British Journal of Pharmacology (1996) 118, 755-761

Presynaptic modulation by L-glutamate and GABA of sympathetic co-transmission in rat isolated vas deferens

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1 The modulatory effects of L-glutamate and its structural analogues, and of γ-aminobutyric acid (GABA), on sympathetic co-transmission were studied in the rat isolated vas deferens exposed to electrical field stimulation (EFS).

2 Application of exogenous L-glutamate caused a concentration-dependent (1 μM–3 mM) inhibition of the rapid twitch component of the biphasic EFS contraction. However, L-glutamate (1 μM–3 mM) had a minimal effect on the phasic contraction induced by exogenous adenosine 5'-triphosphate (ATP, 150 μM) and noradrenaline (50 μM). Unlike L-glutamate, D-glutamate had no effect on the EFS contraction.

3 The L-glutamate-induced inhibition of the EFS contractions was significantly attenuated by the glutamate decarboxylase (GAD) inhibitor 3-mercapto-propionic acid (150 μM) and was abolished in the presence of the GABA transaminase (GABA-T) inhibitor, 2-aminoethyl hydrogen sulphate (500 μM).

4 The L-glutamate-induced inhibition of the electrically evoked contraction was not affected by the adenosine A_1-receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX)(30 nM), reactive blue 2 (30 μM) or the $GABA_A$ receptor antagonist bicuculline (50 μM). However, the $GABA_B$ receptor antagonist 2-hydroxysaclofen (50 μM) significantly inhibited the L-glutamate effect.

5 Similar to L-glutamate, GABA also caused a concentration-dependent (0.1–100 μM) inhibition of the EFS contractions. This GABA-induced inhibition was not affected by either the $GABA_A$ receptor antagonist bicuculline (50 μM) or reactive blue 2 (30 μM). However, a significant attenuation of the GABA-mediated effect was recorded with the $GABA_B$ receptor antagonist 2-hydroxysaclofen (50 μM). Contractions of the vas deferens induced by exogenous ATP and noradrenaline were not affected by GABA (0.1–100 μM).

6 The L-glutamate analogues, N-methyl-D-aspartate (NMDA) (1 μM–1 mM) and quisqualate (Quis 0.1 μM–0.3 mM) had no effect, whilst kainate (Kain, 1 μM–1 mM) caused an inhibition of the EFS-induced contractions. Effects of Kain could be abolished by the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dioxine (CNQX, 10 μM). NMDA, Quis and Kain had no effect on the exogenous ATP- or noradrenaline-induced contractions.

7 It is concluded that the excitatory amino acid L-glutamate modulates the electrically evoked vas deferens contraction through conversion to the inhibitory amino acid GABA by a specific GABA transaminase. The GABA formed may then act on $GABA_B$ receptors and cause inhibition of the contraction through a presynaptic mechanism.

Keywords: vas deferens (rat); L-glutamate; γ-aminobutyric acid (GABA); glutamate decarboxylase; GABA transaminase

Introduction

Glutamate is one of the main amino acid candidates for the role as an excitatory synaptic neurotransmitter in the nervous system (Evans *et al.*, 1982). Besides the well known excitatory actions of glutamate, there is, as yet, no evidence to indicate whether or not this excitatory amino acid acts as a neuromodulator in peripheral organs receiving sympathetic innervation. It has been shown that glutamate receptors are present on the cell bodies of the sympathetic neurones of the rat vas deferens (Lara & Bastos-Ramos, 1988) and stimulation of these receptors by an L-glutamate analogue, kainic acid, caused a decrease in the content of [^{3}H]-noradrenaline. In rabbit brain cortex slices, stimulation of glutamate receptors also inhibits the electrically-evoked release of [^{3}H]-noradrenaline (von Kugelgen *et al.*, 1993).

On the other hand, γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system and it has been shown that GABA inhibits the contractile activity of the urinary bladder by an action on the autonomic nerves (Kusunoki *et al.*, 1984; Maggi *et al.*, 1985; Ferguson & Marchant, 1995). It has also been shown that GABA is present in a high concentration in the reproductive organs (Erdo *et al.*, 1982). These observations suggest that L-glutamate and GABA can modulate the release of other neurotransmitters in both peripheral and central structures. However, the physiological role(s) of these amino acid neurotransmitters on sympathetic neurotransmission in the rat vas deferens has not been clearly defined.

The rat vas deferens has a rich sympathetic innervation (Bell, 1972; Furness & Iwayama, 1972; Anton *et al.*, 1977) and it has been well documented that adenosine 5'-triphosphate (ATP) and noradrenaline are co-transmitters released from the sympathetic varicosities (Brown *et al.*, 1983; Sneddon & Machaly, 1992). Therefore, the vas derefens is a useful model for studying the role(s) of presynaptic glutamatergic and GABAergic mechanisms in male reproduction function (e.g. contraction of the vas deferens and ejaculation of sperm). The prostatic half of the rat vas deferens was used because it has been shown previously that the purinergic mechanism is dominant (Brown *et al.*, 1983). Thus interactions between purinoceptors and the excitatory/inhibitory amino acids can be easily studied. In the present study, modulation by the excitatory amino acid L-glutamate and its analogues, and by the inhibitory amino acid GABA, on the electrical field stimulated and agonist induced contractions of the rat isolated vas deferens was investigated. In addition, the possible existence of

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enzymatic biotransformation of L-glutamate into GABA, as demonstrated in other peripheral organs (White, 1981; Sawaki *et al.*, 1995), was also examined.

