circuit. The right femoral artery and vein were cannulated for the continuous measurement of arterial blood pressure and the administration of fluid or anaesthetic agents. Anaesthesia was maintained during the subsequent course of the experiments with α-chloralose (60 mg/kg, I.V.). Additional α-chloralose was administered when necessary to prevent the return of the corneal reflex. The animals were maintained normocapnic (arterial carbon dioxide tension, P_{a,CO_2}, close to 32 mmHg throughout the course of the experiments). The end-tidal concentration of carbon dioxide was monitored continuously by means of an infra-red analyser, and samples of arterial blood were taken frequently during the experiments for the estimation of P_{a,CO_2}, pH and arterial oxygen tension (Corning blood gas analyser). In each cat, the mean arterial blood pressure was greater than 80 mm Hg. Rectal temperature was maintained at 38 °C with a heating blanket.

The animals were placed in a stereotaxic frame, and a longitudinal incision was made in the scalp which was then retracted and ligated onto a metal ring in such a manner that it formed an intact

pool over the calvarium. The left temporal muscle was retracted and a craniotomy, measuring 2·5 × 1·5 cm, was made over the left parietal cortex with a dental drill which was cooled with saline. The exposed dura was bathed by warmed mineral oil (liquid paraffin) maintained at 38 °C. The depth of the oil pool was approximately 1·5 cm. Thereafter, surgical manipulations were performed with the aid of a Bausch & Lomb stereomicroscope with a zoom lens, the field being illuminated by Schott fibre-optic systems. The dura was removed carefully, and any bleeding from the cut dural edges was sealed using bipolar diathermy.

Vascular calibre was measured using an image-splitting technique (Baez, 1966). Individual pial vessels on the convexity of the brain were viewed in focus through the microscope, and the image was passed through a Vickers image-splitting eye-piece to a closed circuit television camera and displayed on a television monitor. Vascular diameter was measured from the degree of shear applied to the image splitter that had been calibrated against wire and thread of known diameters.

Substance P was dissolved in mock cerebrospinal fluid immediately prior to use. The composition of the mock cerebrospinal fluid was (mM): Na^+145, K^+3, Ca^{2+}2·5, HCO_3^- 11, Cl^-142; adjusted to pH 7·2 by aeration with 5 % CO_2 and 95 % O_2. Glass micropipettes with a tip diameter of 8–10 μm were filled with mock cerebrospinal fluid under mineral oil. Using a micro-manipulator, the micropipettes were inserted through the arachnoid into the perivascular space close to a cerebral arteriole. A minute amount of the mock cerebrospinal fluid (about 5 μl) containing the peptide was injected into the perivascular space over 15 s, and any resulting alterations in vascular calibre were monitored for periods of up to 3 min following the injection. The observed changes in calibre in response to the perivascular microinjection of substance P (expressed as percent changes from the diameter of the vessel prior to drug administration) were compared to those following the administration of mock cerebrospinal fluid alone by means of an analysis of variance (Scheffé, 1959).

RESULTS

Immunohistochemistry. Fine varicose nerve fibres containing substance P immunoreactivity were regularly encountered in the adventitia and at the adventitia-media border of all pial arteries examined. A richer supply of substance P fibres was noted in pial vessels belonging to the rostral portion of the circle of Willis than in the more caudally located arteries. Thus, numerous nerve fibres were found in the wall of the anterior (Pl. 1 *A*) and middle cerebral arteries. Smaller numbers of substance P fibres were present in the posterior cerebral artery, the cerebellar arteries and the basilar artery. Few nerve fibres were observed in material from the vertebral artery. The small pial arteries and arterioles on the cortical surface (Pl. 1 *B*) received substance P immunoreactive fibres, though to a lesser extent than the major cerebral arteries.

