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An analysis of the purinergic component of active muscle vasodilatation obtained by electrical stimulation of the hypothalamus in rabbits

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1 In anaesthetized rabbits, electrical stimulation of the hypothalamus in areas analogous to the defence area in cats produces the 'defence reaction.' This response includes signs of arousal and a large increase in blood flow to skeletal muscle in the hind limb caused by a vasodilatation in the skeletal muscle vasculature.

2 The vasodilatation is a sympathetic response, and it is not dependent upon muscle activity in the hind limb.

3 The muscle vasodilatation is insensitive to α-adrenoceptor, β-adrenoceptor, cholinoceptor and histamine receptor antagonists.

4 Intra-arterial injections of the purinoceptor agonists, adenosine triphosphate (ATP) and adenosine, mimic the vasodilatation produced by electrical stimulation.

5 The P_1-purinoceptor blocker, aminophylline, attenuates adenosine-induced vasodilatation, but it does not affect the vasodilatation produced by ATP or hypothalamic stimulation.

6 The P_2-purinoceptor blocker, antazoline, attenuates the vasodilatation produced by both ATP and hypothalamic stimulation.

7 Our results suggest that the muscle vasodilatation produced by hypothalamic stimulation is mediated by purinergic nerves which release ATP and act on P_2-purinoceptors.

Introduction

The 'defence reaction' (Hess & Brügger, 1943) is a response produced by electrically stimulating the hypothalamus in conscious, unrestrained cats. It includes visible features such as exophthalmos, mydriasis, piloerection, hissing, and changes in respiration, and is thought to be the response which is naturally elicited by threatening or emotionally stressful situations. Abrahams *et al.* (1960) demonstrated that atropine-sensitive active muscle vasodilatation is an important component of the defence reaction. Subsequently, many researchers have regarded the production of active muscle vasodilatation as verification that the defence areas of the brain have been stimulated in their experiments (e.g., Szidon & Fishman, 1971; Kumada *et al.*, 1975; Wennergren *et al.*, 1976).

We have been interested in the effects of emotional arousal on body temperature regulation in rabbits (Stitt, 1976). To investigate these effects, we thought that elicitation of the defence reaction by electrically stimulating the hypothalamus would be a useful method for duplicating the autonomic effects of emotional arousal. However, production of the defence reaction in rabbits has never been clearly demonstrated. Therefore, in the present study, we wished to ascertain whether the defence reaction could be elicited in rabbits by demonstrating the presence of active muscle vasodilatation during hypothalamic stimulation. We discovered that large increases in blood flow to skeletal muscle could be evoked which resembled those described for cats. The increases resulted from a neurogenic vasodilatation. However, unlike cats, the rabbit vasodilatation was a non-adrenergic, non-cholinergic response.

Recently, a growing number of studies are reporting autonomic responses that are both non-adrenergic and non-cholinergic. Burnstock (1972) has suggested that many of these responses are due to another division of the autonomic nervous system which utilizes purinergic nerves to mediate its re-

sponses. Thus, we also investigated pharmacologically active muscle vasodilatation in the rabbit in terms of the current purinergic nerve hypothesis.

Parts of this work have been published in abstract form (Shimada & Stitt, 1979; 1982).

Methods

We used New Zealand white rabbits which ranged in size from about 3.5 to 4.7 kg. Urethane (20% in 0.9% NaCl) was administered for anaesthesia at an initial dose of $1\,g\,kg^{-1}$: half of the dose given intravenously and the remainder, intraperitoneally. Additional doses of urethane were given as needed to maintain anaesthesia. A tracheotomy was performed and an endotracheal cannula was inserted to facilitate spontaneous respiration. An electric heating pad helped to maintain a normal rectal temperature.

Blood flow in the femoral artery was measured with an electromagnetic flowmeter (Model BL 613, Biotronex Laboratory) using a 1.0 or 1.5 mm diameter flow probe. A stout ligature, placed about the ankle, occluded blood flow to the paw, and vessels to fat and skin were tied off or cauterized so that most of the blood flow in the femoral artery perfused skeletal muscle. In 3 experiments, the hind limb was skinned down to the ankle ligature to eliminate all cutaneous contributions to femoral blood flow. The removed skin was sutured in its original position and the entire leg was placed in a polyethylene bag to retain heat and moisture. During some experiments, a second flow probe, 3.0 mm in diameter, was placed on the renal or superior mesenteric artery, close to its origin at the abdominal aorta. Distal occlusion of each artery verified the accuracy of the zero flow level measured by the flowmeter.

In some experiments a concentric, double-lumened catheter was inserted proximally into the femoral artery contralateral to that in which blood flow was measured. The tip was advanced to the bifurcation of the distal aorta. A Statham pressure transducer monitored arterial blood pressure through the outer lumen (V-6 vinyl tubing, 0.86 mm i.d., 1.52 mm o.d.). The inner lumen provided a route for intra-arterial injections of pharmacological agents (V-1 vinyl tubing, 0.28 mm i.d., 0.64 mm o.d.). After injection, these drugs were immediately swept down the iliac artery and into muscle vascular beds whose blood flow was being measured. In other experiments, single lumened catheters were inserted into the contralateral femoral artery (PE 160 tubing) for pressure measurement and into the superficial epigastric artery (V-1 tubing) for close, intra-arterial injections at the site of blood flow measurement. The arterial blood pressure signal drove a Grass Co. tachograph (Model 7P4) for heart rate measurements. The phase relationship between the blood flow and blood pressure signals was retained by using identical Grass Co. preamplifiers (Model 7P1, low pass filter cutoff freq. = 3 Hz).

In some experiments, relative changes in tidal volume and respiration rate were monitored by strapping a latex inflation bag around the thorax of the animal. A Grass Co. volumetric pressure transducer (Model PT5 A) measured volume changes in the applied bag. In 12 animals, electromyograms displayed skeletal muscle activity in the hind limb. A concentric, bipolar electrode inserted into the upper thigh muscles recorded the electrical activity through a Grass Co. 7P5 preamplifier.

In three experiments, the effects of sympathectomy were observed. We performed a sympathectomy by retroperitoneally approaching the left abdominal sympathetic trunk through a flank incision. Ganglia L_1 through L_3 and the interconnecting fibres were excised.

