P₂-purinergic receptors regulate phospholipase C and adenylate cyclase activities in immortalized Schwann cells

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Schwann cells play an important role in both the development and regeneration of peripheral nerves. Proliferation and differentiation of Schwann cells are critically dependent on changes in the levels of cAMP. ATP is a fast excitatory transmitter in the peripheral nervous system, inducing depolarization of the vagus nerve through occupancy of P₂-purinergic receptors. In the present study we demonstrate that extracellular ATP stimulates phospholipase C and inhibits adenylate cyclase activities in cultured Schwann cells. Addition of ATP inhibited, in a concentration-dependent manner, forskolin- or isoprenaline-stimulated adenylate cyclase activity. The rank order of potency corresponding to different purinergic receptor agonists was 2-methylthio-ATP > ATP = ADP ≥ adenosine 5'-[γ-thio]triphosphate (ATP[S]) > UTP, consistent with the involvement of a P_{2y} subtype. Adenosine and adenosine 5'-[α,β-methylene]triphosphate (pp[CH₂]pA) were ineffective. Preincubation with pertussis toxin completely blocked this inhibitory effect. When Schwann cells were pre-labelled with *myo*-[³H]inositol and incubated in Hanks' balanced salt solution containing Ca²⁺ and Mg²⁺, addition of ATP[S] resulted in a concentration-

dependent increase in the release of InsP with a concomitant increase in intracellular free [Ca²⁺] ([Ca²⁺]_i). Under these conditions, the effects of both ATP and UTP were of lower magnitude. Removal of Ca²⁺ and Mg²⁺ from the assay medium resulted in a significant increase in the effects of ATP[S], ATP and UTP. The decreased response observed in the presence of both bivalent cations (1.2 mM Ca²⁺ and 1 mM Mg²⁺) could not be explained either by increased degradation of ATP by Ca²⁺/Mg²⁺-dependent nucleotidases or by cation influx. The rank order of potency for the effects of agonists on phospholipase C activity was ATP[S] = adenosine 5'-[γ-imido]triphosphate > ATP = UTP > ADP, indicating the involvement of a P_{2u} receptor subtype in this response. Adenosine, AMP and pp[CH₂]pA were ineffective. These results demonstrate that immortalized Schwann cells express P_{2u} and P_{2y} purinoceptors, which are coupled to stimulation of phospholipase C and inhibition of adenylate cyclase, respectively. Our observations unveil signal-transduction pathways that may be used by ATP to regulate proliferation and differentiation of Schwann cells, and ultimately to influence nerve homeostasis.

INTRODUCTION

Schwann cells are localized at the nodes of Ranvier and have an important role in the production and maintenance of the myelin sheath. Characterization of receptor-mediated signal-transduction pathways in Schwann cells and their role in Schwann-cell proliferation and myelination is a key element in understanding their influence in nerve function under normal and pathological conditions. Several reports have established that changes in cAMP levels are involved in the regulation of both proliferation and differentiation in primary [1–3] and immortalized [4,5] Schwann cells. It has been proposed that cAMP elevation (likely induced by axon-associated factors), acts as a primary signal that synergizes with growth factors in driving Schwann-cell proliferation. On the other hand, withdrawal from the cell cycle (by poorly understood mechanisms) acts as a secondary signal that, superimposed on increased cAMP levels, induces differentiation and myelination (for review see [6]). The list of physiological agents reported to increase cAMP levels in primary Schwann cells is limited to β₁-adrenergic agonists, vasoactive intestinal peptide and secretin [7]. On the other hand, the mitogenic effects triggered by axolemma-derived fractions and glial growth factor seem to involve protein kinase C activation [8,9].

