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EVIDENCE FOR AN INDIRECT CHOLINERGIC REGULATION OF BLOOD FLOW IN THE HYPOTHALAMUS OF CONSCIOUS RABBITS

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1 The effects of methacholine, atropine and adrenoceptor blockade on hypothalamic blood flow (HBF) were measured in conscious rabbits.

2 A dose of 1 μg methacholine increased HBF while smaller and larger doses had no significant effect.

3 The vasodilatation induced by methacholine was blocked by atropine and by chemical sympathectomy of the hypothalamus with 6-hydroxydopamine.

4 The vasodilatation was reversed by propranolol but was not affected by phenoxybenzamine.

5 These results suggest that the vasodilator action of muscarinic receptor agonists on hypothalamic resistance vessels depends upon the integrity of a noradrenergic system, and is mediated via β-adrenoceptors.

Introduction

It is now well established that intraparenchymal cerebral blood flow is affected by adrenergic nerves (Rosendorff, Mitchell, Scriven & Shapiro, 1976). Adrenergic nerves arising in the medulla cause an indirect β-adrenoceptor-mediated vasodilatation while those originating in the superior cervical ganglia cause a direct α-adrenoceptor-mediated vasoconstriction (Mitchell, Mitchell & Rosendorff, 1978).

Conversely there is little evidence for an effect by cholinergic nerves. Cholinergic nerves have been shown to dilate pial vessels (Lavrentieva, Mchedlishvili & Plechkova, 1968) and to supply other large cerebral arteries (Lee, Su & Bevan, 1975). The close association between adrenergic and cholinergic nerve endings in the extracerebral circulation (Iwayama, Furness & Burnstock, 1970) provides morphological evidence for Burn & Rand's (1959) suggestion that acetylcholine may mediate or facilitate release of noradrenaline from adrenergic nerve terminals. Physiological evidence that this takes place in the extracerebral circulation has been provided recently (Aubineau, Sercombe, Edvinsson & Owman, 1977).

On the other hand, no true perivascular cholinergic innervation has been found on intracerebral vessels although an extensive intracerebral cholinergic pathway exists in association with the vascular bed (Hardebo, Edvinsson, Emson & Owman, 1977). Further, Meyer, Teraura, Sakomoto & Kondo (1971) have suggested that a central cholinergic pathway causes vasodilatation of intraparenchymal blood vessels. Our own previous experiments have shown a vasodilator adrenergic pathway (Mitchell *et al*, 1978).

We have now examined the possibility that the intracerebral cholinergic nerves influence intraparenchymal cerebral blood flow indirectly by causing release of noradrenaline from these intracerebral adrenergic nerves.

Methods

Fifteen New Zealand white rabbits of either sex and weighing between 2 and 3 kg were used in the study. The hypothalamus was chosen as the test region because it is a homogeneously perfused and relatively large region of gray matter. It is also well supplied with extrinsic and intrinsic noradrenergic nerves.

Access to the hypothalamus was gained by a modification of the method of Monnier & Gangloff (1961). Two weeks before any experiments were begun, rabbits were anaesthetized with 30 mg/kg Nembutal (Abbott) and perspex headplates screwed to their skulls. Holes drilled through the headplates at coordinates aB–15 mm allowed stereotaxic access to the hypothalamus. Two weeks after operation, hypothalamic blood flow (HBF) was measured in conscious rabbits by the ^{133}xenon clearance technique (Cranston & Rosendorff, 1971). To measure the effects of various substances and procedures on HBF, one side of the hypothalamus was designated the control side

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and the other the test side. The vascular characteristics of each side of the hypothalamus are discrete and similar (Rosendorff, 1972) so each rabbit acted as its own control. This aspect of the experimental design implies that similar systemic and local conditions exist at any moment for each half of the hypothalamus and any changes in HBF measured must then be dependent on the experimental drug.

At the time of experiments, injection cannulae were placed so that their tips lay in identical positions in the hypothalamus on each side of the midline. Injections into the control side contained 15 μCi ^{133}xenon dissolved in 5 μl 0.9% w/v NaCl solution (saline). The test side received the ^{133}xenon in saline plus the test substances. The final volume injected into the test side was 5 μl. Injections were given into each side alternately at intervals of at least 10 min. After each injection the clearance of the radioactive isotope was measured with an external collimated scintillation counter and recorded on punchtape. HBF was then calculated from the ^{133}xenon clearance curve on an IBM 370 computer using a non-linear regression analysis. HBF (ml 100 g^{-1} tissue min^{-1}) was obtained from the formula $HBF = \lambda\beta$ where β is the decay parameter of the monoexponential clearance curve and λ the tissue-blood partition coefficient for xenon. For the rabbit hypothalamus $\lambda = 0.74$ (Rosendorff & Luff, 1970).

