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Mechanisms underlying ACh induced modulation of neurogenic and applied ATP constrictions in the submucosal arterioles of the guinea-pig small intestine

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1 Role of the vascular endothelium in acetylcholine (ACh) induced modulation of neurogenic and applied ATP (adenosine 5′-triphosphate) constrictions of intestinal submucosal arterioles was investigated.

2 Arteriole constrictions, induced either by exogenous ATP or evoked by perivascular nerve stimulation, were attenuated in the presence of ACh. 100 nM ACh almost completely abolished neurogenic constrictions whereas up to 10 μM ACh reduced constrictions to exogenous ATP by only about 60%.

3 Treatment of the arterioles with 100 μM Nω-nitro-L-arginine (NOLA) and 5 μM indomethacin, to block respectively nitric oxide (NO) and prostanoid release from the endothelium, had no effect on the ACh induced inhibition of neurogenic constrictions but significantly attenuated the inhibitory effects of ACh on constrictions to exogenous ATP.

4 Disruption of the vascular endothelium had no effect on the ACh induced inhibition of neurogenic constrictions but attenuated the inhibitory effects of ACh on applied ATP constrictions to the same extent as after treatment with NOLA and indomethacin. In comparison, endothelial disruption completely abolished the inhibitory effect of substance P (SP) on exogenously applied ATP constrictions.

5 50 nM ACh significantly attenuated the amplitude of neurally evoked excitatory junction potentials (ejps) recorded from the vascular smooth muscle without altering the time constant of decay (τ_{decay}) of the ejps.

6 It is concluded that ACh inhibits neurogenic constrictions by prejunctional modulation of transmitter release from the perivascular sympathetic nerves with no major role for endothelial paracrine factors.

7 Endothelial NO and/or prostanoids mediate some of the ACh induced inhibition of constrictions to exogenous ATP whereas the endothelium independent inhibitory effects of ACh are attributed to a direct action of ACh on the vascular smooth muscle. However, an indirect effect resulting from activation of vasodilator nerves cannot be ruled out.

Keywords: Arteries; acetylcholine; prejunctional autoreceptors; vascular smooth muscle; vascular endothelium; nitric oxide; prostaglandins; EDHF

Abbreviations: ACh, acetylcholine; ATP, adenosine 5′-triphosphate; EDHF, endothelium derived hyperpolarizing factor; Ejp, excitatory junction potential; NO, nitric oxide; NOLA, Nω-nitro-L-arginine; SP, substance P; τ_{decay}, time constant of decay of the excitatory junction potential

Introduction

Factors released from the endothelium have an important role in modulating the tone of blood vessels. In particular endothelial factors that relax blood vessels have received a lot of attention in the past decade. These include NO, prostanoids and endothelium derived hyperpolarizing factor (EDHF). *In vitro* experiments where blood vessels, with an existing tone or tone induced by exogenously applied constrictor agents, dilate in response to one or more of these relaxing factors are commonplace. However, this gives limited information about the physiological effectiveness of the endothelium in situations where the contractile state of the arteriolar muscle is determined by the level of sympathetic nerve activity. In the submucosal arterioles of the guinea-pig small intestine, it has been shown that endothelium dependent vasodilatation of vessels constricted to exogenous application of the thromboxane analogue, U46619, is mostly mediated by NO (Andriantsitohaina & Surprenant, 1992). Recently, in the same arteriolar preparation, Hashitani & Suzuki (1997) have shown hyperpolarizing effects of ACh which they attributed to the release of EDHF from the endothelium. Since NO appears to be almost exclusively implicated in the dilator response of these arterioles to ACh when constricted using exogenously applied U46619 (Andriantsitohaina & Surprenant, 1992) or phenylephrine (Coffa & Kotecha, unpublished data), it is not clear what the role of EDHF would be. However it would appear that the relative importance of NO and EDHF in vasodilator responses can be changed dramatically just by changing the mode of induction of tone (Plane & Garland, 1996). It may be envisaged that EDHF might be more effective than NO against a contractile agonist that relies on voltage-dependent Ca^{2+} influx, such as ATP. In the submucosal arterioles of the guinea-pig small intestine, the transmitter responsible for constrictions to perivascular nerve stimulation

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is ATP (Evans & Surprenant, 1992). Therefore it is of special interest to see if EDHF contributes to modulation of tone evoked by perivascular nerve stimulation or applied ATP.

An additional mechanism that needs to be considered is the modulation of extrinsic perivascular sympathetic nerve activity by the intrinsic vasodilator nerves. Tone in these arterioles can be inhibited by stimulation of intrinsic vasodilator nerves (Kotecha & Neild, 1995a; Neild *et al*., 1990) and is mediated mainly by neurogenic release of ACh. Although it is clear that ACh released from these intrinsic dilator nerves can reach the endothelium to release relaxing factors (Andriantsitohaina & Surprenant, 1992), it is also established that these nerves can attenuate release of transmitter from perivascular sympathetic nerves by prejunctional inhibition (Kotecha & Neild, 1995b). This latter effect is most likely to be mediated *via* the prejunctional muscarinic receptors located on the sympathetic nerve terminals (Fernandes *et al*., 1991; Komori & Suzuki, 1987; Kotecha & Neild, 1995b). Hence, the relative contributions of the endothelium versus that of prejunctional inhibition, in the ACh mediated modulation of neurogenically induced tone, needs to be clarified.

The general aim of this project was to evaluate the mechanisms underlying ACh induced inhibition of constrictions in the submucosal arterioles of the guinea-pig small intestine. The specific aims of this study were twofold. Firstly, to assess the role of the endothelium in mediating the effects of ACh on neurogenically induced and exogenously applied ATP constrictions; secondly, to see if ACh was able to attenuate sympathetic transmitter release evoked by perivascular nerve stimulation. It was hoped that these experiments would shed light on the relative roles of the endothelium versus prejunctional inhibition of transmitter release in the ACh mediated modulation of neurogenic constrictions.

