J. comp. Physiol. 122, 325–345 (1977)

Journal of Comparative Physiology · B

Cholinergic Mechanisms and the Response to ATP in the Systemic Vasculature of the Rainbow Trout

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Received February 4, 1977

Summary. 1. Cholinergic mechanisms in the systemic vasculature of the rainbow trout have been pharmacologically analysed using an isolated trunk preparation perfused at constant flow.

2. Acetylcholine (ACh) causes a two component vasoconstriction comprising a rapid initial peak (response A) and a long-lasting tail on the initial peak (response B). Skeletal muscle contraction is not involved in either response.

3. Response B has a high dose threshold, is nicotinic in nature, and is blocked by α-adrenoreceptor antagonists, procaine, and 0 mM Ca^{++} plus 20 mM Mg^{++}, indicating a classical indirect action of ACh on sympathetic neurons or chromaffin tissue to cause the release of an adrenergic transmitter.

4. Response A has a lower dose threshold, is nicotinic in nature, is resistant to α-adrenergic, muscarinic cholinergic, and adrenergic neuron blockade, is insensitive to tetrodotoxin and 0 mM Ca^{++} plus 20 mM Mg^{++}, but is blocked with some specificity by procaine.

5. The properties of the nicotinic receptors of response A differ from those of the traditional ganglionic and skeletal muscle types of higher vertebrates.

6. Response A is mimicked by ATP, which appears to act by a direct mechanism in the preparation.

7. It is concluded that response A reflects either direct stimulation of nicotinic receptors on vascular tissue or an indirect nicotinic effect resulting in the release of a non-adrenergic, non-cholinergic transmitter such as ATP.

8. Unlike the situation in higher vertebrates, no muscarinic dilatory responses to ACh occur in the preparation.

Introduction

Adrenergic mechanisms regulating vascular resistance have been extensively studied in teleost fish (see Wood, 1976, for references), while cholinergic mechanisms have received rather less attention. Nevertheless, the limited information

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available suggests some differences from the mammalian pattern. There is general accord that ACh constricts the ventral aorta (Kirby and Burnstock, 1969; Klaverkamp and Dyer, 1974) and the branchial vessels (Östlund and Fänge, 1962; Reite, 1969; Richards and Fromm, 1969, 1970; Belaud et al., 1971; Wood, 1975; Chan and Chow, 1976) by stimulation of muscarinic receptors. Little work has been carried out on the systemic circulation, but the available evidence indicates a weak cholinergic vasoconstriction of high dose threshold (Reite, 1969; Stray-Pedersen, 1970; Nilsson, 1972; Holmgren and Nilsson, 1974). At least in the study of Nilsson (1972), the response appeared muscarinic in nature. Nilsson and Grove (1974) and Holmgren and Nilsson (1975) have recently provided good evidence of a muscarinic constrictory mechanism in the spleen of the cod, but it is not clear whether this resides specifically in the splenic vascular tissue. In contrast to mammals (Crossland, 1970; Krnjevic, 1974; Wilson et al., 1975), there exist no reports of either muscarinic dilatory effects or any vascular nicotinic actions of ACh in teleost fish.

The purpose of the present work was therefore to examine in detail the cholinergic pharmacology of the perfused systemic vascular bed of the rainbow trout, *Salmo gairdneri,* to see whether real differences from the mammalian situation exist. The investigation was intended as a parallel study to the previous analysis of the adrenergic pharmacology of the system (Wood, 1976), and similar methods were used. During the course of the project, a cholinergic response of rather unconventional properties was discovered, so the pharmacological nature of the mechanism was pursued at some length.

Materials and Methods

Rainbow trout (125–350 g) were acquired, maintained, and acclimated to $14.5 \pm 1.5°$ C as described previously (Wood, 1976). Experiments were performed in a constant temperature room at $5 \pm 1°$C on pump-perfused preparations of the trunk ($N=89$), the eviscerated trunk ($N=2$), and the visceral circulation alone ($N=5$). The perfusion system, medium, and methodology have been described in detail elsewhere (Wood and Shelton, 1975; Wood, 1976). Changes in vascular resistance were monitored as changes in the perfusion pressure at constant flow, which was set to 0.45–0.55 ml/100 g total body weight·min in the trunk preparation, and 0.30–0.35 ml/100 g total body weight·min in the visceral preparations.

I. The Preparations

In all cases, the animals were anaesthetized in 650 mg/l MS-222 (Sandoz) and injected with 500 i.u./100 g of sodium heparin (Sigma) before sacrifice. In the normal trunk preparation, consisting of the whole body posterior to the heart, the dorsal aorta was cannulated (Portex PP 190 or 200) just posterior to the entry of the last efferent branchial arteries (see Wood, 1976 for details). In the eviscerated trunk preparation, the point of dorsal aortic cannulation was about 1 cm posterior to the origin of the coeliac artery, and all organs comprising or associated with the alimentary tract were excised. The visceral circulation alone was perfused in situ by tying a short length of PP 90 into the origin of the coeliac artery.

In experiments involving electrical stimulation of the whole trunk preparation, enamelled copper wires, connected to a D.C. square wave stimulator, were bared for 1–2 mm at their tips and inserted into either the spinal cord (for neural stimulation) or the cut end of the myotomal muscle mass (for direct muscle stimulation). To prevent movement artifact on the perfusion pressure

record caused by thrashing of the trunk during stimulation, the preparation was confined to a small Perspex chamber inside the saline bath. The anterior end of the trunk was firmly wedged with foam rubber, but the tail was free to move laterally about 1 cm on either side.

II. Drugs

(i) Agonists. l-adrenaline bitartrate (AD), acetylcholine chloride (ACh), dopamine hydrochloride, methacholine chloride, pilocarpine hydrochloride, histamine dihydrochloride, 5-hydroxytryptamine creatine sulfate complex, adenosine 5′-triphosphate disodium salt from equine muscle (ATP) (all Sigma) and nicotine hydrogen (+)-tartrate (British Drug Houses).

(ii) Antagonists. yohimbine hydrochloride, atropine sulfate, hexamethonium bromide, quinidine hydrochloride, procaine hydrochloride, tetrodotoxin, propranolol hydrochloride (all Sigma), phenoxybenzamine hydrochloride (Smith, Kline, and French), guanethidine sulphate (Ciba), d-tubocurarine chloride (Burroughs Wellcome), gallamine triethiodide (May and Baker), and nicotine hydrogen (+)-tartrate (British Drug Houses).

In a few experiments, a perfusate of high magnesium concentration (20 mM Mg^{++}) and deficient in calcium (0 mM Ca^{++}) was made by omitting $CaCl_2 \cdot 2H_2O$ and adding an appropriate amount of $MgCl_2 \cdot 6H_2O$ to the Cortland saline (Wolf, 1963) which formed the basis of the perfusion medium. As the change in total concentration was small, no adjustment was made to the other ions.

Agonists were usually administered by injection as discrete doses (volume = 0.1 ml) through a side-arm into the perfusion line immediately proximal to the preparation. Antagonists (and occasionally agonists) were administered as constant concentrations in the perfusate and allowed to act for at least 60 min before agonists were retested in the continued presence of the antagonist. All drug solutions were stored in the dark at 1°C during use and renewed approximately every 3 h.

