Electrophysiological subtypes of inhibitory P₁ purinoceptors on myenteric neurones of guinea-pig small bowel

¹F.L. Christofi & J.D. Wood

Department of Physiology, College of Medicine, The Ohio State University, Columbus, Ohio 43210, U.S.A.

1 Conventional intracellular microelectrode techniques were used to subclassify P₁ purinoceptors linked to reduction of cell input resistance, steady-state hyperpolarization of the membrane potential, or inhibition of fast e.p.s.ps, in neurones of microdissected myenteric plexus preparations from guinea-pig ileum. The potencies of P₁ purinoceptor agonists were estimated in neurones that were current clamped to a fixed membrane potential.

2 In AH/Type 2 neurones, the A₂ agonist, CGS 21680, the A₁ agonist, CCPA or the mixed A₁–A₂ agonist, NECA, suppressed excitability by reducing input resistance (40–50% max.) and causing hyperpolarization (20–25 mV max.). CGS 21680 (0.1–1 µM) enhanced the after-hyperpolarizing potential.

3 From cumulative dose-response data, the potency order for reducing input resistance was CCPA (IC₅₀ = 5.1 ± 2.2 nM) >>> CGS 21680 (IC₅₀ = 5.6 ± 2.5 µM). This effect was reversed by the A₁ antagonist, CPT (EC₅₀ = 65 ± 11 nM).

4 In contrast, the potency order for membrane hyperpolarization was CCPA (IC₅₀ = 61 ± 23 nM) = CGS 21680 (IC₅₀ = 290 ± 90 nM) ≥ NECA (IC₅₀ = 450 ± 100 nM). Hyperpolarization elicited by CCPA was sensitive to the A₁–A₂ antagonist, DPSPX.

5 Agonists suppressed fast e.p.s.ps, but not DMPP responses, with an order of CCPA (IC₅₀ = 8.1 ± 3.0 nM) >>> CGS 21680 (IC₅₀ = 10 ± 2.9 µM).

6 In conclusion, the excitability of AH/Type 2 neurones is suppressed by activation of high affinity A₁ receptors that may be linked to a cyclic AMP-dependent pathway, leading to increase in calcium-dependent potassium conductance and enhancement of the after-hyperpolarizing potential. Activation of lower affinity non A₁ receptors linked to a cyclic AMP-independent pathway reduces excitability and leads mainly to a steady-state hyperpolarization. Adenosine also suppresses nicotinic cholinergic transmission by activating presynaptic high affinity A₁ receptors.

Keywords: P₁ purinoceptors; adenosine A₁ receptors; myenteric plexus; enteric nervous system; electrophysiology; potassium conductance; cyclic AMP; fast e.p.s.ps; acetylcholine release

Introduction

Burnstock (1978) classified purinoceptors into P₁ and P₂ subtypes; at P₂ sites, the preferred agonists are ATP and ADP and at P₁ sites, they are adenosine and AMP. P₁ purinoceptors were subclassified into A₁ and A₂ adenosine receptors according to the ability of adenosine and related analogues to cause respective inhibition or stimulation of adenylyl cyclase activity (Londos *et al.*, 1977; Van Calcar *et al.*, 1979). A₂ receptors were further subdivided into high affinity A_{2a} and lower affinity A_{2b} (Bruns *et al.*, 1986; Jarvis *et al.*, 1989). The A₁–A₂ definition has come to rely mainly on the potency profile and sensitivity of P₁ purinoceptor agonists or antagonists, since evidence for receptor-cyclase coupling often does not exist and there is evidence for receptor coupling to cyclase-independent pathways (Trussel & Jackson, 1987; Yakel *et al.*, 1988; Broad & Cook, 1993).

Adenosine A₁ receptors which predominate on both cholinergic and tachykinergic terminals of myenteric neurones (Gustafsson *et al.*, 1985; Christofi & Cook, 1986; Broad *et al.*, 1992) are involved in inhibition of neuroeffector transmission in the small bowel (Gustafsson *et al.*, 1991; Christofi & Cook, 1987; Christofi *et al.*, 1990). Gustafsson *et al.* (1991) recently subclassified the peripheral receptor into a lower affinity A_{1b} receptor to distinguish it from central higher affinity A_{1a} receptors.

