Changes in purinergic responses of the rabbit isolated central ear artery after chronic electrical stimulation *in vivo*

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- 1 The effect of chronic (4–16 days) electrical stimulation (5 Hz, 0.3 ms, 4–10 V) of the great auricular nerve *in vivo* on sympathetic cotransmission in the rabbit isolated central ear artery was examined.
- 2 Chronic stimulation had no significant effect on frequency-dependent (4–60 Hz) neurogenic contractions or contractile responses induced by exogenous noradrenaline (0.1–300 μ M).
- 3 In contrast, contractions induced by exogenous α,β -methylene ATP (10.0 μ M) were significantly decreased in preparations from 16-day stimulated animals in comparison with sham-operated, 4-day and 8-day chronically stimulated animal groups.
- 4 It is concluded that chronic electrical stimulation of nerves supplying the ear artery may lead to the selective alteration of postjunctional P_{2x} -purinoceptor mechanisms, while the effects mediated by post-junctional α_1 -adrenoceptors remain unchanged.

Keywords: Rabbit ear artery; chronic electrical stimulation; noradrenaline; sympathetic neurotransmission; purinergic transmission

Introduction

Chronic electrical nerve stimulation is used clinically in the treatment of intractable pain, eye and ear disorders, activation of paralysed skeletal muscles, and more recently for stimulating autonomic nerves to correct bladder control and impotency (Loeb, 1989). Few studies have investigated the effects of chronic electrical stimulation *in vivo* on the local control of the vasculature which is important in terms of how best the procedure can be used to alleviate disorders such as peripheral vascular disease (Tallis *et al.*, 1983). It is known, however, that prolonged electrical stimulation increases tissue blood flow and induces structural changes in the vasculature, including neovascularization (Brown *et al.*, 1976; Cameron *et al.*, 1989; Hudlická *et al.*, 1991). The rabbit isolated central ear artery is a useful model since the great auricular nerve which supplies the artery (Feldberg, 1926) is easily accessible for stimulation *in vivo*. The preparation is also known to show structural and functional changes after chronic activity (Bevan & Tsuru, 1979; Fronek & Alexander, 1986; Tsuru, 1990).

In the present study, changes in the neurogenic contractions and contractions of the ear artery induced by noradrenaline (NA) and α,β -methylene ATP (α,β -MeATP), a stable and potent analogue of ATP which acts as a cotransmitter with NA in this preparation (Kennedy *et al.*, 1986; Saville & Burnstock, 1988), were compared following 4, 8 and 16 days of electrical stimulation of the great auricular nerve and in sham-operated animals.

Methods

Experiments were performed on adult (4–6 months) male New Zealand White rabbits (2.5–3.0 kg).

Procedure for chronic electrical stimulation

Animals were anaesthetized with Hypnorm (fentanyl citrate (0.09 mg kg⁻¹) and fluanisone (3 mg kg⁻¹), injected i.p. collectively, Janssen Pharmaceuticals, U.K., 0.3 mg kg⁻¹, i.p.) and Hypnovel (midazolam chloride, up to 2 mg kg⁻¹, i.p., Roche Products, U.K.). Additional intramuscular injections of 0.4 mg Hypnorm were given every 30–40 min as necessary.

Two multistranded, stainless steel electrodes were sutured 2–3 mm from either side of, and parallel to, the right great auricular nerve. The length of the insulation-free part of the electrodes was approximately 1–2 mm. The teflon-coated, silicone-insulated stainless steel wires were run subcutaneously and attached to a silicone-coated external plug which was sutured onto the back of the animal. All animals were given approximately 3 mg kg⁻¹ gentamycin intramuscularly and allowed to recover.

Stimulation began not earlier than 48 h after postoperative recovery. Continuous electrical stimulation parameters of 5 Hz, 0.3 ms pulse width, 4–10 V with a Grass SD9 stimulator were given for 6–12 h each day for 4, 8 and 16 days with rest periods at weekends. Animals had freedom of movement, were given food and water *ad libitum* and monitored hourly to ensure that stimulation parameters were not inducing undue stress during periods of stimulation. Upon the initial onset of stimulation, most animals either intermittently shook their heads from side-to-side or stroked the stimulated ear for approximately 1–2 min. Thereafter the animals appeared unconcerned. A few animals initially attempted to disconnect themselves but they subsequently adapted to the chronic stimulation. None of the animals showed signs of discomfort during the course of the experiments. Conditions of these experiments were examined at depth and applied under the current Animals (Scientific Procedures) Act, 1986, UK. Sham-operated control animals had electrodes implanted and were attached to the stimulator, but not stimulated, for an 8-day period.

