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Modulation of extracellular GABA levels in the retina by activation of glial P2X-purinoceptors

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1 In the rat retina, γ-aminobutyric acid (GABA) released as a transmitter is inactivated by uptake mainly into glial cells (Müller cells). Activation of P2-purinoceptors in Müller cells increases $[Ca^{2+}]_i$ and the present study was undertaken to see whether this action affected the glial release of [^{3}H]-GABA from the superfused rat isolated retina.

2 Adenosine 5′-triphosphate (ATP) and the P2X-purinoceptor agonists, α,β-methylene-ATP (α,β-meATP) and β,γ-methyleneATP (β,γ-meATP) significantly increased the KCl-evoked release of [^{3}H]-GABA from the retina.

3 Adenosine and the P2Y-purinoceptor agonist, 2-chloroATP, had no effect on the KCl-evoked release of [^{3}H]-GABA from the retina. However, 2-methylthioATP (2-Me-S-ATP) significantly enhanced the evoked release of [^{3}H]-GABA.

4 The effect of ATP on the glial release of [^{3}H]-GABA was abolished by the P2-antagonist, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS).

5 When the superfused retina was exposed to the GABA uptake inhibitor, SKF89976A, the enhancing effect of α,β-meATP on the KCl-evoked release of GABA was abolished.

6 The KCl-evoked release of [^{3}H]-GABA from the frog retina and rat cerebrocortical slices, which take up GABA mainly into neurones, was not affected by ATP or α,β-meATP.

7 We concluded that the glial Müller cells in the rat retina possess P2-receptors, activation of which increases the 'release' of preloaded [^{3}H]-GABA apparently by reducing uptake. On balance, the results suggest the involvement of P2X-purinoceptors, although we cannot exclude the possibility that P2Y-purinoceptors may be involved. Our results suggest that ATP, as well as being a conventional transmitter in the retina, may be involved in neuronal-glial signalling and modulate the extracellular concentration of GABA.

Keywords: Glia; Müller cells; GABA release; GABA uptake; ATP; retina

Introduction

There is much evidence that adenosine 5′-triphosphate (ATP) is a transmitter in the autonomic nervous system (Burnstock, 1997), but until recently the evidence that ATP was a transmitter in the central nervous system was largely circumstantial. However, by using whole-cell recording from medial habenula slices with patch-clamp electrodes, ATP-mediated synaptic currents were discovered (Edwards *et al*., 1992). These results provide strong evidence that ATP is a fast transmitter in the brain, acting at ligand-gated P2X-purinoceptors. In the retina, much more attention has been given to the possibility that adenosine is a transmitter (Blazynski & Perez, 1991), but ATP is by far the most abundant purine in the retina, from which it can be released by potassium depolarization (Perez *et al*., 1988). The localization of ATP in the retina is unknown but adenosine is co-located with acetylcholine (ACh) in displaced amacrine cells (Blazynski, 1989), and since adenosine in the retina is rapidly phosphorylated to ATP (Perez *et al*., 1986), it is highly probable that ATP and ACh are co-localized in these neurones. This view is supported by evidence from release experiments which suggest that endogenous ATP acting on P2X-purinoceptors modulates the light-evoked release of ACh from the rabbit retina (Neal & Cunningham, 1994). This release study provided the first evidence that ATP might be a retinal transmitter. In addition, ATP may be involved in neuronal-glial interactions in the retina, because in isolated salamander Müller cells and in rat Müller cells, ATP stimulates the release of calcium from internal stores (Keirstead & Miller, 1997; Newman & Zahs, 1997). The Müller cells in the rat retina take up γ-aminobutyric acid (GABA) and in the presence of a GABA-T inhibitor, the transmitter accumulates in these glial cells (Neal & Iversen, 1972). In the present study, we have found that P2X-purinoceptor agonists increase [^{3}H]-GABA release from Müller cells apparently by reducing GABA uptake. Thus, in addition to acting as a conventional transmitter in the rat retina, ATP may also interact with glia to modulate the extracellular concentration of GABA.