A stereotaxic apparatus (David Kopf) immobilized the head of the rabbit. A craniotomy was trephined, and an agar plug sealed the opening but still permitted electrode penetration into the brain. The stereotaxic atlas of Fifková & Marsala (1967) provided guidance for electrode positioning. Stainless steel stimulating electrodes had diameters of 0.020 or 0.022 inches (about 0.50 or 0.55 mm). Formvar enamel (General Electric Co.) insulated the electrode except for the tip which remained exposed for a length of 0.2 to 0.5 mm. We also manufactured electrodes from stainless steel insect pins (0.28 mm dia.) with tip exposures between 0.15–0.20 mm. Stimulation was monopolar; an indifferent electrode was attached to skin near the craniotomy wound. Two optically isolated stimulators (Model CCIU-8, Frederick Haer) driven by a pulse generator (Model Pulsar 6b, Frederick Haer) provided biphasic, constant current stimulation. The most commonly used stimulus parameters were a 1–2 ms pulse duration at 77 Hz for 20 s with the current usually in the range 50–500 μA. We did not perform either a systematic study of stimulation parameters or a detailed mapping of effective brain sites; rather, we were interested in the response to stimulation and, thus, we generally employed parameters and stimulation loci known to be effective. We performed histological examinations on 17 rabbits. After each of these experiments, the brain was perfused with saline through the carotid arteries and then fixed with 10% formalin. Sections (50 μm) were cut in the region of the hypothalamus, mounted on glass microslides and stained for Nissl substance with cresyl violet.

Drugs were dissolved in sterile 0.9% saline on the

day of the experiment. Two series of pharmacological experiments were preformed. For the first series involving cholinergic and adrenergic substances, blocking agents were delivered in volumes of a few ml while vasocative drugs were injected in volumes of 25 or 50 μl. Injections were followed by a 200 μl heparinized (30 u ml^{-1}) saline flush (catheter volume was 20 to 30 μl). Responses to the saline flush alone were not perceptible in any of our measurements. Injections of appropriate agonist, before and after administration of a blocking agent, verified the presence of an effective pharmacological blockade in the hind limb vasculature. Such a blockade was indicated by the absence of any blood flow response to agonist injection. If the response to agonist was unaltered or only attenuated, then additional doses of antagonist were administered until abolition. The agonists employed were acetylcholine chloride (ACh), adrenaline (Ad, (−)-epinephrine bitartrate) and noradrenaline (NA, (−)-arterenol bitartrate). We used the following antagonists: atropine sulphate, phenoxybenzamine ('Dibenzyline', courtesy of Smith, Kline and French Laboratories, Philadelphia PA., USA), (±)-propranolol hydrochloride, phentolamine mesylate ('Regitine', Ciba Pharm. Co., Summit, N.J., USA), (−)-scopolamine hydrobromide and propantheline bromide. In the second series of experiments, a purinergic mechanism was investigated. The injection volume for the agonists, ACh, adenosine triphosphate (ATP), adenosine, inosine, and histamine, was 10 μl. We chose concentrations of blocking drugs so that their injection volumes were a few ml. A flush with 100 μl heparinized saline followed all injections. The following additional drugs were used: adenosine-5′-triphosphate (disodium salt), adenosine, inosine, histamine dihydrochloride, aminophylline, antazoline phosphate, pyrilamine maleate, metiamide (Smith, Kline and French), and cimetidine (Smith, Kline and French). With the exceptions noted, all drugs were purchased from Sigma Chemical Co. (St. Louis, MO., USA). Where appropriate, drug doses refer to the salt.

Vascular resistance was calculated by dividing mean arterial blood pressure by femoral artery blood flow. Venous outflow pressure was assumed to be negligible. The magnitude of a vasodilatation was calculated as a difference in vascular resistance (expressed in units of PRU, i.e., peripheral resistance units or mmHg ml^{-1} min^{-1}) before and during hypothalamic stimulation. The vascular resistance before stimulation was calculated just prior to stimulation while the vascular resistance during stimulation was acquired at the point of maximal blood flow. The magnitude of a vascular response depends upon the initial amount of tone present in the vascular bed (Myers & Honig, 1969). To quantify this dependence, we calculated a linear regression (Figure 2a) of the maximal changes in vascular resistance obtained from all rabbits on initial resistance, using the least squares method (Snedecor & Cochran, 1967). When a drug treatment was administered, vascular resistance usually changed. Thus, the vasodilatations elicited before and after drug treatment arose from different initial resistances, rendering a direct comparison of these responses improper. Using the regression equation, the magnitude of the vasodilatation after drug treatment was 'adjusted' to that magnitude which would have been produced if it had arisen from the same initial resistance as the pre-drug vasodilatation. The adjusted value (Snedecor & Cochran, 1967) for the response after drug treatment was:

$$\triangle R_{adj} = \triangle R + b(R_{control} - R_{drug}) \qquad (1)$$

where $\triangle R$ = vasodilatation after drug treatment, b = regression coefficient (0.82), $R_{control}$ = initial resistance for the vasodilatation elicited before drug treatment and R_{drug} = initial resistance for the vasodilatation produced after drug treatment. After correcting for the difference in initial resistance, the responses before and after drug treatment could be properly compared. We then used a paired t test to determine if treatment with a pharmacological antagonist had a statistically significant effect.

During experiments which involved purinergic agents, we wished to produce log dose-response curves for drug injections and current-response curves for electrical stimulation in the hypothalamus. To this end, we normalized all vasodilatations to the maximal vasodilatation producible by electrical stimulation as described by the regression line in Figure 2. We have expressed each vasodilatation as a percentage of the maximal change in resistance (% ΔRm) which would be expected given the initial resistance for the vasodilatation. Figure 2b illustrates an example of this procedure. Since all vasodilatations are normalized by the same regression equation, a vasodilatation produced by drug injection can be directly compared with one produced by electrical stimulation, e.g., a 50% ΔRm vasodilatation produced by an injection of ATP is the same size as a 50% ΔRm response produced by hypothalamic stimulation. A probit transformation linearized the log dose-response curves acquired for the injection of drugs. Current-response curves acquired for electrical stimulation were linearized by plotting current on a logarithmic scale. After transformation of both types of curves we used linear regression analysis and an analysis of covariance to determine the statistical significance of any shifts of curves or changes in slope produced by blocking drugs (Armitage, 1971). We selected a 95% level of significance.

Results

Electrical stimulation of the hypothalamus evoked symptoms of widespread sympathetic nervous system arousal accompanied by somatic motor effects. Responses included mydriasis, exophthalmos, widening of the palpebral fissures and changes in respiration. Sometimes, profuse salivation and movements of the jaw, tongue, and vibrissae were evident. Occasionally, stimulation also produced perking of the ears and piloerection on the shoulders and along the spine. Cardiovascular changes were also induced: arterial blood pressure altered, heart rate rose, and visceral vascular beds constricted. Most importantly, blood flow to skeletal muscle increased substantially.