Methods

Guinea-pigs (Monash outbred strain) of either sex and weighing 200–300 g were killed by a heavy blow to the head followed by exsanguination, and a piece of ileum was removed. The ileum was slit open and pinned out mucosa uppermost, and the mucosa peeled off. A sheet of connective tissue containing the submucosal arterioles and nerve plexus was then separated from the circular muscle and pinned out in a small chamber with a transparent base. The sheet was superfused continuously with warmed oxygenated physiological saline, composition (mM): NaCl 120; KCl 5; $CaCl_2$ 2.5; $MgSO_4$ 2; $NaHCO_3$ 25; NaH_2PO_4 1; glucose 11, equilibrated with 95% O_2/5% CO_2. The preparation was viewed with an inverted compound microscope equipped with a television camera, and arteriole diameter was monitored by computer analysis of the television images (Neild, 1989). Nerves were stimulated using a bevelled glass pipette of tip diameter 50–80 μm (Neild *et al*., 1990). The pipette was filled with the same physiological saline that was used to superfuse the preparation, and connected to the cathode of a constant current stimulator. The anode of the stimulator was connected to an indifferent electrode in the recording chamber. A pulse duration of 0.1 ms was used. Pipettes were placed on the surface of an arteriole to stimulate perivascular sympathetic nerves. Intracellular recordings of smooth muscle membrane potential were made using conventional glass microelectrodes filled with 2 M KCl and with resistances in the range 100–200 MΩ. Ejps (excitatory junction potentials) were obtained by stimulation of the perivascular nerves using a single pulse once every minute.

Brief constrictions (once every 4 mins) of the arteriole were obtained by one of two methods. Arterioles were constricted either by stimulating the perivascular nerves (10 Hz, 1 s) or by ionotophoretic application of 10 μM ATP (10 Hz, 1 s). The effect of ACh and SP (substance P) on the constriction was obtained by superfusing the desired concentration in the bath for 4 min prior to obtaining the constriction. With the superfusion rate of 10 mls per min and the bath volume of 0.5 ml, this was ample time for the ACh or SP concentration in the bath to reach the required level.

Vascular endothelium was disrupted by cannulating the parent mesenteric artery, that give rise to an arteriole network, at its point of entry to the intestine. Disruption of the endothelium was obtained by perfusing arterioles with physiological saline containing 0.1% CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-propane-sulphonate). Endothelial damage was assessed by staining with a fluorescent dye, ethidium bromide, which stains nuclei of damaged cells (Daly *et al*., 1992). Effectiveness of endothelial disruption was further established by evaluating the inhibitory effect of SP before and after perfusion of CHAPS, thus eliminating any uncertainty about endothelial function. Muscle integrity was assessed by comparing the constrictions obtained by perivascular nerve stimulation or ionotophoretic application of ATP before and after the endothelium was disrupted.

Drugs used were: acetylcholine, adenosine 5′-triphosphate, indomethacin, (3-[(3-cholamidopropyl)dimethylammonio]-propane-sulphonate), ethidium bromide and Nω-nitro-L-arginine (Sigma); substance P (Auspep, Australia); suramin hexasodium and tetrodotoxin (Research Biochemicals International).

Analysis and statistics

Experiments were carried out on arterioles with diameters in the range 60–110 μm. Amplitude of arteriole constriction was measured in μm and expressed as a fraction of the resting arteriole diameter. Inhibition of constrictions were measured as a reduction in the amplitude of the constrictions (in μm) and expressed as per cent of the uninhibited constriction. EC_{50} value was obtained from sigmoid concentration response curves fitted to each set of data. The amplitude of the ejp was measured in control and ACh-containing physiological saline. The time constant of decay ('τ_{decay}') of the ejp was measured by fitting a straight line to a semilogarithmic plot of the ejp decay (see Kotecha & Neild, 1995b for details). 'τ_{decay}' is a good estimate of the membrane time constant (Hirst & Neild, 1978).

All means are given with the standard error of the mean and the number of observations. In most cases two-tailed '*P*' values were obtained using paired or unpaired Student's *t*-test. Two factor (dose and treatment) repeated measures ANOVA was used to evaluate the effect of SP. A probability value of <0.05 was considered significant in all cases.

Results

General observations

The resting diameters of the arterioles chosen for neurally released (78 ± 6.8 μm) and exogenous ATP (76.4 ± 5.4 μm) experiments were not significantly different ($P = 0.86$; unpaired *t*-test; $n = 12$). There was also no significant difference in the amplitude of constrictions to perivascular nerve stimulation (0.40 ± 0.06) or exogenous ATP (0.37 ± 04; $P = 0.38$; unpaired

t-test; $n=6$). Neurogenic constrictions were reversibly abolished in the presence of the sodium channel blocker tetrodotoxin (1 μM), whereas constrictions to exogenous ATP were unaffected. Application of suramin (200 μM), 2 min prior to testing, attenuated the constrictions to both perivascular nerve stimulation ($n=4$) and exogenous ATP ($n=3$) to less than 10% of the original amplitude confirming that the neurogenic transmitter was ATP (Evans & Surprenant, 1992).

Inhibitory effect of ACh on neurogenic and exogenous ATP constrictions

The protocol used for assessing the effects of ACh on neurogenic and exogenous ATP induced constrictions is shown in Figure 1. Figure 2 shows the summarised effect of ACh on constrictions obtained by the two different methods. The maximum inhibition by ACh on exogenous ATP constrictions was $61.6\pm6.3\%$ which was significantly different from that on neurogenic constrictions ($102.6\pm1.7\%$; $P<0.0001$; unpaired *t*-test; $n=6$). The EC_{50}s of the ACh concentration-response relationship for reduction of neurogenic constrictions (12.2 ± 4.0 nM) and exogenous ATP constrictions (25.7 ± 7.9 mM) were not significantly different ($P=0.19$; unpaired *t*-test; $n=6$).