Methods

Measurement of contractile activity

Sprague–Dawley rats (250–300 g) were killed by stunning and decapitation. Vasa deferentia were rapidly removed and placed in a Petri dish containing gassed (95% O_2 and 5% CO_2, pH 7.4) modified Tyrode solution of the following composition (mM): NaCl 137, KCl 2.68, $CaCl_2$ 1.8, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, $MgCl_2$ 1.03, D-glucose 5.55. The tissues were freed of connective tissue and adhering fat, and cut transversely and only the prostatic portion (about 1 cm in length) was used. The preparations were mounted with surgical thread in 5 ml organ baths under 0.5 g resting tension. Isometric tension changes were recorded with a force-displacement transducer (Grass FT 03) coupled to either a Macintosh computer using the MacLab Chart v 3.3.3 programme or a Thermal Arraycorder (WR 7400, Graphtec). The preparations were equilibrated under 0.5 g resting tension for a period of 60 min with frequent washes and the resting tension was re-adjusted, if necessary, before commencement of the experiments.

Electrical field stimulation (EFS, delivered from a Grass Stimulator, S8800), by a pair of platinum electrodes, was achieved by trains of pulses of supramaximal voltage, 8 Hz, 0.3–0.5 ms pulse duration for 10 s every 5 min. Agonists (L-glutamate, D-glutamate, N-methyl-D-aspartate, quisqualate, kainate and GABA) were added to the organ bath cumulatively 30 s before the vas deferens was stimulated electrically. The effects of L-glutamate, D-glutamate, N-methyl-D-aspartate, quisqualate, kainate and GABA on contraction of the vas deferens elicited by the application of exogenous ATP and noradrenaline were studied in a non-cumulative manner. Only one concentration-response curve to L-glutamate and its analogues, and GABA, was constructed in each preparation. Pre-incubations with different antagonists and enzyme inhibitors were performed 90 min before the vas deferens was stimulated. In response to EFS, a two-component biphasic response was recorded. The response consisted of an initial 'twitch' which declined within 500 ms and was followed by a 'maintained' contraction (Brown *et al.*, 1983). The fast twitch which reached a maximum approximately 200 ms after the application of stimulation was considered as the maximum of the purinergic component (Anton *et al.*, 1977). The time course to peak of this fast twitch was similar to that observed in the phentolamine-pretreated vas deferens. To get some idea about the adrenergic component (sustained response) of the vas deferens bi-phasic contraction, the height of the sustained component just before the termination of the EFS was considered as the adrenergic component. Because of some 'overlapping' of the initial fast twitch (purinergic) and maintained (adrenergic) contractions of the vas deferens in response to trains of EFS (Brown *et al.*, 1983; Todorov *et al.*, 1994), it was decided to perform an accurate measurement of the adrenergic component in the α, β-methylene-ATP pretreated vas deferens. A concurrent time-matched control experiment was performed to determine the reproducibility of the EFS-induced contraction and results have shown that there was no apparent change over the time course of the experiments (Figure 1a). The vas deferens contraction (height of the fast and slow responses) before the administration of different agonists (L-glutamate, D-glutamate, N-methyl-D-aspartate, quisqualate, kainate and GABA) was taken as 100% and any change observed in the presence of drug was expressed as a percentage change of the pre-drug controls.

Drugs

L-Glutamate hydrochloride, D-glutamic acid, γ-aminobutyric acid (GABA), adenosine 5′-triphosphate (ATP) disodium, (±)-noradrenaline hydrochloride (NA), phentolamine hydrochloride, α,β-methylene-adenosine 5′-triphosphate Lithium, N-methyl-D-aspartate acid (NMDA), kainic acid (Kain), reactive blue 2, 3-mercapto-propionic acid and 2-aminoethyl hydrogen sulphate were purchased from Sigma Chemicals Company (U.S.A.). They were dissolved in de-ionised water and stored at −30°C. (+)-Bicuculline, (±)-baclofen, 2-hydroxysaclofen, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and L-quisqualic acid (Quis) were purchased from Research Biochemicals International (U.S.A.). (+)-Bicuculline and 2-hydroxysaclofen were dissolved in dimethyl sulphoxide (DMSO) and prepared just before use, whilst DPCPX was dissolved in absolute ethanol and used within one month after preparation. (±)-Baclofen was dissolved in de-ionised water, and Quis was dissolved in 0.001 M hydrochloric acid. Tetrodotoxin (TTX) was purchased from Calbiochem-Novabiochem Corporation (U.S.A.) and dissolved in de-ionised water. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) was purchased from Tocris Cookson (U.K.) and dissolved in DMSO. The effects of equivalent amounts of the different solvents (DMSO, ethanol and 0.001 M hydrochloric acid) were also tested in the preparations and results showed that these solvents had no effect on the contraction of the vas deferens.

