Differentiation by pyridoxal 5-phosphate, PPADS and IsoPPADS between responses mediated by UTP and those evoked by α,β -methylene-ATP on rat sympathetic ganglia

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1 The effect of pyridoxal 5-phosphate, and the 2',4' and 2',5'-disulphonic acid isomers of 6-azophenyl-pyridoxal 5-phosphate (PPADS and IsoPPADS respectively) on depolarization of the rat superior cervical ganglion evoked by α,β -methylene-adenosine 5'-triphosphate (α,β -Me-ATP) and uridine 5'-triphosphate (UTP) were determined by a grease-gap recording technique.

2 Pyridoxal 5-phosphate (10–100 μ M) and PPADS (10–100 μ M) enhanced UTP- and depressed α,β -Me-ATP-evoked depolarizations but did not significantly alter depolarizations evoked by potassium or hyperpolarizations evoked by adenosine. IsoPPADS (10 μ M) depressed α,β -Me-ATP-evoked depolarizations but did not alter depolarizations evoked by UTP. Depolarizations evoked by muscarine were depressed by IsoPPADS but not by pyridoxal 5-phosphate.

3 It is concluded that pyridoxal 5-phosphate, PPADS and IsoPPADS are antagonists at P_{2X} -purinoceptors but not at the receptors that mediate UTP-evoked depolarization of the rat superior cervical ganglion. These observations substantiate the recent proposal that the rat superior cervical ganglia possess distinct receptors for purine and pyrimidine 5'-nucleotides, i.e. P_{2X} -purinoceptors and pyrimidinoceptors respectively.

Keywords: Rat superior cervical ganglion; UTP; pyridoxal-5-phosphate; PPADS; IsoPPADS; pyrimidinoceptor; P_{2X} -purinoceptor

Introduction

It has been suggested that some tissues contain extracellular receptors that recognise the purine nucleotide, adenosine 5'-triphosphate (ATP) and the pyrimidine nucleotide, uridine 5'-triphosphate (UTP), having equal affinity for ATP and UTP (Davidson *et al.*, 1990; reviewed by O'Connor *et al.*, 1991), i.e. a P_{2U} -purinoceptor. In addition it has also been proposed that ATP and UTP can activate distinct receptors, i.e. P_2 -purinoceptors and pyrimidinoceptors respectively (reviewed by Seifert & Schultz, 1989). Pyrimidinoceptors have been suggested to exist on some vascular tissues, e.g. rabbit basilar and ear arteries (von Kugelgen *et al.*, 1987; von Kugelgen & Starke, 1990), the perfused isolated rat liver (Haussinger *et al.*, 1987), and on these tissues UTP rather than ATP activates pyrimidinoceptors. In contrast to these studies there have been few studies to examine the effects of pyrimidines on the nervous system or to determine if their effects are due to activation of pyrimidinoceptors.

Recently it has been proposed that neurones of the rat superior cervical ganglion (SCG) possess pyrimidinoceptors and that this preparation is a model tissue for the study of pyrimidinoceptors within the nervous system (Connolly & Harrison, 1993; Connolly *et al.*, 1993b; Connolly & Harrison, 1994; Connolly, 1994a). This hypothesis was based upon the observation that there are distinct receptors that are activated by UTP but not by ATP or α,β methylene adenosine 5'-triphosphate (α,β -Me-ATP) (a selective P_{2X} -purinoceptor agonist (Burnstock & Kennedy, 1985)). In addition SCG neurones possess P_1 -purinoceptors of the A_1 -subtype, which upon activation by adenosine or ATP (which presumably activates these receptors due to its rapid metabolism to adenosine) hyperpolarize ganglia. In contrast both α,β -Me-ATP and UTP depolarized rat SCG neurones and depolarizations elicited by UTP were not antagonized, rather they were enhanced in the presence of either suramin (a

P_{2X} -purinoceptor antagonist) or reactive blue 2 (a P_{2Y} -purinoceptor antagonist) (see Connolly *et al.*, 1993b; Connolly & Harrison, 1994 respectively). Recently it has been proposed that pyridoxal 5-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) is a novel and selective antagonist of P_{2X} -purinoceptors (Lambrech *et al.*, 1992; Ziganshin *et al.*, 1993; McLaren *et al.*, 1994) however, the effect of PPADS on UTP-mediated responses has yet to be reported. Here the effects of pyridoxal 5-phosphate (a precursor of PPADS), PPADS and an isomer of PPADS, namely the 2',5'-disulphonic acid derivative of 6-azophenyl-pyridoxal 5-phosphate (IsoPPADS) on the responses of the rat SCG evoked by α,β -Me-ATP, adenosine and UTP are described. Preliminary reports of this study have been presented elsewhere (Connolly *et al.*, 1994; Connolly, 1994b).