The first series of experiments was designed to establish a dose-response curve and the effect of a cholinomimetic drug (methacholine chloride, Merck) on HBF. Methacholine (MCh) was chosen as it has a longer *in vivo* half-life than acetylcholine (Lindmar, Loffelholz & Muscholl, 1968). After the effect of MCh had been established the next series of experiments was designed to determine the effect of various receptor antagonists on the response. In this series of experiments atropine (M.L. Laboratories), propranolol (Inderal, ICI) and phenoxybenzamine (Dibenyline, S.K. and F.) were used. In addition chemical sympathectomy of the hypothalamus was achieved with 6-hydroxydopamine (Ungerstedt, 1971), 300 μg of which were injected into the hypothalamus 4 days before injections of 1 μg MCh.

There appeared to be appreciable inter-individual differences in basal HBF. The results were therefore analysed by two-way analysis of variance to account separately for inter-individual and inter-treatment differences. Tests of significance were based on the residual variance calculated using the several observations obtained on each animal for each treatment (F test). Each rabbit was used once only for each experiment and was not used more than four times in total. In each experiment equal numbers of trials were performed on each animal.

Results

In all groups of rabbits the analysis of variance revealed highly significant inter-individual variation in HBF ($P < 0.01$). The analysis also revealed the following effects of the various test procedures.

Methacholine chloride

Table 1 shows the dose-response relationship for 6 doses of MCh. A dose of 1 μg MCh per injection increased HBF significantly ($P < 0.01$) by 10 ml 100 g^{-1} min^{-1}. None of the other doses had any significant effect.

Table 1 Response of hypothalamic blood from (HBF) in conscious rabbits to different doses of methacholine (MCh)

Treatment	*No. of animals*	*No. of trials*	*HBF ($\bar{X} \pm$ s.e.) (ml 100 g^{-1} min^{-1})*	*Change in HBF*	P (*F test*)
^{133}Xenon in saline	3	18	35.6 ± 4.1	+3.3	NS
^{133}Xenon in saline + 0.1 ng MCh		18	38.9 ± 3.4		
^{133}Xenon in saline	3	15	29.6 ± 3.1	−0.9	NS
^{133}Xenon in saline + 1.0 ng MCh		15	28.7 ± 3.6		
^{133}Xenon in saline	4	24	48.5 ± 4.8	+3.6	NS
^{133}Xenon in saline + 10 ng MCh		24	52.1 ± 4.6		
^{133}Xenon in saline	3	15	23.7 ± 3.2	+10.3	<0.01
^{133}Xenon in saline + 1 μg MCh		15	34.0 ± 3.5		
^{133}Xenon in saline	3	18	45.5 ± 2.8	+4.4	NS
^{133}Xenon in saline + 10 μg MCh		18	49.9 ± 3.8		
^{133}Xenon in saline	4	20	55.7 ± 4.2	+5.8	NS
^{133}Xenon in saline + 100 μg MCh		20	61.5 ± 4.8		

Table 2 Effect of receptor blockade on methacholine (MCh) vasodilatation

Treatment	*No. of animals*	*No. of trials*	*HBF* ($\bar{X} \pm$ *s.e.*) (*ml 100* g^{-1} min^{-1})	*Change in HBF*	P (*F test*)
^{133}Xenon in saline	3	15	31.3 ± 3.8	−1.4	NS
^{133}Xenon in saline + atropine		15	29.9 ± 2.8		
^{133}Xenon in saline	3	15	27.7 ± 2.1	+1.5	NS
^{133}Xenon in saline + 1 μg MCh + atropine		15	29.2 ± 2.4		
^{133}Xenon in saline	3	18	34.8 ± 2.6	−1.8	NS
^{133}Xenon in saline + 1 μg MCh + 6-OHDA		18	33.0 ± 2.7		
^{133}Xenon in saline	3	15	37.1 ± 2.1	−7.9	<0.01
^{133}Xenon in saline + 1 μg MCh + propranolol		15	29.2 ± 3.2		
^{133}Xenon in saline	3	18	22.3 ± 0.7	+8.9	<0.01
^{133}Xenon in saline + 1 μg MCh + phenoxybenzamine		18	31.2 ± 2.1		

Receptor antagonists

The increase in HBF produced by the 1 μg dose of MCh could be abolished by the intrahypothalamic injection of 0.65 μg atropine. Atropine itself had no effect on HBF (Table 2). The increase in HBF due to MCh (1 μg) could also be blocked by chemical sympathectomy of the hypothalamus using 6-hydroxydopamine (Table 2). Further, the vasodilatation due to MCh could be blocked and even reversed, by intrahypothalamic injection of 20 μg of the β-adrenoceptor antagonist, propranolol (Table 2). The vasodilatation however was not affected by intrahypothalamic injections of 50 μg of an α-adrenoceptor antagonist, phenoxybenzamine (Table 2). Neither propranolol nor phenoxybenzamine had any effect on HBF when injected alone.