III. Treatment of Data

As the elicitation of maximal constrictory responses interfered with the subsequent responsiveness of the preparation, only the lower 60–70% of complete dose/response relationships were commonly determined. Unlike adrenergic effects (Wood, 1976), the sizes of responses to ACh, nicotine, and ATP were independent of the level of baseline vascular resistance (Rs) in the systemic circulation. Thus these responses were measured as simply the maximum absolute increases in Rs (units = cm $H_2O \cdot min \cdot 100\,g \cdot ml^{-1}$). Responses to a test dose of AD (10 nmoles) were routinely recorded as a control measure and have been expressed as percentage increases in baseline Rs (Wood, 1976). Results have been reported as means ± 1 S.E., and an experimental design appropriate for the application of the paired Student's *t*-test (two-tailed) to responses at the same dose level has been employed. Where only the results of individual experiments are given, at least 3 preparations showed qualitatively identical results and no contradictory data were obtained, unless otherwise stated. In all original perfusion pressure records shown, 2–5 cm H_2O of the pressure was due to the resistance of the perfusion cannula.

Results

I. The Basic Response to ACh

Injection of ACh caused a dose-dependent vasoconstriction in the whole trunk preparation (Fig. 1A) with a threshold of 1–32 nmoles. Doses of 10 μmoles or greater commonly caused a persistent rise in baseline vascular resistance (Rs) which was superimposed on the normal response and resistant to all forms of blockade employed in this study, indicating a non-specific effect. At doses

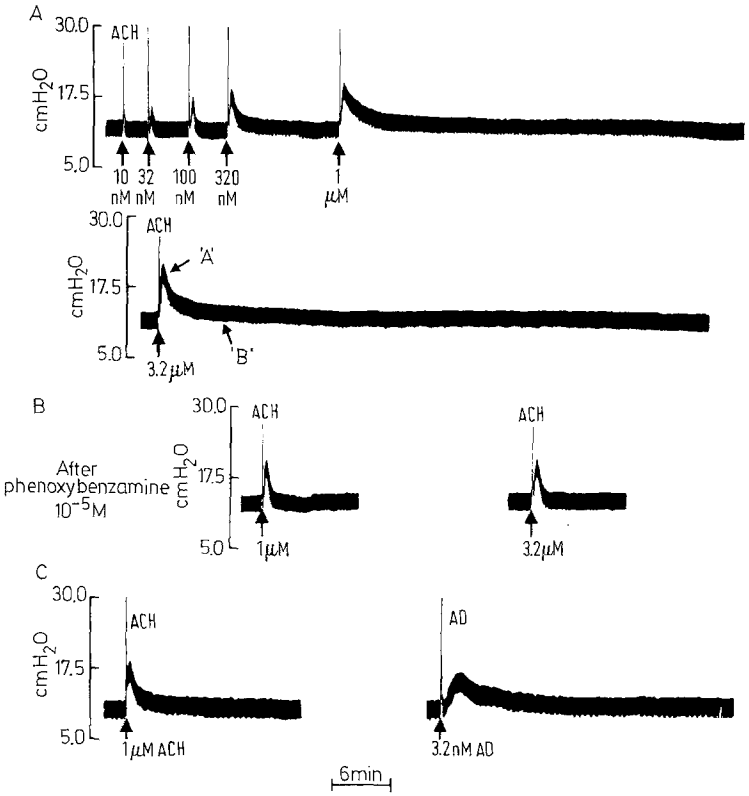

Fig. 1. A Typical perfusion pressure records of a constrictory dose/response curve to ACh. Note the two components of the vasoconstriction: the initial rapid constriction occurring at all doses (response A) and the long-lasting tail at doses $\geq$320 nmoles (response *B*). **B** The responses to 1 and 3.2 μmoles ACh in the same preparation after the α-adrenergic antagonist phenoxybenzamine (10^{-5} M). Note abolition of response B but persistence of response A. The slight decreases in size of the latter relative to **A** reflect the depressant effects of time and tachyphylaxis. **C** A comparison of the forms of the constrictory responses to approximately equi-pressor doses of ACh (1 μmole) and AD (3.2 nmoles) in the same preparation (different from **A** and **B**). Note the much faster peak of the ACh response

below about 320 nmoles, the response to ACh followed a simple monophasic pattern. Above 320 nmoles, the pressor response continued to increase in height with the dose, but a second component appeared. The latter was manifested as a tail on the initial peak which greatly prolonged the total period of the response (eg. 30–60 min at 3.2 μmoles) (Fig. 1A). The initial pressor peak has been termed response A, and the tail response B.

Relative to an AD response of similar magnitude, the initial peak of the ACh constriction was extremely rapid (15–40 s as opposed to 60–150 sec after injection, Fig. 1C). ACh (threshold = 1–32 nmoles) was generally a much less potent agonist than AD (threshold = 320 pmoles – 1 nmole) and exhibited a dose/response curve of much lower slope than the AD relationship (compare Fig. 1A with Fig. 1 of Wood, 1976). There was no correlation between the magnitudes

of adrenergic and cholinergic responses in individual fish, and over the 8 month period during which experiments were performed (November–June), there occurred no significant variation in cholinergic reactivity. Pronounced changes in adrenergic reactivity were seen over the same period (Wood, 1976).

Successive injections of ACh caused a gradual decrease in the size of response A although complete tachyphylaxis was never observed. In some cases, a partial recovery was seen after leaving the preparation for 30–60 min. Cholinergic responsiveness also tended to decrease with time alone, so that old preparations were less reactive than freshly set up ones. To minimize the influence of these complications in blocking studies, the following approach was adopted. In 8 trunks, a dose/response A curve to ACh (10 nmoles – 3.2 μmoles) and the response to 10 nmoles AD were initially recorded; the preparation was then perfused with drug-free medium for 80 min and the procedure repeated. The response to AD remained unchanged, but the ACh dose/response A curve was significantly depressed by a constant proportion at all dose levels. The average response A was $65.9 \pm 2.3\%$ (8) of the initial response A. The tail component (response B) was only marginally reduced. In experiments in which blocking agents were administered, an identical protocol was followed, the antagonist being added to the perfusate for 80 min (60 min at the nominal concentration due to the dead space of the perfusion system) after completion of the initial control determinations. All control responses (type A) to ACh were adjusted to 65.9% before averaging and statistical analysis to correct for the depressant effects of time and tachyphylaxis. In all Figures, this correction has been applied to control data, unless otherwise stated. No adjustment was made to the control value for 10 nmoles AD as this remained unaffected.

II. Studies with Adrenergic and Cholinergic Antagonists

Phenoxybenzamine (10^{-5} M), a non-equilibrium antagonist of α-adrenoreceptors, had no effect on the ACh dose/response A curve (Fig. 2A). However phenoxybenzamine abolished the second tail-like component (response B) so that at high dose levels of ACh the form of the constriction resembled that at low dose levels (Fig. 1B). Yohimbine had a similar effect, although a slight tail sometimes persisted. As expected (Wood, 1976), both agents eliminated the response to 10 nmoles AD (Fig. 2A). These results provide a clear separation of the two components of the cholinergic pressor effect, and imply that the second phase reflects an indirect action of ACh to cause in some way the release of an adrenergic transmitter substance which stimulates vascular α-adrenergic receptors. Propranolol (10^{-5} M), a competitive β-adrenergic blocking agent, had no effect on either component of the ACh response or the response to 10 nmoles AD (2 experiments).