Purinergic receptors are widely distributed and mediate several responses, including vasodilation, proliferation, muscle con-

traction or relaxation, secretion and modulation of ion transport. In the nervous tissue, ATP can either act as a neurotransmitter or modulate the release and action of other neurotransmitters (for review see [10]). The action of extracellular ATP on the modulation of neuronal signal-transduction systems involves an increase in Ca²⁺ uptake, activation of phospholipase A₂, C and D, and changes in the levels of cAMP and cGMP. In peripheral nerve, ATP can be released at the nerve terminals and also from damaged nerves. However, little is known about the role of this nucleotide in the regulation of Schwann-cell function. We now report that ATP and adenosine 5'-[γ-thio]triphosphate ([ATP[S]]) activate the release of inositol phosphates and inhibit adenylate cyclase activity by a pertussis-toxin-sensitive mechanism in a rat Schwann cell line. A preliminary account of these results has been presented in abstract form [11].

MATERIALS AND METHODS

Materials

myo-[2-³H]Inositol (20 mCi/mmol) and [α-³²P]ATP were purchased from DuPont-NEN Products (Boston, MA, U.S.A.). Pertussis toxin was obtained from List Laboratories (Campbell, CA, U.S.A.). Polyethyleneimine-cellulose plates were obtained from Sigma (St. Louis, MO, U.S.A.). Analytical-grade anion-exchange resin AG1-X8 (200–400 mesh, chloride form) was obtained from Bio-Rad. Immortalized Schwann cells were kindly

Abbreviations used: ATP[S], adenosine 5'-[γ-thio]triphosphate; 2MeSATP, 2-methylthioadenosine 5'-triphosphate; pp[CH₂]pA, adenosine 5'-[α,β-methylene]triphosphate; p[NH]ppA, adenosine 5'-[γ-imido]triphosphate; HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's Modified Eagle Medium; [Ca²⁺]_i, intracellular free Ca²⁺ concentration.

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provided by Dr. Seth Porter (Syntex, Palo Alto, CA, U.S.A.). The anti-cAMP antiserum was kindly given by Dr. Kevin Catt (NICHD/NIH, Bethesda, MD, U.S.A.).

Cell culture

Immortalized Schwann cells were routinely grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal-calf serum in 75 cm² flasks, as previously described [12]. These cells, although defective in growth control, associate with axons in culture, deposit a basal lamina and ensheath and myelinate axons, retaining most, if not all, of the functional capabilities of primary Schwann cells [13]. Cells were subcultured into 12-well plates for experiments aimed at measuring cAMP accumulation or phosphoinositide breakdown. For measurement of intracellular free Ca²⁺ ([Ca²⁺]_i), cells were plated on 12 mm glass cover-slips. To minimize variations, cells were routinely used at the same passage number, 48–72 h after plating. Under these conditions, the cultures were consistently 80–100% confluent, with few or no cell aggregates.

Determination of phosphoinositide metabolism

Cells were labelled with [³H]inositol (1 µCi/well) in serum- and inositol-free DMEM for approx. 16–20 h before the experiment. In some experiments, pertussis toxin (0.1 µg/ml) was also added during the labelling period. After labelling, cells were washed for 1 h in DMEM, followed by removal of media and addition of 1 ml of Hanks' Balanced Salt Solution (HBSS) containing 10 mM LiCl and 10 mM glucose. Unless otherwise indicated, the concentrations of Mg²⁺ and Ca²⁺ in the HBSS solution were 0.9 and 1.2 mM respectively. Cells were preincubated for 15 min, followed by addition of the desired agonists (10 µl) and further incubation for an additional 30 min. The reaction was stopped by removal of the media and addition of 500 µl of methanol/HCl (100:1, v/v). Cells were scraped and transferred to glass tubes containing 1 ml of chloroform and 25 µg of phytic acid hydrolysate to give final proportions of chloroform:methanol:water of 10:5:4 (by vol.). After separation, the upper phase was removed and subjected to chromatography on AG1-X8 Dowex columns (chloride form) as previously described [14]. Briefly, InsP₁, InsP₂ and InsP₃ were eluted with 30 mM, 90 mM and 0.5 M HCl respectively, and radioactivity in each fraction was measured by scintillation spectroscopy. Lower phases were washed with 500 µl of theoretical upper phase (chloroform:methanol:water, 3:47:48, by vol.) containing 1 mM *myo*-inositol, and dried down under nitrogen. Dried extracts were resuspended in 300 µl of chloroform:methanol:water (75:25:2, by vol.) and duplicate 30 µl samples were counted for radioactivity.