Figure 1 shows a response to electrical stimulation. Femoral artery blood flow increased from 5 ml min^{-1} to almost 20 ml min^{-1}. This was accompanied by minor changes in arterial blood pressure, clearly indicating a large fall in vascular resistance. During stimuli which produced increases in muscle blood flow, tachycardia was usually present. The response of arterial blood pressure was variable; generally, weak stimuli produced hypotension while strong stimuli produced hypertension. Figure 1 also shows that superior mesenteric artery blood flow nearly ceased, indicating an intense splanchnic vasoconstriction during stimulation. When renal artery blood flow was measured, it behaved similarly. Muscle blood flow increased within 2–3 s after stimulation began, suggesting a neurogenic mediation. It rose only during the stimulation period and fell immediately after stimulation ceased. We observed increases in respiration rate as well as a tendency towards a decreased tidal volume. Strong stimuli resulted in brief periods of apnoea. When recovery periods of about 5 min were allowed to elapse, we could elicit identical responses for hours. Stimulation of either side of the hypothalamus produced vasodilatation in both hind limbs.

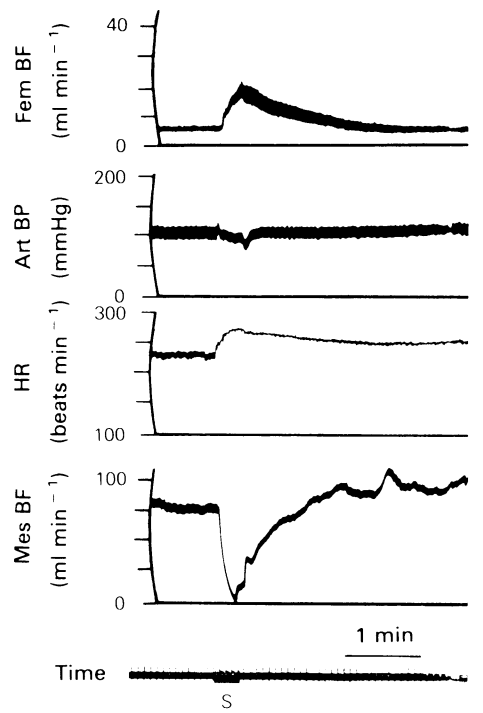


Figure 1 Effects of hypothalamic stimulation. The variables are femoral artery blood flow (Fem BF), arterial blood pressure (Art B.P.), heart rate (HR) and superior mesenteric artery blood flow (Mes BF). Bar below time marker indicates electrical stimulation period (S). A 20 s stimulation (475 μA, 77 Hz, 1 ms pulse duration) produces a sharp increase in femoral blood flow accompanied by a slight hypotension, tachycardia and a striking fall in mesenteric blood flow.

The dependence of the magnitude of the vasodilatation upon the initial resistance of the vascular bed is quantified in Figure 2a. It shows the maximal vasodilatation obtained from each of 39 rabbits plotted against its initial resistance. The solid line is the regression line determined for the 36 normally prepared hind limbs, and the pair of broken lines delimits the 95% confidence band for this regression line. At the average value for initial resistance of 20.1 PRU, the decrease in vascular resistance caused by electrical stimulation is 14.4 PRU. This is equivalent to an increase in conductance of about 350%. The complete removal of skin from 3 hind limbs produced no significant differences in either resting blood flow or evoked vasodilatation. The average basal blood flow was 1.50 ml min^{-1} kg^{-1} body wt. ± 0.161 (s.e., $n = 36$) for the normally prepared limbs and 1.50 ml min^{-1} kg^{-1} body wt. ± 0.239 (s.e., $n = 3$) for the completely skinned limbs. Figure 2a shows that the vasodilatations observed in the skinned limbs were similar to those found in the normally prepared limbs; removal of the cutaneous circulation did not abolish the vasodilatation. The vasodilatation was occurring in the skeletal muscle vasculature.

In three experiments, we observed the effects of sympathectomy on the vasodilatation produced by stimulation. Blood flows in each hind limb were measured, and arterial blood pressure was monitored from a carotid artery catheter. Electrical stimulation produced vasodilatation in both hind limbs. A left sympathectomy was then performed resulting in systemic hypotension due, in part, to a loss of vasoconstrictor tone in the left hind limb. Administration of dextran restored blood pressure. Sympathetic innervation to the left hind limb was interrupted, but its motor innervation remained intact. When the hypothalamus was restimulated we found that the vasodilatation was abolished in the left hind limb. An

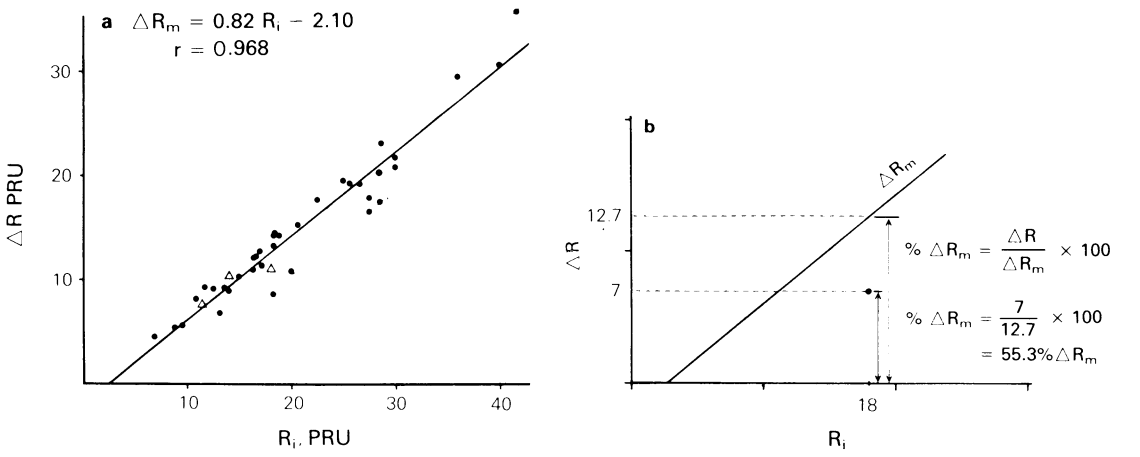


Figure 2 (a) Relationship between change in hind limb vascular resistance (ΔR) produced by hypothalamic stimulation and the initial resistance (R_i) just prior to stimulation. Each data point represents the maximal vasodilatation elicited from 36 rabbits with normally prepared hind linbs (solid circles) and 3 rabbits with completely skinned hind limbs (triangles). The equation and the solid line represent the regression of ΔR on R_i for the normally prepared hind limbs and the dotted lines demarcate the 95% confidence band for the regression. (b) Illustrated is our procedure for calculating the magnitude of an evoked vasodilatation. The line labelled ΔRm represents the linear regression from (a). The dot represents a hypothetical vasodilatation which produces a change of resistance equal to 7, starting from an initial resistance of 18. From the equation for ΔRm, the maximum change of resistance expected at an initial resistance of 18 would be 12.7. Thus, the vasodilatation is equal to 55.3% ΔRm. This procedure compensates for changing initial resistance during an experiment (PRU = peripheral resistance unit = $mmHg\ min^{-1}\ ml^{-1}$).

increase in blood flow to the intact right hind limb verified that electrical stimulation was still able to produce vasodilatation. These results were observed in all sympathectomy experiments. In one experiment, one further manoeuvre was performed. The fifth lumbar vertebra was breached, and the spinal cord was severed. This procedure disrupted the motor innervation to the right hind limb while leaving its sympathetic innervation intact. Stimulation still produced vasodilatation in the right hind limb. These results demonstrated not only that the vasodilatation is sympathetic in nature, but also that the increase in blood flow does not require skeletal muscle activity or motor innervation to the hind limb.