Statistics

Data are given as mean ± s.e. mean for n experiments with statistical comparisons based on Student's unpaired t test at a $P<0.05$ level of significance. When no error bar is presented in a figure, it is smaller than the size of the symbol.

Results

Effects of L-glutamate on EFS contractions

Electrical field stimulation (EFS) of the rat isolated vas deferens produced a biphasic response: a rapid component followed by a sustained component (Figure 1a); and this biphasic response, but not exogenous 150 μM ATP- and 50 μM NA-mediated contractions (2.1 ± 0.6 g and 1.8 ± 0.4 g, respectively) ($n=5$ for each drug), was abolished by TTX (50–100 nM). Incubation with L- but not D-glutamate caused a concentration-dependent (1 μM–3 mM) inhibition of the biphasic response ($n=11$) (Figure 1b). A high concentration of L-glutamate (5 mM) was not used because it caused an increase in the basal tension of the vas deferens ($n=7$).

In order to evaluate which component (fast twitch or sustained responses) of the biphasic response was inhibited by L-glutamate, the vas deferens was either pretreated with the α-adrenoceptor antagonist phentolamine (10 μM) or rendered tachyphylactic to ATP with the stable nucleotide analogue α, β-methylene ATP (10 μM). Exogenous noradrenaline (50 μM) caused a reproducible rapid, phasic contraction (1.8 ± 0.4 g) ($n=10$), which was abolished in the presence of phentolamine (10 μM). In the EFS vas deferens, the sustained component of the biphasic response was reduced by phentolamine ($n=9$) (Figure 1c), and subsequent challenge with exogenous noradrenaline (50 μM) caused no contraction. The remaining non-adrenergic component of the biphasic response showed a 19.4 ± 3.2% increase in size, compared to control ($n=9$) (Figure 1c).

Exogenous ATP (150 μM) elicited a reproducible rapid, phasic contraction (2.1 ± 0.6 g) ($n=9$). Consecutive application of α,β-methylene ATP (10 μM) at 2 min intervals, without washouts, caused tachyphylaxis and the size of the α,β-methylene ATP-mediated contraction decreased dramatically (first dose: 4.1 ± 0.8 g, second dose: 1.1 ± 0.2 g and third dose: 0.5 ± 0.2 g tension) and no detectable contraction was recorded after the third dose of α,β-methylene ATP (10 μM) ($n=7$). Subsequent applications of exogenous ATP (150 μM) caused no contraction ($n=6$) and the result indicated that the desensitization of P_2-purinoceptors had been successfully