Fura-2 loading and microfluorimetry

Schwann cells were washed once with HBSS and incubated at 37 °C with 1 µM fura-2 acetoxymethyl ester (fura-2/AM) for 45–60 min to allow entrapment and hydrolysis of the fura-2 ester. After loading, the cover-slips were washed and mounted in a perfusion chamber on the stage of an upright fluorescence microscope. The loaded cells were assayed within 90 min from the end of loading. Microfluorimetry was performed as previously described [15]. Briefly, cells were illuminated alternatively with 340 and 380 nm-wavelength light through the epifluorescence optics of the microscope. The fluorescence signal was normalized by calculating the ratio of the emission intensity after excitation at 340 and 380 nm. This ratio is a function of the [Ca²⁺]_i and independent of the fura-2 concentration. Conversion of the fura-2 ratio into [Ca²⁺]_i was accomplished by a calibration curve

which was constructed with fura-2 in a solution containing 0.15 mM KCl, 1 mM EGTA, 10 mM Mops buffer, pH 7.4, and appropriate concentrations of CaCl₂ to yield 50–1000 µM free Ca²⁺.

Measurement of cAMP levels

Schwann cells were serum-deprived 3–6 h before the start of experiments. In some cases, cells were treated with pertussis toxin for 16–20 h, as described for measurement of phosphoinositide metabolism. At the beginning of the experiment, the medium was removed and 1 ml of 500 µM isobutylmethylxanthine in DMEM was added. After a 5 min preincubation, the desired agonists were added and the cells further incubated for 15 min. At the end of this period, the media were removed, followed by three washes with ice-cold Dulbecco's PBS and addition of 500 µl of boiling 50 mM sodium acetate, pH 6.0. Cells were scraped and transferred to microfuge tubes. The tubes were placed in boiling water for 10 min. cAMP was measured by radioimmunoassay [16].

Determination of ecto-nucleotidase activity

[α -³²P]ATP breakdown was measured as an estimation of ecto-nucleotidase activity. Cells were subcultured in 12-well plates as described above. After 48 h, the medium was replaced by serum-free DMEM and cells were incubated for 15 h. After removal of the media, 500 µl portions of HBSS containing 10 mM LiCl, 10 mM glucose and variable amounts of Mg²⁺ and Ca²⁺ were added. After 15 min preincubation, a second 500 µl portion of the respective HBSS solutions containing 0.36 µCi of [α -³²P]ATP and increasing concentrations of unlabelled ATP was added. Samples of the media (2.5 µl) were removed at 0, 5, 15, 30 and 60 min. Separation of hydrolysis products was performed by TLC on polyethyleneimine-cellulose plates, with 1 M LiCl as running solvent (internal standards of unlabelled nucleotides were added to each sample). Radioactive spots were detected by autoradiography, cut out and counted by liquid scintillation.

RESULTS

cAMP accumulation

Schwann cells incubated in the presence of forskolin or isoprenaline displayed a significant increase in the accumulation of cAMP (Table 1). Addition of ATP or ATP[S], ineffective by itself (results not shown), resulted in approx. 50–75% inhibition of isoprenaline- or forskolin-stimulated cAMP production respectively (Table 1). This inhibitory effect of ATP was concentration-

Table 1 Effect of ATP on forskolin- or isoprenaline-stimulated cAMP accumulation in immortalized Schwann cells

Schwann cells were incubated for 12 h in serum-free DMEM. Cells were subsequently washed and incubated for 15 min with the indicated concentrations of forskolin or isoprenaline in the presence or absence of ATP. Samples were processed as described in the Materials and methods section. Each value represents the mean $\pm$ S.E.M. of triplicate incubations. Symbols show significant differences from control (**) or from forskolin or isoprenaline (†) at $P < 0.01$, as determined by Student's *t* test.