Guanethidine (10^{-5} M), an adrenergic neuron blocker in higher forms (Nickerson and Collier, 1975), did not depress response A and in fact seemed to potentiate it slightly at the upper end of the dose/response curve (Fig. 2B). Guanethidine also had no apparent effect on response B. This agent significantly potentiated the response to AD (Fig. 2B), an effect typical of its action on

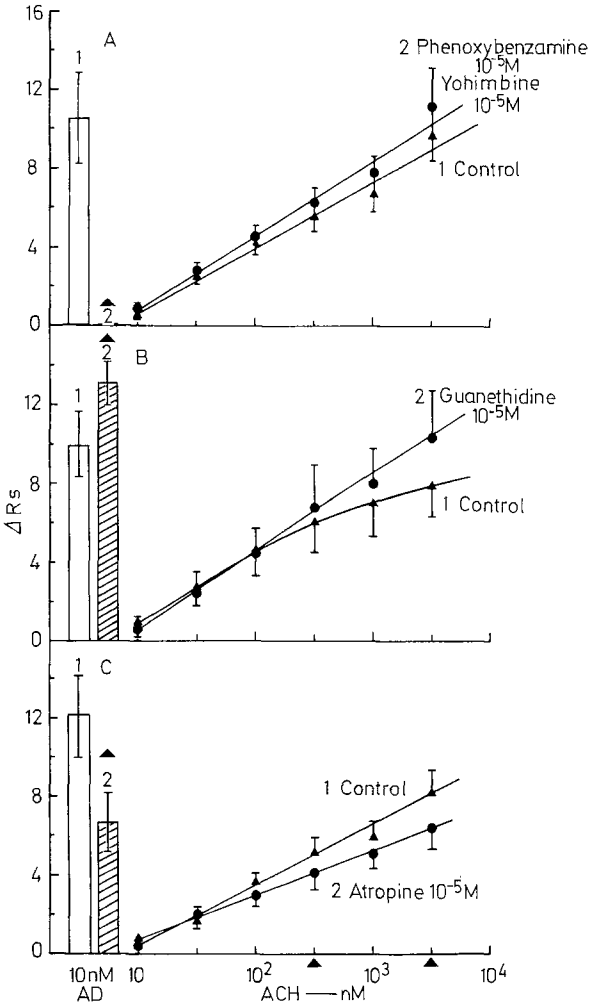

Fig. 2A–C. The effects of (**A**) the α-adrenergic antagonists phenoxybenzamine (10^{-5} M) ($N=3$) or yohimbine (10^{-5} M) ($N=3$), (**B**) the adrenergic neuron blocking agent guanethidine (10^{-5} M), and (**C**) the muscarinic cholinergic antagonist atropine (10^{-5} M) on the constrictory dose/response curve to ACh and the response to a test dose of AD (10 nmoles). Means ±S.E. ($N=6$). As the effects of the two agents in **A** were apparently identical, their results have been combined. *Note:* The ACh dose/response curve reflects the magnitude of response A only. The control data for ACh have been expressed as 65.9% of the actual initial responses to correct for the depressant effects of time and tachyphylaxis (see text for details). Responses are expressed as absolute increases in Rs (units = cm $H_2O \cdot min \cdot 100\,g \cdot ml^{-1}$) for ACh (also for nicotine and ATP in other Figs.) and percentage increases in Rs for AD (units = percent increase in $Rs \times 10^{-1}$). 1, ▲ control; 2, ● after experimental treatment. Large triangle = $p < 0.05$

mammalian preparations at this concentration (Maxwell, 1965). The failure of α-adrenoreceptor and adrenergic neuron blocking agents to inhibit response A seems to exclude the involvement of adrenergic nerves and/or adrenergic transmitter release in its genesis.

Atropine (10^{-5} M), a selective antagonist of muscarinic cholinoreceptors, slightly depressed the ACh dose/response A curve, and this effect was significant at two dose levels (Fig. 2C). However atropine is a classical competitive antagonist (Innes and Nickerson, 1975), yet there was no evidence of a parallel shift of the curve. On the contrary, the nature of the inhibition was characteristic of a non-competitive effect (Ariens, 1964) and the response to 10 nmoles AD was reduced to an even greater extent, indicating non-specificity of antagonism. There was no evidence of a differential effect on response B. At 10^{-6} M, a concentration highly effective in competitively antagonizing the muscarinic effects of ACh in the perfused gills of *S. gairdneri* (Wood, 1975), atropine was without effect on both the ACh and AD responses in the trunk (2 experiments). Thus a muscarinic element does not appear to be involved in the ACh responses.

Three specific antagonists of nicotinic cholinoreceptors with different selectivity were tested. d-Tubocurarine has a relatively broad spectrum of action, exerting a classical competitive antagonism at the nicotinic receptors of both autonomic ganglia and skeletal muscle (Koelle, 1975a). At 10^{-5} M, d-tubocurarine induced a marked parallel displacement of the ACh dose/response A curve to the right (Fig. 3A) in typical competitive fashion. The difference was significant at all overlapping doses. This agent also severely reduced or abolished response B. The AD vasoconstriction was unaffected. Gallamine, a competitive antagonist with a reportedly much greater potency on the nicotinic receptors of skeletal muscle than on those of ganglia (Koelle, 1975c) had a very similar effect to d-tubocurarine in the trunk (Fig. 3B). The shift of the dose/response A curve was somewhat smaller, but the difference was again significant at all doses. The AD response was unaffected, and response B of the cholinergic constriction inhibited. Hexamethonium exhibits supposedly opposite selectivity to gallamine, exerting a potent competitive blockade on ganglionic nicotinic receptors with only a weak action on those of skeletal muscle (Volle and Koelle, 1975). Hexamethonium (10^{-5} M) had a similar effect to gallamine and d-tubocurarine on the ACh dose/response A curve, but was obviously less potent (Fig. 3C). Statistical significance of the separation of control and experimental relationships with this agent was only obtained at the lower doses of ACh. The AD response was again unaffected, and response B inhibited. These results clearly indicate that both components of the ACh constriction are mediated through nicotinic receptors. At least for response A the blocking potency order d-tubocurarine > gallamine > hexamethonium implies that the receptors involved are perhaps closer to the mammalian skeletal muscle type.

III. The Effects of Skeletal Muscle Contraction

The preceding data suggested that response A might result from a mechanical effect on Rs caused by skeletal muscle contraction. High doses of ACh