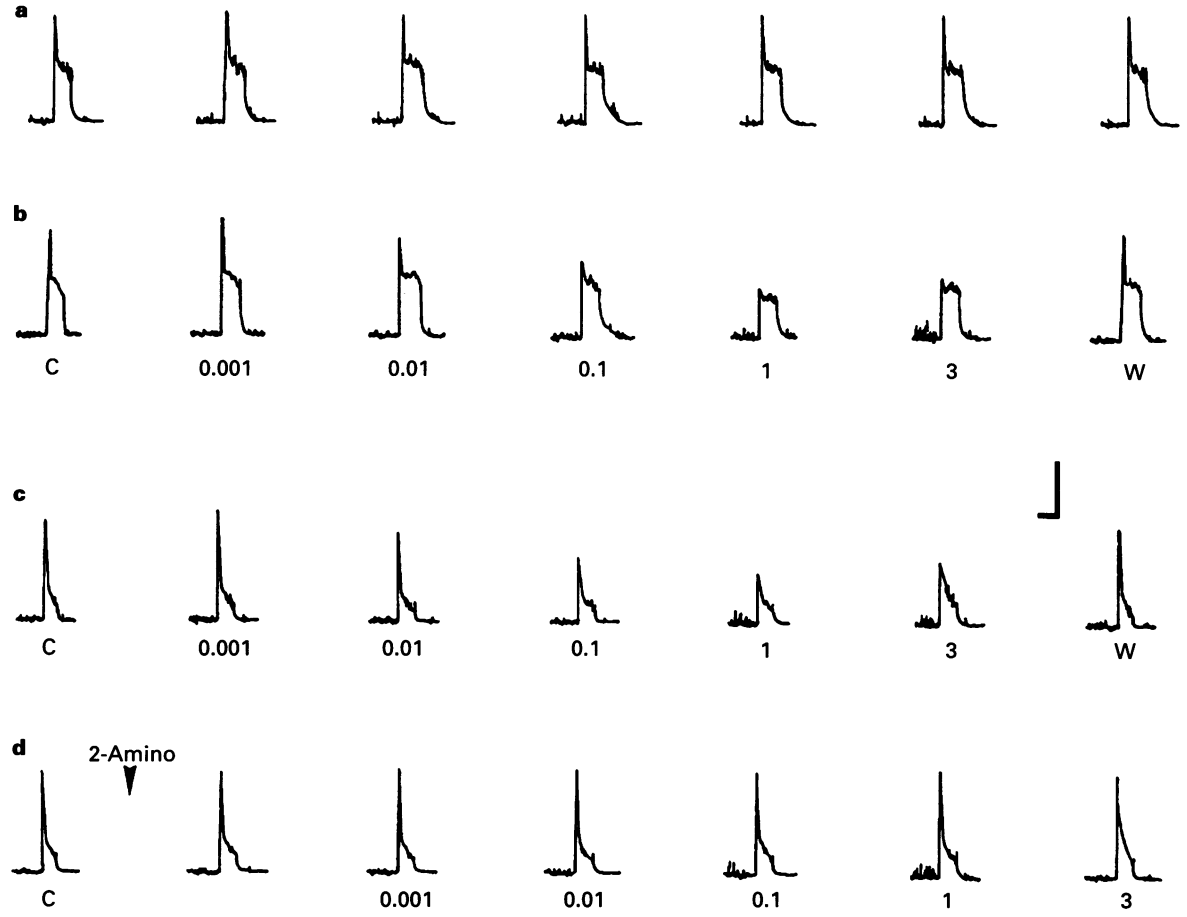


Figure 1 Representative tracings of the neurogenic contractions of the electrically field-stimulated (supramaximal voltage, 8 Hz, 0.3–0.5 ms duration for 10 s) rat isolated vas deferens. (a) Time-matched controls, and responses to increasing concentrations of exogenous L-glutamate in (b) control, (c) phentolamine (10 μM)-treated and (d) phentolamine (10 μM) and 2-aminoethyl hydrogen sulphate (500 μM) (2-Amino)-treated preparations. Final bath concentrations in mM are shown; C and W indicate control and washes, respectively. Calibration bar: 1 g and 10 s.

achieved. Following the tachyphylaxis procedure, all that remained was a small, slow, monophasic response (250 ± 17 mg tension, $n=20$) in response to the EFS. In the presence of both α,β-methylene ATP (10 μM) and phentolamine (10 μM), the electrically evoked biphasic contraction was abolished ($n=15$).

For the purinergic component (fast twitch) of the vas deferens, exogenous L-glutamate also caused inhibition of electrically induced contraction in a concentration-dependent manner and the % inhibition of contraction was similar, when compared to the untreated preparation (% inhibition caused by 3 mM L-glutamate: $42 \pm 13\%$ vs $44 \pm 17\%$) ($n=7$) (Figure 1b,c). D-Glutamate (1 μM–3 mM) had no effect on the EFS contraction ($n=4$). The inhibitory effect of L-glutamate could be reversed after washout and the electrically stimulated contraction was not significantly different from the control response ($n=7$) (Figure 1b). In contrast, application of L-glutamate had no apparent effect on the adrenergic component of the contraction ($n=7$) (control: 250 ± 17 mg; 3 mM L-glutamate: 227 ± 33 mg tension).

Effects of 3-mercapto-propionic acid, a L-glutamate decarboxylase inhibitor, on L-glutamate-induced inhibition of EFS contractions

Incubation with a L-glutamate decarboxylase (GAD) inhibitor, 3-mercapto-propionic acid (150 μM), significantly attenuated the L-glutamate-induced inhibition of the fast twitch of the biphasic response, such that only a maximal inhibition of $20 \pm 4.6\%$ was observed ($n=11$, $P<0.05$) (Figure 2). Similar effects were produced by 3-mercapto-propionic acid (150 μM) on the rapid monophasic response in the phentolamine pretreated vas deferens ($n=10$) (maximum inhibition: $24 \pm 4.1\%$). Application of 3-mercapto-propionic acid (150 μM), alone, had no effect on either the fast twitch or the sustained components of the biphasic response, the purinergic response alone, or on the basal tension of the vas deferens ($n=11$).

Effects of 2-aminoethyl hydrogen sulphate, a GABA transaminase inhibitor, on L-glutamate-induced inhibition of EFS contractions

In contrast to the GAD inhibitor, incubation with a GABA transaminase (GABA-T) inhibitor, 2-aminoethyl hydrogen sulphate (500 μM), almost abolished the L-glutamate-induced inhibition of the fast twitch of the biphasic response in 16 out of 20 preparations ($n=16$, $P<0.05$) (Figure 2); 2-aminoethyl hydrogen sulphate had no apparent effect on the other four preparations. Similar observations were recorded with 2-aminoethyl hydrogen sulphate (500 μM) on the non-adrenergic component of the vas deferens contraction in 10 out of 13 preparations ($n=10$, $P<0.05$) (Figure 1d). 2-Aminoethyl hydrogen sulphate (500 μM) alone had no effect on the fast twitch or the sustained components of the biphasic response, the purinergic component alone, or on the basal tension of the vas deferens ($n=11$).

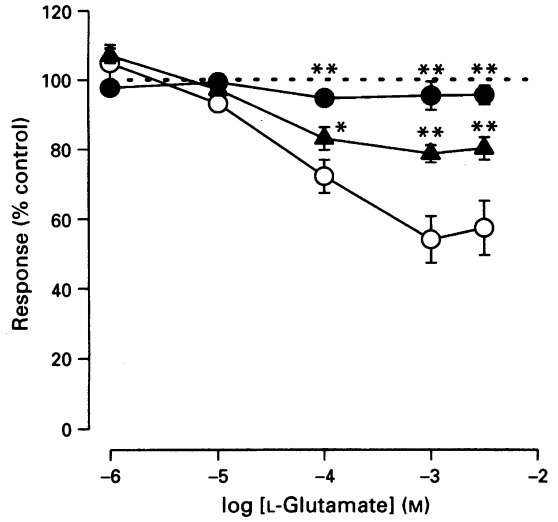


Figure 2 Cumulative concentration-response curves to L-glutamate on the fast-twitch (purinergic) neurogenic response (supramaximal voltage, 8 Hz, 0.3–0.5 ms duration for 10 s) in control (○), 3-mercaptopropionic acid (150 μM)-treated (▲) and 2 aminoethyl hydrogen sulphate (500 μM)-treated (●) preparations. Phentolamine 10 μM was present throughout the experiments. All data are expressed as mean ± s.e. mean of 11–16 preparations. The dotted line represents 100%. *$P<0.05$ and ** $P<0.001$ compared to controls.