	cAMP (pmol/mg of protein)
Control	7.1 $\pm$ 0.3
Forskolin (5 µM)	72.0 $\pm$ 5.9**
Forskolin (5 µM) + ATP (100 µM)	37.9 $\pm$ 5.0†
Isoprenaline (10 µM)	49.8 $\pm$ 5.8**
Isoprenaline (10 µM) + ATP (100 µM)	18.6 $\pm$ 0.9†

Figure 1 Concentration–response curves corresponding to the effects of nucleotides on isoprenaline-stimulated cAMP accumulation in Schwann cells

Cells were serum-deprived for 12 h and incubated in the presence of 100 μ M isoprenaline and the indicated concentrations of nucleotides. Results shown represent cAMP accumulation relative to that measured with 100 μ M isoprenaline alone. Samples were processed as described in the Materials and methods section.

Table 2 Reversal by pertussis toxin of the inhibitory effect of ATP on forskolin-stimulated cAMP levels in Schwann cells

Schwann cells were incubated for 12 h in serum-free DMEM with or without 100 ng/ml pertussis toxin (PT). At the end of the incubation, cells were washed and incubated for 15 min with forskolin in the presence or absence of ATP. Samples were processed as described in the Materials and methods section. Each value represents the mean $\pm$ S.E.M. of triplicate incubations: ** significantly different from their corresponding controls at $P < 0.01$, as determined by Student's t test.

	cAMP (pmol/mg of protein)	
	– PT	+ PT
Control	15.0 $\pm$ 1.9	16.5 $\pm$ 0.6
Forskolin (5 μ M)	45.6 $\pm$ 2.7**	42.0 $\pm$ 8.9**
Forskolin (5 μ M) + ATP (100 μ M)	22.8 $\pm$ 3.2	37.8 $\pm$ 7.4**

dependent, displaying an IC₅₀ value of approx. 1 μ M (Figure 1). We also examined the pharmacological specificity of this inhibitory effect. The rank order of potency corresponding to different ATP analogues was 2-methylthio-ATP (2MeSATP) > ATP = ADP $\geq$ ATP[S] > UTP, consistent with the involvement of a P_{2y} purinergic-receptor subtype (Figure 1). Adenosine and adenosine 5'-[α,β -methylene]triphosphate (pp[CH₂]pA) were ineffective (results not shown). Incubation of cells in the presence of pertussis toxin (100 ng/ml) completely blocked the inhibitory effect of ATP on forskolin- and isoprenaline-stimulated cAMP accumulation (only forskolin is shown in Table 2). These results indicate that a pertussis-toxin-sensitive G-protein mediates the effects triggered by occupancy of the extracellular ATP receptor present in Schwann cells, and that this receptor probably belongs to the P_{2y} subtype.

Phosphoinositide metabolism

When [³H]inositol-labelled Schwann cells were incubated with ATP[S] in HBSS containing 1.2 mM Ca²⁺ and 1 mM Mg²⁺ (HBSS⁺) and 10 mM LiCl for 30 min, there was a significant

Table 3 Ca²⁺ and Mg²⁺ influence the ability of nucleotides to release InsP in Schwann cells

Cells were prelabelled with myo-[³H]inositol, washed and incubated with 100 μ M of the indicated nucleotides in HBSS in the presence (HBSS⁺) or absence (HBSS[−]) of 1.2 mM CaCl₂ and 1 mM MgCl₂. Samples were processed as described in the Materials and methods section. Each value represents the mean $\pm$ S.E.M. of triplicate determinations: **significantly different from their corresponding controls at $P < 0.01$ as determined by Student's t test. Abbreviation: PPI, phosphoinositides.