Methods

Ganglia were prepared for recording of d.c. potentials via the internal carotid nerve and the body of the ganglion as described before (Connolly *et al.*, 1993a,b). SCG were removed from male Sprague-Dawley rats (200–330 g) killed with a lethal dose of urethane, desheathed and submerged between the greased barriers of a three compartment recording bath. The central chamber containing the ganglion body was superfused (about 2 ml min⁻¹) with a physiological salt solution (PSS) at pH 7.4 and 25 $\pm$ 1°C, containing (mM): NaCl 125, NaHCO₃ 25, KCl 1, KH₂PO₄ 1, MgSO₄ 1, glucose 10, CaCl₂ 0.1 and pre-oxygenated (5% CO₂/95% O₂). Potential differences between the earthed central chamber and the internal carotid nerve were recorded with Ag/AgCl electrodes, the signals being filtered through a low pass filter, amplified via d.c. pre-amplifiers and recorded on a pen recorder.

Ganglia were equilibrated for at least 2 h before use. The effect of potassium and muscarine or the effects of purines and UTP on the d.c. potentials were determined by applying

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these substances for 1 or 2 min respectively. To avoid desensitization a minimum of 15 min was allowed between each application and responses to all agonists were reproducible with time (e.g. see results Figure 1).

With the exception of pyridoxal solutions all drugs were prepared in PSS as 10 mM stock solutions and frozen as aliquots (-20°C). Pyridoxal solutions were prepared immediately before use (as 10 mM stock solutions in PSS) and protected from light. Experiments with pyridoxal 5-phosphate were done in near darkness. In some experiments the effect of pyridoxal 5-phosphate (at 30 or $100\text{ }\mu\text{M}$) on agonist-induced responses was determined and because after washing out ($>25\text{ min}$) pyridoxal 5-phosphate there was no significant difference in the control and subsequent responses (e.g. see results Figure 1), these ganglia were also used to determine the effect of PPADS. There was no significant difference between the effects of PPADS when determined on either ganglia previously exposed to pyridoxal 5-phosphate or when only PPADS was examined. In all other experiments the effect of only pyridoxal 5-phosphate or one of its derivatives at one concentration was examined on each ganglion. Responses in the presence of pyridoxal 5-phosphate, PPADS or IsoPPADS (30 to 150 min incubation) were compared (Student's paired t test) to control responses without

each drug. P values were considered statistically significant if less than 0.05. Differences between responses are denoted by: $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Adenosine hemisulphate, α,β -Me-ATP and UTP were obtained from Sigma Chemical Co. U.K. Pyridoxal 5-phosphate, PPADS and IsoPPADS were a gift from Tocris-Cookson, Langford, Bristol, U.K. ($\pm$)-Muscarine was bought from Research Biochemicals Inc. (Semat, U.K.) and salts for PSS were of Analar grade.

Results

Pyridoxal 5-phosphate, PPADS or IsoPPADS did not significantly alter the basal d.c. potential of ganglia (data not presented, but see Figure 1), depolarizations evoked by potassium, or hyperpolarizations evoked by adenosine (Figure 1 and Table 1). Depolarizations evoked by muscarine were significantly depressed by IsoPPADS but not PPADS (Table 1). In the presence of IsoPPADS ($10\text{ }\mu\text{M}$) of three out of fifteen ganglia that were depolarized by muscarine (control), two produced a hyperpolarization ($-55\text{ }\mu\text{V}$ and $-140\text{ }\mu\text{V}$) preceding a depolarization and on a third ganglion muscarine evoked only a hyperpolarization (control = $140\text{ }\mu\text{V}$