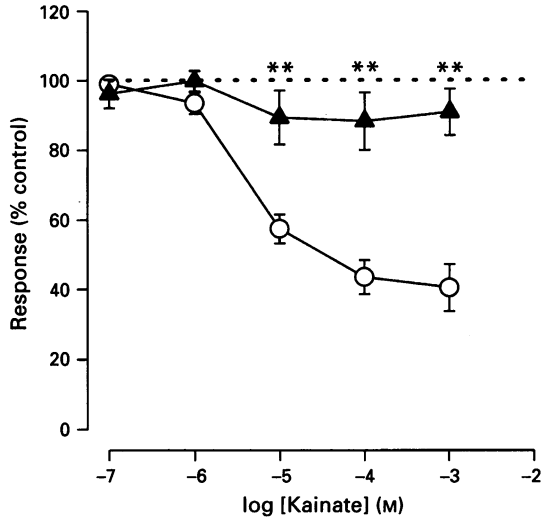


Figure 3 Cumulative concentration-response curves to kainate on the fast-twitch (purinergic) neurogenic response (supramaximal voltage, 8 Hz, 0.3–0.5 ms duration for 10 s) in control (○) and 6-cyano-7-nitroquinoxaline-2,3-dioxine (10 μM)-treated (▲) preparations. Phentolamine 10 μM was present throughout the experiments. All data are expressed as mean ± s.e. mean of 5–8 preparations. The dotted line represents 100%. **$P<0.001$ compared to controls.

Effects of D-glutamate, N-methyl-D-aspartate, quisqualate and kainate on EFS contractions

Effects of L-glutamate analogues: D-glutamate, NMDA, Quis and Kain were also examined in the vas deferens. The biphasic (both the fast twitch and the sustained components) and purinergic components of the EFS contractions were not affected by D-glutamate (1 μM–3 mM) ($n=5$), NMDA (1 μM–1 mM) ($n=6$) and Quis (0.1 μM–0.3 mM) ($n=5$). However, Kain caused a concentration-dependent (1 μM–1 mM) inhibition of the fast twitch of the biphasic response and the purinergic components of the vas deferens contractions (Figure 3) ($n=8$, $P<0.05$). The Kain-induced inhibition was abolished in the presence of the selective non-NMDA receptor antagonist CNQX (10 μM) (Figure 3) ($n=5$, $P<0.05$). D-Glutamate (1 μM–3 mM) ($n=5$), NMDA (1 μM–1 mM) ($n=6$), Quis (0.1 μM–0.3 mM) ($n=5$) and Kain (1 μM–1 mM) ($n=8$) had no effect on the adrenergic component of the EFS-induced contractions (control: 250 ± 17 mg; 3 mM D-glutamate: 223 ± 18 mg; 1 mM NMDA: 258 ± 30 mg; 0.3 mM Quis: 239 ± 20 mg and 1 mM Kain: 252 ± 28 mg tension).

Possible mediators of L-glutamate-induced inhibition

In a search for possible mediators responsible for the inhibitory effects of L-glutamate, interactions with the adenosine A_1-receptor antagonist, DPCPX (30 nM) were studied. Incubation with DPCPX (30 nM) had no apparent effect on the L-glutamate-induced inhibition of electrically evoked vas deferens contraction (non-adrenergic and biphasic responses) ($n=7$) (Figure 4). DPCPX (30 nM) itself had no effect on either the basal tension or the non-adrenergic component, or on the fast twitch of the biphasic response of the vas deferens.

The effects of the GABA receptor antagonists on L-glutamate-induced inhibition were also examined. Incubation with the $GABA_A$ antagonist, bicuculline (50 μM) ($n=7$), had no effect on the L-glutamate-induced inhibition of electrically evoked vas deferens contraction (Figure 4). However, in the presence of the $GABA_B$ antagonist, 2-hydroxysaclofen (50 μM), the L-glutamate-induced inhibition of the fast twitch was significantly attenuated ($n=8$, $P<0.05$) (Figure 4). Application of baclofen (0.1–10 μM), a $GABA_B$ receptor agonist, also caused a concentration-dependent inhibition of the fast twitch of the biphasic response and the inhibitory effect of baclofen was abolished in the presence of 2-hydroxysaclofen (50 μM) ($n=5$) (data not shown). None of these GABA receptor antagonists had an effect on either the basal tension, the non-adrenergic component, or on the fast twitch of the biphasic response of the vas deferens.