	InsP released (%) [100 $\times$ InsP d.p.m./ (InsP d.p.m. + PPI d.p.m.)]	
	HBSS [−]	HBSS ⁺
Control	1.38 $\pm$ 0.15	1.31 $\pm$ 0.09
ATP	2.43 $\pm$ 0.12**	1.59 $\pm$ 0.12
UTP	2.66 $\pm$ 0.16**	1.68 $\pm$ 0.08
ATP[S]	3.82 $\pm$ 0.77**	2.32 $\pm$ 0.20**

increase in the release of InsP (Table 3), but no changes were observed for InsP₂ and InsP₃ (results not shown). This effect was concentration-dependent, with a threshold at 30 μ M agonist (Figure 2a). When ATP was replaced by ATP[S], the increase in InsP release was of a lower magnitude (no more than 30% over control) and observed in most, but not all, experiments (Table 3). Single-cell measurements of [Ca²⁺]_i transients showed that addition of ATP[S] to Schwann cells elicited a concentration-dependent increase in [Ca²⁺]_i (Table 4). This increase closely followed the kinetics of InsP₃ release (Figure 3). On the other hand, and similarly to what was observed during InsP-release experiments, addition of either 0.1 mM or 1 mM ATP was ineffective in increasing [Ca²⁺]_i (results not shown).

Several ATP analogues were assayed in order to identify further the putative purinergic-receptor subtype involved in the InsP response. The effects of UTP (Table 3) and ADP were similar to that of ATP, whereas AMP and adenosine were ineffective (results not shown). Incubation of Schwann cells in the presence of pp[CH₂]pA or 2MeSATP, selective agonists for the P_{2x} and P_{2y} purinoceptor subtypes respectively, were also ineffective (results not shown).

Interestingly, we observed that removal of Ca²⁺ and Mg²⁺ from the HBSS increased the potency and the efficacy of the effect triggered by ATP and analogues (Table 3 and Figure 2). However, addition of 1 mM each of EGTA and EDTA to a nominally Ca²⁺- and Mg²⁺-free medium (to chelate bivalent cations arising from water and reagents) significantly decreased basal release of InsP and abolished nucleotide effects (results not shown). Determination of the order of potency of different ATP analogues in prelabelled Schwann cells suspended in HBSS without Ca²⁺ and Mg²⁺ (HBSS[−]) revealed that ATP, UTP or ATP[S] elicited a concentration-dependent increase in the release of InsP, with similar EC₅₀ values in the 7–12 μ M range (Figures 2a and 2b). Under these conditions, ATP[S], adenosine 5'-[γ -imido]triphosphate (p[NH]ppA), ATP and UTP (at a concentration of 100 μ M) were the most effective agonists stimulating InsP release (Table 5). ADP had a moderate effect, whereas 2MeSATP, pp[CH₂]pA and adenosine were ineffective (Table 5).

Experiments in which Schwann cells were assayed in HBSS⁺ in the presence of up to 2 mM ATP (to generate a concentration of ATP^{4−} equivalent to that in HBSS[−] containing 100 μ M ATP) did not mimic the effect of ATP in HBSS[−] (results not shown).

Preincubation of the cells with pertussis toxin (100 ng/ml) did not alter significantly the maximum amount of InsP release by

Figure 2 Concentration–response curves corresponding to the effects of nucleotides on InsP release in the presence or absence of Ca^{2+} and Mg^{2+}

Schwann cells were prelabelled with $\text{myo-}[^3\text{H}]\text{inositol}$, washed and incubated with increasing concentrations of nucleotides. After incubation, samples were processed as described in the Materials and methods section. The release of InsP is expressed as $[100 \times \text{InsP d.p.m.}/(\text{InsP d.p.m.} + \text{PPI d.p.m.})]$. Abbreviation: PPI, phosphoinositide. The results represent means $\pm$ S.E.M. of triplicate determinations. (a) Schwann cells were incubated with increasing concentrations of ATP[S] in HBSS⁺ or HBSS[−], as indicated. (b) Schwann cells were incubated with increasing concentrations of ATP or UTP in HBSS[−].