Effect of NOLA plus indomethacin on the ACh concentration-response relationship

After obtaining a control set of data (in the experiments above), the arterioles were incubated in 100 μM NOLA plus 5 μM indomethacin for at least 30 min before repeating the experiment. Treatment with NOLA and indomethacin had no significant effect on the ACh mediated inhibition of neurogenic constrictions as shown in Figure 3 (maximum inhibition after treatment was $104\pm1\%$ compared with the control value of 102.6 ± 1.7; $P=0.38$; paired *t*-test; $n=6$). After treatment with NOLA + indomethacin, the EC_{50} of the ACh concentration-response relationship for neurogenic constrictions (9.9 ± 3.3 nM) was not significantly different from control (12.2 ± 4.0 mM; $P=0.74$; paired *t*-test; $n=6$). On the other hand, NOLA + indomethacin significantly decreased the maximum inhibition, by ACh, of exogenous ATP induced constrictions from 61.6 ± 6.3 to $33.9\pm6.9\%$ ($P=0.02$; paired *t*-test; $n=6$) and significantly increased the EC_{50} of the ACh concentration-response relationship from 25.7 ± 7.9 to 103.2 ± 31.3 nM ($P=0.02$; paired *t*-test; $n=6$) as shown in Figure 4.

Effect of endothelial disruption on the ACh concentration-response relationship

In a separate set of experiments, the endothelium of the arterioles was disrupted using the detergent CHAPS. Figure 5A shows a photo of the arteriole where the endothelium was successfully disrupted. The nuclei of the damaged endothelium have been stained with the fluorescent dye, ethidium bromide. There was no significant difference in the amplitude of constrictions to perivascular nerve stimulation (0.37 ± 0.42; $P=0.62$; unpaired *t*-test; $n=6$) or exogenous ATP (0.32 ± 0.26; $P=0.32$; unpaired *t*-test; $n=6$) after damage to the endothelium as compared with normal arterioles (see General observations), thus providing evidence for arteriole smooth muscle integrity. On deliberate overexposure to CHAPS, the nuclei of the damaged arteriole muscle cells were stained as shown in Figure 5B.

The concentration-response relationship of ACh induced inhibition of neurogenic constrictions was unchanged by disruption of the endothelium with an EC_{50} of 10.1 ± 0.7 nM compared with the control value of 12.2 ± 4.0 nM ($P=0.79$) and a maximum inhibition of $105.2\pm0.8\%$ as compared with the control inhibition of $102.6\pm1.7\%$ ($P=0.32$; unpaired *t*-test; $n=6$; Figure 3). Disruption of the endothelium decreased the ACh induced maximum inhibition of exogenous ATP constrictions to the same extent ($28.4\pm3.4\%$) as that obtained after NOLA + indomethacin ($33.9\pm6.9\%$; $P=0.71$; unpaired *t*-test; $n=6$; Figure 4).

Additionally, the EC_{50} of the concentration-response relationship of ACh induced inhibition of constrictions to exogenous ATP after disruption of the endothelium (113 ± 22.4 nM) was not significantly different to that obtained

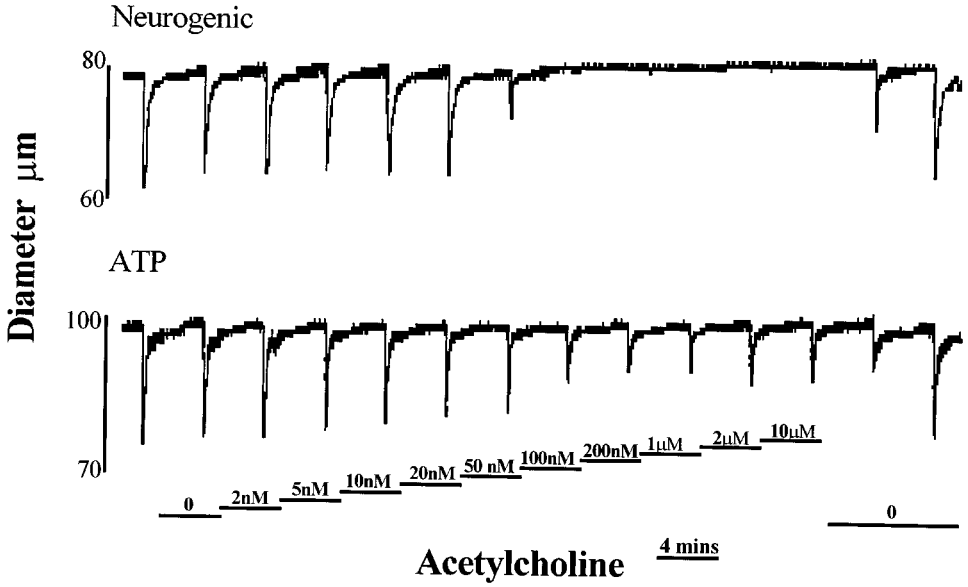

Figure 1 Protocol used to assess the inhibitory effect of ACh on neurogenic and exogenous ATP constrictions. After obtaining consistent constrictions to either neurogenic or exogenous ATP, ACh was superfused in the organ bath in increasing concentrations, from 2 nM to 10 μM, and then washed out. 100 nM ACh completely abolished the neurogenic constrictions whereas up to 10 μM failed to completely inhibit the constrictions to exogenous application of ATP.