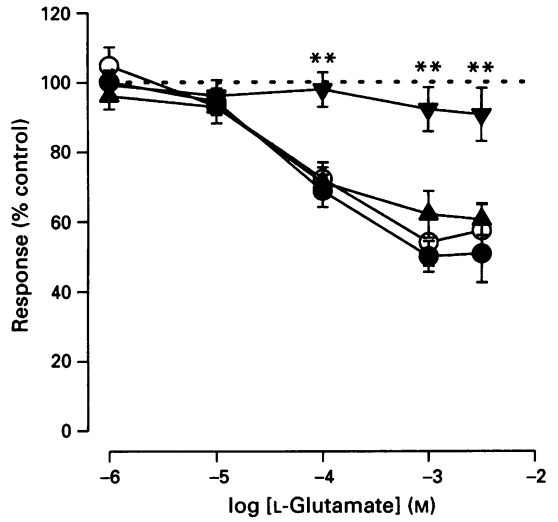


Figure 4 Cumulative concentration-response curves to L-glutamate on the fast-twitch (purinergic) neurogenic contractions (supramaximal voltage, 8 Hz, 0.3–0.5 ms duration for 10 s) in control (○), 8-cyclopentyl-1,3-dipropylxanthine (30 nM)-treated (●), bicuculline (50 μM)-treated (▲) and 2-hydroxysaclofen (50 μM)-treated (▼) preparations. Phentolamine 10 μM was present throughout the experiments. All data are expressed as mean ± s.e. mean of 7–8 preparations. The dotted line represents 100%. ** $P<0.001$ compared to controls.

In addition, the effect of the P_{2Y}-receptor blocker, reactive blue 2 (30 μM), was tested. Reactive blue 2 (30 μM) itself had no effect on either the basal tension or the non-adrenergic component and biphasic response of the vas deferens. The

presence of reactive blue 2 (30 μM) did not affect the L-glutamate-induced inhibition of electrically evoked vas deferens contraction ($n=4$) (data not shown).

Effects of GABA on EFS contractions

Similar to L-glutamate, GABA caused a concentration-dependent (0.1–100 μM) inhibition of the electrically stimulated vas deferens contraction (both the biphasic and the non-adrenergic phasic components) ($n=13$) (Figure 5). The GABA-induced inhibition was not affected by the $GABA_A$ antagonist bicuculline (50 μM) ($n=7$) but was significantly attenuated by the $GABA_B$ antagonist 2-hydroxysaclofen (50 μM) ($n=8$, $P<0.05$) (Figure 5). Similar to L-glutamate, pre-incubation with GABA (0.1–100 μM) had no effect on the adrenergic component of the contraction ($n=7$). The presence of reactive blue 2 (30 μM) did not affect the GABA-induced inhibition of electrically evoked vas deferens contraction ($n=4$) (data not shown).

Effects of L-glutamate, D-glutamate, NMDA, Quis, Kain and GABA on the exogenous ATP-induced vas deferens contraction

Exogenous application of ATP (150 μM) caused a reproducible rapid, phasic contraction (2.1±0.6 g) ($n=9$). Incubation with L-glutamate (1 μM–1 mM) had no apparent effect on the exogenous ATP-induced contraction ($n=7$). With the highest concentration of L-glutamate tested (3 mM), the ATP-induced contraction was reduced by 18.8±3.2%, compared to the control response ($n=7$, $P<0.05$). The presence of D-glutamate (1 μM–3 mM) ($n=4$), GABA (0.1–100 μM) ($n=8$), NMDA (1 μM–1 mM) ($n=8$), Quis (0.1 μM–0.3 mM) ($n=7$) and Kain (1 μM–1 mM) ($n=5$) had no effect on the exogenous ATP-induced contraction (control: 2.1±0.6 g; 3 mM D-glutamate: 1.8±0.6 g; 100 μM GABA: 2.0±0.4 g; 1 mM NMDA: 1.8±0.8 g; 0.3 mM Quis: 2.3±0.5 g and 1 mM Kain: 1.9±0.5 g tension).

Effects of L-glutamate, D-glutamate, NMDA, Quis, Kain and GABA on the exogenous noradrenaline-induced vas deferens contraction

Exogenous noradrenaline (50 μM) caused a reproducible, phasic contraction (1.8±0.4 g) ($n=10$) of the rat vas deferens. Incubation with either L-glutamate (1 μM–3 mM) ($n=8$), D-glutamate (1 μM–3 mM) ($n=4$), GABA (0.1–100 μM) ($n=7$), NMDA (1 μM–1 mM) ($n=8$), Quis (0.1 μM–0.3 mM) ($n=7$) and Kain (1 μM–1 mM) ($n=5$) had no effect on the exogenous noradrenaline-induced vas deferens contraction (control: 1.8±0.4 g; 3 mM L-glutamate: 1.7±0.5 g; 3 mM D-glutamate: 1.5±0.8 g; 100 μM GABA: 2.0±0.5 g; 1 mM NMDA: 1.6±0.7 g; 0.3 mM Quis: 2.0±0.7 g and 1 mM Kain: 1.7±0.7 g tension).