Figure 1 Effect of pyridoxal 5-phosphate (PP) and pyridoxal 5-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) on the d.c. potential and responses of a single rat superior cervical ganglion evoked by potassium, adenosine, uridine 5'-triphosphate (UTP) and α,β -methylene adenosine 5'-triphosphate (α,β -Me-ATP). Responses evoked by 5 mM potassium (K^+), $100\text{ }\mu\text{M}$ adenosine, $100\text{ }\mu\text{M}$ UTP and $100\text{ }\mu\text{M}$ α,β -Me-ATP in (a) the absence (control), (b) presence of $100\text{ }\mu\text{M}$ PP, (c) after washing out PP (washout) and (d) in the presence of $100\text{ }\mu\text{M}$ PPADS. The scale in the bottom right hand corner of the trace applies to all the traces, which are a continuous record from a single ganglion. The bars refer to the time of application of drugs and depolarization is shown as an upward deflection of the trace. The open, solid and double open arrows show the times at which PSS was changed to PSS containing PP, from PP to PSS and then to PSS containing PPADS. N.B. The d.c. potential did not alter upon changing from one superfusate to another and the depolarizations evoked by α,β -Me-ATP were reduced in the presence of PP and reversed to a hyperpolarization in the presence of PPADS.

Table 1 Effect of pyridoxal 5-phosphate, PPADS and IsoPPADS on the response of the rat superior cervical ganglion evoked by adenosine, potassium and muscarine

Compound	Concentration (μM)	n	Control ($0 \mu\text{M}$)	Pyridoxal 5-phosphate ($100 \mu\text{M}$)	PPADS ($10 \mu\text{M}$)	PPADS ($100 \mu\text{M}$)	IsoPPADS ($10 \mu\text{M}$)
Adenosine	100	4	-116 ± 15	-138 ± 22			
	100	4	-134 ± 27		-131 ± 8		
	100	4	-156 ± 31			-181 ± 37	
	100	15	-124 ± 14				-126 ± 18
Potassium	5000	4	169 ± 15	199 ± 22			
	5000	4	146 ± 18		134 ± 24		
	5000	4	165 ± 41			175 ± 29	
	5000	15	181 ± 22				165 ± 25
Muscarine	0.1	4	243 ± 23		223 ± 43		
	0.1	15	207 ± 26				$115 \pm 28^{***}$

Responses are $\mu\text{V} \pm \text{s.e. mean}$ and n = number of ganglia. Statistical differences between responses (paired t test) in the absence (control) and presence of pyridoxal 5-phosphate, PPADS or IsoPPADS are indicated by: $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Figure 2 Effect of pyridoxal 5-phosphate-6-azophenyl-2',5'-disulphonic acid (IsoPPADS) on concentration-response curve of the depolarization of rat superior cervical ganglion evoked by uridine 5-triphosphate (UTP) or α,β -methylene-adenosine 5'-triphosphate (α,β -Me-ATP). Summary of the response of a minimum of eight ganglia evoked by UTP (circles) or α,β -Me-ATP (triangles) in the absence ($\circ, \Delta$) and presence ($\bullet, \blacktriangle$) of $10 \mu\text{M}$ IsoPPADS respectively. Statistical differences between depolarizing responses in the absence and presence of IsoPPADS are indicated by: $*P < 0.05$ and $***P < 0.001$.

cf in IsoPPADS = $-140 \mu\text{V}$). Depolarizations evoked by $100 \mu\text{M}$ α,β -Me-ATP were significantly depressed by IsoPPADS ($10 \mu\text{M}$) by $43 \pm 6\%$ ($n = 15$) whereas depolarizations evoked by $100 \mu\text{M}$ UTP were not significantly altered ($-2 \pm 11\%$, $n = 15$). IsoPPADS ($10 \mu\text{M}$) significantly depressed concentration-dependent depolarizations evoked by α,β -Me-ATP but not depolarizations evoked by UTP (Figure 2).