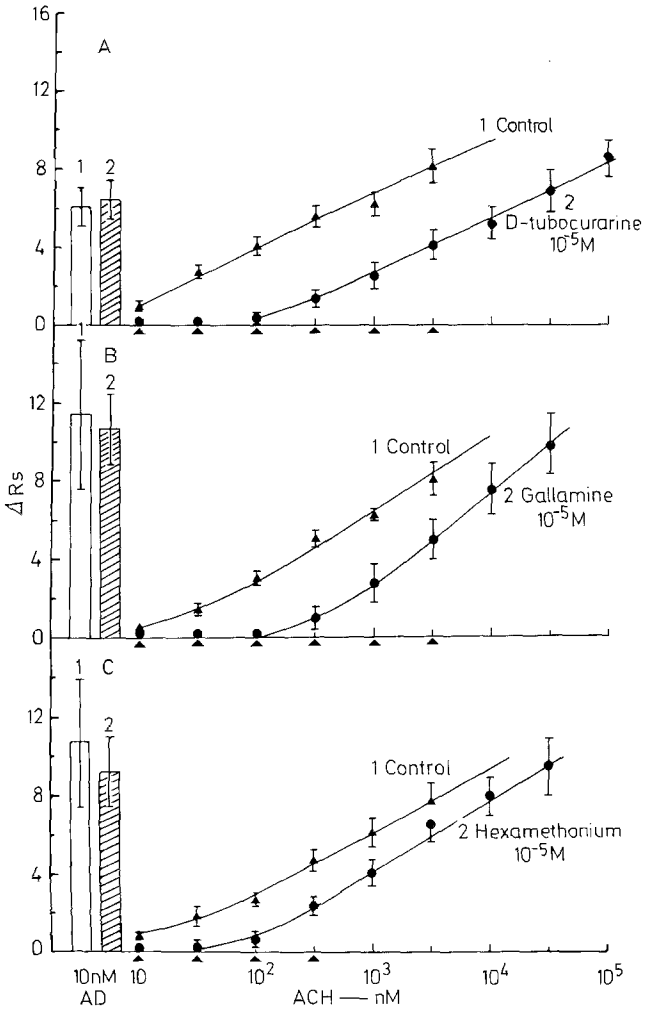

Fig. 3A–C. The effects of the nicotinic cholinergic antagonists **A** d-tubocurarine (10^{-5} M), **B** gallamine (10^{-5} M), and **C** hexamethonium (10^{-5} M) on the dose/response curve to ACh and the response to a test dose of AD (10 nmoles). Means ± 1 S.E. ($N=6$). Other details as in Figure 2

(> 1 μmole) did in fact sometimes cause a visible twitch of the trunk but the effect was instantaneous and extremely short-lived (< 1 sec) relative to the initial constrictory response (up to 3 min, Fig. 1B). To check this possibility, skeletal muscle contraction was experimentally elicited. Stimulation of either the spinal cord or the cut end of the myotomal muscle mass with 10 msec pulses (0.5 V–10 V) caused voltage dependent contractions of the trunk ranging from violent single twitches at 1 Hz to a fused response at 10–30 Hz. With a single exception, skeletal muscle contraction under all stimulus conditions was accompanied by either a slight decrease (up to 15%) in Rs or no effect on Rs (5 preparations). This sole pressor effect (10% increase in Rs) in one trunk (in 2 of 6 instances

only at 6 V, 15 Hz applied to the spinal cord) was coincident with the onset of stimulation and of very short duration (< 15 s) despite continued stimulation. All of these preparations showed the typical response to ACh injections. Consequently skeletal muscle contraction does not appear to be involved in the pressor effect of ACh.

IV. Studies with Muscarinic Agonists and Nicotine

Methacholine and pilocarpine are specific muscarinic stimulants with negligible potency on nicotinic cholinoreceptors (Crossland, 1970; Koelle, 1975b). Both agents were totally without effect on the systemic vasculature of the trout in doses up to 10 μmoles. Nicotine has virtually no effect on muscarinic receptors but a dual action on nicotinic receptors, first stimulating and later blocking them by a persistent depolarization (Volle and Koelle, 1975). In the perfused trunk, nicotine (10 nmoles – 1 μmole) caused a constriction identical in form to that produced by ACh (Fig. 4A); both responses A and B were definitely present. Nicotine seemed only slightly less potent than ACh, although self-antagonism precluded accurate potency comparisons. Successive injections (6) of 320 nmoles nicotine caused complete self-blockade (Fig. 5). The response to an equal dose of ACh was severely depressed but persisted, while that to 10 nmoles AD was completely unaffected. These results with specific agonists and antagonists are in complete agreement that both components of the ACh constriction are wholly nicotinic, non-muscarinic phenomena.

Studies were carried out to determine if the agonist action of nicotine in producing response A paralleled that of ACh in its susceptibility to antagonism by other agents. Because of the problem of self-blockade, it was possible to test only whether or not the response A to a certain dose of nicotine (320 nmoles), known to be always effective in untreated preparations, was present after administration of a blocking agent. The results showed that nicotine exhibits a blockade profile with respect to response A almost identical to that of ACh (Table 1). The variable effect of hexamethonium (10^{-5} M) probably reflected this agent's rather weak antagonism of the ACh dose/response A curve as well (Fig. 3C).

When nicotine was applied as an antagonist via a constant concentration in the perfusate, the drug had little or no effect on the ACh dose/response A curve at 10^{-5} M (Fig. 4B), but completely abolished it at 10^{-4} M (Fig. 4C). At both concentrations, self-blockade (to 320 nmoles nicotine) occurred (Table 1). These results are in agreement with nicotine's mode of action in higher vertebrates (Volle and Koelle, 1975). The response to 10 nmoles AD was depressed by a variable extent (always less than 50%) at both concentrations (Fig. 4B, 4C, Table 1), indicating a non-specific element in nicotine's activity.

V. Studies with Other Agonists

The preceding results narrow down the mechanism of action of ACh (and nicotine) in causing response A to either a direct effect on nicotinic receptors at the vascular end-organ or an indirect action on nicotinic receptors elsewhere

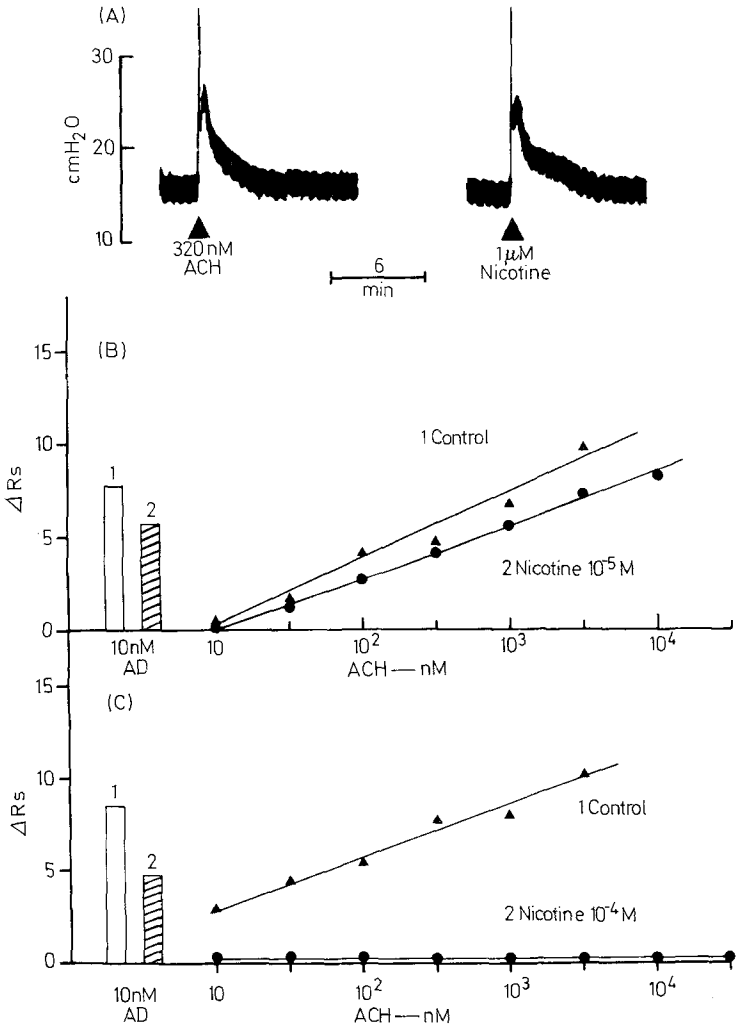

Fig. 4A–C. The actions of nicotine. **A** A comparison of the constrictory responses to approximately equi-pressor doses of nicotine (1 μmole) and ACh (320 nmoles) in the same preparation. Note the similarity in form of the constrictions. **B** and **C** Results of typical experiments showing the effects of nicotine at **B** 10^{-5} M and **C** 10^{-4} M in the perfusate on the dose/response curve to ACh and the response to a test dose of AD (10 nmoles). Other details as in Figure 2

leading in some way to the release of a non-adrenergic, non-cholinergic transmitter substance which in turn acts at the vascular end-organ. Both of these possibilities are unconventional in the mammalian context.