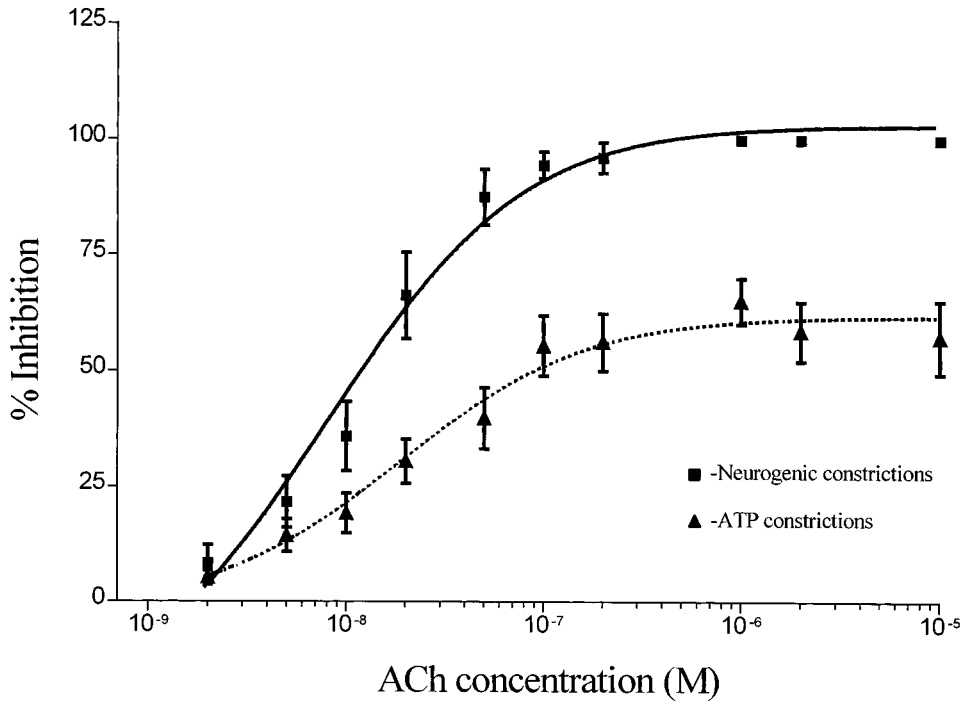

Figure 2 Summary of the inhibition produced by ACh on the constrictions to perivascular nerve stimulation and iontophoretically applied ATP in the guinea-pig submucosal arteriole. Relationship between the change in the size of constrictions (plotted as per cent inhibition) of neurogenic ATP (maximum inhibition: 100%; EC_{50}: 12.2±4.0 nM: sigmoid curve in solid line) and exogenous ATP (maximum inhibition: 61.6±6.3%; EC_{50}: 25.7±7.9 nM: sigmoid curve in dashed line) and the concentration of ACh. Each point is the mean±s.e.mean of six observations and the sigmoid curves were obtained from an average of individual sigmoid curves fitted to each set of data.

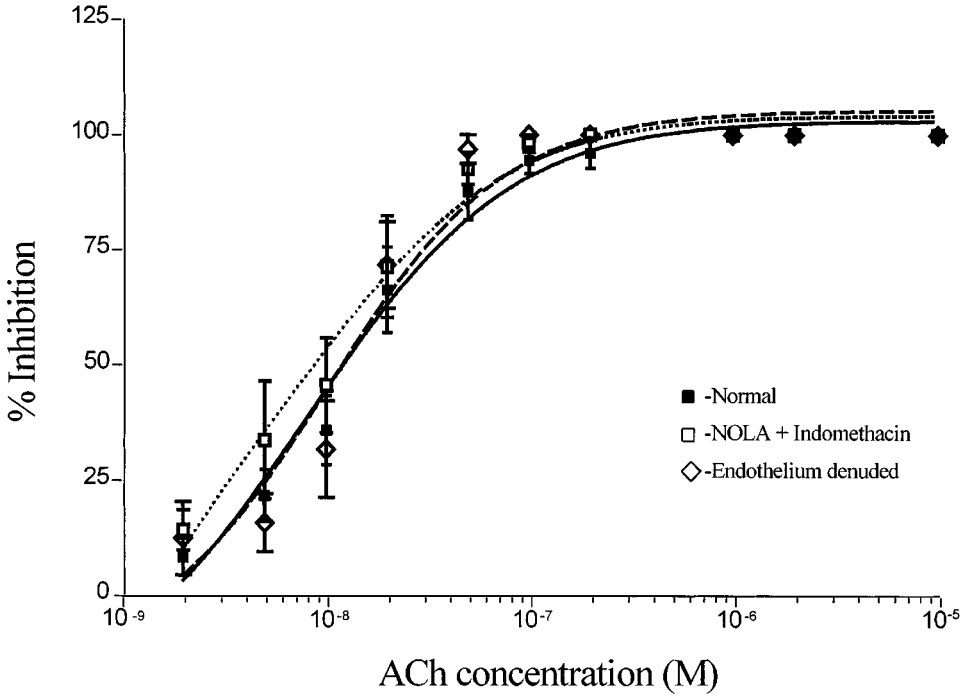

Figure 3 Summary of the inhibition produced by ACh on the constrictions to perivascular nerve stimulation in control solution and after treatment with NOLA and indomethacin or disruption of the vascular endothelium. Comparison between the inhibitory effect of ACh on the amplitude of constrictions to neurogenic ATP before (maximum inhibition: 100%; EC_{50}: 12.2±4.0 nM: sigmoid curve in solid line) and after treatment with 100 μM NOLA+5 μM indomethacin (maximum inhibition: 100%; EC_{50}: 9.9±3.3 nM: sigmoid curve in short dashed line). NOLA+indomethacin did not have any significant effect on the ACh induced inhibition of neurogenic constrictions. Disruption of the vascular endothelium also failed to change the concentration-response relationship of ACh on the neurogenic constrictions (maximum inhibition: 100%; EC_{50}: 10.1±0.7 nM: sigmoid curve in long dashed line). Each point is the mean±s.e.mean of six observations and the sigmoid curves were obtained from an average of individual sigmoid curves fitted to each set of data.

after treatment with NOLA + indomethacin (103.2 ± 31.3 mM; $P = 0.68$; unpaired t-test; $n = 6$).