Both pyridoxal 5-phosphate and PPADS produced a concentration-dependent enhancement of depolarizations evoked by UTP and a concentration-dependent depression of depolarizations evoked by α,β -Me-ATP (Figure 3). On some

Figure 3 Effect of pyridoxal 5-phosphate and pyridoxal 5-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) on the depolarizations of the rat superior cervical ganglion evoked by $100 \mu\text{M}$ α,β -Me-ATP (α,β -Me-ATP) and $100 \mu\text{M}$ uridine 5-triphosphate (UTP). Summary of the response of four to eight ganglia evoked by UTP (circles) or α,β -Me-ATP (triangles) in the presence of pyridoxal 5-phosphate (PP) ($\circ, \Delta$) or PPADS ($\bullet, \blacktriangle$) respectively. Responses are expressed as a percentage of the response evoked by UTP or α,β -Me-ATP in the absence of pyridoxal 5-phosphate or PPADS. Statistical differences between responses in the absence and presence of pyridoxal 5-phosphate or PPADS are indicated by $*P < 0.05$ and $**P < 0.01$.

ganglia, pyridoxal 5-phosphate containing compounds as well as potentiating the peak response evoked by UTP, sharpened the time-course of the depolarization.

Discussion

The inability of pyridoxal 5-phosphate, PPADS or IsoPPADS to alter responses evoked by sub-maximal concentrations of either adenosine (which activates P_1 -purinoceptors; Connolly *et al.*, 1993a) or potassium (a nonspecific depolarizing agonist) suggests that these drugs do not antagonize A_1 -adenosine receptors or produce nonspecific effects on rat SCG neuronal membranes respectively. The inactivity of PPADS on adenosine-induced hyperpolarizations of the rat SCG is consistent with its reported lack of antagonism of A_1 -adenosine receptors on the rabbit vas deferens (Lambrecht *et al.*, 1992).

The ability of PPADS, a putatively selective P_{2X} -purinoceptor antagonist (Lambrecht *et al.*, 1992; McLaren *et al.*, 1994) to depress depolarizations of the rat SCG evoked by α,β -Me-ATP (a selective P_{2X} -purinoceptor agonist, Burnstock & Kennedy, 1985) indicates these depolarizations were mediated by P_{2X} -purinoceptors. Similarly the ability of

pyridoxal 5-phosphate and IsoPPADS to depress depolarizations of the rat SCG evoked by α,β -Me-ATP indicates that these compounds are also antagonists of P_{2X} -purinoceptors. The receptor responsible for the hyperpolarization evoked by α,β -Me-ATP in the presence of PPADS is unknown. This hyperpolarization is unlikely to involve P_{2X} - or P_{2Y} -purinoceptors because of its resistance to a high concentration of PPADS and the virtual inactivity of 2-methylthio-ATP (a P_{2Y} -purinoceptor selective agonist) on the rat SCG (Connolly *et al.*, 1993b).

The similar degree of antagonism of depolarizations evoked by α,β -Me-ATP by both PPADS and IsoPPADS suggests that the position of the sulphonic acid group at the 4'- or 5'- position on 6-azophenylpyridoxal 5-phosphate is not particularly critical for the antagonistic activity of these compounds. Pyridoxal 5-phosphate had some, albeit weak antagonistic activity at P_2 -purinoceptors, indicating that the pyridoxal 5-phosphate moiety of both PPADS and IsoPPADS is partly responsible for their antagonism at P_2 -purinoceptors. However, the addition of the 6-azophenyl-disulphonic acid moiety clearly enhances the antagonistic potency of pyridoxal 5-phosphate at P_{2X} -purinoceptors.

IsoPPADS but not pyridoxal 5-phosphate altered depolarizations evoked by muscarine suggesting that IsoPPADS is an antagonist of muscarinic receptors of the rat SCG. The ability of IsoPPADS to depress depolarizations evoked by muscarine and reveal a muscarine-induced hyperpolarization on some ganglia is consistent with a selective depression of M_1 -muscarinic receptor-mediated depolarization but not M_2 -muscarinic receptor-mediated hyperpolarization. The observation that IsoPPADS depressed depolarizations of the rat SCG evoked by muscarine but not those produced by UTP also confirms the proposal that muscarine and UTP activate distinct receptors on the rat SCG (Connolly *et al.*, 1993b).