Animals were killed the day after the last period of stimulation with an overdose of sodium pentobarbitone followed by exsanguination. The ipsilateral (stimulated) and

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contralateral (non-stimulated) proximal segments of the rabbit central ear artery were isolated, cleaned of connective tissue and prepared for *in vitro* organ bath pharmacological investigations as previously described (Bevan & Osher, 1972).

Pharmacology

Experiments were conducted as outlined by Maynard *et al.* (1991). Briefly, proximal isolated ring segments (4 mm), two rings from each ear of the rabbit (i.e. ipsi- and contralateral ears), were bathed in modified Krebs solution in organ baths under isometric conditions with a resting tension of 0.5–1.0 g for at least 1 h. The Krebs solution was composed of the following (mM): NaCl 133, KCl 4.7, NaHPO₄ 1.35, NaHCO₃ 16.3, MgSO₄ 0.61, CaCl₂ 2.52 and glucose 7.8 and was gassed with 95% O₂ and 5% CO₂ and maintained at 37°C. Electrical field stimulation was provided by a Grass SD9 stimulator and carried out with two platinum wire electrodes placed parallel to, and on either side of, the rabbit ear artery rings. The stimulator was set at supramaximal voltage (60 V), 0.3 ms pulse width for 1 s train duration at 4, 8, 16, 30 and 60 Hz. Responses to electrical field stimulation were elicited and recorded at 2 min intervals, and were abolished in the presence of tetrodotoxin (1 µM) which confirms that the contractions elicited were neurogenic and not due to direct muscle stimulation. Cumulative concentration-response curves were established for noradrenaline (0.1–300 µM), whilst α,β-MeATP (0.5, 1.0 and 10 µM) was added as single applications 45 min apart to avoid desensitization of the P_{2x}-purinoceptors. The latter drug was washed out once a maximum response was established; consequently exposure time was approximately 1–2 min. Each rabbit central ear artery ring was investigated by electrical field stimulation to obtain frequency-response data and responses to exogenous noradrenaline and α,β-MeATP in that order. There was a minimum of 20 min and three changes of Krebs solution between each series of contractions (e.g., between neurogenic contractions and noradrenaline-induced contractions). Potassium chloride (120 mM) was added to each preparation at the end of the experimental protocol as a control for the contractile capacity of the vascular smooth muscle in each preparation.

Drugs

Salts for Krebs solution, (–)-noradrenaline bitartrate (NA), α,β-MeATP and tetrodotoxin were obtained from Sigma Chemical Company, Poole, Dorset. NA (0.1 M) in 100 µM ascorbic acid was made up each day and diluted in distilled water. All other drugs were salts made in aqueous solution.

Expression of results and statistical methods

Throughout this study *n* refers to the number of animals investigated. Data from each animal (*n* = 1) were calculated as the average from two preparations per animal. All results are expressed as mean ± standard error of the mean (s.e.mean). All contractile responses are expressed as a percentage of the response induced by KCl (120 mM) in the same preparation. pD₂ values were calculated as –log[NA] which produced 50% of the maximal response. Statistical significance was determined by analysis of variance followed by Tukey's statistic where appropriate, using the Crunch Statistical Package (Crunch Software Corporation, California, 1987). A probability level of *P* < 0.05 was considered significant.

Results

KCl- (120 mM) induced contractions did not differ either between groups of animals, or between the ipsi- versus contralateral side within any one animal group. In sham-operated (control) animals, KCl (120 mM)-induced contractions amounted to 2.7 ± 0.4 g (*n* = 4). Since data obtained from ipsi- and contralateral preparations from sham-operated animals were not significantly different, the values were pooled (Tables 1, 2; Figure 1).

Frequency-response data

Electrical field stimulation of the perivascular nerves of the rabbit central ear artery preparations at basal tone elicited frequency-dependent (4–60 Hz) contractions which were blocked by tetrodotoxin (1 µM). There were no significant differences either across all the animal groups, or between ipsilateral and contralateral sides within each group investigated (Table 1).

Responses to exogenous noradrenaline

Cumulative application of exogenous NA (0.1–300 µM) induced concentration-dependent contractile responses in isolated ear artery preparations. These responses were not significantly different in their pD₂ values or maximal responsiveness, either across all the groups or between ipsi- and contralateral sides within each group investigated (Table 2).