Responses of isolated middle cerebral arteries. Under resting conditions (i.e. when the arteries had reached a steady tension of approximately 3 mN), substance P effected only a minimal relaxation of middle cerebral arteries. Administration of prostaglandin $F_{2\alpha}$ resulted in a pronounced, persistent contraction (14·7 ± 0·2 mN) (mean ± S.E. of mean) of the vessels similar to the maximum elicited by 127 mM-K^+ (15·7 ± 2·4 mN). In vessels contracted with prostaglandin $F_{2\alpha}$, the administration of substance P resulted in a dose-dependent relaxation of the cerebral artery (Fig. 1). The mean effective concentration of substance P was $1{\cdot}4 \pm 1{\cdot}2 \times 10^{-9}$ M, and the maximum relaxation obtained in vessels with active tension induced by prostaglandin $F_{2\alpha}$ was 1·6 ± 0·3 mN. The presence of 10^{-6} M-atropine, 2×10^{-6} M-cimetidine or 10^{-6} M-propranolol in the organ bath did not affect the relaxation of middle cerebral arteries induced by the administration of substance P.

Responses of pial arterioles in situ. The perivascular injection of mock cerebrospinal fluid has minimal effects upon pial arteriolar calibre (mean alteration in calibre:

$-0{\cdot}7 \pm 1{\cdot}5\%$). The mean resting arteriolar calibre was 139 ± 11 μm (mean $\pm$ S.E. of mean). Perivascular application of substance P solution resulted in a dose-dependent increase in arteriolar calibre, significant responses being observed in the concentration range 10^{-9}–10^{-6} M (Fig. 2). The maximum increase in arteriolar calibre ($19{\cdot}3 \pm 3{\cdot}3\%$) was observed with 10^{-6} M. The concomitant administration of 10^{-6} M-atropine or 10^{-6}

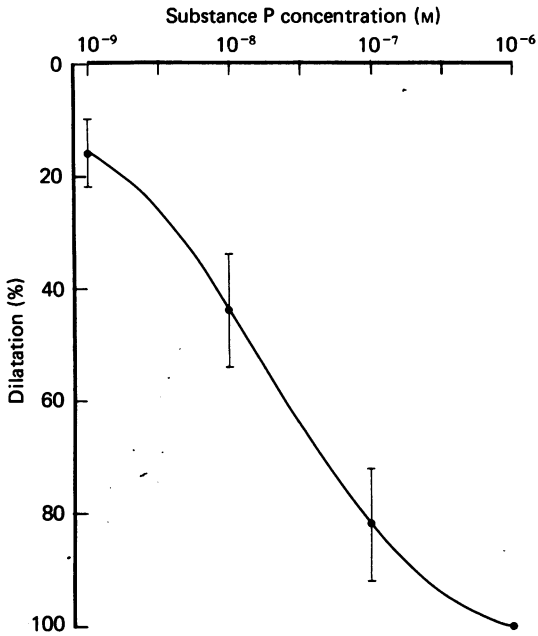

Fig. 1. Dose–response relaxation of the middle cerebral artery achieved by the addition of substance P to the organ bath. The vessels had been constricted by the prior administration of $2{\cdot}5 \times 10^{-6}$ M-prostaglandin $F_{2\alpha}$. Data are presented as mean values $\pm$ S.E. of mean of four individual tests.

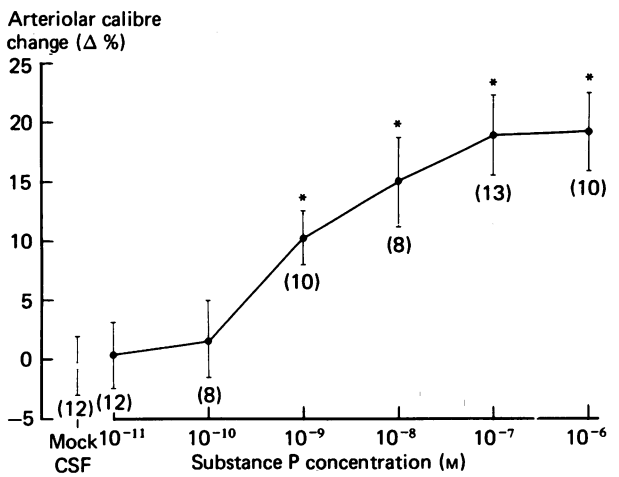

Fig. 2. Pial arteriolar calibre alterations associated with perivascular microapplication of substance P. Data are presented as mean values $\pm$ S.E. of mean and the number of vessels examined with each concentration is shown in parentheses. $^*P < 0{\cdot}01$.