Several lines of evidence suggest that inhibitory adenosine receptors, distinct from A₁ receptors, may also exist on enteric neurones (Buckley & Burnstock, 1983; Gustafsson *et al.*, 1985; Christofi & Cook, 1987; Christofi *et al.*, 1990;

Barajas-Lopez *et al.*, 1991). The neuronal non A₁ inhibitory receptor in myenteric ganglia is likely to be located on the somas of AH/Type 2 neurones which display postsynaptic responses to adenosine, because the other predominant neuronal type, the S/Type 1 neurone, apparently lacks postsynaptic inhibitory adenosine receptors (Zafirov *et al.*, 1985; Palmer *et al.*, 1987a), but displays excitatory responses to both adenosine and ATP (Katayama & Morita, 1989).

Adenosine reduces neuronal excitability in both central neurones (Green & Haas, 1991) and myenteric AH/Type 2 neurones (Palmer *et al.*, 1987a) by eliciting a steady-state hyperpolarization of the membrane potential and by enhancing postspike after-hyperpolarizing potentials. Both responses are accompanied by a reduction in cell input resistance.

In cortical neurones, two distinct adenosine receptors may mediate enhancement of after-hyperpolarizing potentials and steady-state hyperpolarization of the membrane potential, since there is a potency difference for adenosine between these two responses (Greene & Haas, 1985; 1991). Furthermore, adenosine affects two different potassium conductances in striatal (Trussel & Jackson, 1987) and hippocampal neurones (Gerber *et al.*, 1989), which further suggests the expression of two distinct receptors. The electrophysiology of adenosine in myenteric AH/Type 2 neurones is similar to that in central nervous neurones, raising the possibility that two distinct inhibitory receptors may also be expressed on AH/Type 2 neurones.

The after-hyperpolarizing potential is modulated by intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels and adenosine enhances the AHP in both central (Haas, 1984; Madison & Nicoll, 1986) and myenteric neur-

¹ Author for correspondence.

ones (Zafirov *et al.*, 1985; Palmer *et al.*, 1987a,b; Xia *et al.*, 1993; Christofi & Wood, 1993a). The available evidence fits the hypothesis that the enhancement of the afterhyperpolarizing potential and reduction in cell input resistance observed in AH/Type 2 neurones, may involve adenosine A₁-mediated reduction in neuronal cyclic AMP. However, the receptor subtype involved in these responses remains unknown.

The recent availability of more selective agonists for A₁ (Lohse *et al.*, 1988; Christalli *et al.*, 1988) and A₂ (Hutchinson *et al.*, 1989; Jarvis *et al.*, 1989) receptors is proving useful in further identification and subclassification of P₁ purinoceptors. Electrophysiological findings indicate that P₁ purinoceptor agonists suppress excitatory postsynaptic potentials (e.p.s.ps) in all categories of myenteric neurones, by acting at presynaptic receptors. Agonists also unmask prominent slow i.p.s.ps by suppressing slow e.p.s.ps in myenteric AH/Type 2 neurones (Christofi & Wood, 1993b). Limited structure-activity data with selective adenosine analogues suggest an interaction at presynaptic A₁ receptors. Selective A₁ and A₂ agonists also depress neuronal excitability in myenteric AH/Type 2 neurones (Christofi *et al.*, 1993). A more rigorous analysis of the potency profile of agonists at pre- and postsynaptic receptors has not been carried out.

The aim of this study was to use intracellular recording techniques to test the general hypothesis that adenosine suppresses neuronal excitability in myenteric neurones by activating two distinct P₁ purinoceptors. Receptors were classified according to relative potencies (IC₅₀ values) of selective A₁ and A₂ agonists in causing a reduction in cell input resistance, steady-state hyperpolarization or inhibition of fast e.p.s.ps. Adenosine antagonists were used to verify the P₁ purinoceptor nature of the interaction(s). Some of the results were previously presented in abstract form (Christofi & Wood, 1993c).

Methods

Intact myenteric plexus preparation

Adult male guinea-pigs, weighing 300–500 g, were killed by stunning and subsequent exsanguination. This method was approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University. The segment of ileum was pinned flat with the mucosal side up to Sylgard 184 encapsulating resin (Dow Corning, Midland, MI, U.S.A.) in a dissection dish containing ice-cold Krebs solution. Fine forceps were used to remove the mucosa, submucosal plexus, and inner muscle layers and to expose the myenteric plexus on the underside of the longitudinal muscle layer. A 2.0 × 1.0 cm segment of the preparation was pinned to Sylgard in a 2.0-ml recording chamber. The tissue was superfused with Krebs solution warmed to 37°C and gassed with 95% O₂:5% CO₂ (pH 7.3–7.4), at a rate of 10–15 ml min⁻¹. The composition of the Krebs solution was (in mM): NaCl 120, CaCl₂ 2.5, MgCl₂ 1.2, NaH₂PO₄ 1.35, NaHCO₃ 14.4 and glucose 11.5.