In the search for such a non-adrenergic, non-cholinergic messenger as required in the second of these hypotheses, several putative neurotransmitters were tested in the perfused trunk: dopamine (Goldberg, 1972); histamine and 5-hydroxytryptamine (Reite, 1972; Douglas, 1975); and ATP (Burnstock, 1972,

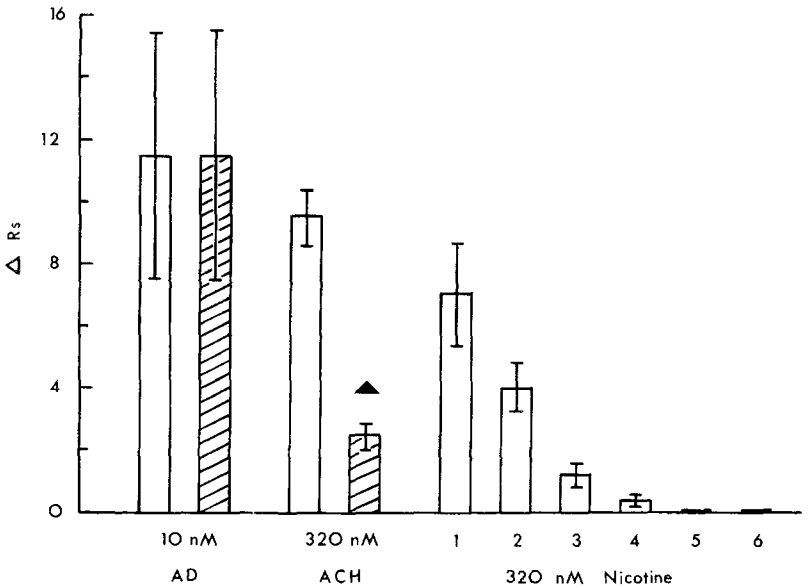

Fig. 5. The effects of 6 successive injections, 10–15 min apart, of a test dose of nicotine (320 nmoles) on the response to itself, and on the responses to test doses of AD (10 nmoles) and ACh (320 nmoles). Means ± 1 S.E. ($N=5$). The responses to AD and ACh were recorded before and after the 6 doses of nicotine. No correction has been applied to the control data for ACh. Other details as in Figure 2

Table 1. A comparison of the susceptibility to blockade of the constrictory responses to AD, ACh (response A and B), nicotine, and ATP by various agents

Agent	Concentration	AD	ACh response A	ACh response B	Nicotine[a]	ATP
Phenoxybenzamine	10^{-5} M	×	–	×	–	–
Yohimbine	10^{-5} M	×	–	×	–	–
Guanethidine	10^{-5} M	–	–	–	–	–
Atropine	10^{-5} M	↓	↓	↓	–	–
d-Tubocurarine	10^{-5} M	–	×	×	×	–
Gallamine	10^{-5} M	–	×	×	×	–
Hexamethonium	10^{-5} M	–	×	×	×[b]	–
Nicotine	10^{-5} M	↓	–	–	×	–
Nicotine	10^{-4} M	↓	×	×	×	–
Quinidine	10^{-5} M	↓	–	–	–	–
Quinidine	$\geqq 10^{-4}$ M	↓	↓	↓	×	↓
O mM Ca^{++} plus 20 mM Mg^{++}		↓	↓	×	–	↓
Tetrodotoxin	1.6×10^{-6} M	–	–	–	–	–
Tetrodotoxin	6.3×10^{-6} M	↓	–	–	–	–
Procaine	$\geqq 3.2 \times 10^{-4}$ M	↓	×	×	×	–

– = no effect; × = blockade, apparently specific; ↓ = depression or blockade, apparently non-specific

[a] Refers to only the initial rapid constrictory response ("response A") caused by nicotine. Due to the self-blockade exerted by nicotine, it was only possible to determine whether a response was present (–) or not (×) after the experimental treatment. Non-specific depression (↓) could not be detected. A test dose of 320 nmoles nicotine was used, which was known to always produce a response in untreated preparations

[b] Blockade in 2 out of 3 experiments

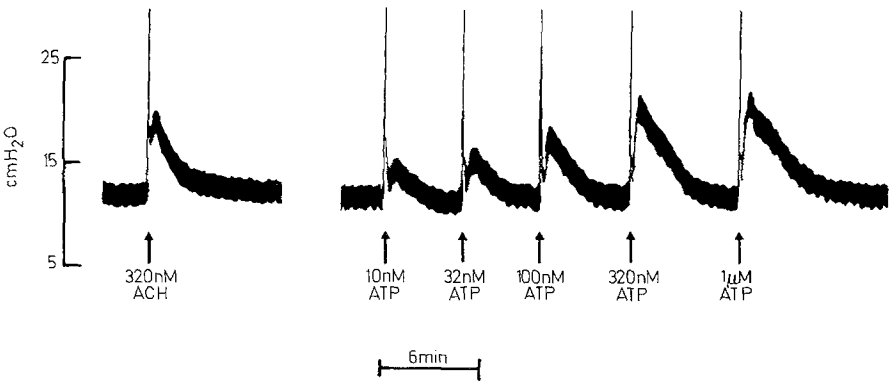

Fig. 6. Typical perfusion pressure records of a constrictory dose/response curve to ATP and the response A to a test dose of ACh (320 nmoles) in the same preparation. Note the similarity of the ACh and ATP responses, and the rapidity and relatively short duration of the latter even at high doses

1975; Boyd, 1973). In agreement with previous findings (Wood, 1976), dopamine (in doses up to 10 μmoles) caused only a small, slow increase in Rs which could be antagonized by α-adrenergic blocking agents. Neither histamine nor 5-hydroxytryptamine had any effect on Rs in doses up to 10 μmoles. However ATP caused a dose dependent vasoconstriction in the trunk, the threshold sensitivity varying widely between preparations (1 nmole – 1 μmole). The form of the response was remarkably similar to that of the initial constrictory response A to ACh, with a rapidly developing peak (15–60 s) and relatively short duration (less than 6 min even at high doses) (Fig. 6). The slope of the ATP dose/response curve and its efficacy were also similar to those of ACh (cf. Fig. 1A) and far less than those of AD. In light of these similarities, further studies were carried out on the mechanism of action of ATP in the trunk.

VI. Studies with ATP

In single experiments, the susceptibility of the ATP response to blockade by a range of antagonists effective against ACh or AD was tested. The ATP constriction was completely unaffected by adrenergic neuron and receptor blocking agents, competitive nicotinic and muscarinic antagonists, and nicotine (Table 1). These results indicate a direct mechanism of action for ATP at the vascular end-organ, and thus in no way oppose the possibility that ATP or a related substance may be the transmitter ultimately released by the indirect nicotinic action of ACh.