During some of our experiments, we observed electromyographic (EMG) records acquired from the hind limb in which blood flow was measured. In twelve animals, we induced shivering by shaving their torsos. The presence of shivering verified the proper performance of our EMG equipment. Results of all experiments were similar. When electrical stimulation in the hypothalamus started, shivering activity was immediately suppressed. The EMG revealed no muscle activity during stimulation. The large increase in blood flow to the skeletal muscle was still observed along with the usual arterial blood pressure and heart rate responses. After stimulation terminated, shivering activity slowly returned, and basal blood flow to the hind limb rose as shivering set in. These EMG records showed that shivering is suppressed by hypothalamic stimulation. Furthermore, they demonstrated that muscle activity can be totally absent during the vasodilator response, and that muscle activity is not required for the vasodilatation to occur. These experiments rule out the possibility that the vasodilatation is caused by a muscular reactive hyperaemia.

Histological examination revealed that most of the stimulation points were in the perifornicular areas of the hypothalamus. Figure 3 shows coronal brain sections, containing histologically verified stimulation sites. One point was in the medial preoptic area, two were in the medial portion of the anterior hypothalamus, and the remainder were close to the fornix as it coursed through the medial regions of the dorsomedial, ventromedial, and posterior hypothalamus.

Our initial pharmacological experiments tested three possible mechanisms for the increase in blood flow: a release of α-adrenergic vasoconstrictor tone, a cholinergic vasodilator mechanism such as that found in carnivores, and a β-adrenergic vasodilator mechanism. In the experiment shown in Figure 4 and described below, all three possibilities were tested. Control responses to electrical stimulation of the hypothalamus (S), to adrenaline (Ad), and to norad-

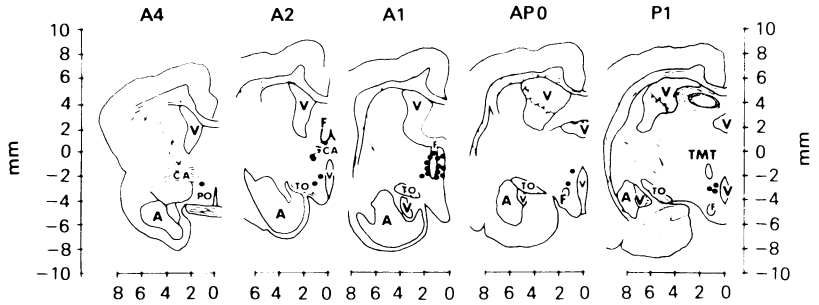


Figure 3 Brain stimulation sites from 17 rabbits. The solid circles are 23 histologically verified stimulation sites from which large increases in muscle blood flow were elicited. These coronal sections were adapted from the atlas of Fifková & Marsala (1967). The scales are in mm. Abbreviations: A, amygdala; CA, commisura anterior; F, fornix; PO, preoptic area; TMT, tractus mammillo-thalamicus; TO, tractus opticus; V, ventricle.

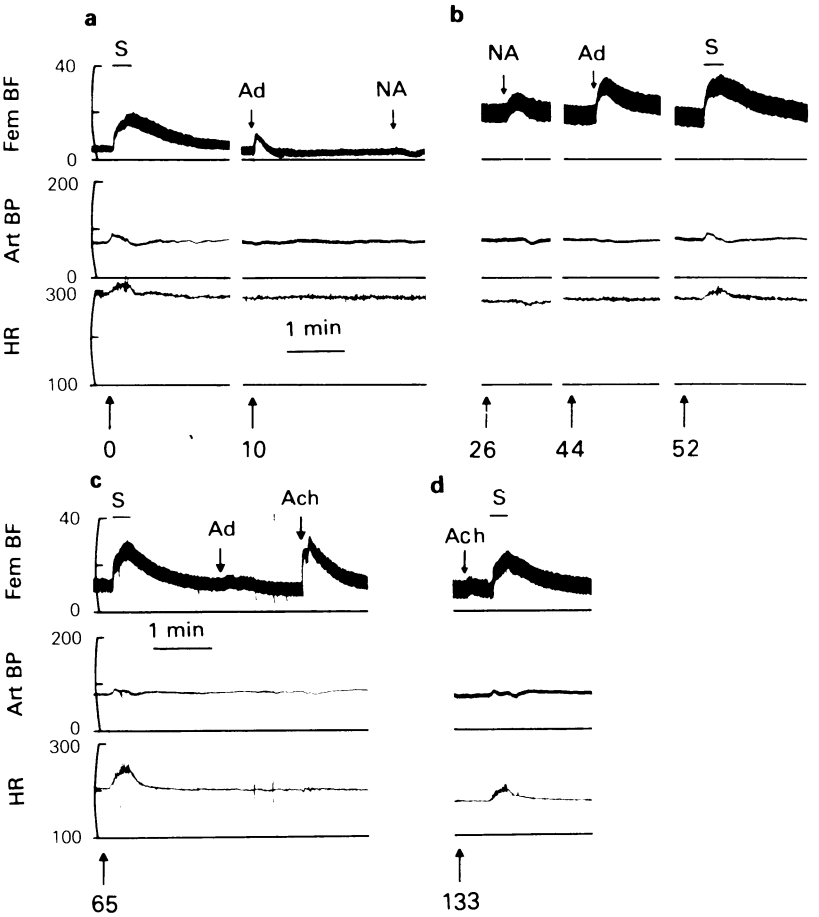
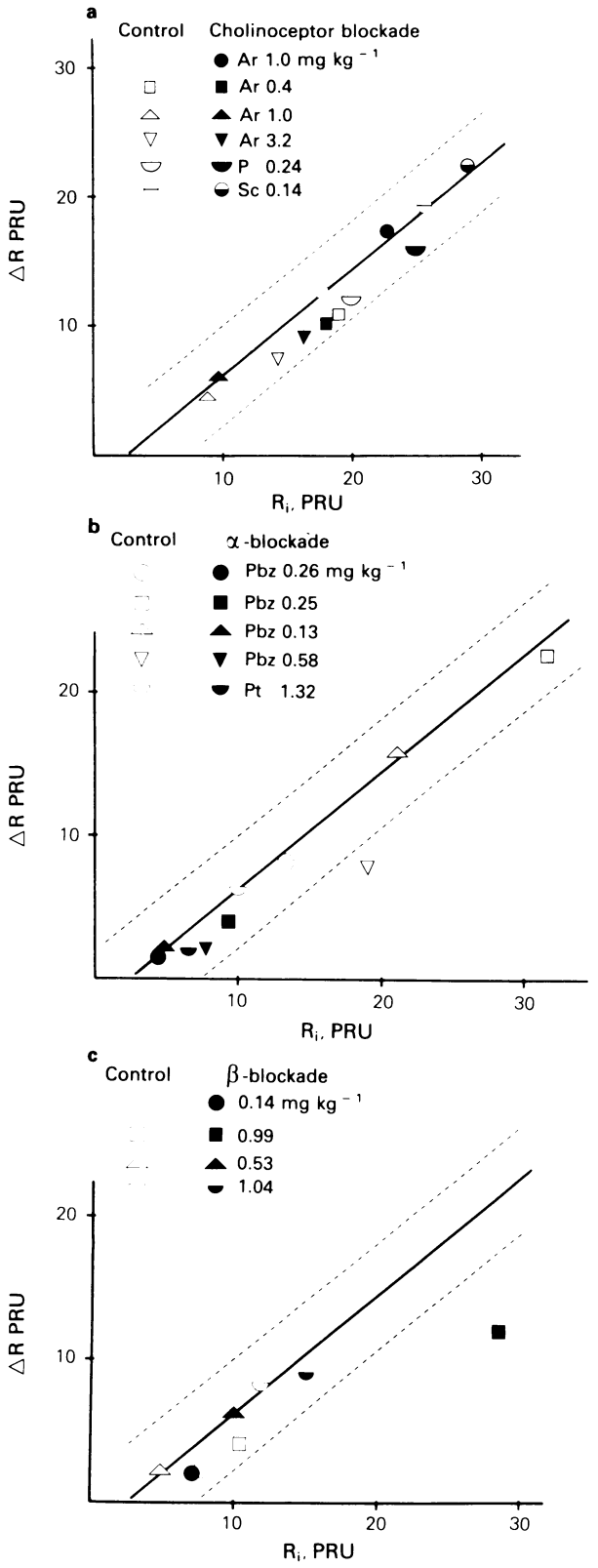