Methods

[^{3}H]-GABA release from rat retina (glial release)

Male Wistar rats (weight 200–250 g) were killed by cervical dislocation and the eyes enucleated. The retinas were dissected and placed in 5 ml of oxygenated (95% O_2/5% CO_2) Krebs-bicarbonate Ringer (see below for composition) containing aminooxyacetic acid (100 μM) at 32°C. After a preliminary incubation for 10 min, [^{3}H]-GABA was added to give a final concentration of 11 nM

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and the incubation was continued for a further 30 min in a shaking water bath.

Each retina (approximately 10 mg) was transferred to a small superfusion chamber (200 μl) and retained between two filter paper discs (Brandel Superfusion System). Six retinas were simultaneously superfused with oxygenated medium at a flow rate of 0.7 ml min^{-1}. The retinas were washed for 15 min and then 2 min (1.4 ml) fractions were collected. In order to evoke (submaximal) GABA release, the retinas were exposed for 2 min to medium containing elevated KCl (60 mM). Two periods of high KCl exposure (S_1 and S_2) were separated by 8–10 samples. The effect of drugs was studied by adding them to the medium with the second pulse of KCl (S_2). In controls, which were run simultaneously, no drug was added to the second pulse of KCl.

[3H]-GABA release from frog retina (neuronal release)

Male or female common frogs (*Rana temporaria*) weighing approximately 20 g were briefly dark-adapted (20 min) and killed by pithing. The retinas were removed and incubated with [3H]-GABA at room temperature in Frog Ringer (see below for composition), which was also used to superfuse the tissue as described previously. [3H]-GABA is accumulated by neurones in the frog retina and the Müller cells lack the GABA transporter (Neal *et al*., 1979).

[3H]-GABA release from cortical slices (neuronal release)

Cortical slices were prepared from rat brain as described previously (Neal & Bowery, 1979). The slices (250 μm thick) were loaded with [3H]-GABA and then superfused as described for rat retina. Cortical slices accumulate [3H]-GABA predominantly in neurones (Iversen & Bloom, 1972).

At the end of each experiment, the retinas or cortical slices and their surrounding filter papers were removed from the collection chambers and dissolved in 0.5 ml of Soluene 350, which was then neutralized with 200 μl of glacial acetic acid. The radioactivity in the tissue and the superfusion samples was measured following the addition of scintillant (Ultima Gold, 3 ml) by a Wallac 1409 liquid scintillation counter.

The release of [3H]-GABA is expressed as the fractional release. In each superfusion experiment, the ratio of the two KCl-evoked releases (S_2/S_1) was calculated. Results were compared between controls and experiments, where a drug was present during S_2, by use of Student's unpaired t test. Significant differences were assumed at a level of $P<0.05$.

Materials

Krebs-bicarbonate medium had the following composition (mM): NaCl 118, KCl 4.84, $CaCl_2$ 2.4, $NaHCO_3$ 25, $MgSO_4$ 1.8, KH_2PO_4 1.2 and glucose 9.5. It was oxygenated with 95% O_2/5% CO_2. Frog Ringer of the following composition was used (mM): NaCl 105, KCl 4.21, $CaCl_2$ 2.24, $MgSO_4$ 1.05, KH_2PO_4 1.06, $NaHCO_3$ 22.2 and glucose 8.4.

[3H]-GABA (specific activity 80.0 Ci $mmol^{-1}$) was obtained from Amersham International 2-Chloro-ATP (2-chloroadenosine triphosphate tetrasodium) and 2,Me-S-ATP (2-methylthioATP tetrasodium) were obtained from RBI. PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid) was obtained from Tocris Cookson. All other chemicals were obtained from Sigma or Merck (U.K.). SKF89976A [(RS)-N-(4,4,diphenyl-3-butenyl)nipecotic acid] was a gift from Nova Pharmaceutical Corp., USA.

Results

[3H]-GABA release from rat retina

When the retinas were superfused, there was a steady resting efflux of [3H]-GABA and as we have shown previously (Neal & Bowery, 1979), exposure of the retina to KCl (60 mM) (S_1) evoked a striking increase in [3H]-GABA release (Figure 1). A second pulse of 60 mM KCl (S_2) evoked a smaller release of [3H]-GABA (Figure 1). The ratio of S_2/S_1 in one typical set of control experiments was 0.63 ± 0.022 ($n=13$).