Strong support for this "purinergic" hypothesis would be gained if an experimental treatment inhibited the response to both ACh (response A) and ATP without affecting that to AD, thereby indicating specificity. Unfortunately specific blocking agents for "ATP receptors" are not yet available, but quinidine is known to antagonize some purinergic effects with selectivity (Burnstock,

1972, 1975), In the trunk preparation, quinidine at 10^{-5} M had no effect on the dose/response curve to ATP or the response to ACh (1 μmole) but abolished that to 10 nmoles AD. At 10^{-4} M quinidine, the constriction to 1 μmole ACh also disappeared while the ATP dose/response curve was severely depressed but persisted at all doses. Only at 10^{-3} M did quinidine eliminate the ATP response. Consequently no conclusions about the mode of action of ACh or ATP on the systemic vasculature of the trout could be drawn from the use of quinidine. A similar lack of specificity by quinidine has been noted by other workers in situations where ATP is the postulated transmitter (Burnstock et al., 1970; Sneddon et al., 1973).

Specific desensitization to ATP has been suggested as an alternative to specific blockade (Burnstock, 1972). However, the response to ATP in the trunk was exceptionally resistant to tachyphylaxis as attempted by a variety of procedures, and the response to AD always disappeared before the ATP constriction was significantly reduced. A similar failure to produce tachyphylaxis to ATP has been reported in other preparations where ATP is the suspected transmitter (Burnstock et al., 1970, 1972).

VII. Studies with Neural Blocking Agents

If the ACh response A involves a neural link between the stimulation of nicotinic receptors and the release of a non-adrenergic, non-cholinergic transmitter, then nerve blockade might be expected to selectively inhibit the response.

In general, the release of transmitter from nerve endings is blocked by low calcium levels and/or high magnesium levels (Krnjevic, 1974). One hour's perfusion with a 0 mM Ca^{++} plus 20 mM Mg^{++} medium depressed the dose/response A curve to ACh in the trunk by up to 50%, but constrictions persisted at all doses (Fig. 7A). The response to a test dose of ATP (1 μmole) was reduced by a similar proportion, and that to 10 nmoles AD by a greater extent. The tail of the ACh constriction (response B) was totally eliminated. All effects were partially reversed by 60 min washout with regular perfusion medium (Fig. 7A). The relative resistance of response A tends to indicate the absence of a neural element.

Tetrodotoxin is a potent blocker of nervous conduction (Takata et al., 1966); 1.6×10^{-6} M is the concentration normally used to selectively block nerves while leaving muscle unaffected (Carter, 1969). Due to its expense, sufficient tetrodotoxin was available for only 3 experiments. At 1.6×10^{-6} M, tetrodotoxin had no effect on the responses to ACh (A and B), AD, and ATP (Fig. 7B). However the characteristic twitch of the whole trunk preparation elicited by electrical stimulation of the spinal cord was abolished by this level of tetrodotoxin, indicating an effective neural blockade. Except for a 40% depression of the AD (10 nmoles) response, results were identical in a single trial at 6.3×10^{-6} M tetrodotoxin. These data again indicate the non-involvement of nervous conduction in the initial ACh constriction.

The local anaesthetic procaine also blocks nervous conduction but is less potent than tetrodotoxin and has a broad range of actions on excitable tissue

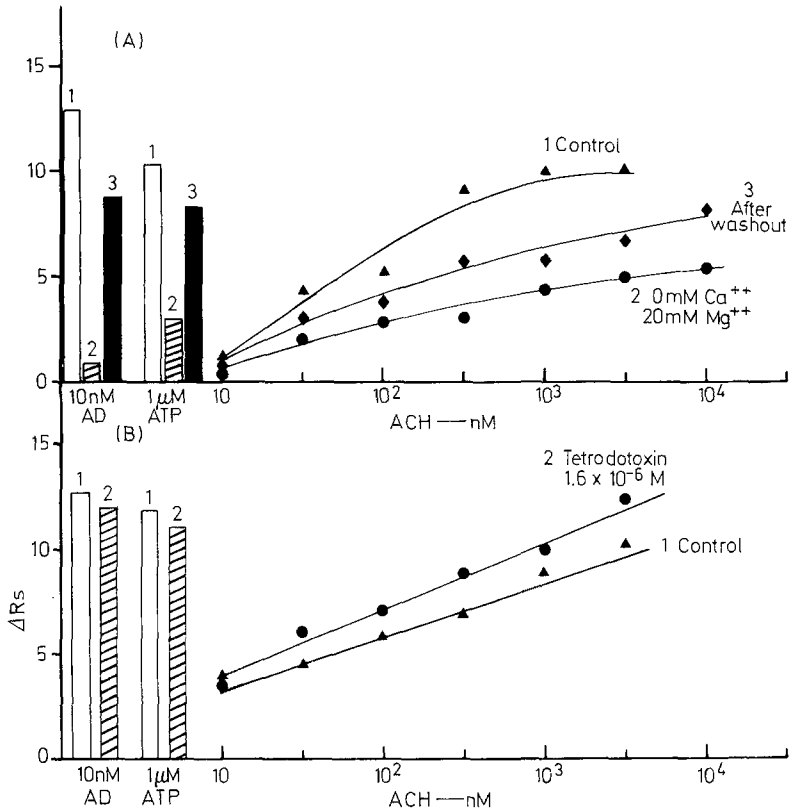

Fig. 7A and B. Results of typical experiments showing the effects of **A** a perfusion medium containing 0 mM Ca^{++} plus 20 mM Mg^{++} and **B** tetrodotoxin (1.6×10^{-6} M) on the dose/response curve to ACh and the responses to test doses of AD (10 nmoles) and ATP (1 μmole), 3, ◆ after return to normal medium for 1 h. Other details as in Figure 2

(Ritchie and Cohen, 1975). At 10^{-4} M, this drug slightly depressed the ACh dose/response A curve and the response to AD (10 nmoles) while that to ATP (1 μmole) was unaffected (Fig. 8A). However at 3.2×10^{-4} M, an apparently selective inhibition of the ACh constriction occurred (Fig. 8B). The entire cholinergic constriction (responses A and B) was abolished at all doses up to 32 μmoles ACh (2 experiments) or persisted in a very reduced form at 10–32 μmoles only (2 experiments). The response to AD (10 nmoles), although depressed, still occurred, while that to ATP (1 μmole) was unaffected. At 10^{-3} M procaine, the response to 10 nmoles AD also disappeared, although 100 nmoles AD still elicited a pressor reaction and the effect of ATP (1 μmole) remained undiminished (Fig. 8C). These results therefore demonstrate a much greater susceptibility to procaine blockade of the cholinergic constrictory mechanisms than of the adrenergic mechanism. This phenomenon was unassociated with any action of procaine on effector organ contractility for the ATP response remained constant. As such, the evidence favours a neural link and thus seems to conflict with the findings involving tetrodotoxin and 0 mM Ca^{++} plus 20 mM Mg^{++}.