Intracellular recording technique

The myenteric ganglia were visualized with Hoffman Modulation Contrast optics and epi-illumination. Ganglia were immobilized with 100 µm L-shaped stainless steel wires placed on either side of the ganglion, perpendicular to the longitudinal muscle axis. Transmembrane electrical potentials were recorded with conventional intracellular microelectrodes filled with 3 M KCl and having resistances of 80–120 MΩ. The preamplifier (M767, World Precision Instruments) had bridge circuitry for intraneuronal injection of electrical current through the recording electrode. All data were recorded on magnetic tape for later analysis. The input resistance of the cells was estimated from the slopes of ohmic current-

voltage plots as described previously (Grafe *et al.*, 1980). Current injection was controlled by a programmed pulse generator that delivered a sequence of six 200-ms rectangular hyperpolarizing pulses that increased with constant increments at 1 s intervals. Impaled neurones were classified as S/Type 1, AH/Type 2 or Type 3 neurones according to reported criteria (Hirst *et al.*, 1974; Wood, 1989). AH/Type 2 neurones have a characteristic long-afterhyperpolarizing potential lasting from 4–20 s and 5–20 mV in amplitude that is absent in S/Type 1 neurones. Type 3 neurones do not display action potentials with intrasomal injection of depolarizing current of any magnitude, but have fast and slow e.p.s.ps. S/Type 1 neurones always have fast e.p.s.ps.

Experimental protocols

Impaled neurones were equilibrated and membrane potentials stabilized for 15 min before they were exposed to adenosine compounds. To determine the potency profiles of agonists in causing a reduction in input resistance and hyperpolarization in AH/Type 2 neurones, the membrane potential was initially current clamped to –56 mV. Cells were exposed to increasing concentrations of P₁ purinoceptor agonists in a cumulative fashion at 7 min intervals. In cumulative drug-addition experiments, AH/Type 2 neurones which displayed less than 12% spontaneous recovery (tachyphylaxis) from the maximum effect at each concentration were used in the analysis. In 60% of AH/Type 2 neurones, dose-response data was collected for both input resistance and hyperpolarization. The relative potency of agonists determined from cumulative-dose response data was compared with that determined from non-cumulative-dose response data for a restricted number of drug concentrations.

Fast e.p.s.ps were evoked in S/Type 1, AH/Type 2 and Type 3 neurones, by applying single electrical shocks (0.5–1.0 ms duration) to interganglionic connectives with electrodes made from Teflon-insulated Pt-wire (20 µm diam.) and an S-88 square-wave stimulator (Grass Instruments). To determine the inhibitory potency of agonists on fast e.p.s.ps, the membrane potentials of myenteric neurones was current-clamped to –75 mV. For fast e.p.s.ps, data were pooled from all three cell types and no differences in adenosine receptors between cell types were assumed. Cumulative-dose response curves were constructed for each agonist, at 7 min intervals. Six fast e.p.s.ps were averaged in control and test conditions and data were normalized to % of control fast e.p.s.p. amplitude.

Abbreviations

The following abbreviations are used: ACh, acetylcholine; DMPP, (1,1-dimethyl-4-phenyl-piperazinium iodide); CCPA, (2-chloro-N⁶-cyclopentyladenosine); CGS 21680, (2-[p-(carboxyethyl) phenylethylamino]-5'-N-ethylcarboxamidoadenosine); NECA, (5'-N-ethyl-carboxamidoadenosine); CPT, (8-cyclopentyl-1,3-dimethylxanthine); DPSPX, (1,3-dipropyl-8-p-sulphophenylxanthine).

Drugs

The neurones were exposed to adenosine and its synthetic analogues CCPA, NECA and CGS 216180, the antagonists CPT and DPSPX, forskolin (H₂O soluble form) and hexamethonium, by adding the substances to the superfusion solution. Forskolin and DMPP were also applied by micro-ejection from fine-tipped pipettes (tip diameter, 10–20 µm) with nitrogen pulses of controlled pressure and duration (Picospritzer, General Valve Corp., East Hanover, NJ, U.S.A.). The pipettes contained fast green to visualize the distribution of the ejected substances in the perfusion chamber. The tips of the pipettes were positioned 20–50 µm from the impaled neurone. All drugs were dissolved in distilled water. Adenosine compounds and forskolin were pur-

chased from Research Biochem Inc. (Natick, MA, U.S.A.). CGS 21680 that was used in some studies was a kind gift from Dr R.A. Lovell at Ciba-Geigy Corporation (Summit, NJ, U.S.A.). Agents acting on cholinergic receptors were purchased from Sigma (St. Louis, MO, U.S.A.).