Figure 4 Effects of pharmacological blockade on muscle vasodilatation. (a) Control; (b) during α-adrenoceptor blockade; (c) during β-adrenoceptor blockade; (d) during cholinoceptor blockade. The variables displayed are femoral artery blood flow (Fem BF) (ml min^{-1}), arterial blood pressure (Art B.P.) (mmHg), heart rate (HR) (beats min^{-1}) and elapsed time during the experiment (min). Bars above the blood flow record indicate 20 s periods of electrical stimulation (S) in the hypothalamus. Close, intra-arterial injections of 1 μg adrenaline (Ad), 2 μg noradrenaline (NA) or 125 ng acetylcholine (ACh) are indicated by arrows above the blood flow record. See text for a description of this experiment.



renaline (NA) are shown in the first panel of Figure 4. Hypothalamic stimulation (450 μA, 77 Hz, 1 ms pulse duration for 20 s) increased femoral arterial blood flow from 5 ml min^{-1} to about 15 ml min^{-1}. One μg of Ad produced a slight vasodilatation whereas 2 μg of NA produced vasoconstriction. If the mechanism for the muscle vasodilatation were a reduction of α-adrenergic vasoconstrictor tone, then prior elimination of that tone should abolish the vasodilatation. Therefore, we administered 0.13 mg kg^{-1} of the α-adrenoceptor blocking agent, phenoxybenzamine. Such a small quantity directly into the hind limb was used to produce a blockade at this site while minimizing widespread reductions in vascular tone and the subsequent systemic hypotension. The effectiveness of the α-blockade in the hind limb vasculature is evident in the second panel of Figure 4. First, basal femoral blood flow was markedly increased over that measured before phenoxybenzamine administration, indicating a decrease in vascular tone. Second, the response to a subsequent injection of NA was reversed from vasoconstriction to vasodilatation, and the vasodilatation in response to Ad was greatly enhanced. Despite the loss of vascular tone, the increase in blood flow produced by S was still present except that it now arose from a higher level of basal blood flow. The vasodilatation was not due to a release of vasoconstrictor tone. Propranolol (0.53 mg kg^{-1}) was then administered between panels (b) and (c) to impose a β-adrenoceptor blockade in the hind limb vasculature. The systemic effect of propranolol is evident in panel (c) as a reduction in resting heart rate. Again, S produced vasodilatation, indicating that the mechanism was not β-adrenergic. A subsequent injection of Ad resulted in no response, verifying the presence of an effective β-adrenoceptor blockade. A control injection of 125 ng acetylcholine (ACh) elicited a large vasodilatation. Between panels (c) and (d), atropine, (1 mg kg^{-1}) was used to produce a cholinoceptor blockade. No response to ACh (panel d)) verified the effectiveness of this blockade. However, S still re-

Figure 5 Effects of (a) cholinoceptor, (b) α-adrenoceptor, and (c) β-adrenoceptor blockades on the muscle vasodilatation produced by hypothalamic stimulation. The vasodilatation, ΔR, is plotted against R_i, the vascular resistance just prior to stimulation. The open symbols show the control responses produced by stimulation before pharmacological blockade while the solid symbols indicate the responses after blockade. Each pair of identically shaped symbols represents one experiment. The solid line and the dashed lines are the regression line and confidence band from Figure 2. Propranolol was used for β-adrenoceptor blockade. Abbreviations: Ar, atropine; P, propantheline; Sc, scopolamine; Pbz, phenoxybenzamine; Pt, phentolamine.