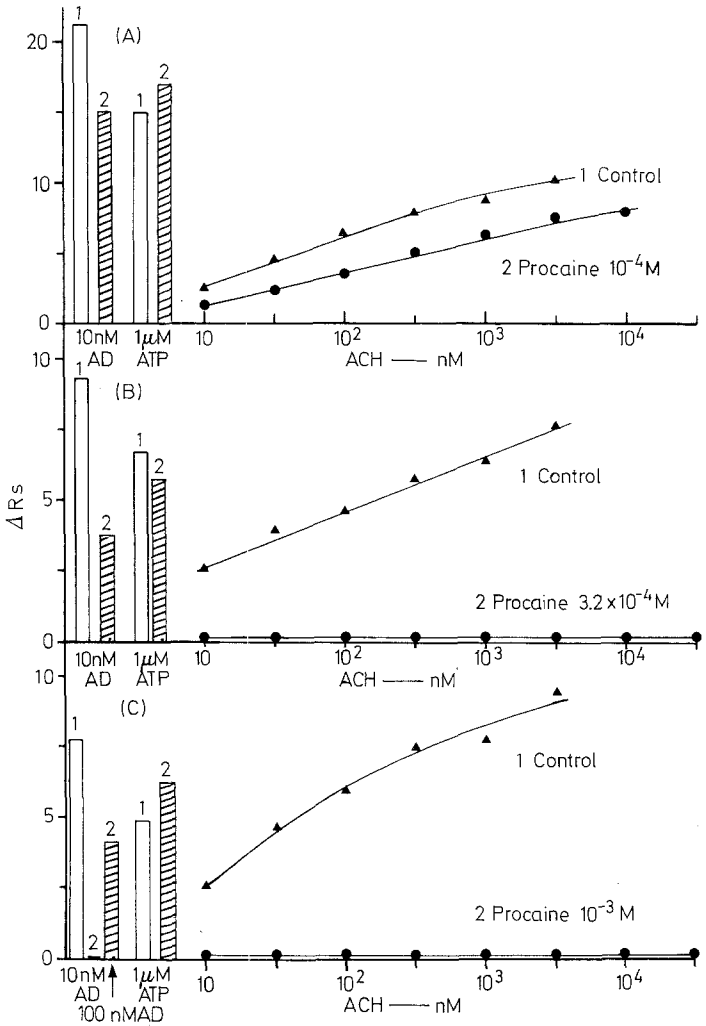

Fig. 8A–C. Results of typical experiments showing the effects of the local anaesthetic procaine at **A** 10^{-4} M, **B** 3.2×10^{-4} M, and **C** 10^{-3} M on the dose/response curve to ACh and the responses to test doses of AD (10 nmoles and 100 nmoles) and ATP (1 μmole). Other details as in Figure 2

VIII. Responses to ACh during α-Adrenergic Tone

If any dilatory effects of ACh occur in the systemic vasculature of the trout, their existence might only be demonstrable against a background of high vasomotor tone, as with β-adrenergic dilatory effects (Wood, 1976). However, when a high level of tone was induced by perfusion with 5.5×10^{-6} M AD, the basic constrictory response to ACh persisted in similar magnitude and was again sensitive to competitive blockade by d-tubocurarine but not by atropine. The only observed vasodilatory effects occurred with injections of 3.2 μmoles ACh or greater, and these relaxations were resistant to both atropine and d-tubo-

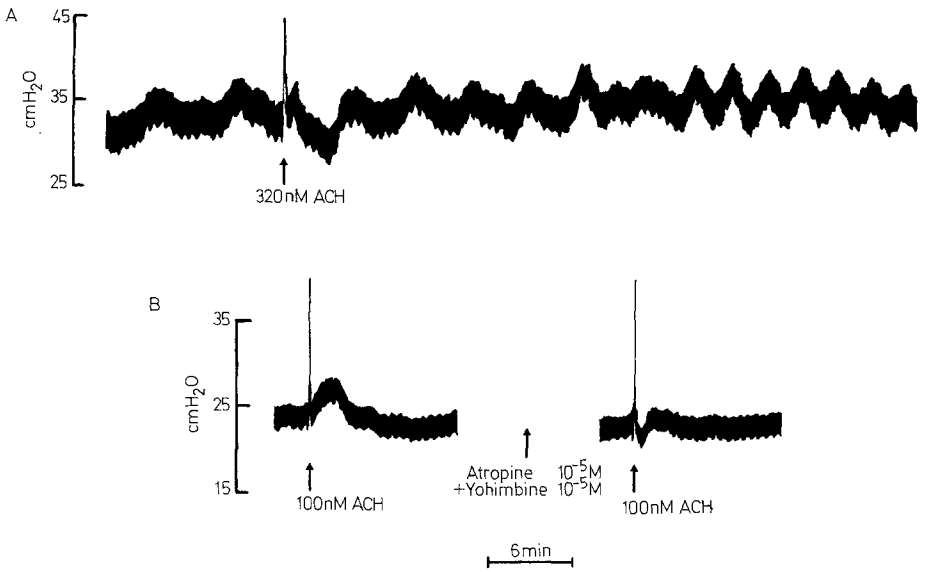

Fig. 9A and B. Representative perfusion pressure records from perfused visceral preparations. **A** Trace from a preparation showing large spontaneous fluctuations in resistance which effectively obscured any response to the injection of ACh (320 nmoles). **B** Trace from a preparation showing less background fluctuation. ACh (100 nmoles) caused a definite constriction which, although reduced, definitely persisted after combined treatment with atropine (10^{-5} M) and yohimbine (10^{-5} M)

curarine. As such they probably resulted from a non-specific depressant effect of very high ACh doses on α-adrenergic tone.

IX. Eviscerated and Visceral Preparations

These preparations were employed in an attempt to localize the cholinergic mechanisms in the systemic vasculature. In the 2 eviscerated trunks examined, the form and sensitivity of both components of the ACh constriction appeared identical to those in the whole trunk, as did the response to AD (10 nmoles). In 3 of the 5 perfused visceral circulations, a definite dose-dependent constrictory response to ACh (10 nmoles upwards) occurred, though the form was complicated by spontaneous variations in baseline resistance (Fig. 9B). The response was reduced but persisted to some degree after combined treatment with atropine (10^{-5} M) and yohimbine (10^{-5} M), and therefore bore some similarity to response A in the whole trunk (Fig. 9B). A definite vasoconstriction to AD (10 nmoles) also occurred in these 3 preparations.

Cholinergic and adrenergic responses were difficult to detect (in 2 preparations impossible to detect) because of large oscillations in baseline resistance (Fig. 9A) which presumably reflected the mechanical effects of spontaneous intestinal motility on blood vessel calibre in the visceral circulation. As in mammals (Hanson and Moore, 1969), pressure in the gut and distension in its walls may be important determinants of coeliac vascular resistance in vivo in fish.

X. The Effects of Antagonists on Vascular Resistance

All of the neural and nicotinic cholinergic blockers employed in this study tended to elevate baseline Rs in the whole trunk preparation by up to 10%, though this effect was significant ($p < 0.05$) only with d-tubocurarine (10^{-5} M) and hexamethonium (10^{-5} M). As all of these agents could act at various points to block any latent neural control of skeletal muscle, a simple mechanical effect on Rs due to a loss of skeletal muscle tone may have been involved. As none of the drugs dilated the preparation, these results, together with earlier findings (Wood, 1976), indicate a complete absence of vasomotor tone of any kind in the perfused systemic vasculature of the trout at baseline Rs.

Discussion

The present investigation has identified two ACh mediated responses of different mechanism in the systemic vasculature of *S. gairdneri*. The initial rapid vasoconstriction (response A) presents unusual pharmacological properties, while the tail-like component (response B) occurring only at high dose levels of ACh follows a pattern commonly seen in higher vertebrates.