Discussion

Since the pioneering work of Sneddon and Burnstock (1984) on guinea-pig vas deferens, it has been generally agreed that ATP and noradrenaline are co-transmitters and are probably stored and released from the same exocytotic vesicles. Therefore, according to this hypothesis, any substance which affects the release of ATP from the vesicles should also affect the release of noradrenaline. However, in our study, L-glutamate, kainate and GABA only affected the purinergic but not the adrenergic component of the vas deferens contraction in response to EFS. The 'differential effect' of L-glutamate, GABA as well as kainate on the two components of the EFS-induced contraction could indicate that ATP and noradrenaline are released from two vesicles (Todorov *et al.*, 1994), instead of a single exocytotic vesicle.

In the present study, the excitatory amino acid L-glutamate, instead of producing its well known excitatory effects, caused a concentration-dependent inhibition of the vas deferens contraction (both the fast twitch of the biphasic response and non-adrenergic components) elicited by EFS. The involvement of physiological prejunctional auto-inhibition caused by the released ATP on P_2- purinoceptor (von Kugelgen *et al.*, 1994) is ruled out in the present study because the presence of reactive blue 2, a prejunctional P_2-purinoceptor antagonist, did not affect the L-glutamate mediated inhibition of vas deferens contraction.

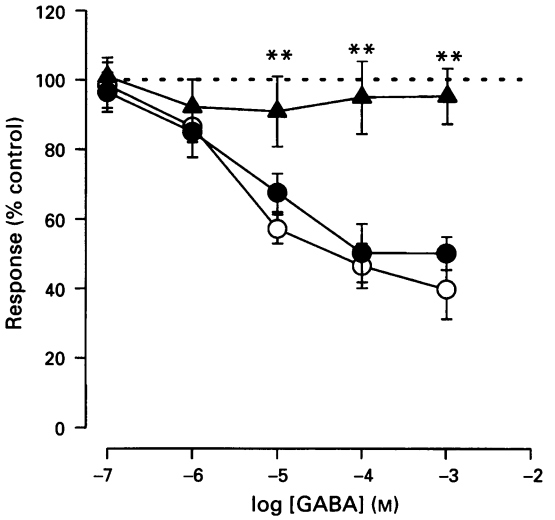
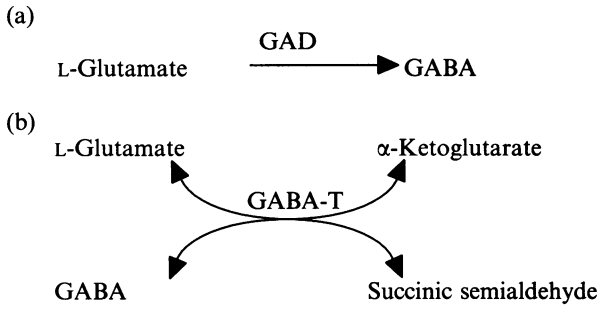


Figure 5 Cumulative concentration-response curves to GABA on the fast-twitch (purinergic) neurogenic contractions (supramaximal voltage, 8 Hz, 0.3–0.5 ms duration for 10 s) in control (○), bicuculline (50 μM)-treated (●) and 2-hydroxysaclofen (50 μM)-treated (▲) preparations. All data are expressed as mean±s.e. mean of 7–13 preparations. Phentolamine 10 μM was present throughout the experiments. The dotted line represents 100%. ** $P<0.001$ compared to controls.

It has been reported that L-glutamate inhibits the release of a neurotransmitter via adenosine (von Kugelgen *et al.*, 1992b) derived from neurogenically released ATP (Daly, 1982). Adenosine activates the presynaptic A_1-purinoceptors (von Kugelgen *et al.*, 1992a), causing inhibition of neurotransmitter release (Taylor *et al.*, 1983; von Kugelgen *et al.*, 1992b). In our study, the presence of the selective A_1 receptor antagonist DPCPX (Lohse *et al.*, 1987) did not reverse the inhibitory effects of L-glutamate on electrically evoked vas deferens contraction. Therefore, the involvement of presynaptic inhibition via the activation of an A_1 receptor is very unlikely.

Modulation by L-glutamate of the postsynaptic P_2-purinoceptor- and α_1-adrenoceptor-mediated contractions of the vas deferens was also investigated. However, in contrast to EFS experiments, contraction induced by exogenous noradrenaline was not affected by L-glutamate over the same concentration range (1 μM–3 mM) used. On the other hand, L-glutamate did not affect the exogenous ATP-induced contraction with concentrations below 3 mM, with a 19% decrease of the ATP-induced contraction being observed with the highest concentration of L-glutamate (3 mM) tested. These results strongly suggest that L-glutamate only has a minimal effect on the postsynaptic P_2-purinoceptors, and no effect on the α_1-adrenoceptors.