Effect of P2-receptor agonists on spontaneous resting release of [3H]-GABA

Exposure of the rat retina for 2 min to ATP (1 mM), α,β-methyleneATP (α,β-meATP) (100 μM), β,γ-methyleneATP (β,γ-meATP) (100 μM), 2-chloroATP (100 μM) and 2-methylthio-ATP (2-Me-S-ATP) (1 mM) did not have any effect on the resting release of [3H]-GABA.

Effect of P2-receptor agonists on KCl-evoked [3H]-GABA release

Exposure of the rat retina for 2 min to ATP (1 mM), α,β-meATP (100 μM) and β,γ-meATP (100 μM) during the second high KCl exposure (S_2) significantly increased the KCl-evoked release of [3H]-GABA (Figure 2). Thus, the S_2/S_1 ratio in the presence of these P2X-agonists was increased by 22% (ATP and β,γ-meATP) and 37% (α,β-meATP) compared with the ratio obtained in control experiments (no drug). When ATP and α,β-meATP were tested over a range of concentrations, it was found that the maximum effect of both drugs was similar (Table 1). The concentrations of ATP and α,β-meATP that produced 50% of the maximal response were approximately 220 μM and 65 μM, respectively.

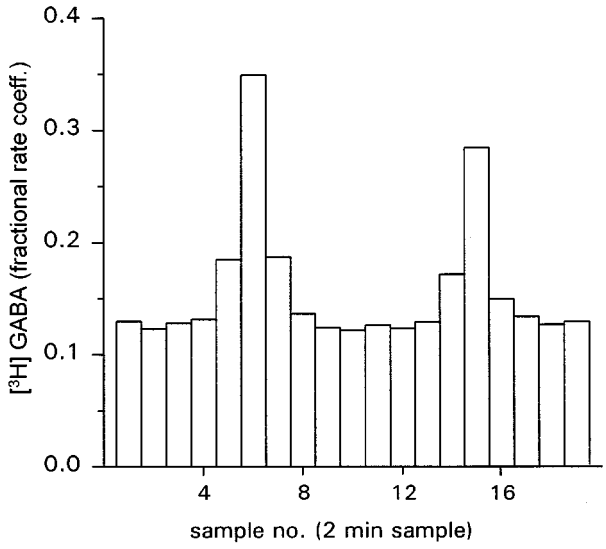

Figure 1 Typical superfusion experiment showing the 'glial' release of [3H]-GABA from a rat isolated retina. The retina was exposed to high-K (KCl 60 mM) during samples 5 (S_1) and 14 (S_2) and in each experiment the ratio S_2/S_1 was calculated. Six separate retinas were superfused simultaneously, 3 of which were controls (no drug) and 3 of which were exposed to drug during the second pulse of high-KCl. The ratio S_2/S_1 for control retinas was 0.63 ± 0.022 (mean ± s.e.mean, $n=13$). This ratio is increased by drugs that enhance the KCl-evoked release of [3H]-GABA.

Exposure of the retina to adenosine (1 mM) or the P2Y-receptor agonist, 2-chloroATP (100 μM), had no effect on the KCl-evoked release of [^{3}H]-GABA from the retina (Figure 2). 2-Me-S-ATP (100 μM) also had no effect on the evoked release of [^{3}H]-GABA. However, this compound is susceptible to ecto-nucleotidases and when the concentration was raised to 1 mM, it enhanced the KCl-evoked release of [^{3}H]-GABA to the same extent as ATP (1 mM) (Figure 2).

Effect of the P2-antagonist PPADS on KCl-evoked [^{3}H]-GABA release

PPADS (100 μM) had no effect on the KCl-evoked release of [^{3}H]-GABA from the rat retina but it completely blocked the enhancing effect of ATP (1 mM) on the KCl-evoked release of [^{3}H]-GABA (Table 2).