Effect of endothelial disruption on the SP induced inhibition of exogenous ATP constrictions

From previous work in the laboratory, it was established that 10 nM SP produced maximal inhibition in these arterioles. Hence, to be absolutely certain of eliciting a full endothelial response, three concentrations of SP (10, 20 and 50 nM) were chosen for these experiments. SP induced modulation of arteriole constrictions were examined in three experiments before and after endothelial disruption. SP inhibited the applied ATP constrictions to about 65% ($n = 3$; Figure 6). As expected, the inhibitory effects of SP were not dose dependent ($P = 0.33$; repeated measure ANOVA; $n = 3$) in the chosen range of concentrations. Disruption of the endothelium, using CHAPS, significantly attenuated ($P = 0.023$; repeated measure ANOVA; $n = 3$) the inhibitory effect of SP on the ATP induced constrictions (Figure 6). The inhibition produced by SP after endothelial disruption was not significantly different from zero ($P = 0.91$).

There was no significant difference in the amplitudes of the ATP induced constrictions before (0.37 ± 0.05) or after endothelial disruption (0.36 ± 006; $P = 0.78$; paired t-test, $n = 3$), in the above experiments, thus indicating that the ATP

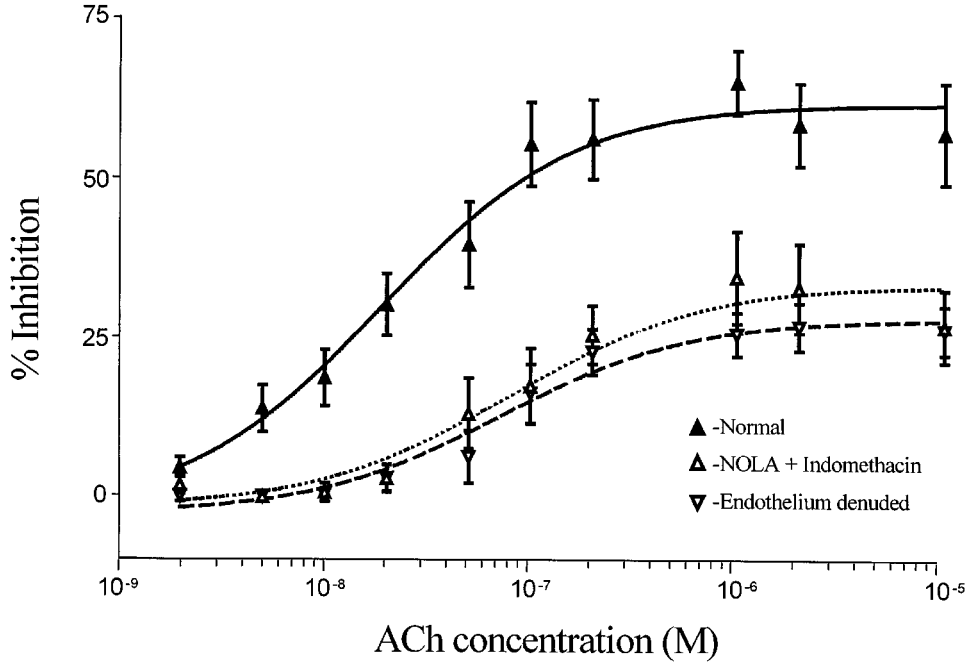

Figure 4 Summary of the inhibition produced by ACh on the constrictions to iontophoretic ATP in control solution and after treatment with NOLA and indomethacin or disruption of the endothelium. Comparison between the inhibitory effect of ACh on the amplitude of constrictions to exogenous ATP before (maximum inhibition: 61.6 ± 6.3%; EC_{50}: 25.9 ± 7.9 nM: sigmoid curve in solid line) and after treatment with 100 μM NOLA + 5 μM indomethacin (maximum inhibition: 33.9 ± 6.9%; EC_{50}: 103.2 ± 31.3 nM: sigmoid curve in short dashed line). NOLA + indomethacin significantly attenuated the effect on the ACh induced inhibition of applied ATP constrictions. Disruption of the vascular endothelium attenuated the inhibitory effect of ACh on applied ATP constrictions (maximum inhibition: 28.4 ± 3.4%; EC_{50}: 113 ± 22.4 nM: sigmoid curve in long dashed line) to the same extent as that observed after treatment with NOLA + indomethacin. Each point is the mean ± s.e.mean of six observations and the sigmoid curves were obtained from an average of individual sigmoid curves fitted to each set of data.

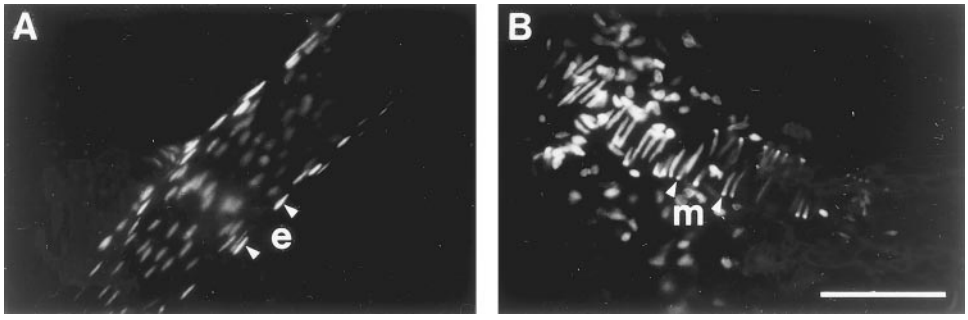

Figure 5 Picture showing evidence of endothelium and muscle damage in arterioles perfused with CHAPS. (A) Picture showing successful disruption of vascular endothelium. Nuclei of damaged endothelial cells (e) are stained with the fluorescent dye, ethidium bromide. In (B), the arterioles were overexposed to CHAPS causing extensive muscle damage as shown by staining nuclei of muscle cells (m) with ethidium bromide. Calibration bar: 100 μM.

induced constrictions were not complicated by contribution from the endothelium, as found in other preparations (Kennedy *et al*., 1985).