Statistical analysis

Mean values $\pm$ s.e.mean are reported. For some experiments, the IC₅₀ value (half-maximal inhibitory concentration) for each agonist was obtained from individual sigmoid cumulative dose-response curves (5 to 7 agonist concentrations), fitted by a non-linear curve fitting programme (DeLean *et al.*, 1978). Mann-Whitney nonparametric unpaired two tail *t*-tests were used to assess differences between IC₅₀ values. In addition, all cumulative dose-response data obtained in each neurone (2 to 8 agonist concentrations) were pooled for each agonist response and plotted using slidewrite software programme (4.1 version, Advanced Graphics Software, Inc., Carlsbad, CA, U.S.A.). For multiple comparisons between corresponding agonist concentrations on these dose-response curves, the significance of differences was evaluated by ANOVA followed by the modified *t* test according to Bonferroni. Statistical significance was inferred with corrected Bonferroni *P* values of <0.05 . Illustrated curves represent the spline function through the data points.

Results

Results were analysed for 126 of 136 neurones from 59 guinea-pigs. These neurones represented 95 AH/Type 2, 16 Type 3 and 25 S/Type 1 neurones.

Postsynaptic effects of agonists acting on adenosine receptors in AH/Type 2 neurones

AH/Type 2 neurones used in the intracellular dose-response analysis had a mean resting membrane potential of -59 ± 1.4 mV ($n = 32$, 95% confidence limits between -56 and -62 mV). The mean input resistance of AH/Type 2 neurones that were current clamped at -56 mV was 92 ± 5.7 M Ω ($n = 31$, 95% confidence limits between 80 and 102 M Ω).

The agonists CGS 21680, CCPA or NECA (1 nM–10 μ M) suppressed excitability in forskolin-responsive AH/Type 2 neurones (Figure 1). Agonists suppressed excitability by reducing cell input resistance, and causing hyperpolarization (Figure 1). CGS 21680 enhanced postspike after-hyperpolarizing potentials at concentrations ranging from 0.1–1 μ M ($n = 10$, Figure 2).

P₁ purinoceptor agonists elicited hyperpolarization in 80 of 95 (84%) AH/Type 2 neurones. There was a linear 1:1 inverse relationship between hyperpolarization and the resting membrane potential of the neurones (Figure 3). The

Figure 1 Inhibitory action of the A₂ agonist, CGS 21680, on the excitability characteristics of a myenteric AH/Type 2 neurone of the guinea-pig small bowel. (a) CGS 21680 (200 nM) suppressed excitability by causing a 24 mV hyperpolarization of the membrane potential which was associated with a reduction in cell input resistance. A decrease in the amplitude of the electrotonic potentials evoked by the hyperpolarizing current pulses indicates a reduction in input resistance. The anodal-break action potentials which occurred at the offset of the hyperpolarizing current pulses were suppressed by CGS 21680. (a(i)) Expanded time scale showing a single anodal-break response (current parameters: 100 ms duration; 0.5 nA) in the absence of CGS 21680. (a(ii)) Inhibited anodal-break response with CGS 21680. (b) Excitatory response to the adenylyl cyclase activator forskolin (5 μ M) in the same neurone. The resting membrane potential (-60 mV) was current-clamped to -56 mV. The horizontal bars represent the length of time the drug was applied to the superfusion solution. Calibrations: vertical, 30 mV; horizontal, 15 s.

Figure 2 Action of CGS 21680 on the postspike after-hyperpolarizing potential of a myenteric AH/Type 2 neurone of the guinea-pig small bowel. Action potentials were evoked by intrasomal injection of depolarizing current pulses. (a) Control excitability; (b) a concentration of $0.2 \mu\text{M}$ CGS 21680 caused an increase in the duration of the afterhyperpolarizing potential (record begins after 1 min superfusion of CGS 21680). (c) A higher concentration of CGS 21680 ($1 \mu\text{M}$) applied at the arrow, quickly abolished the action potentials. At the offset of the final action potential, the afterhyperpolarizing potential did not recover and was observed as a sustained 23 mV hyperpolarization of the membrane potential. (d,e) Continuous records showing recovery beginning with a 3 min washout of CGS 21680 in Krebs solution. The resting membrane potential (-64 mV) of the recorded neurone was current-clamped at -56 mV . Calibrations: vertical, 20 mV; horizontal, 5 s.