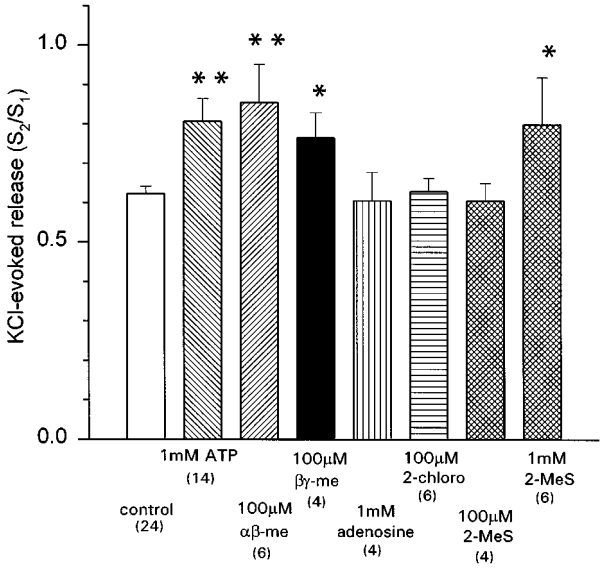

Figure 2 Effect of P2-receptor agonists and adenosine on the KCl-evoked release of [^{3}H]-GABA from glial cells in the rat retina. ATP (1 mM) and the P2X-agonists α,β-methylene ATP (α,β-me) (100 μM) and β,γ-methylene ATP (β,γ-me) (100 μM) significantly enhanced the KCl-evoked release of [^{3}H]-GABA as did 2-methylthioATP (1 mM) (2-Mes). In contrast, adenosine (1 mM) and the P2Y-agonist 2-chloroATP (100 μM) (2-chloro) had no effect on the evoked [^{3}H]-GABA release. Each column is the mean$\pm$s.e.mean (vertical lines) of the S_2/S_1 ratio. *$P<0.05$, **$P<0.02$, Student's t test. Numbers in parentheses indicate number of superfusions.

Table 1 Effect of different concentrations of ATP and α,β-meATP on the KC1-evoked release of [^{3}H]-GABA from glial cells of the rat retina

		KCl-evoked release of [^{3}H]-GABA (% of control)	n	P
ATP	0.1 mM	1.6$\pm$0.29	3	NS
	0.5 mM	39.8$\pm$5.63	4	<0.001
	1.0 mM	30.6$\pm$2.0	14	<0.001
	4.0 mM	24.7$\pm$2.24	11	<0.01
α,β-meATP	50 μM	8.1$\pm$1.02	4	NS
	100 μM	36.5$\pm$4.12	6	<0.001
	250 μM	22.6$\pm$2.87	5	<0.05

Each result is the mean$\pm$s.e.mean. P indicates significantly different from control, NS not significant; n indicates the number of superfusions.

Effect of SKF89976A on the action of α,β-meATP on [^{3}H]-GABA release

Under normal physiological conditions, Müller fibres in the rat retina do not accumulate GABA because the glial cells possess GABA-T, the enzyme responsible for GABA degradation. However, uptake by the Müller fibres is thought to be involved in regulating the extracellular concentration of GABA in the rat retina (Newman & Reichenbach, 1996). Since the 'release' of [^{3}H]-GABA in the present experiments represents the balance of release and reuptake, we tested the effect of α,β-meATP (100 μM) in the presence of the GABA uptake inhibitor SKF89976A (30 μM). The results are summarized in Figure 3 and show that whilst the inhibitor of GABA uptake did not itself affect the S_2/S_1 ratio, it abolished the enhancing effect of α,β-meATP on the KCl-evoked glial release of [^{3}H]-GABA. Thus, the increase in evoked GABA release caused by activation of P2X-receptors seems to result from a decrease in GABA uptake by the glial cells. This conclusion was supported by a preliminary uptake experiment in which rat retinas were incubated with [^{3}H]-GABA in the presence of ATP (1 mM) or α,β-meATP (100 μM). Both compounds significantly inhibited [^{3}H]-GABA uptake ($P<0.01$), the tissue:medium ratios being: controls 14.4$\pm$1.20 ($n=8$); ATP 10.2$\pm$1.56 ($n=6$); α,β-

Table 2 Effect of PPADS on the enhanced KC1-evoked release of [^{3}H]-GABA induced by ATP