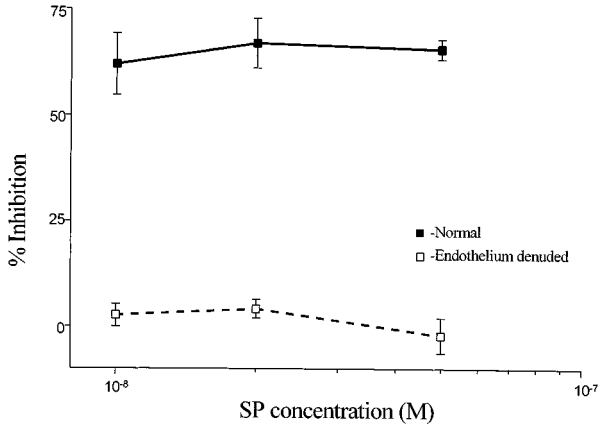

Figure 6 Summary of the inhibition produced by SP on the constrictions to iontophoretic ATP in control solution and after disruption of the endothelium. The inhibitory effect of SP on ATP induced constriction was not dose-dependent for the three chosen concentrations ($62\pm7.2\%$ at 10 nM; $67\pm5.9\%$ at 20 nM and $66\pm2.4\%$ at 50 nM). Disruption of the endothelium significantly attenuated the inhibitory effect of SP ($3\pm2.7\%$ at 10 nM; $4\pm2.3\%$ at 20 nM and $-2\pm4.1\%$ at 50 nM). The inhibition produced by SP after endothelial disruption was not significantly different from 0%. Each point is the mean$\pm$s.e.mean of three observations.

Effect of ACh on the amplitude and decay time constant of ejps

In agreement with the results obtained by Hashitani & Suzuki (1997), it was found that ACh (up to 100 nM) had no detectable effect on the resting membrane potential (72.5 ± 7.76 mV; $n=12$) of the arteriole smooth muscle. In the example shown in Figure 7, summation of ejps evoked by a train of perivascular nerve stimuli at 10 Hz, 1 s, with the same parameters that lead to neurogenic constrictions are shown. In the presence of 50 nM ACh, a blunted response to perivascular nerve stimuli is obtained. The blunted response in the presence of 50 nM ACh could be due to a decrease in the amount of neurotransmitter released from the sympathetic nerves (as would be indicated by an attenuation in the amplitude of ejps) and/or a fall in membrane resistance in the arteriole smooth muscle, which would affect the decay of the ejps ('τ_{decay}') (Kotecha & Neild, 1995b). In order to differentiate between the two possibilities, ejps were evoked by a single stimulus to the perivascular nerves once every 60 s. Presence of 50 nM ACh in the bathing medium significantly attenuated the amplitude of evoked ejps (from 12.23 ± 0.81 to 7.90 ± 0.90 mV; $P<0.0001$) without significantly altering 'τ_{decay}' (291.9 ± 15.36 versus 295.1 ± 11.26 ms; $P=0.76$; paired *t*-tests; $n=12$) as exemplified in Figure 8.

Discussion

The present study shows that there are differences in the mechanisms underlying ACh induced inhibition of neurogenic constrictions as opposed to constrictions obtained by exogenous application of ATP. Recently Plane & Garland

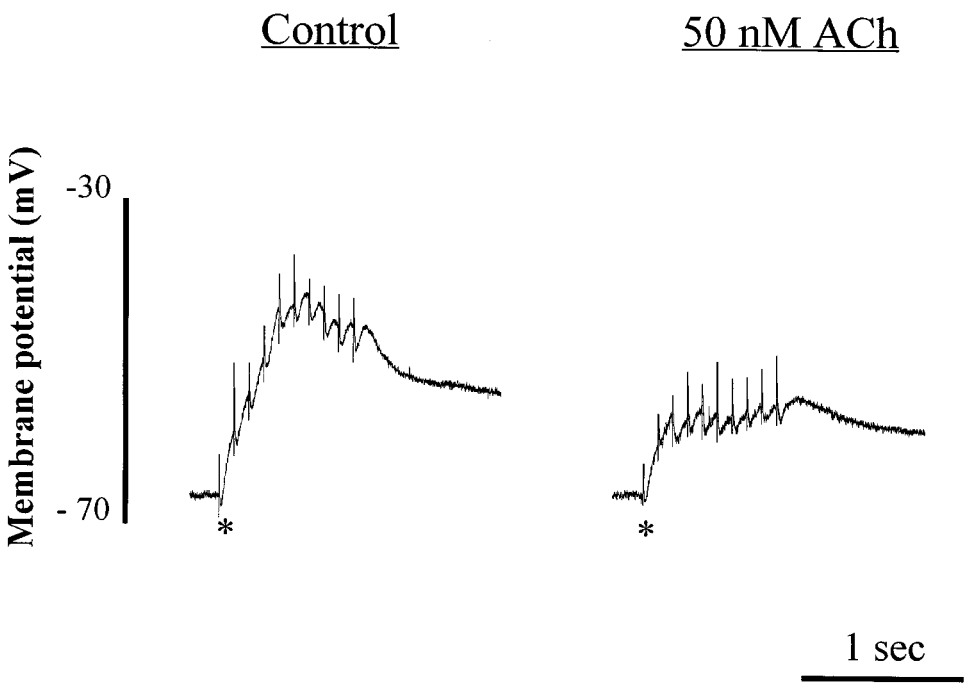

Figure 7 Effect of ACh on the summation of ejps evoked by a train of perivascular nerve stimulation. Perivascular nerves were stimulated at 10 Hz, 1 s to give ejps which summed to give a peak value of 26 mVs. Each ejp is preceded with a stimulus artefact. In the same experiment, presence of 50 nM ACh reduced the peak amplitude of the summed ejps to 14 mVs. For measurement of change in ejp amplitude and 'τ_{decay}' the effect of ACh on single ejps was observed.