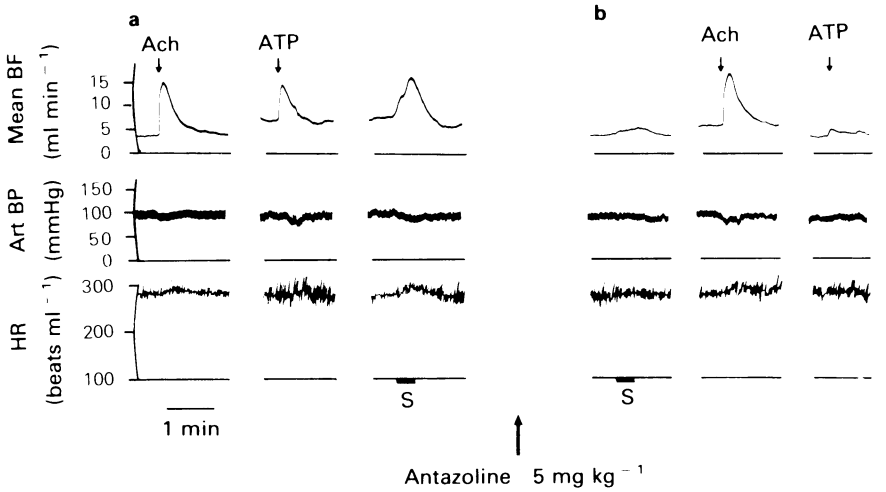


Figure 6 Effects of antazoline. The variables illustrated are from the top: mean blood flow through the femoral artery, systemic arterial blood pressure, and heart rate. (a) Control responses to 50 ng acetylcholine (ACh), 1 $\mu g\,kg^{-1}$ ATP, and 250 μA electrical stimulation (S) in the hypothalamus for 20 s. All produced an increase in blood flow with no rise in arterial blood pressure. Antazoline was administered, i.a., and the responses were re-examined. After antazoline (b) only ACh produced vasodilatation. Responses to S and ATP were attenuated.

sulted in muscle vasodilatation. Thus, the vasodilator mechanism was not cholinergic.

Figure 5 illustrates the results from all cholinoceptor, α-adrenoceptor, and β-adrenoceptor blockade experiments. In no instance was the vasodilatation abolished ($\Delta R = 0$) by any treatment. We verified the atropine experiments by using the cholinoceptor blockers, scopolamine and propantheline. Phentolamine was also administered to confirm the phenoxybenzamine results. Nearly all responses after drug treatment fell within the 95% confidence band. Statistical analysis by paired t test revealed that there was no significant difference between control and cholinoceptor ($P > 0.9$), α-adrenoceptor ($P > 0.78$), or β-adrenoceptor ($P > 0.15$) blockades. We conclude from these data that the muscle vasodilatation is not due to either a cholinergic vasodilator system, release of α-adrenergic vasoconstrictor tone or a β-adrenergic vasodilator mechanism.

In our next set of experiments we investigated the possible involvement of a purinergic mechanism in skeletal muscle vasodilatation. Intra-arterial injections of ATP produced vasodilatations which closely resembled those produced by 20 s electrical stimuli

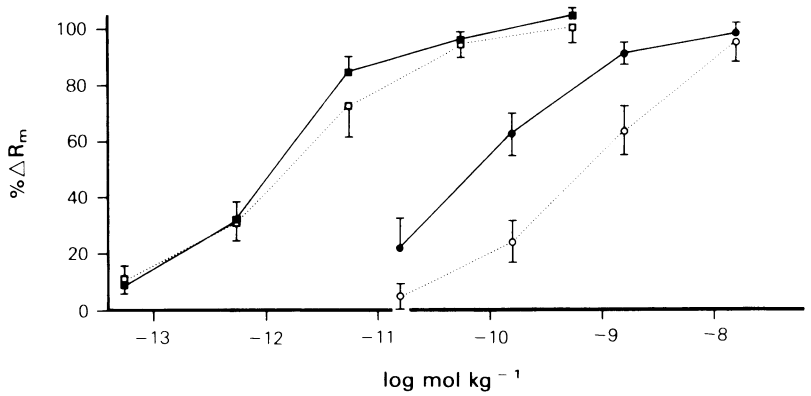


Figure 7 Log dose-response curves for hind limb vasodilatation in rabbit produced by ATP and ACh, both before (solid symbols) and after antazoline (5 $mg\,kg^{-1}$ i.a.) (open symbols): ATP (●, ○); ACh (■, □). The symbols illustrate the mean ± s.e. for data from five experiments. Antazoline has no effect on ACh but it significantly shifts the ATP curve to the right in a parallel manner.

(Figure 6). The vasodilatations elicited by ATP were prompt, short lived, and the maximal response was similar to that produced by electrical stimulation. A log dose-response curve for ATP is presented in Figure 7. One can see that the vasodilatations are dose-dependent with a threshold dose near 0.01 nmol kg^{-1} ATP and a maximal response produced by approximately 1 nmol kg^{-1} ATP. Adenosine injections also produced vasodilatations resembling those produced by electrical stimulation, but with a potency somewhat less than ATP (See Figure 9). Inosine was inactive when injected intra-arterially even at doses of up to 100 $\mu g\, kg^{-1}$.

Antazoline, a P_2-purinoceptor antagonist, was able to attenuate vasodilatations produced by both injections of ATP and electrical stimuli. Figure 6 shows portions of an experiment where these effects of antazoline are demonstrated. In Figure 6a, responses to injections of ACh and ATP, as well as to electrical stimulation were elicited. All three agents evoked vasodilatation in the rabbit hind limb. We then administered antazoline 5 mg kg^{-1} i.a. Not shown in this figure is a vasodilatation produced by antazoline itself, perhaps due to its local anaesthetic effect or a possible α-adrenoceptor antagonistic property. This vasodilatation produced by antazoline required 10–15 minutes to subside. After recovery occurred, the responses to ACh, ATP, and electrical stimulation were re-examined. Part (b) of this figure shows that the vasodilatations due to injection of ATP and electrical stimulation were markedly attenuated. On the other hand, ACh vasodilatation was unaffected.

The effect of antazoline on log dose-response curves for both ATP and ACh are shown in Figure 7. Data from 5 experiments demonstrated that antazoline shifts the ATP log dose-response curve to the right by almost one order of magnitude. Statistical analysis revealed no significant difference between the slopes of each curve. Thus, antazoline blocked ATP in a competitive manner. As an indication of antazoline's attenuation, these curves predict that an 80% ΔRm vasodilatation would be reduced to a response with a magnitude of 47% ΔRm. Antazoline did not affect the dose-response curve for ACh; the curves before and after antazoline were not statistically different.

Figure 8 illustrates the effects of antazoline on current-response curves for electrical stimulation in the hypothalamus. The 5 experiments shown are the same experiments as those shown in Figure 7 which demonstrated antazoline's action on ATP vasodilatations. One can see that in every experiment, antazoline shifted the curve to the right, indicating an attenuation of the hypothalamically induced vasodilatation. These shifts were statistically significant, and they occurred in a parallel manner. Based on data for all experiments, regression analysis indicated that an 80% ΔRm vasodilatation produced by electrical stimulation would be attenuated to a 41% ΔRm response by 5 mg kg^{-1} antazoline.