Figure 3 Linear 1:1 inverse relationship between the resting membrane potential and NECA-induced hyperpolarization of the membrane potential in myenteric AH/Type 2 neurones of the guinea-pig small bowel. The numbers in parentheses denote the range of resting membrane potentials pooled to calculate x-axis values. The data were fit by linear regression with a correlation coefficient of 0.999. Each point on the line represents the mean $\pm$ s.e.mean from 9 to 10 AH/Type 2 neurones.

maximum hyperpolarization for each agonist is shown in Table 1. At maximally-effective concentrations ($1 \mu\text{M}$), agonists caused a decrease in cell input resistance in 89 of 95 (93%) AH/Type 2 neurones. In nine of these responsive neurones (11.1%), there was no apparent hyperpolarization associated with the reduction in input resistance. Other AH/Type 2 neurones which responded with a depolarization and

increase in excitability (Christofi *et al.*, 1993) with A_2 agonists were excluded from this drug analysis.

Potency profile of P_1 purinoceptor agonists

The membrane hyperpolarization and reduction in cell input resistance persisted in the continued presence of P_1 purinoceptor agonist in the Krebs superfusate. This permitted the relative potencies of agonists to be determined by cumulative drug addition. Figure 4a illustrates the cumulative-dose hyperpolarization curves for CGS 21680, CCPA and NECA. The IC_{50} value for each agonist in causing hyperpolarization is shown in Table 1. The potency profile of agonists was CGS 21680 = CPA $\geq$ NECA. In each of four additional AH/Type 2 neurones, in which stable recordings were made for 2 h, complete agonist-hyperpolarization dose-response relations were obtained for both CGS 21680 and CCPA. CGS 21680 was nearly equipotent with CCPA (Figure 4b).

Cumulative dose-response analysis for adenosine agonists in reducing cell input resistance (Figure 5a,b) revealed a potency order of CCPA $\gg$ CGS 21680 (Figure 5c). The IC_{50} values for agonists are stated in Table 2 along with potency ratios. CCPA was also more potent in reducing input resistance than in causing hyperpolarization in the same AH/Type 2 neurones ($n = 3$, Figure 5d).

Non-cumulative dose-response analysis for P_1 purinoceptor agonists

In another series of experiments, each neurone was exposed to a single concentration of each agonist for 5 min. Other

Figure 4 Cumulative dose-hyperpolarization curves for P₁ purinoceptor agonists in myenteric AH/Type 2 neurones of the guinea-pig small bowel: (□) CGS 21680; (○) CCPA; (◆) NECA. The potency order of agonists is CGS 21680 = CCPA ≥ NECA. The resting membrane potential of the neurones was current clamped at -56 mV. Data from each preparation were normalized to the percentage of the control current-clamped membrane potential at -56 mV. Two to six concentrations of agonist were exposed to each of 49 neurones in a cumulative fashion at 7 min intervals. Each data point represents the mean ± s.e.mean for six to eight neurones. (b) Dose-response relations for the A₁ agonist CCPA (●) and the A₂ agonist CGS 21680 (□) in eliciting hyperpolarization of the membrane potential in the same AH/Type 2 neurone. The IC₅₀s for CCPA and CGS 21680 were 100 nM and 250 nM, respectively; the potency order remained as CCPA = CGS 21680.

Table 1 Potency of P₁ purinoceptor agonists in causing hyperpolarization in AH/Type 2 neurones of the guinea-pig small intestinal myenteric plexus

Agonist	Hyperpolarization (% control) ^a IC ₅₀	^b Maximum hyperpolarization (mV)	n
CCPA	61 ± 23 nM	25 ± 3.5	10
CGS 21680	290 ± 90 nM	27 ± 3.0	10
NECA	450 ± 100 nM	20 ± 2.3	9
^c CGS 21680:CCPA	4.8		
NECA:CCPA	7.4		
NECA:CGS 21680	1.6		

^aThe data represents the mean ± s.e.mean of the IC₅₀ values estimated from the best fit (De Lean, 1978) of 5 to 6 individual dose-response curves constructed with cumulative addition of 5 to 7 concentrations of agonist.

^bNeurones were exposed to a 10 μM concentration of agonist; n, number of neurones used to estimate maximum hyperpolarization; the membrane potential was current-clamped to -56 mV.

The Mann-Whitney unpaired 2 tail *t* test was used to compare differences between IC₅₀ values; CCPA vs CGS 21680, *P* > 0.05; CCPA vs NECA, *P* < 0.05; CGS 21680 vs NECA, *P* > 0.05.

^cRatio of IC₅₀ values = potency ratio. For abbreviations, see text.

agonists were tested at 15 min intervals only in those cells that completely recovered from the effect of the previous agonist. Non-cumulative analysis gave the same results as cumulative analysis.