Over the range of concentrations examined, pyridoxal 5-phosphate, PPADS and IsoPPADS were never observed to depress responses evoked by UTP, only depressing depolarizations mediated by α,β -Me-ATP on the same ganglion. These results show the selectivity of these pyridoxal 5'-phosphate containing compounds for purine- rather than pyrimidine-nucleotide evoked depolarizations of the rat SCG, supporting the proposal that α,β -Me-ATP and UTP activate distinct receptors for nucleotides.

The mechanism by which pyridoxal 5-phosphate containing compounds potentiate UTP-evoked depolarizations of the rat SCG remains obscure, but it is unlikely to be due to an antagonism of an opposing P_{2Y} -purinoceptor mediated hyperpolarization, because of the virtual absence of P_{2Y} -purinoceptors on the rat SCG (Connolly *et al.*, 1993a). Interestingly, an enhancement of depolarizations of the rat SCG evoked by UTP was observed in the presence of the P_2 -purinoceptors antagonists, suramin (Connolly *et al.*, 1993b) and reactive blue 2 (Connolly & Harrison, 1994). Thus one explanation of the potentiating effect of P_2 -purinoceptor antagonists on UTP-evoked depolarizations is that this phenomenon is coupled to their ability to depress depolarizations evoked by α,β -Me-ATP. However the link between this potentiating effect and P_2 -purinoceptor antagonism may be coincidental.

Evidence for these effects being independent of each other is suggested by the ability of IsoPPADS to depress depolarizations evoked by α,β -Me-ATP without any significant effect on UTP-evoked depolarizations. Here it should be noted that the effect of IsoPPADS was examined at only one concentration (10 μ M) and thus it is possible that at higher concentrations IsoPPADS, like PPADS, would also have enhanced UTP-evoked depolarizations. Conversely the ability of reactive blue 2 (Connolly & Harrison, 1994, see Figure 1d) and uniblue A (unpublished observations) to enhance UTP-evoked depolarizations without altering depolarization evoked by α,β -Me-ATP suggests these two effects may be independent of each other. Alternative explanations for the potentiation of UTP-evoked depolarizations include antagonism of an underlying UTP-evoked hyperpolarization, a change in the metabolism of UTP or its metabolites or an allosteric interaction. These effects remain to be investigated, but some evidence in favour of the latter hypotheses is suggested by the observation that on some ganglia pyridoxal 5-phosphate containing compounds as well as potentiating the peak response evoked by UTP, sharpened the time-course of the response. Because unlike UTP, α,β -Me-ATP is relatively resistant to degradation by ectonucleotidases (Welford *et al.*, 1986; 1987), the ability of pyridoxal phosphate and PPADS to enhance depolarizations evoked by UTP (but depress α,β -Me-ATP-evoked depolarizations) could have arisen from the net effect of inhibition of ectonucleotidase activity and an antagonism of P_{2X} -purinoceptors, thus raising the possibility that UTP and α,β -Me-ATP activate the same receptor. An inhibition of ectonucleotidase activity by suramin (Hourani & Chown, 1989) could also account for the ability of suramin (but not reactive blue 2, which does not inhibit ectonucleotidases, Hourani & Chown, 1989) to enhance UTP-evoked depolarizations. Apart from studies using P_2 -purinoceptor antagonists there is additional evidence that ATP and UTP activate distinct receptors, which is based upon desensitization studies (Connolly, 1994a). Further experiments to examine the effect of specific inhibitors of ectonucleotidases on purine and pyrimidine evoked responses of the rat SCG should help resolve these issues.

Because there are no suitable antagonists of the effects of UTP at pyrimidinoceptors (Seifert & Schultz, 1989) further studies to examine the modulatory effects of pyridoxal 5-phosphate and its derivatives on responses evoked by pyrimidines may lead to further discoveries on how pyrimidines (and purines) work on the nervous system.

It is concluded that pyridoxal 5-phosphate, PPADS and IsoPPADS are antagonists at P_{2X} -purinoceptors and like other P_2 -purinoceptor antagonists they distinguish between purine and pyrimidine 5'-triphosphate-evoked depolarizations of the rat superior cervical ganglion. These results provide further justification for the proposal that SCG neurones possess both P_{2X} -purinoceptors and pyrimidinoceptors.

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