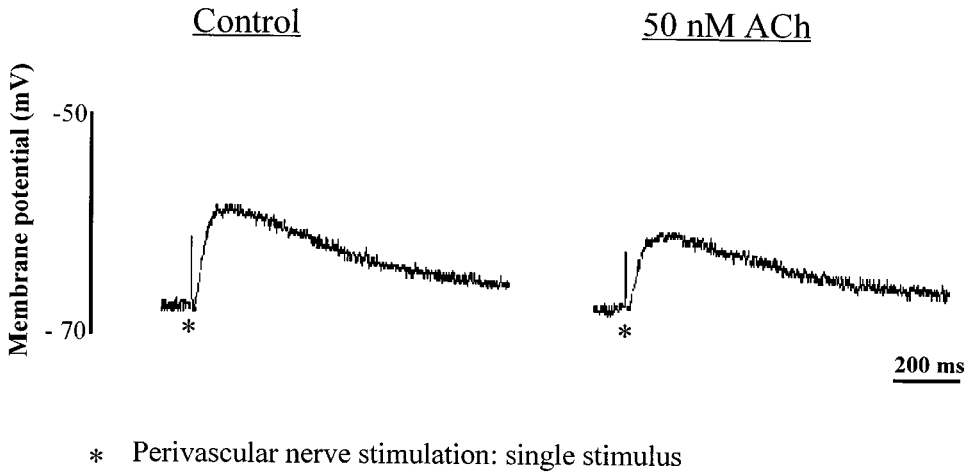

Figure 8 Effect of ACh on the amplitude and time constant of decay of ejp evoked by perivascular nerve stimulation. For measurement of change in ejp amplitude and 'τ_{decay}' the effect of ACh on single ejps was observed. The ejps were evoked by stimulation of the perivascular sympathetic nerves with single pulses. The control ejp had an amplitude of 11.5 mV and 'τ_{decay}' of 322 ms. In the same experiment, presence of 50 nM ACh reduced the amplitude of the ejp to 7.1 mV with a 'τ_{decay}' of 327 ms.

(1996) have demonstrated that the predominant vasodilator mediator, of ACh induced relaxations, can be changed dramatically from NO to EDHF just by changing the mode of induction of tone. Hence in this study, to identify any differences in the mechanisms involved in ACh induced inhibition of neurally mediated versus agonist induced constrictions, the exogenous agonist used (i.e. ATP) was kept the same as the neurally released transmitter. In the guinea-pig submucosal arterioles, it has been shown that both neurally released and applied ATP exerts its effect by binding to the P_{2x}-purinoceptor (Evans & Surprenant, 1992; Galligan *et al*., 1996). The P_{2x}-purinoceptors are ligand-gated cation selective ion channels with relatively higher permeability for Ca^{2+} as compared with Na^{+} (Bean, 1992; Valera *et al*., 1994). Four subclasses of P_{2x}-purinoceptor have been identified with the main difference between them being the unitary conductances of the channels (Abbracchio & Burnstock, 1994; Benham, 1992). It is not known if there are differences between the subclasses of P_{2x} receptors mediating neural and applied ATP responses. One clear result to emerge is that P_{2x}-purinoceptors mediate fast receptor responses, directly linked to an ion channel (Abbracchio & Burnstock, 1994). In the guinea-pig submucosal arterioles, ATP (neurally released or exogenously applied) activates P_{2x}-purinoceptors (Evans & Surprenant, 1992; Galligan *et al*., 1996) and the ATP activated current mimics the known properties of the conductance underlying the excitatory junction current (Benham, 1992; Finkel *et al*., 1984; Valera *et al*., 1994). It would thus seem logical to assume that the same relaxants are involved in the ACh induced inhibition of neurogenic and applied ATP constrictions at the postjunctional level. Therefore the differences seen in this study reflect a role for prejunctional inhibitory mechanism mediated by ACh.

Role of the vascular endothelium in the ACh induced inhibition of constrictions

Prevention of NO and prostanoid formation attenuated the inhibitory effect of ACh on exogenous ATP constrictions. Disruption of the endothelium attenuated the ACh induced inhibition of the exogenous ATP constrictions to the same extent as after treatment with NOLA and indomethacin (see Figure 4) thus providing confirmation of functional damage to the endothelium. It is unlikely that endothelial damage would affect the release of NO and prostanoids without affecting the release of EDHF. Further support for the functional completeness of endothelial damage is provided by the use of SP instead of ACh to cause inhibition of exogenous ATP constrictions. The finding that disruption of the endothelium completely abolished the inhibitory effects of SP eliminates any uncertainty of the method used since SP is known to be a totally endothelium dependent vasodilator. Therefore, NO and/or prostanoids appear to be the only endothelial candidates that partially mediate ACh induced inhibition of exogenous ATP constrictions and the remnant inhibitory effects of ACh are endothelium independent.