Data for membrane hyperpolarization were expressed as a percentage of the current-clamped membrane potential of -56 mV. The hyperpolarizations produced by 5 nM CCPA (-7.8 ± 3.1%, *n* = 6), 5 nM CGS 21680 (-8.4 ± 3.0%, *n* = 6) and 5 nM NECA (-9.3 ± 4.1, *n* = 6) were not significantly different from each other (*P* > 0.05). At a 200 nM concentration of each agonist, CCPA (-41.6 ± 4.9, *n* = 6) caused the same hyperpolarization (*P* > 0.05) as CGS 21680 (-35.4 ± 4.9, *n* = 6). NECA (-19.3 ± 3.3, *n* = 6) caused a significantly smaller hyperpolarization than CCPA or CGS 21680 (*P* < 0.05). The apparent potency order for agonists on causing hyperpolarization (*n* = 25) remained as CGS 21680 = CCPA ≥ NECA.

The reduction in cell input resistance by agonists was expressed as a percentage of the resting cell input resistance of each neurone that was current-clamped at -56 mV. A concentration of 5 nM CCPA reduced the cell input resistance by -22.0 ± 4.3% (*n* = 6), whereas 5 nM CGS 21680 did not affect the cell input resistance (-0.0 ± 0.0%, *n* = 6); differences between agonist are significantly different (*P* < 0.003). At a 200 nM concentration, CCPA (-45.8 ± 5.9%, *n* = 6) was significantly more effective (*P* < 0.002) than CGS 21680 (-9.2 ± 7.2%, *n* = 7) in reducing input resistance. The apparent order of potencies of agonists for reducing the input resistance remained as CCPA >>> CGS 21680.

Competitive blockade with xanthine derivatives

The hyperpolarization induced by 0.2 μM CCPA (-40.6 ± 5.0%, *n* = 6) was blocked in the presence of the mixed A₁-A₂ adenosine receptor antagonist DPSPX (-7.3 ± 2.5%, 2 μM, *n* = 6). DPSPX also reversed the effect on cell input resistance (data not shown). The reduction in cell input resistance by CCPA was dose-dependently reversed by the A₁ selective antagonist, CPT (*P* < 0.001). The EC₅₀ for CPT was 65 ± 11 nM (*n* = 3); a small membrane hyperpolarization (2 to 6 mV) or no hyperpolarization occurred in these cells, and therefore the effect of the antagonist was mostly to block the reduction in input resistance mediated by CCPA.

Presynaptic inhibition of fast e.p.s.ps

S/Type 1 neurones had relatively low resting membrane potentials (-38 to -53 mV) and higher input resistances (135 to 300 MΩ) than AH/Type 2 neurones. Type 3 neurones had membrane potentials ranging from -64 to -78 mV and input resistances ranging between 40 and 65 MΩ throughout recording periods (0.5-1.5 h). The nicotinic cholinergic nature of the fast e.p.s.ps was confirmed by hexamethonium (20 μM) blockade in all cell types. CGS 21680 or CCPA dose-dependently and reversibly suppressed or abolished fast e.p.s.ps in 17 of 21 S/Type 1, 4 of 4 AH/Type 2 and 8 out of 10 Type 3 neurones that received fast cholinergic inputs, without affecting the postsynaptic action of the nicotinic agonist DMPP (*n* = 16). The potency order of agonists was CCPA >>> CGS 21680 in suppressing fast e.p.s.ps (Figure 6, Table 2). In 2 S/Type 1 neurones, in which complete dose-inhibition curves on fast e.p.s.ps were obtained for both CCPA and CGS 21680, the potency order of CCPA >>> CGS 21680 was retained (data not shown). The receptor nature of the adenosine inhibition of fast e.p.s.ps has been established (Christofi & Wood, 1993b).

Discussion

P₁ purinoceptor subtypes on myenteric neurones

The present study revealed that a unitary P₁ purinoceptor mediates both presynaptic inhibition of fast e.p.s.ps in