Table 4 Effect of ATP[S] on the levels of $[\text{Ca}^{2+}]_i$

Fura-2-loaded Schwann cells were exposed to the indicated concentrations of ATP[S] in HBSS containing 1 mM MgCl_2 and 1.2 mM CaCl_2 . ATP[S]-induced $[\text{Ca}^{2+}]_i$ transients were measured as described in the Materials and methods section. The changes in $[\text{Ca}^{2+}]_i$ were expressed as $\% \Delta [\text{Ca}^{2+}]_i$, as means $\pm$ S.E.M. $[100 \times (\text{peak} - \text{basal})/\text{basal}]$ and include the responsive and non-responsive cells.

ATP[S] (μM)	% $\Delta [\text{Ca}^{2+}]_i$	Total no. of cells	% responsive cells
10	23 ± 7	12	67
30	123 ± 30	33	79
100	242 ± 40	33	100
300	178 ± 18	53	62

100 μM ATP[S] or ATP in HBSS[−] (results not shown). These results indicate that the purinoceptor subtype and the G-protein coupling to phospholipase C activation are different from those involved in the inhibition of adenylate cyclase.

Determination of ecto-nucleotidase activity

Several reports in the literature have established that various cells in culture exhibit a significant Ca^{2+} - and Mg^{2+} -dependent ecto-nucleotidase activity which is able to hydrolyse ATP to ADP, AMP and adenosine [17]. Such activity could decrease the concentration of ATP during the assays and explain the small effects of this nucleotide in the presence of cations. This possibility was investigated by studying the rate of degradation of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ by Schwann cells incubated in HBSS in the presence or absence of 1.2 mM CaCl_2 and 0.9 mM MgCl_2 . As shown in Figure 4(a), when 100 μM $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ was added in the presence of both cations we observed a significant degradation of the nucleotide, with only 30 % remaining after a 30 min incubation. Omission of Ca^{2+} and Mg^{2+} from the HBSS decreased the degradation by 10 % only at 15 min; no significant differences were observed at other time points. However, addition of EGTA and EDTA (1 mM each), to chelate Ca^{2+} arising from water and reagents in the medium, completely prevented the breakdown of

Figure 3 Effect of ATP[S] on $[\text{Ca}^{2+}]_i$ in single Schwann cells

Fura-2-loaded cells were exposed to 300 μM ATP[S] in HBSS containing 1.2 mM CaCl_2 and 1 mM MgCl_2 . $[\text{Ca}^{2+}]_i$ was determined by measuring fura-2-related fluorescence as described in the Materials and methods section. The inset shows the kinetics of ATP[S]-stimulated InsP₃ release in Schwann cells from one independent experiment (repeated three times); results shown represent fold stimulation by addition of 100 μM ATP[S] (relative to control), for each time point.

ATP throughout the 30 min period (results not shown). Degradation of ATP occurred in parallel with a significant increase in the production of ADP and small amounts of AMP (Figure 4b). Increasing the concentration of unlabelled ATP up to 1 mM did not result in significant changes in the rate of degradation (results not shown). These results suggested that the decreased effect of ATP on InsP release in HBSS⁺ does not result from a decrease in the concentration of this nucleotide during the assay or formation of ADP. Furthermore, when Schwann cells were simultaneously exposed to ATP and ADP (100 μM each) in HBSS[−], the release of InsP was similar to that observed in the

Table 5 Stimulation of InsP release in Ca²⁺- and Mg²⁺-free HBSS (HBSS⁻)

Prelabelled Schwann cells were washed and preincubated for 15 min in HBSS⁻ containing 10 mM LiCl. Agonists were added and incubation continued for another 30 min. Reaction was stopped and samples were processed as described in the Materials and methods section. InsP released was calculated as the fraction of total labelled phosphoinositides. The effect of nucleotides was expressed as % of InsP released under control conditions. Values are means $\pm$ S.E.M.: significantly different from control at $P < 0.01$ (**) and at $P < 0.05$ (*) as determined by Student's *t* test. Abbreviation: GTP[S], guanosine 5'-[γ -thio]triphosphate