M-propranolol, which themselves were without effect on arteriolar calibre, did not significantly alter the dilatations induced by substance P (Fig. 3). No relationship could be demonstrated between the pre-injection calibre of the arterioles and the magnitude of their dilatation in response to 10^{-7} M-substance P (Fig. 4).

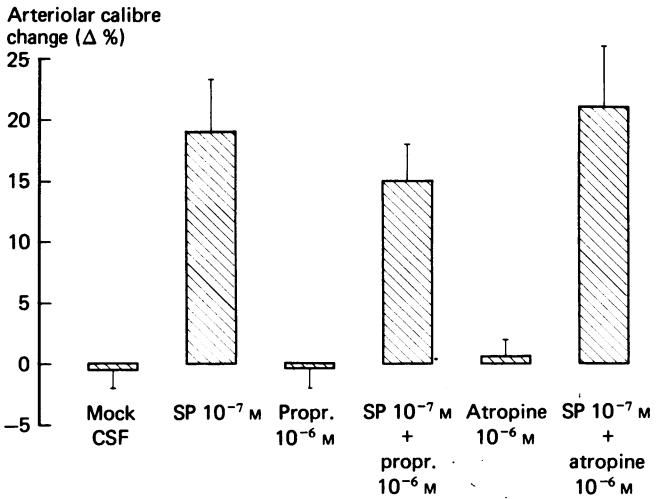

Fig. 3. The effects of 10^{-6} M-propranolol and 10^{-6} M-atropine upon the pial arteriolar responses to 10^{-7} M-substance P. Data are from ten to twelve tests with each drug combination.

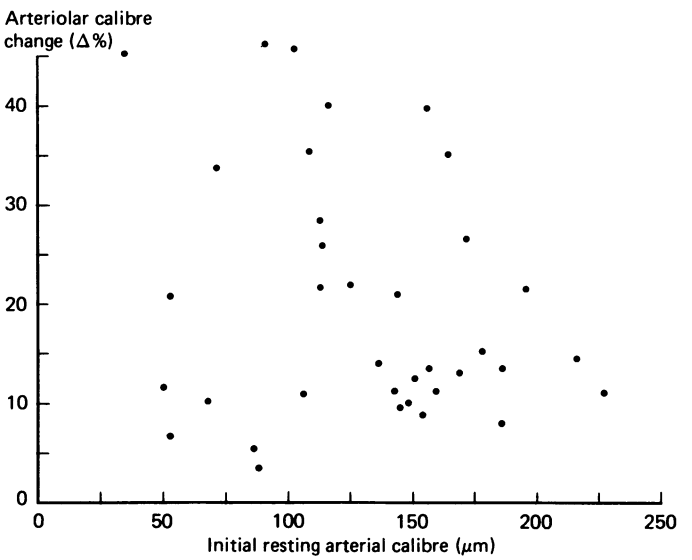

Fig. 4. Pial arteriolar calibre alterations associated with perivascular microinjection of 10^{-7} M-substance P in relation to pre-injection calibre of the arteriole. Each point represents the response of a single vessel. The responses to 10^{-7} M-substance P and to the peptide in combination with either 10^{-6} M-propranolol or 10^{-6} M-atropine have been pooled.