Discussion

In all groups of animals the analysis of variance revealed marked interindividual variability in HBF. Several factors contribute to this variability (Mitchell *et al*, 1978). However, the inter-individual variances are not considered to be important because each animal served as its own control: flows in half the hypothalamus are compared with flows in the other half virtually simultaneously.

It is possible that intrahypothalamic injections of the test substances change HBF by altering arterial blood pressure. However, the cerebral autoregulatory range in the rabbit is 41 to 140 mmHg (Cranston & Rosendorff, 1971) so for intrahypothalamic injections to change HBF through a systemic mechanism they would have to raise or lower mean arterial pressure considerably. Furthermore these changes would have to last less than 10 min, the time of xenon clearance, otherwise the change in blood pressure would be reflected in a similar change in HBF in the 'control' hypothalamus. It is therefore unlikely that blood pressure changes are responsible for changes in HBF observed in our experiments. Further, we have previously shown that intrahypothalamic injections do not change mean arterial pressure (Rosendorff *et al*, 1976; Mitchell *et al*, 1978). Nevertheless the effect of intrahypothalamic injections of 1 μg, 10 μg and 100 μg MCh on mean arterial blood pressure was measured. None of these doses had any effect on mean arterial blood pressure. The changes in HBF observed in the experiments described here are therefore likely to be dependent on the local effects of the experimental drugs.

Although there is evidence that extracerebral vessels are supplied by cholinergic nerves (Lavrentieva *et al*, 1968) and that these nerves influence vascular tone (Aubineau *et al*, 1977), there is no equivalent evidence for cholinergic influence on intracerebral vessels.

However, our experiments suggest that exogenous cholinomimetic drugs can affect intraparenchymal cerebrovascular tone. A dose of 1 μg MCh caused a significant vasodilatation. Larger doses had no significant effect. This lack of significant responses to higher doses of MCh may be due to receptor blockade. Burn (1974) has suggested that a single receptor can produce a dose-dependent excitation or inhibition. An excitatory response is produced at low doses of cholinoceptor agonist and an inhibitory response at high doses.

However, it is also possible that the lack of significant responses to higher doses of MCh is due to the higher control values of HBF recorded at these doses. The higher control values could be explained if some MCh diffused into the contralateral control hypothalamus where it also caused a vasodilatation. It is possible therefore that the 10 ng, 10 μg and 100 μg doses all produced an increase in HBF, but because flow increased on both sides of the hypothalamus our method of calculation failed to show a significant effect except for the 1 μg dose.

The increase in HBF caused by the 1 μg dose of MCh could be due to a direct effect of the drug on cholinoceptors on blood vessels, or it could be due to an effect of MCh on neighbouring adrenergic nerves. Further, if the effect of MCh is to regulate release of noradrenaline, then the noradrenaline could influence vascular diameter either by stimulating blood vessel receptors directly or indirectly via neuronal cell body receptors with a resulting increase in firing rate and the evolution of vasodilator metabolites (Mitchell *et al*, 1978).

Our experiments suggest that the effect of MCh on intraparenchymal blood vessels is indirect. The evidence for this is that the effect of MCh is blocked by chemical sympathectomy and β-adrenoceptor blockade. If the blood vessels themselves contained cholinoceptors the sympathetic blockade would not have abolished the MCh-induced vasodilatation. It is also unlikely that the MCh effect is nonspecific since its effect is blocked by a specific cholinoceptor antagonist, atropine. Further the effects are not due to the antagonists themselves. All of these observations are compatible with the idea that the injection of MCh causes the release of noradrenaline from adrenergic neurones. The released noradrenaline in turn, activates a β-adrenoceptor causing vasodilatation. It also seems likely that the adrenoceptor lies on neuronal cell bodies and that the vasodilatation is due, at least in part, to the production of metabolites (Mitchell *et al*, 1978).

Our experiments also suggest that the cholinoceptor is muscarinic. MCh is a specific muscarinic agonist (Lindmar *et al*, 1968) and atropine is a specific

muscarinic antagonist (Edvinsson & Owman, 1977). However, Lindmar *et al* (1968) have suggested that muscarinic receptors are inhibitory on effector organs. An explanation for our results may then be that MCh stimulates muscarinic receptors which inhibit the release of noradrenaline thereby reducing vasoconstrictor tone. This explanation is unlikely. Neither propranolol nor phenoxybenzamine has any effect on HBF which implies that there is no measurable autonomic vasoconstrictor tone in hypothalamic blood vessels. It seems possible therefore that in the autonomic control of cerebral blood flow, muscarinic receptors may be excitatory. Further evidence to support this suggestion is that atropine reduces hypercapnia-induced vasodilatation (Kawamura, Meyer & Hiromoto, 1975).

Finally, our results do not necessarily suggest that endogenous acetylcholine in the hypothalamus has similar effects. However, the present data are consistent with the idea that cholinoceptors are present, that they can influence intraparenchymal cerebral blood vessel diameter and that they are likely to act on adrenergic fibres and not on the blood vessels directly.

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