Role of the vascular smooth muscle in the ACh induced inhibition of constrictions

The inhibitory effects of ACh on exogenous ATP constrictions after endothelium damage are best explained by a direct action of ACh on the vascular smooth muscle. This direct effect on the smooth muscle requires significantly higher concentration of ACh ($EC_{50} = 113$ nM) as compared with the concentration of ACh required to release endothelial factors ($EC_{50} = 25.7$ nM). Hyperpolarization of barium depolarized arteriole smooth muscle to ACh in the presence of NOLA and indomethacin was attributed to the release of EDHF by Hashitani & Suzuki (1997) although recent experiments from the same laboratory suggests that there is no evidence for the presence of EDHF in these arterioles (Suzuki, personal communication). The results of the present study confirms that endothelium dependent inhibitory effects of ACh can be fully accounted for by NO and prostanoid formation with no evidence for a role of EDHF as shown by experiments in which the endothelium had been damaged. Endothelium independent relaxation and hyperpolarization has been shown in vessels of the cephalic circulation, such as the lingual artery of the rabbit and the posterior auricular artery of the cat (Brayden & Bevan, 1985; Brayden & Large, 1986). Dilatations produced by stimulation of cholinergic, presumably parasympathetic fibres in these vessels were unaffected by removal of the endothelium. Hence, they were mediated by muscarinic receptors present on the vascular smooth muscle (Brayden & Bevan, 1985; Brayden & Large, 1986). Alternatively, activation of prejunctional

nicotinic receptors on vasodilator nerves may underlie the endothelium independent inhibitory effects of ACh (Toda *et al*., 1997). It could be envisaged that endothelium independent ACh induced smooth muscle hyperpolarization would most likely underlie the modulation of constrictions that are primarily caused by depolarization of the smooth muscle membrane. Constrictions produced by ATP acting on a P_{2x} receptor *via* the opening of voltage operated Ca^{2+} channel (Reilly & Hirst, 1996) would be reduced by hyperpolarization induced reduction in Ca^{2+} influx across the membrane. In the absence of production of endothelial factors, the EC_{50} for inhibition of exogenous ATP constrictions by ACh (113 nM) in this study correlates well with the EC_{50} of the ACh induced hyperpolarization (125 nM) obtained by Hashitani & Suzuki (1997) in barium depolarized arterioles. Hence, it is conceivable that the ACh induced hyperpolarization in the absence of NO and prostanoid productions, seen by Hashitani & Suzuki (1997), is a result of direct action of ACh on the arteriole smooth muscle. Additionally, using a spectrofluorometer with a Ca^{2+} sensitive probe, fura-2 (Suzuki, personal communication) has shown that there is a decrease in intracellular calcium levels in the vascular smooth muscle of the guinea-pig intestinal arterioles, induced by a direct action of ACh on the muscle.

Role of prejunctional muscarinic receptors in the ACh induced inhibition of constrictions

The profile of ACh mediated inhibition of neurogenic constrictions is different from that of the ACh mediated inhibitions of constrictions to exogenously applied ATP. The maximum inhibition obtained by ACh on exogenous ATP constrictions was about 60% even with ACh concentrations as high as 10 μM, which gave complete inhibition of neurogenic constrictions (see Figures 1 and 2). The inhibition of neurogenic constrictions, produced by ACh, was not affected by agents which prevent the formation of either NO or prostanoids, nor was it affected by disruption of the endothelium. Therefore, endothelial paracrine factors do not mediate the ACh induced inhibition of neurogenic constrictions. Additionally, after endothelial damage, the EC_{50} of ACh induced inhibition of neurogenic constriction was much lower (10 nM) compared to the EC_{50} for a direct effect of ACh on the smooth muscle (113 nM), thus suggesting that ACh induced inhibition of neurogenic constrictions does not result from a direct effect on the vascular smooth muscle. Overall, postjunctional mechanisms (i.e., ACh induced release of endothelial factors or a direct action of ACh on the arteriole smooth muscle) fail to account for the inhibition of neurogenic constrictions. There is clearly another powerful inhibitory mechanism involved.

The involvement of an endothelium independent prejunctional mechanism is further substantiated by the data showing a significant attenuation in the amplitude of ejps seen in the presence of 50 nM ACh. Decrease in ejp amplitude reflects a decrease in the amount of transmitter released from the perivascular sympathetic nerves (Kotecha & Neild, 1995b). Decrease in sympathetic outflow mediated *via* the prejunctional muscarinic receptors has been known for some time (Eglen & Whiting, 1990; Fernandes *et al*., 1991; Komori & Suzuki, 1987; Kotecha & Neild, 1993). The failure of this concentration of ACh to affect the 'τ_{decay}' suggests that any factors released from the endothelium by this concentration does not cause any major conductance changes at the muscle membrane. The finding that there was no apparent contribution from postjunctional mechanisms towards the inhibition of neurogenic constrictions, over the range of ACh concentrations used, was surprising. However, since the reduction in transmitter release is the first step of action of ACh, the resultant constriction is reduced before it is affected by any contribution from postjunctional mechanisms. We have shown in a previous study that even a very small change in transmitter release has a dramatic effect on constrictions resulting from trains of stimuli (Kotecha & Neild, 1995b). One might expect to see some contribution from postjunctional mechanisms at lower concentrations of ACh but as the concentration of ACh increases, the constriction resulting from a decrease in transmitter release would be markedly reduced such that contribution from postjunctional mechanisms becomes insignificant. This predominant effect of ACh is presumably what determines the ACh induced inhibition of neurogenic constrictions since in the overall scheme, the EC_{50} values and sigmoid curves are generated for the whole range of ACh concentrations.

Hence, there appears to be a very powerful role for prejunctional inhibitory mechanisms. This mechanism may be the one that is physiologically important as these arterioles have an intrinsic cholinergic vasodilator innervation (Brookes *et al*., 1991) that appears to be sparse but very potent with regard to its effect on sympathetic activity (Kotecha & Neild, 1995b).

In conclusion, ACh induced release of endothelial relaxing factors (NO and/or prostanoids) mediate the inhibition of exogenous ATP constrictions. ACh induced endothelium independent inhibition of applied ATP constrictions is accounted for by a direct action of ACh on the vascular smooth muscle. Neither the endothelium nor the vascular smooth muscle are involved in the ACh induced inhibition of neurally mediated constrictions. Prejunctional inhibitory effect of ACh on the sympathetic nerve terminals is the exclusive mechanism by which ACh attenuates neurally mediated constrictions.

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