The intriguing inhibitory effect of the excitatory amino acid L-glutamate may be due to L-glutamate causing either the release of another neurotransmitter (Pin & Bockaert, 1989; Hofmann & Mockel, 1991; Ferreira *et al.*, 1994) or the enzymatic biotransformation of L-glutamate into the inhibitory amino acid GABA (White, 1981; Sawaki *et al.*, 1995), as occurs in the Krebs' cycle. In the Krebs' cycle, L-glutamate can be transformed into GABA by two pathways:



The biotransformation of exogenous L-glutamate into GABA via the above enzymatic reactions has been detected in peripheral organs (White, 1981; Sawaki *et al.*, 1995). To explore the possible pathway(s) involved in the formation of GABA in the present L-glutamate experiments, enzyme inhibitors for both pathways were used. 3-Mercapto-propionic acid inhibits glutamate decarboxylase (GAD) activity in rat liver ($IC_{50} = 100$ μM, White, 1981) and 2-aminoethyl hydrogen sulphate causes an irreversible inhibition of GABA-T *in vitro* in rat kidney ($IC_{50} = 300$ μM) and *in vivo* (Fowler & John, 1972; White, 1981; Qume *et al.*, 1995). Pre-incubation with 3-mercapto-propionic acid reduced L-glutamate responses by about 50–60%. However, the L-glutamate-induced inhibition of vas deferens contraction was almost abolished in the presence of 500 μM 2-aminoethyl hydrogen sulphate (90 min pre-incubation). It has been shown that pre-incubation with 2-aminoethyl hydrogen sulphate for 90 min caused more than 90% inactivation of GABA-T activity (Fowler & John, 1972). Therefore, these results are consistent with the observations that GABA-T plays a more significant role than GAD in the formation of GABA from L-glutamate in the peripheral organs e.g. liver and kidney (White, 1981). Involvement of a specific enzymatic reaction in the biotransformation of exogenous L-glutamate into GABA is further supported by the observations that the L-glutamate analogues: D-glutamate, N-methyl-D-aspartate and quisqualate had no effect on the vas deferens contraction. This is probably due to the fact that the enzyme involved cannot convert these compounds into GABA.

To confirm the involvement of GABA and the type(s) of GABA receptor on the inhibitory effects of L-glutamate on EFS-induced vas deferens contractions, the $GABA_A$ receptor antagonist, bicuculline (Curtis *et al.*, 1970), and $GABA_B$ receptor antagonist, 2-hydroxysaclofen (Kerr *et al.*, 1988), were examined. In the present study, the L-glutamate-mediated inhibition of electrically evoked vas deferens contraction was not affected by bicuculline but was abolished by 2-hydroxysaclofen (50 μM). In addition, administration of the $GABA_B$ agonist baclofen also inhibited the electrically evoked vas deferens contraction and this inhibitory effect was blocked by 2-hydroxysaclofen (50 μM). Similar observations have been made in guinea-pig vas deferens preparations (Kerr *et al.*, 1988). These results provide another piece of evidence to suggest that the L-glutamate-mediated inhibition of EFS induced vas deferens contraction involves the biotransformation of L-glutamate into GABA, and that $GABA_B$ receptors are involved.

In contrast to NMDA, Quis and D-glutamate, administration of kainate, another L-glutamate analogue, inhibited electrically evoked vas deferens contraction. This analogue probably acts through a mechanism different from L-glutamate (Gonzales *et al.*, 1995), as the inhibitory effect of kainate, but not L-glutamate, could be abolished by the non-NMDA receptor antagonist CNQX (Watkins *et al.*, 1990). These results suggest that the inhibitory effect of kainate on the EFS vas deferens contraction is mediated by non-NMDA receptors.

In addition, the present results suggest that effects of L-glutamate and GABA are mediated through a presynaptic mechanism because exogenous ATP- (except with the highest concentration of L-glutamate) and noradrenaline-induced contractions of the vas deferens were not affected by these drugs. In addition, the electrically stimulated vas deferens contraction was abolished by tetrodotoxin, thereby indicating that the responses are neurogenic, as tetrodotoxin suppresses neuronal depolarization (Narahashi, 1974). However, it has been shown that sympathetic nerve stimulation elicits an overflow of purines including ATP from the innervated tissue. The overflow is partly neural in origin and partly from the effector cells (Westfall *et al.*, 1978; Rump & von Kugelgen, 1994). In our present study, the 'incomplete' inhibition by L-glutamate, GABA and kainate on the electrically stimulated vas deferens contraction, at its highest concentration, may represent the residual fast twitch response of the vas deferens which is due to the release of ATP from the vas deferens smooth muscle cells (Kurz *et al.*, 1994).

Nevertheless, results obtained from the present study suggest that exogenous L-glutamate inhibits EFS-induced vas deferens contraction via the enzymatic formation of the inhibitory amino acid, GABA. The GABA formed and the exogenous GABA inhibit only the neuronal purinergic component by activating presynaptic $GABA_B$ receptors (Schlicker *et al.*, 1984; Gray & Green, 1987). However, our results at the present stage cannot provide any conclusion about the underlying mechanism(s) involved in the presynaptic $GABA_B$ receptor-mediated inhibition of the purinergic component of the vas deferens contraction. On the other hand, the L-glutamate analogue, kainate, produces its inhibitory effect via the activation of non-NMDA receptors.

The authors wish to thank Prof. R.L. Jones for his critical comments on the earlier version of the manuscript and Mr. G. Stenton for proof reading. The financial support from the Shaw College Student Summer Work Scheme is also acknowledged (K.-Y. T).

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(Received December 5, 1995
Revised February 5, 1996
Accepted February 13, 1996)