DISCUSSION

Substance P was originally detected by von Euler & Gaddum (1931) and later identified chemically as an undecapeptide (Chang, Leeman & Niall, 1971). Substance P immunoreactive nerve fibres occur both in the central and the peripheral nervous systems (Nilsson, Larsson, Håkanson, Brodin, Pernow & Sundler, 1975; Pearse & Polak, 1975; Hökfelt, Johansson, Kellerth, Ljungdahl, Nilsson, Nygards & Pernow, 1977; Alm, Alumets, Brodin, Håkanson, Nilsson, Sjöberg & Sundler, 1978; Hökfelt, Johansson, Ljungdahl, Lundberg & Schultzberg, 1980). In the periphery, substance P fibres are associated with ganglia, smooth muscle cells, blood vessels, glandular acini and epithelia (Hökfelt *et al.* 1977; Alm *et al.* 1978; Hökfelt *et al.* 1980). The potent vasodepressor properties of substance P were noted by von Euler & Gaddum (1931) when they isolated the material, and the dilatations which substance P can effect have been investigated in several, peripheral vascular beds (Maxwell, 1968; Hallberg & Pernow, 1975; Eklund, Jogestrand & Pernow, 1977).

A number of recent investigations have been pointed towards a possible role for peptides in the control of cerebral tissue perfusion. A dense supply of VIP-containing nerve fibres is present in the walls of cerebral blood vessels (Larsson *et al.* 1976; Lindvall, Alumets, Edvinsson, Fahrenkrug, Håkanson, Hanko, Owman, Schaffalitzky de Muckadell & Sundler, 1978; Edvinsson *et al.* 1980). VIP can elicit the dilatation of cerebral arteries (Larsson *et al.* 1976) and pial arterioles (McCulloch & Edvinsson, 1980). The systemic administration of VIP can result in large increases in cerebral blood flow, although the interpretation of the primary mechanism underlying this vasodilatation is complicated by the concomitant alterations in cerebral metabolic activity which were observed (McCulloch & Edvinsson, 1980).

In the present study, substance P-containing fibres have been demonstrated around pial vessels in the cat, confirming and extending the original observations of Chan–Palay (1977) in the ventral medulla of the rat. Perivascular substance P fibres have a widespread distribution in the brain and are not confined to functionally specialized regions such as the ventral medulla. The substance P fibres were more numerous in vessels from the rostral portion of the circle of Willis and were less frequently observed in caudally located vessels. The distribution of the substance P reactive fibres thus resembles the pattern observed previously in feline pial vessels for sympathetic, acetylcholine-esterase-positive and VIP-containing perivascular nerves (Edvinsson, Nielsen, Owman & Sporrong, 1972; Edvinsson & MacKenzie, 1976; Larsson *et al.* 1976; Edvinsson *et al.* 1980). Moreover, cerebral blood vessels in other mammalian species, including man, are invested with substance P immunoreactive nerve fibres (Uddman, Edvinsson, Owman & Sundler, 1981).

In the present study, substance P was shown to induce dose-dependent relaxation of isolated middle cerebral arteries. The mean effective concentration was comparable with that previously shown for VIP (Larsson *et al.* 1976), although the magnitude of the maximum relaxation was slightly lower for substance P than for VIP. The minimum effective concentration of both substance P and VIP in eliciting dilatations is considerably lower than those of monoamines examined under similar conditions (see Edvinsson & MacKenzie, 1976). The dilatatory effect of substance P would not appear to be mediated by histaminergic H_2, cholinergic or β-adrenergic receptors, all

of which are known to be present in cerebral blood vessels (Edvinsson & MacKenzie, 1976), since the response to substance P was unaffected by the presence of cimetidine, atropine or propranolol. Essentially similar observations were made with substance P and the responses of pial arterioles *in situ*. The arteriolar responses complement the *in vitro* observations and extend the observations of cerebrovascular vasomotor effects of substance P to an *in vivo* condition and to vascular elements which contribute significantly to cerebrovascular resistance (Stromberg & Fox, 1972), as well as avoiding the necessity to contract the vessels actively with prostaglandins as was the case *in vitro*.

Thus, the presence of perivascular nerve fibres containing substance P, together with the demonstrations of its potent cerebrovascular actions, provides evidence for an additional peptidergic neuronal system by which alterations in cerebral tissue perfusion might be achieved.