	KCl-evoked release of [^{3}H]-GABA (S_2/S_1)	n
Control	0.63$\pm$0.02	24
ATP (1 mM)	0.77$\pm$0.05*	12
PPADS (100 μM)	0.62$\pm$0.05	4
ATP + PPADS	0.57$\pm$0.01	6

Each result is the mean$\pm$s.e.mean. *Indicates significantly different from control, $P<0.02$; n indicates the number of superfusions

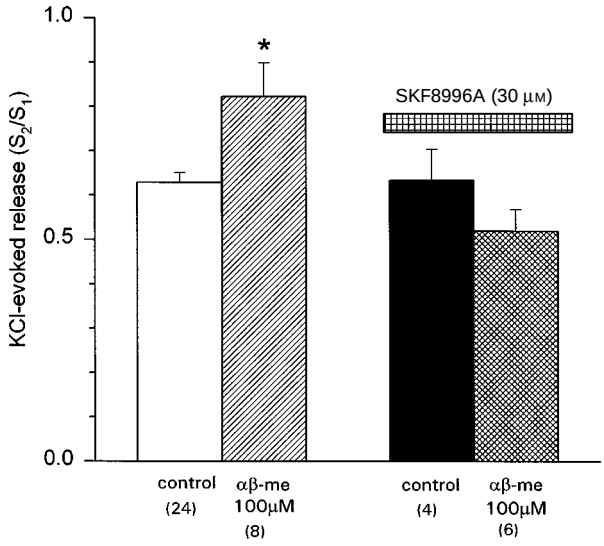

Figure 3 Effect of the GABA uptake inhibitor (SKF89976A) on the enhanced KCl-evoked release of [^{3}H]-GABA from the rat retina induced by α,β-meATP. The presence of SKF89976A (30 μM) in the superfusion medium did not affect the control values of S_2/S_1 but abolished the enhancing effect of α,β-meATP (100 μM). Vertical lines indicate s.e.mean, numbers in parentheses indicate number of superfusions. *$P<0.02$.

meATP 10.6±0.75 ($n=5$). The inhibitory effect of ATP was blocked by PPADS (100 μM) which itself had no effect on [^{3}H]-GABA uptake. Thus, the inhibitory effect of ATP on GABA uptake was receptor-mediated rather than a direct effect on the GABA transporter.

Effect of ATP and α,β-meATP on neuronal GABA release

In contrast to the rat retina in which GABA uptake is predominantly glial, the uptake by frog retina and rat cortical slices is mainly neuronal (Neal & Bowery, 1979). Thus, we used these tissues to compare the effects of P2X-purinoceptor agonists on the 'glial' and 'neuronal' release of [^{3}H]-GABA. The results are summarized in Figure 4 and show that ATP and α,β-meATP have no effect on the KCl-evoked neuronal release of [^{3}H]-GABA from cortical slices or frog retina.

Discussion

The present experiments show that ATP, but not adenosine, increases the KCl-evoked release of [^{3}H]-GABA from the rat retina. Since autoradiographic studies have shown that in the presence of the GABA-T inhibitor AOAA, [^{3}H]-GABA is accumulated predominantly by the glial Müller cells (Neal & Iversen, 1972), these experiments strongly suggest that ATP enhances [^{3}H]-GABA release by activating P2-purinoceptors located on the Müller cells.

P2 purinoceptors have been found previously on isolated salamander Müller cells (Keirstead & Miller, 1997) and on the Müller cells in the rat acutely isolated retina (Newman & Zahs, 1997). In both preparations, activation of these receptors by