In 5 experiments, we examined the effects of ad-

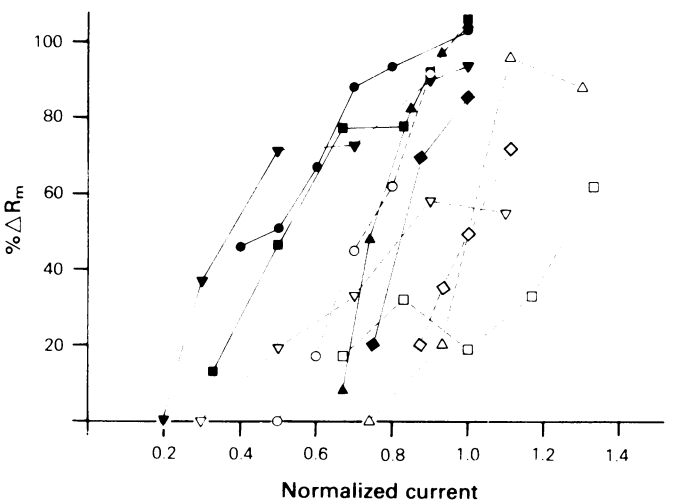


Figure 8 Current-response (vasodilatation) curves for five experiments. There are two curves for each experiment, one before (solid symbols) and one after (open symbols) antazoline (5 mg kg^{-1}). For each experiment, the current has been normalized to that current producing the maximal vasodilatation. These currents are 200 μA (●, ○); 300 μA (■, □); 270 μA (▲, △); 100 μA (▼, ▽); 80 μA (◆, ◇). This transformation allows the control experiments to be plotted close to each other so that the effect of antazoline can be seen more readily.

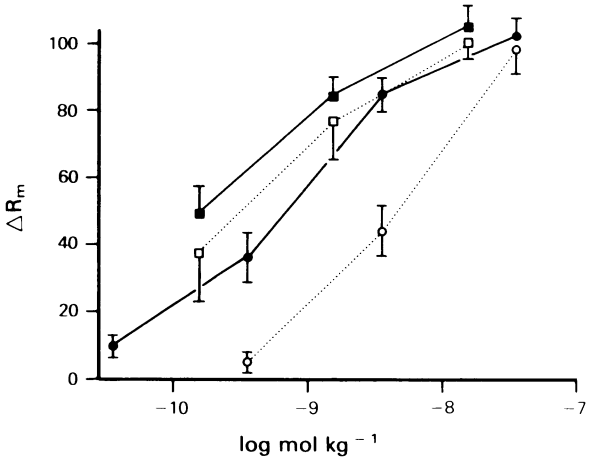


Figure 9 Log dose-response (vasodilatation) curves for ATP (■, □) and adenosine (●, ○). The effect of aminophylline on these curves are demonstrated: control symbols, solid; after aminophylline, open symbols. At a dose of 1 mg kg^{-1}, this drug has no significant effect on vasodilatation evoked by injection of ATP, but it does significantly shift the adenosine curve. The symbols illustrate mean values with vertical lines showing s.e. for data from five experiments.

ministering 1 mg kg^{-1} of the P_1-purinoceptor blocker, aminophylline, into the hind limb. The effects on the ATP and adenosine log dose-response curves are illustrated in Figure 9. Aminophylline moves the adenosine curve to the right by nearly an order of magnitude. On the other hand, P_1-blockade had no significant effect on ATP-induced vasodilatation. Furthermore, in 3 additional experiments, aminophylline could not attenuate the vasodilatation caused by hypothalamic stimulation.

Antazoline is an antihistamine, and we wondered whether this action could explain its inhibition of active muscle vasodilatation instead of an antagonism of ATP. Therefore, in 5 rabbits, we examined the effects of both H_1- and H_2-histamine receptor antagonists on the vasodilatation elicited by hypothalamic stimulation. The results from each experiment were the same. When injected intra-arterially, histamine caused either vasoconstriction or no change in hind limb blood flow. After H_1-receptors were blocked with pyrilamine, histamine then caused a marked vasodilatation, but the response to electrical stimulation remained the same. Then, an H_2-receptor blocker, either metiamide or cimetidine, was administered into the hind limb. After this treatment, histamine injection caused no effect, demonstrating the effectiveness of the H_2-blockade. Even with H_1- and H_2-receptors blocked, the vasodilatation produced by stimulation was unchanged, showing that it was not histaminergically mediated.

Discussion

An important discovery arising from our experiments is the existence of active muscle vasodilatation in rabbits; this phenomenon has often been denied in the past. Bülbring & Burn (1937) discounted the existence of a vasodilator system in the skeletal muscle of both monkeys and rabbits, and no evidence for sympathetic vasodilator fibres in rabbits could be uncovered by Rosenblueth & Cannon (1935). Uvnäs and co-workers examined numerous species for the presence of active muscle vasodilatation (Uvnäs, 1966; Bolme *et al.*, 1970) but most, including 5 types of monkey, the hare, and the rabbit, were reported to be without skeletal muscle vasodilator fibres. Contrary to these early studies, Schramm *et al.* (1971) provided evidence for sympathetic muscle vasodilatation in the monkey. Increases in muscle blood flow could be produced by electrically stimulating areas of the brain which were analogous to those stimulated in carnivores. However, unlike carnivores, this sympathetic vasodilatation was not cholinergic. Large doses of atropine could not block the blood flow increases. The mediator for this response was not determined.

Contrary to the earlier findings of Eliasson *et al.* (1952), Hilton *et al.* (1979) have recently claimed that muscle vasodilatation produced by motor cortical stimulation in cats is not due to an active vasodilatation but, instead, is secondary to muscular contractions: a functional hyperaemia. Hilton's work poses the question of whether or not our vasodilatation might also be a functional hyperaemia. Our evidence indicates that it is not. There are marked differences in the characteristics of blood flow responses evoked in the experiments of Hilton *et al.* (1979) and those which we observed. Even during powerful muscle contractions, the magnitude of their hyperaemic blood flow responses was minor when compared with the magnitudes of both active muscle vasodilatation in cats and the vasodilatation which we observed. Muscle contractions always caused an initial reduction in blood flow before the hyperaemia set in, a response not observed in our experiments. In addition, we know that our vasodilatation requires sympathetic but not motor innervation. Finally, our EMG recordings showed that vasodilatation can occur without any muscle activity. The vasodilatation is not due to a functional hyperaemia.