Figure 5 Effect of P₁ purinoceptor agonists on cell input resistance in myenteric AH/Type 2 neurones of guinea-pig small bowel. (a) Effect of CCPA on current-voltage (*I-V*) plots, that were obtained by intrasomal injection of incremental increases in hyperpolarizing current. *I-V* relations were constructed in the presence of cumulative increases in CCPA concentration at 7 min intervals. The decrease in the slope of the *I-V* plots was used to calculate the decrease in cell input resistance produced by CCPA. (●) Control; (Δ) 1 nM CCPA; (○) 5 nM CCPA; (+) 50 nM CCPA; (▲) 200 nM CCPA; (▽) 1 μM CCPA; (■) 10 μM CCPA; (□) recovery after 20 min washout in Krebs solution. (b) Dose-response curve for CCPA extrapolated from the slopes of individual *I-V* plots in (a). (c) Cumulative dose-response relations for adenosine agonists on reducing cell input resistance in AH/Type 2 neurones: (●) CCPA; (□) CGS 21680. Two to seven concentrations of each agonist were exposed to each of 30 neurones. Each data point represents the mean $\pm$ s.e. mean of six to nine neurones. ANOVA indicated a $P < 0.0001$ between data points. Post-tests between pairs of values at each concentration of agonist on the curves yielded the following Bonferroni P values: ns, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (d) Potency of the A₁ agonist CCPA in reducing input resistance (○) and causing hyperpolarization (●) in the same AH/Type 2 neurone. CCPA was 20 fold more potent in reducing input resistance than in causing hyperpolarization. The resting potential of the AH/Type 2 neurones was current clamped to -56 mV.

Figure 6 Dose-response relations for selective adenosine analogues as inhibitors of fast e.p.s.ps in neurones of the guinea-pig small intestinal myenteric plexus. Seventeen neurones were exposed to two to five concentrations of agonist in a cumulative fashion, at 7 min intervals. Each data point is the mean $\pm$ s.e. mean for 5–7 neurones. The resting potential was current clamped at -75 mV. (●) CCPA; (○) CGS 21680. ANOVA indicated significant differences ($P < 0.0001$) between data points at concentrations greater than 1 nM. Post-tests between pairs of values at each concentration of agonist on the curves yielded the following Bonferroni P values: NS, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

myenteric neurones, and postsynaptic inhibition of excitability in AH/Type 2 neurones. Our findings support the existence of high affinity adenosine A₁ receptors like those in the brain (Lohse *et al.*, 1988; Jarvis *et al.*, 1989; Lupica *et al.*, 1990; Gustafsson *et al.*, 1991). This conclusion is supported by data showing micromolar potency for the selective A₂ agonist CGS 21680, nanomolar potency of the highly selective A₁ agonist CCPA and a potency order of CCPA $\gg$ CGS 21680 at both pre- and postsynaptic receptors. Furthermore, the potency of the selective A₁ antagonist, CPT (Daly *et al.*, 1987) at somal receptors on AH/Type 2 neurones is the same as its K_D at high affinity A₁ receptors on hippocampal neurones (Haas & Green, 1991).

This study provided novel electrophysiological evidence for heterogeneity of inhibitory P₁ purinoceptors on AH/Type 2 neurones. The pharmacology of adenosine analogues strongly suggests that hyperpolarization occurs mainly by activating a receptor that is distinct from A₁ receptors. The potency profile of CGS 21680 = CCPA $\geq$ NECA does not fit the profile of an A₁ or an A₂ inhibitory P₁ purinoceptor subtype, and is different from the profile obtained for high affinity A₁ sites. This different agonist profile could not be attributed to methodology, because differences in relative potencies of agonists were verified: (1) in cells with current clamped mem-

Table 2 Potency of adenosine analogues at pre- and postsynaptic P₁ purinoceptors on neurones of the guinea-pig small intestinal myenteric plexus

Agonist	% reduction in input resistance IC ₅₀	^b % inhibition of fast e.p.s.ps IC ₅₀
CCPA	5.2 ± 2.2 nM	8.1 ± 3.1 nM
CGS 21680	5.6 ± 2.5 µM	^c 1.0 ± 2.9 µM
^d CGS 21680: CCPA	Potency ratios A ₂ :A ₁ 1087	> 1260
^d CCPA		

^aInput resistance estimated from current-voltage plots according to Grafe *et al.* (1980). The membrane potential of AH/Type 2 neurones was current-clamped at -56 mV.

^bThe membrane potential of S/Type 1 and Type 3 neurones was current-clamped at -75 mV.

^{c,d}Data represent the mean ± s.e.mean of IC₅₀ values estimated from the best fit of six individual dose-effect curves constructed with cumulative additions of 5 to 7 concentrations of agonists; the difference between the IC₅₀ values of CCPA and CGS 21680 is statistically significant; the Mann-Whitney unpaired 2 tail *t* test gave a *P* = 0.033.

^cIC₅₀ value is the maximum (30%) inhibition of fast e.p.s.ps observed with CGS 21680 at 10 µM concentration.