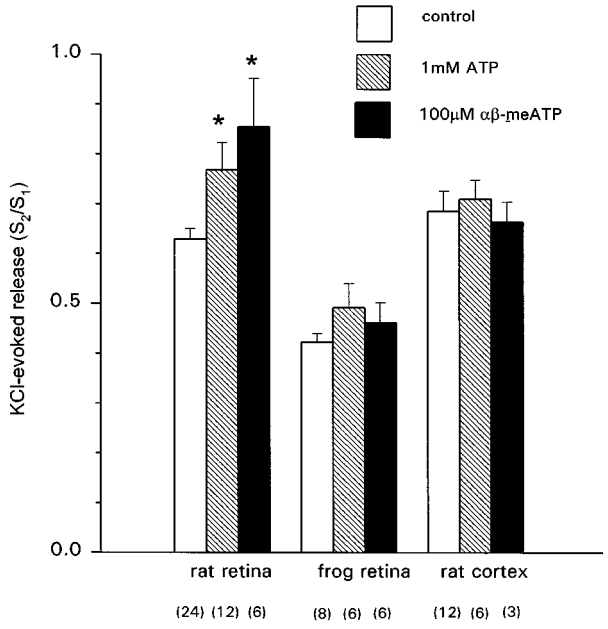

Figure 4 Effects of ATP (1 mM) and α,β-meATP (100 μM) on the KCl-evoked release of [^{3}H]-GABA from rat retina (glial release), frog retina and rat cortical slices (neuronal release). Rat retinas were exposed to KCl (60 mM) but frog retinas and rat cortical slices were exposed to 25 mM KCl because preliminary experiments showed this concentration to evoke a suitable submaximal increase in [^{3}H]-GABA release. Only the evoked release from rat retina was enhanced by ATP and α,β-meATP. Vertical lines indicate s.e.mean, numbers in parentheses indicate number of superfusions, $*P<0.02$.

ATP resulted in a release of calcium from internal stores. Because 2-Me-S-ATP had the same effect as ATP on $[Ca^{2+}]_i$ and because the effect of ATP was blocked by neomycin (a phospholipase C inhibitor), Keirstead & Miller (1997) suggested that the purinoceptors on salamander glial cells are of the P2Y subtype. Similarly, Newman & Zahs (1997) concluded that the effect of ATP on rat Müller cells was mediated, at least in part, by inositol 1,4,5-trisphosphate (IP_3) receptors and therefore presumably involved P2Y receptors. ATP has been shown to release calcium from the internal stores of other glial cells including cultured spinal cord astrocytes (Salter & Hicks, 1994), cultured striatal astrocytes (Centemeri *et al*., 1997) and Schwann cells (Robitaille, 1995; Ansselin *et al*., 1997). All these cells possess P2Y receptors although the perisynaptic glial cells of the frog neuromuscular junction also have P2X receptors (Robitaille, 1995).

In our experiments, one P2Y agonist, 2-chloroATP did not enhance the KCl-evoked release of [^{3}H]-GABA from Müller cells, but another, 2-Me-S-ATP, did. This may indicate the involvement of a particular P2Y-subtype but since 2-Me-S-ATP is also a good P2X agonist (Humphrey *et al*., 1995), it may reflect the involvement of P2X receptors. This possibility is supported by the fact that the P2X agonists, β,γ-meATP and α,β-meATP, were as effective as ATP in enhancing the KCl-evoked glial release of [^{3}H]-GABA. However, high concentrations of these agonists were required to enhance [^{3}H]-GABA release, raising the possibility that they may have produced their effects by activating P2Y receptors. Furthermore, the existence of P2Y receptors responsive to α,β-meATP has been demonstrated (Windscheif *et al*., 1995; Bolego *et al*., 1997).

The effects of ATP were abolished by the antagonist, PPADS. It has been suggested that PPADS is selective for P2X purinoceptors (Ziganshin *et al*., 1993; Windscheif *et al*., 1994), but it seems this is not always so because Boyer *et al*. (1994) found that PPADS potently inhibited P2Y purinoceptor-mediated activation of phospholipase C in turkey erythrocytes. Thus, the fact that PPADS inhibited the action of ATP in our study does not necessarily indicate the presence of P2X purinoceptors on retinal glial cells, especially bearing in mind the fact that the ATP-mediated release of calcium from internal stores in Müller cells seems to involve activation of phospholipase C (Keirstead & Miller, 1997; Newman & Zahs, 1997).