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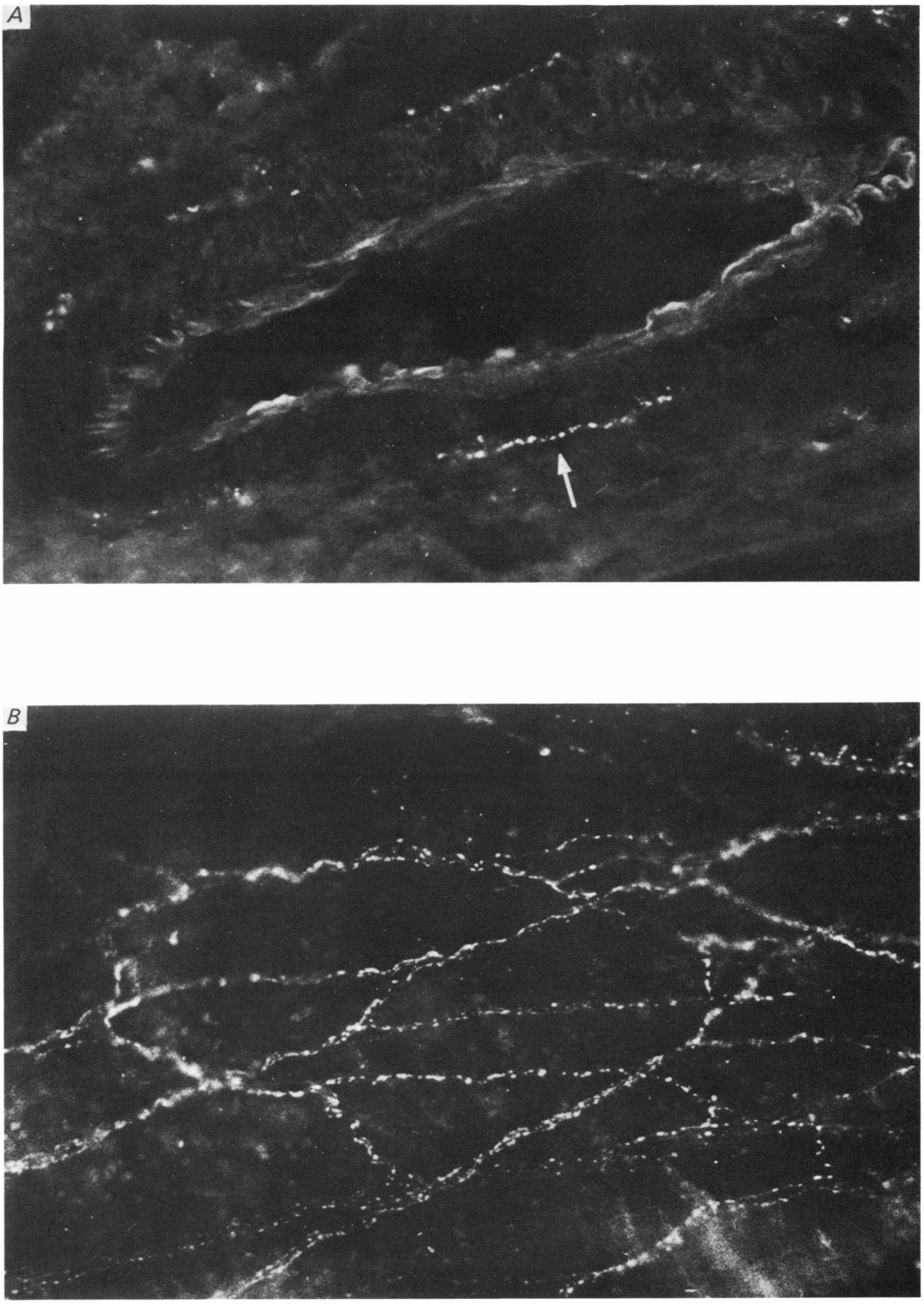
EXPLANATION OF PLATE 1

A, feline anterior cerebral artery. Substance P immunofluorescent nerve fibre (arrow) at the border between media and adventitia layers (× 125). *B*, stretch preparation. Pial artery. Several nerve fibres displaying substance P immunoreactivity are located on the surface of the arterial wall (× 125).



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