Antagonism of the latter response by α-adrenergic and nicotinic, but not by muscarinic, blocking agents, its imitation by nicotine but not by specific muscarinic agonists, and its blockade by procaine and by 0 mM Ca^{++} plus 20 mM Mg^{++} all indicate a classical indirect action of ACh. This mechanism would comprise an interaction with nicotinic receptors resulting in the release of an adrenergic transmitter (adrenaline, noradrenaline, or perhaps both – Abrahamsson and Nilsson, 1975, 1976) which stimulates α-adrenoreceptors on the vascular end-organ to cause vasoconstriction, as in mammals. It is not clear whether ACh causes this release of adrenergic transmitter by nicotinic actions on the post-synaptic membrane of post-ganglionic sympathetic neurons, on their nerve endings directly, or on chromaffin tissue. However the resistance of response B to guanethidine tends to rule out the ganglionic site of action. Chan and Chow (1976) have recently described an in vivo pressor response to high doses of ACh in the eel *Anguilla japonica*. This effect was similar to the present response B in its sensitivity to α-adrenoreceptor blockade and resistance to atropine, but other aspects of its pharmacology (eg. insensitivity to nicotinic blockade) appeared to differ.

The initial constrictory response A to ACh is, however, the more intriguing and unusual, for it is nicotinic in nature, yet resistant to adrenergic and muscarinic cholinergic blockade. The simple lack of a brain in the present preparation rules out any reflex mechanism, such as the response to intra-arterial nicotine in mammals mediated via stimulation of aortic and carotid bodies (Comroe and Mortimer, 1964). The present results seem to exclude all but two possible mechanisms for the genesis of response A: either direct stimulation by ACh of nicotinic receptors on the vascular end-organ, or an indirect effect (analogous to that for response B) resulting in the release of a non-adrenergic, non-cholinergic transmitter.

The former would be by far the simpler explanation; its major drawback is that of singularity. Nicotinic receptors have never been shown to exist on vascular muscle, or indeed on any autonomically controlled smooth muscle, in higher forms, although their presence has occasionally been suggested in non-vascular tissue (eg. Sjöstrand et al., 1972). However there may be some precedent in the cholinergic receptors which mediate positive chronotropism in the lamprey heart. As in the present study, these receptors are nicotinic in nature, antagonized potently by d-tubocurarine, but blocked weakly by hexamethonium (Augustinsson et al., 1956; Falck et al., 1966; Lukomskaya and Michelson, 1972).

An indirect nicotinic action of ACh releasing an unknown transmitter would constitute a more complex explanation, but the experimental results provide several arguments in its favour. Firstly, the partial tachyphylaxis caused by successive doses of ACh is characteristic of an indirect mechanism and can be explained by a gradual depletion of the unknown transmitter (Koppanyi and MacFarlane, 1967), thought it could also be explained by desensitization at the cholinergic receptor (Rang and Ritter, 1970). Secondly, the preferential blockade of the ACh response by procaine (Fig. 8) may indicate the involvement of a neural link. Finally, the remarkable similarity between the form of the ACh response A and that to ATP (Fig. 6) is suggestive that ATP, or a related compound, may be the unknown transmitter involved. Burnstock (1972, 1975) has assembled a substantial body of evidence that a purine compound (most probably ATP) is the transmitter in a number of autonomically innervated preparations (including the teleost gut) where non-adrenergic, non-cholinergic transmission occurs. As in the present study, the administration of exogenous ATP invariably mimics stimulation of the neural mechanism in the temporal form of the mechanical response. In accord with this theory, ATP seems to act by a direct mechanism in the present preparation. The insensitivity of response A to tetrodotoxin and 0 mM Ca^{++} plus 20 mM Mg^{++} (Fig. 7) does not necessarily weigh against this hypothesis, for there exist reports that, in some cases, apparent "purinergic transmission" is resistant to both tetrodotoxin (Carter, 1969; Sneddon et al., 1973) and low Ca^{++}, high Mg^{++} (Hidaka and Kuriyama, 1969).

On the present evidence, it is impossible to choose between the two possible mechanisms. A number of stringent criteria (Burnstock, 1972, 1975) must be satisfied before the existence of a purinergic mechanism can be accepted. It is unfortunate that a specific blocker for purinergic receptors is not yet available. Very recently, 2–2′ pyridylisatogen has been exployed for this purpose with encouraging results (Spedding and Weetman, 1976); the use of this compound may eventually be informative in analyzing the present cholinergic mechanism.

A further unusual feature of response A is that the properties of the nicotinic receptors involved appear peculiar with respect to the actions of nicotinic antagonists. The relative potencies of d-tubocurarine, gallamine, and hexamethonium have been computed from the data of Figure 3 by the dose-ratio method (Ariens, 1964) and compared in Table 2 with analogous results from other studies. Inasmuch as d-tubocurarine was the most potent, there is agreement with the reported characteristics of both skeletal muscle and ganglionic nicotinic receptors. However, the greater potency of gallamine than hexamethonium indicates

Table 2. The relative potencies of the competitive nicotinic antagonists d-tubocurarine, gallamine, and hexamethonium against constrictory response A to ACh in the systemic vasculature of the rainbow trout. Comparable values for the blocking potencies of these agents on typical skeletal muscle and ganglionic receptors of higher vertebrates are presented

Receptor	d-Tubocurarine[a]	Gallamine	Hexamethonium	Reference
Trout vasculature	1000	225	114	Present study
Skeletal muscle	1000	100	4	Ariens (1964)
Skeletal muscle	1000	440	3	Bowman and Webb (1972)
Ganglionic	1000	41	186	Bowman and Webb (1972)

[a] The potency of d-tubocurarine has been arbitrarily set to 1000

a closer similarity to the typical skeletal muscle receptor than to the typical ganglionic type. On the other hand, the potency of hexamethonium was by no means negligible; relative to d-tubocurarine, hexamethonium had a similar activity to that reported on ganglia in mammals. Consequently the receptors in the systemic vasculature of the trout do not seem to fit either of the classical mammalian patterns.

The present study agrees with the few previous observations on the systemic vasculature of teleosts in showing only a constrictory response to ACh (Reite, 1969; Stray-Pedersen, 1970; Nilsson, 1972; Holmgren and Nilsson, 1974). However in *S. gairdneri* this event was totally nicotinic, whereas in the perfused gas gland vessels of the cod, *Gadus morhua,* the ACh vasoconstriction was muscarinic (Nilsson, 1972). No explanation other than the differences in species and preparations can be offered for this disagreement. In none of the studies, including the present, has a muscarinic dilatory effect been demonstrated; this would seem to indicate a basic difference between teleosts and higher vertebrates. In mammals, sympathetic and sacral parasympathetic cholinergic fibres innervate muscarinic receptors which cause depressor effects (Burnstock, 1969). The apparant lack of such a dilatory mechanism in teleosts may reflect both the absence of the sacral parasympathetic outflow (Campbell, 1970) and the fact that sympathetic cholinergic vasodilation appears to be a later evolutionary development (Burnstock, 1969).

I wish to thank Dr. G. Shelton for his advice and encouragement, Dr. D.J. Aidley for useful discussions, and Mr. B. Burgoyne for technical assistance. Phenoxybenzamine hydrochloride was kindly donated by Smith, Kline and French Laboratories Ltd. I am grateful to Dr. G. Duncan for the gift of tetrodotoxin. Financial support was provided by grants from the University of East Anglia, the Commonwealth Scholarship Commission, and the National Research Council of Canada.

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