Nevertheless, taken together, the results of the present study suggest that the purinoceptors involved in enhancing the KCl-evoked release of [^{3}H]-GABA from rat retina are more likely to be of the P2X-type rather than the P2Y-type. Certainly, the rat retina possesses P2X receptors because previous binding studies with [^{3}H]-α,β-meATP revealed that the retinas of several species, including rat, possess two to four times more α,β-meATP binding sites than brain (Neal *et al*., 1995). The rat retina possesses both high and low affinity binding sites for α,β-meATP with similar values to those found in brain (unpublished results). At least seven different P2X-receptor subtypes exist and show considerable variation in the locations where they are expressed (North, 1996), but the number of subtypes in the retina is unknown. In the rat retina, Greenwood *et al*. (1997) found mRNA for the $P2X_2$ receptor subtype in the soma of photoreceptors and in neurones in the inner nuclear and ganglion cells layers. Using an antiserum specific for the $P2X_2$-receptor subunit, they confirmed the expression of protein in these cells and showed that the receptor subunit was particularly abundant in the inner plexiform layer. Northern blot screening suggests that $P2X_1$-receptors are also expressed in the retina (Valera *et al*., 1994). $P2X_2$ receptors have been demonstrated in dorsal root ganglia

satellite cells (Vulchanova *et al*., 1997), but since homomeric $P2X_2$-receptors are insensitive to α,β-meATP (Evans & Surprenant, 1996), it is more probable that the ATP receptors on Müller cells are constructed from $P2X_1$-subunits. However, $P2X_1$ receptors have not yet been localized in the retina and we cannot rule out the possibility that other subunits exist. The $P2X_2/P2X_3$ heteropolymer seems a likely possibility since it is a non-desensitizing subtype that is sensitive to α,β-meATP and has been found in sensory neurones (Vulchanova *et al*., 1997).

In rat isolated retinas, ATP causes the propagation of calcium waves in Müller cells. These waves are propagated through coupled astrocytes that form a syncytium at the vitreal surface of the retina and it has been proposed that these glial waves might constitute an extraneuronal signalling pathway (Newman & Zahs, 1997). An increase in $[Ca^{2+}]_i$ would provide a convenient explanation for our finding that ATP increases [3H]-GABA release from rat Müller cells. This type of mechanism has been shown to be involved in bradykinin-evoked release of glutamate from cultured dorsal root ganglia cells (Parpua *et al*., 1995). Unfortunately, the receptors involved in triggering the release of calcium in Müller cells seem to be mainly P2Y subtypes, rather than the P2X type found in our experiments. Furthermore, it seems unlikely that calcium waves are normally involved in GABA release, because in the absence of GABA-T inhibition, Müller cells do not possess measurable quantities of GABA (Neal *et al*., 1989). However, Müller cells in mammalian retina do have a GABA uptake mechanism (GAT-3; Honda *et al*., 1995) and our experiments suggest that ATP increases [3H]-GABA 'release' by reducing the efficacy of this process. The mechanisms involved are unclear but one possibility is that the sodium gradient across the cell membrane, on which GABA transport depends, is reduced by an influx of sodium ions following activation of the ligand-gated P2X receptor. Newman & Zahs (1997) found that ATP caused only weak depolarizations in rat Müller cells but this is not surprising because the membrane conductance of these cells is dominated by inward-rectifier K-channels (Newman & Reichenbach, 1996). The spontaneous resting release of [3H]-GABA from rat glial cells was not affected by P2-agonists in our study. However, the significance of this result is uncertain because the GABA uptake inhibitor SKF89976A also had no effect on the resting release of [3H]-GABA. Presumably, the superfusion rate used in our experiments was sufficiently slow to allow the spontaneously released [3H]-GABA to be transported back into the tissue, even when the uptake system was partially inhibited.

GABA is a major inhibitory transmitter in the retina, being released from up to one third of the amacrine cells. There is much evidence for a tonic release of GABA from these cells. For example, the spontaneous resting release of ACh from cholinergic amacrine cells, which possess GABA receptors (Zhou & Fain, 1995), is strikingly increased by bicuculline (Cunningham & Neal, 1983). However, the mechanisms controlling this tonic release of GABA in the retina are poorly understood. The present experiments suggest that ATP may be involved in modulating endogenous GABAergic tone by reducing the rate of uptake of GABA by the glial Müller cells. If we are correct in our supposition that ATP and ACh are co-released (Neal & Cunningham, 1994), then an ATP-induced increase in GABA tone would be activated by transiently changing light conditions rather than by changes in background illumination.

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