Active muscle vasodilatation in carnivores is abolished by atropine and, therefore, is considered to be cholinergically mediated. In our experiments atropine had no effect on active muscle vasodilatation in rabbits. Certain strains of rabbits possess the enzyme, atropinesterase, which rapidly hydrolyzes atropine (Sawin & Glick, 1943). Thus, one might suppose that our cholinoceptor blockade with atropine

was evanescent in nature. Despite using ACh injection to demonstrate the presence of a cholinoceptor blockade by atropine, we nevertheless examined the effects of the alternative cholinoceptor blockers, scopolamine and propantheline. The results were the same as with atropine, indicating that active muscle vasodilatation in rabbits is non-cholinergic. Coote *et al.* (1973) concluded that muscle vasodilatation produced by electrical stimulation in the pontomedullary defence area of cats resulted from an inhibition of vasoconstrictor tone. They speculated that species without cholinergic vasodilator nerves could, nevertheless, increase muscle blood flow through such a mechanism. A release of α-adrenergic vasoconstrictor tone does not underly muscle vasodilatation in rabbits since prior pharmacological removal of that tone with α-blockers does not prevent active muscle vasodilatation. Some researchers have suggested the existence of sympathetic, β-adrenergic vasodilatation (e.g., Honig & Myers, 1968; Viveros *et al.*, 1968). Our results with propranolol do not support such a mechanism for rabbit skeletal muscle vasodilatation produced by hypothalamic stimulation.

The current purinergic hypothesis (Burnstock, 1980) envisages nerves which release a purine nucleotide, most likely ATP. The action of ATP is terminated by its breakdown to ADP, AMP, and then, adenosine. Adenosine is either taken up by the purinergic neurone for resynthesis of ATP, or it is broken down further to inosine which is removed by diffusion and the circulation. The postsynaptic target cells of ATP possess P_1-and/or P_2-purinoceptors which mediate the effects of the neurotransmitter (Burnstock, 1976). The P_1- and P_2-receptors are differentiated by the ranking of potencies for various purinoceptor agonists and by the types of drugs which act as antagonists for each receptor. For P_1-receptors, adenosine is the most potent agonist while, in descending order, AMP, ADP, and ATP are relatively less potent. For P_2-receptors, the order of potencies is reversed. They are most sensitive to ATP and least sensitive to adenosine. P_1-receptors are blocked by methylxanthines, such as caffeine and aminophylline. P_2-receptors are resistant to these drugs, but they are thought to be antagonized by others such as quinidine, 2′-substituted imidazolines, such as phentolamine and antazoline (Satchell *et al.*, 1973; Burnstock, 1980), apamin (Shuba & Vladimirova, 1980), arylazido aminopropionyl adenosine triphosphate (Hogaboom *et al.*, 1980; Fedan *et al.*, 1981; 1983), and 2-2′-pyridylisatogen (Hooper, *et al.*, 1974).

In the rabbit hind limb, we found that both ATP and adenosine could evoke vasodilatation while inosine was inactive. Aminophylline's attenuation of vasodilatation elicited by adenosine implies the presence of P_1-receptors in the skeletal muscle vasculature. After blockade of the hind limb with aminophylline, ATP was much more potent than adenosine in producing vasodilatation (Figure 9), and antazoline attenuated vasodilatations produced by injections of ATP. Both observations suggest the existence of P_2-receptors in the hind limb. Finally, antazoline reduced the vasodilatation produced by hypothalamic stimulation. The amount of attenuation was similar to that exhibited by antazoline when it antagonized ATP vasodilatation. Our observations are consistent with the existence of a purinergic vasodilator innervation in skeletal muscle of rabbits which utilizes ATP as its transmitter substance and whose effects are mediated by P_2-receptors.

A problem which has hampered investigations of purinergic nerves is that purinergic antagonists are generally nonspecific. Antazoline is no exception for it is a drug with other known properties. However, we have attempted to eliminate antazoline's other actions as the mechanism for its blockade of active muscle vasodilatation in rabbits. Antazoline is a weak local anaesthetic and weak anticholinoceptor agent (Meier, 1950; Naranjo & Naranjo, 1958). Vasodilatations elicited by ACh injections persist after antazoline treatment. Therefore, antazoline does not block active muscle vasodilatation through a local anaesthetic action by rendering the vasculature unresponsive to all vasodilators. Furthermore, antazoline 5 mg kg^{-1} apparently is less than the dose required to reveal its anticholinoceptor properties. Antazoline belongs to the imidazoline family of compounds which also include the α-adrenoceptor blocker, phentolamine (Meier, 1950). However, possession of an α-adrenoceptor blocking property cannot explain antazoline's action on active muscle vasodilatation. We have previously shown that the α-blockers, phenoxybenzamine and phentolamine, have no effect on active muscle vasodilatation.

Antazoline is probably most noted for its clinical usage as an antihistamine. We wondered whether this property could underly antazoline's blocking effect, and, furthermore, whether active muscle vasodilatation in rabbits was, in fact histaminergic. Suggestions have been made regarding the existence of neurogenic histamine vasodilatation in some species (Brody, 1966). In addition, H_1- and H_2-histamine receptors are known to exist in the hind limb vasculature of rabbits (Angus & Korner, 1977; Angus *et al.*, 1977). Thus, we examined the effects of H_1- and H_2-receptor antagonists. Our results confirmed the work of Angus and his colleagues who found that H_1-receptors mediate vasoconstriction and H_2-receptors mediate vasodilatation. However, neither type of antagonist affected the vasodilatation produced by hypothalamic stimulation. Thus, active muscle vasodilatation is not histaminergic, and an-

tazoline does not antagonize active muscle vasodilatation because of its antihistamine action.

We have observed a separation of antazoline's anti-ATP and antihistamine effects. This drug is thought to be a stable compound which lasts for many months when stored at room temperature. We found this to be true for its antihistamine action. However, the P_2-receptor blocking effect of antazoline lasts for only a few months, at best. In fact, upon arrival in our laboratory, one batch of drug was unable to block the vasodilator effects of ATP. At present, we cannot explain this observation.

When given in therapeutic doses, many antihistamines lead to drowsiness and other central nervous system effects, demonstrating their penetration into the brain. In our experiments, antazoline enters the general circulation, and our experimental preparation does not preclude a central site of action for antazoline in blocking active muscle vasodilatation produced by hypothalamic stimulation. While a central blocking action of antazoline would itself be a significant observation, we believe that antazoline acts at the peripheral vascular site in the skeletal muscle. In several experiments, we have measured blood flow in each hind limb. In the limb which receives antazoline intra-arterially, the electrically evoked vasodilatation exhibits a greater attenuation than the vasodilatation observed in the contralateral hind limb which receives antazoline only indirectly after passage through the circulation. If antazoline acted centrally rather than peripherally, then one would expect an equal attentuation of vasodilatation in both hind limbs. In other experiments, we have introduced up to 200 μg of antazoline directly into the lateral cerebral ventricle. This amount represents 1/80 of the peripheral dose which we used to block ATP and active muscle vasodilatation. An approximate calculation indicates that this amount would result in a concentration in cerebrospinal fluid which would exceed that in plasma when 5 mg kg^{-1} of antazoline is administered systemically. When we gave antazoline centrally, it had no effect on active muscle vasodilatation. We conclude that antazoline blocks active muscle vasodilatation at the same location as it blocks ATP vasodilatation: in the hind limb skeletal muscle vasculature.

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