Agonist (100 μ M)	InsP release (% of control)
Vehicle	100
ATP	176 $\pm$ 18**
ATP[S]	234 $\pm$ 43**
UTP	179 $\pm$ 15**
ADP	139 $\pm$ 3*
AMP	105 $\pm$ 7
Adenosine	95 $\pm$ 21
2MeSATP	87 $\pm$ 3
pp[CH ₂]pA	88 $\pm$ 24
p[NH]ppA	217 $\pm$ 8**
GTP[S]	128 $\pm$ 2*

presence of ATP alone (results not shown), indicating that ADP was not acting as an antagonist.

Influence of Ca²⁺ influx on ATP-mediated InsP release

We studied whether influx of Ca²⁺ was responsible for the differential effects of ATP/UTP on InsP release in HBSS⁺ and HBSS⁻ media. To this end, we carried out experiments in which Gd³⁺, a potent Ca²⁺-channel blocker, was added to Schwann cells incubated in either buffer (HBSS⁺ or HBSS⁻). As shown in Table 6, addition of 100 μ M Gd³⁺ decreased the basal and nucleotide-stimulated release of InsP, although it did not alter significantly the effect of ATP on this response in Schwann cells incubated in HBSS⁻. On the other hand, Gd³⁺ completely abolished the nucleotide effect observed in HBSS⁺ (Table 6). This result eliminated Ca²⁺ influx as responsible for the decreased effect of ATP in HBSS⁺. The response to ATP[S] was slightly

Table 6 Effect of a Ca²⁺-channel blocker (Gd³⁺) on basal and ATP-stimulated InsP release

Schwann cells were incubated in HBSS⁻ or HBSS⁺ with or without 100 μ M GdCl₃. The effect of ATP on InsP release was determined as described in the Materials and methods section. The results shown represent means $\pm$ S.E.M. of triplicate determinations: ** significantly different from the corresponding basal at $P < 0.01$; † significantly different from the corresponding controls at $P < 0.05$ as determined by Student's *t* test. Abbreviation: PPI, phosphoinositide.

		InsP released (%) [100 $\times$ InsP d.p.m./ (InsP d.p.m. + PPI d.p.m.)]	
		Basal	ATP (100 μ M)
HBSS ⁻	Control	1.26 $\pm$ 0.18	1.91 $\pm$ 0.05**
	Gd ³⁺	0.78 $\pm$ 0.06†	1.25 $\pm$ 0.07†
HBSS ⁺	Control	1.68 $\pm$ 0.18	2.12 $\pm$ 0.19**
	Gd ³⁺	1.45 $\pm$ 0.13	1.38 $\pm$ 0.15†

decreased in either medium (HBSS⁻ or HBSS⁺) (results not shown). Separate addition of Ca²⁺ and Mg²⁺ significantly decreased the ATP-mediated response in HBSS⁻ (but not that of ATP[S]). Additional experiments were carried out in which several bivalent cations, also known to affect Ca²⁺ influx, were added to HBSS⁻ media. Addition of 1 mM Co²⁺ completely abolished the effects of ATP and ATP[S] (Table 7). A similar inhibition of the nucleotide-dependent stimulation was observed when Co²⁺ was replaced by 1 mM Mn²⁺ or Cd²⁺. As mentioned above, addition of EGTA and EDTA (1 mM each) to HBSS⁻ significantly decreased basal release of InsP and abolished nucleotide effects. These results indicate that optimal activity of phospholipase C requires threshold concentration of bivalent cations and that higher levels of these cations impair nucleotide-mediated stimulation of this enzyme, by an as yet undefined mechanism.