^dRatio of IC₅₀ values = potency ratio.

brane potentials, (2) by both cumulative and non-cumulative drug addition, (3) in the same AH/Type 2 neurone, as well as, (4) in some cells which had a reduction in cell input resistance, without any apparent hyperpolarization.

The postspike after-hyperpolarizing potential of cortical neurones is enhanced at relatively low concentrations of adenosine, whereas at higher concentrations, the steady-state hyperpolarization predominates (Green & Haas, 1985; 1991), suggesting the existence of two distinct receptors. In our studies, the concentrations of CGS 21680 (0.1–1 µM) needed to enhance the after-hyperpolarizing potential are consistent with its activation of high affinity A₁ receptors. In fact, activation of high affinity A₁ receptors reduces input resistance and therefore must also enhance the after-hyperpolarization, since these responses represent the same ionic conductance (Wood, 1989). Higher concentrations of agonists than those needed to saturate A₁ sites mainly elicit a steady-state hyperpolarization via a lower affinity non A₁ receptor. Since hyperpolarization and reduction in input resistance are inextricably linked responses, we suggest that overlapping activation of the two predominant receptors by A₁ and A₂ agonists, especially at higher concentrations, is the reason for the lack of difference between agonist potencies on hyperpolarization. The non A₁ receptor may possibly represent an A₃ receptor subtype (Jacobson, 1994).

Signalling mechanisms coupled to P₁ purinoceptors

Enhancement of the postspike after-hyperpolarizing potential and reduction in input resistance in AH/Type 2 neurones may represent the physiological correlates of adenosine-mediated reduction in neuronal cyclic AMP (Haas, 1984; Madison & Nicoll, 1986; Zafirov *et al.*, 1985; Palmer *et al.*, 1987a). It was recently confirmed that nanomolar concentrations of the A₁ agonist, CCPA, were also sufficient to inhibit cyclic AMP formation, and CPT completely blocked this inhibition in isolated myenteric ganglia (Xia *et al.*, 1993).

In our study, the prolonged hyperpolarization occurs mainly via a non A₁ receptor and is therefore, by definition, also linked to a cyclic AMP-independent pathway, as is the case in central neurones (Trussell & Jackson, 1987; Green & Haas, 1991). As shown in other systems (Yakel *et al.*, 1988), including the enteric nervous system (Broad & Cook, 1993; Christofi *et al.*, 1993), multiple signalling mechanisms may

contribute to the actions of adenosine in AH/Type 2 neurones. Furthermore, selectivity of action of adenosine at pre- and post-synaptic receptors may arise from the coupling of different signalling pathways to the respective A₁ receptors (Bruns *et al.*, 1987; Dunwiddie & Fredholm, 1989) on myenteric neurones.

The linear 1:1 inverse relationship shown to exist between P₁ purinoceptor-mediated hyperpolarization and the resting membrane potential of each AH/Type 2 neurone suggests that the ionic mechanism involves opening of potassium channels, as reported previously (Palmer *et al.*, 1985; 1987a). Adenosine hyperpolarization and reduction in cell input resistance involves a steady-state potassium current (Haas & Green, 1988). High affinity A₁ and lower affinity non A₁ receptors on AH/Type 2 neurones may be linked to two different potassium conductances (Trussell & Jackson, 1987; Gerber *et al.*, 1989). The A₁ receptor is likely to be linked to a G_iprotein-adenylyl cyclase complex (Zgombick *et al.*, 1989). Activation of the receptor would lead to a reduction in intraneuronal cyclic AMP levels and eventual increase in potassium conductance to reduce excitability in AH/Type 2 neurones. We speculate that the non A₁ receptor (steady-state hyperpolarization) is linked directly to a potassium channel, or to other, as yet unidentified, intracellular signalling pathways.

Conclusions

Adenosine inhibits excitability in AH/Type 2 neurones, by acting at high affinity A₁ inhibitory receptors that are probably linked to a cyclic AMP-dependent pathway. A₁ activation leads to enhancement of the postspike after-hyperpolarization and increase in potassium conductance. Activation of lower affinity non A₁ receptors that are linked to a cyclic AMP-independent pathway leads mainly to a steady-state hyperpolarization produced by an increase in potassium conductance. Adenosine also inhibits ACh release and suppresses nicotinic cholinergic transmission to myenteric neurones by activating high affinity A₁ receptors. The findings establish adenosine as a critical inhibitory neuromodulator in myenteric ganglia, with an intricate neuropharmacology.

This research was supported by National Institutes of Health (NIH) Grants R29 DK-44179 to F.L.C. and RO1 DK37238 to J.D.W.

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(Received December 3, 1993
Revised May 25, 1994
Accepted June 20, 1994)