Table 1 Neurogenic contractions of the rabbit central ear artery induced by electrical field stimulation (60 V, 0.3 ms pulse width, 1.0 s train duration)

	Frequency (Hz)				
	4	8	16	30	60
Sham	8.0 ± 3.5	14.7 ± 3.8	39.5 ± 8.8 (4)	70.0 ± 6.2	98.9 ± 11.1
4 Days ipsilateral	0.6 ± 0.6	7.4 ± 1.0	29.0 ± 4.4 (4)	64.3 ± 13.6	97.9 ± 19.6
Contralateral	0.2 ± 0.2	6.2 ± 2.7	24.6 ± 7.3	54.0 ± 6.4	84.4 ± 11.2
8 Days ipsilateral	3.9 ± 1.9	14.7 ± 5.3	29.3 ± 8.2 (6)	45.9 ± 10.0	65.3 ± 11.4
Contralateral	8.6 ± 4.1	17.9 ± 7.8	37.1 ± 10.5	57.5 ± 9.9	78.8 ± 9.7
16 Days ipsilateral	1.4 ± 1.4	17.2 ± 6.2	40.0 ± 8.9 (4)	60.5 ± 10.4	72.4 ± 11.6
Contralateral	3.3 ± 1.1	23.4 ± 4.3	48.4 ± 7.2	72.5 ± 8.9	87.8 ± 9.5

Preparations from sham-operated animals and animals subjected to chronic electrical stimulation of the great auricular nerve were examined. Unit of measurement is % of the response to KCl (120 mM) and data are expressed as the mean ± s.e.mean. There is no significant difference either across all the groups at each frequency examined, or between ipsi- (stimulated) and contralateral (not stimulated) sides within each group. The number in parentheses refers to the number of animals studied.

Table 2 pD_2 values and maximum contractile responses induced by exogenous noradrenaline (0.1–300 μM) added cumulatively to rabbit isolated ear artery preparations

	pD_2 values	(n)	Noradrenaline maximum response (% of responses to 120 mM KCl)
Sham	6.4 ± 0.2	4	161.5 ± 4.6
4 Days ipsilateral	6.5 ± 0.3	3	157.3 ± 4.6
Contralateral	6.3 ± 0.2	3	183.3 ± 36.5
8 Days ipsilateral	6.2 ± 0.2	6	165.2 ± 15.9
Contralateral	6.6 ± 0.1	6	155.9 ± 6.0
16 Days ipsilateral	6.9 ± 0.4	4	140.6 ± 9.1
Contralateral	6.9 ± 0.2	4	144.3 ± 9.6

Preparations from sham-operated animals and animals subjected to chronic electrical stimulation of the great auricular nerve were examined. Values represent mean $\pm$ s.e.mean, where n is the number of animals. There was no statistical significance either across all the groups investigated using either parameter, or between ipsi- and contralateral segments within each group.

Responses to exogenous α, β -methylene ATP

A single addition of α, β -MeATP (0.5, 0.1 or 10 μM) to preparations at resting tone produced a rapid concentration-dependent contraction that was not maintained. There was a tendency towards an increase in contractile responses to exogenous α, β -MeATP at all three concentrations examined in preparations from animals 4 and 8 days after chronic electrical stimulation *in vivo*, in comparison with sham-operated animals. These increases, however, were not significantly different. There were also no significant differences between ipsilateral and contralateral sides across any of the animal groups. There was, however, a significant reduction in the contractile responses induced by α, β -MeATP (10 μM) in preparations from 16-day chronically stimulated animals. Contractile responses in both ipsilateral and contralateral segments in these animals were significantly reduced in comparison with preparations from corresponding sides of 8-day chronically stimulated animals. In addition, contractile responses induced by α, β -MeATP (10 μM) on the contralateral preparations of 16-day chronically stimulated animals were also significantly attenuated in comparison with the comparable side from 4-day stimulated animals and sham-operated animals (Figure 1).

Discussion

It is evident from this study that chronic electrical stimulation of the great auricular nerve, which supplies the rabbit central ear artery with sympathetic perivascular nerves (Feldberg, 1926), does not induce changes in frequency-dependent neurogenic contractions in rabbit isolated ear artery ring preparations. This suggests that there were no changes in the prejunctional and/or postjunctional events during sympathetic cotransmission. Since, however, neurogenic contractions in the ear artery have both noradrenergic and purinergic components (Kennedy *et al.*, 1986; Saville & Burnstock, 1988), responses to exogenous NA and α, β -MeATP were also examined to see whether there were any postjunctional receptor-related changes.

The findings from exogenous cumulative concentration-response curves to NA indicate that there were no changes in either the sensitivity, represented by the pD_2 values, or the maximal responsiveness to exogenous NA. We therefore concluded that postjunctional α_1 -adrenoceptors, known to mediate the noradrenergic component of vasoconstriction in the rabbit central ear artery (Kennedy *et al.*, 1986) were unaffected by chronic electrical stimulation.

Although there were significant differences in the responses to exogenous α, β -MeATP only at the highest concentration, these trends were apparent at all the concentrations

Figure 1 Contractions induced by α, β -methylene ATP (0.5 (a), 1.0 (b) and 10.0 μM (c)) on rabbit isolated central ear artery preparations from sham-operated animals ($n = 4$) and animals subjected to chronic electrical stimulation of the great auricular nerve for 4 ($n = 4$), 8 ($n = 6$) and 16 ($n = 4$) days. Both ipsilateral (stimulated preparations represented by hatched columns) and contralateral (unstimulated preparations represented by open columns) sides are compared with sham-operated control animals (solid columns). Responses were calculated as a percentage of the response to KCl (120 mM) for each preparation and are represented as the mean ($\pm$ s.e.mean, vertical bars). Statistical analysis was performed using analysis of variance with Tukey's statistic where necessary. * $P < 0.05$; ** $P < 0.01$.

examined. The reduction in responses after 16 days of electrical stimulation in comparison with all other groups suggests that postjunctional P_{2x} -purinoceptors may have been down-regulated in this group of animals. Down-regulation is likely to have occurred due to the increased sympathetic activity (Finberg & Kopin, 1986) and release of sympathetic cotransmitters NA and ATP produced by chronic electrical nerve stimulation. An alternative explanation for the reduction in responses to exogenous α, β -MeATP is that there may have been changes in the P_{2x} -purinoceptor pharmacomechanical coupling mechanism.

Why postjunctional P_{2x} -purinoceptors appear to be selectively affected in this study is unclear. It has been reported however, that repetitive ionophoretic application of ATP results in a decrease in the amplitude of the ATP response in the rabbit ear artery (Suzuki, 1985). In addition, purine nucleosides and nucleotides interact with normal and subsensitive α -adrenoceptors to promote a more rapid rate of α -adrenoceptor resensitization (Holck & Marks, 1978). Taken together, these findings might explain why, particularly after chronic electrical stimulation, contractions to exogenous α, β -MeATP were reduced, yet neurogenic contractile responses, predominantly mediated by α_1 -adrenoceptors except at low frequencies (Kennedy *et al.*, 1986), were unchanged in our study.

The reduction of α, β -MeATP-induced responses in the unstimulated contralateral as well as the stimulated ipsilateral ear preparations may have occurred since stimulation of the great auricular nerve is likely to have increased neural activity both ortho- and antidromically in sensory and motor nerves comprising the great auricular nerve (Feldberg, 1926). Consequently, the increased activity may have been transmitted centrally. Indeed, during electrical stimulation, both ipsi- and contralateral ears of some rabbits were occasionally seen

to exhibit motor responses consistent with the frequency of stimulation.

We have previously shown that when sympathetic cotransmission is impaired, such as after a single massive dose of x-irradiation (Stewart-Lee *et al.*, 1991) or chronic acrylamide treatment (Maynard *et al.*, 1991), responses to exogenous ATP are usually increased. In this procedure, the opposite effect is observed, i.e. there is increased sympathetic activity which appears to attenuate responses to exogenous ATP. Whether there is a basic underlying mechanism involving the purinergic and noradrenergic components of sympathetic co-transmission after manipulation or injury requires further investigation. Regardless, this study supports the hypothesis that postjunctionally mediated responses to exogenous ATP are more sensitive and selectively affected initially, in contrast to noradrenergic responses which may be affected later under pathophysiological conditions (p.11 of Stjärne, 1989; Burnstock, 1990; Maynard *et al.*, 1991; Stewart-Lee *et al.*, 1991).

It is reported that transcutaneous electrical nerve stimulation reduces sympathetic tone in man (Owens *et al.*, 1979), and it has been suggested that increased physiological nerve impulses might result in a selective desensitization of α_1 -postjunctional receptors (Finberg & Kopin, 1986). Our results show that chronic electrical stimulation selectively impairs postjunctional P_{2x} -purinoceptor-mediated responses. The mechanism by which this occurs has yet to be determined.

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