DISCUSSION

In the present study, we demonstrated that immortalized Schwann cells express P₂ purinergic receptors linked to at least two signalling systems: stimulation of phosphoinositide metab-

Figure 4 Effect of Ca²⁺ and Mg²⁺ on breakdown of ATP by Schwann cells

Cells were incubated in HBSS + 100 μ M [α -³²P]ATP (sp. radioactivity 3.6 μ Ci/ μ mol), in the absence or presence of 1.2 mM CaCl₂ and 1 mM MgCl₂. Samples of the reaction mixtures were taken at the indicated times and separated as described in the Materials and methods section. The results shown represent the average of three independent experiments. Results are expressed as the percentage of total added [α -³²P]ATP remaining after incubation for the indicated times (a) or as the percentage of [α -³²P]ADP and [α -³²P]AMP formed from [α -³²P]ATP (b).

Table 7 Effect of different bivalent cations on the release of *InsP* stimulated by ATP and ATP[S]

Schwann cells were incubated in HBSS containing either no cations (control) or 1 mM of the indicated bivalent cations, in the absence or presence of ATP or ATP[S]. Each value represents the mean $\pm$ S.E.M. of 2–3 independent experiments carried out in triplicate. Statistical significances of differences between means were determined by ANOVA, followed by Dunnett's test: ** significantly different from the corresponding basal at $P < 0.01$. Abbreviation: PPI, phosphoinositide.

	InsP released (%) [$100 \times \text{InsP d.p.m.} / (\text{InsP d.p.m.} + \text{PPI d.p.m.})$]		
	Basal	ATP (100 μM)	ATP[S] (100 μM)
Control	1.33 $\pm$ 0.05	2.21 $\pm$ 0.38**	2.76 $\pm$ 0.51**
Co ²⁺	0.97 $\pm$ 0.07	0.97 $\pm$ 0.06	1.09 $\pm$ 0.14
Mn ²⁺	1.12 $\pm$ 0.32	1.16 $\pm$ 0.05	1.44 $\pm$ 0.08
Cd ²⁺	1.25 $\pm$ 0.52	1.66 $\pm$ 0.52	1.51 $\pm$ 0.72
Mg ²⁺	1.24 $\pm$ 0.09	1.44 $\pm$ 0.15	2.31 $\pm$ 0.11**
Ca ²⁺	1.70 $\pm$ 0.08	1.83 $\pm$ 0.28	3.15 $\pm$ 0.12**

olism, with concomitant increase in $[\text{Ca}^{2+}]_i$, and inhibition of agonist-stimulated adenylate cyclase activity.

Extracellular ATP has been reported to stimulate phosphoinositide hydrolysis and to inhibit cAMP accumulation in various cell types. Studies carried out in diverse cell types, including hepatocytes, myocytes and C6 glioma cells, have identified P_2 -purinergic receptors that stimulate phosphoinositide hydrolysis and inhibit cAMP accumulation through different receptor subtypes or different signalling pathways [18–21]. Sato et al. [22] have observed that ATP inhibits adenylate cyclase and stimulates phospholipase C through different subtypes of P_2 -purinergic receptors in FRTL-5 thyroid cells. Recently, Boyer et al. [23] reported that a P_2 -purinergic receptor in C6-2B glioma cells is coupled to inhibition of forskolin- or isoprenaline-stimulated adenylate cyclase activity, but does not regulate inositol lipid metabolism. Pharmacological studies have supported the existence of five different receptor subtypes, termed P_{2x} , P_{2y} , P_{2u} , P_{2t} and P_{2z} [10,17]. ATP, UTP and ADP are endogenous agonists for the P_{2x} , P_{2y} and P_{2u} subtypes. Both methylenephosphonate and phosphorothioate derivatives have been used to distinguish these subtypes. The accepted rank order of potencies is as follows: P_{2x} : $\text{pp}[\text{CH}_2]\text{pA} > \text{ATP} \geq 2\text{MeSATP}$; P_{2y} : $2\text{MeSATP} \geq \text{ATP} = \text{UTP} = \text{ATP[S]}$; P_{2u} : $\text{ATP[S]} \geq \text{UTP} = \text{ATP} \geq \text{ADP}$; P_{2z} : the free form of ATP (ATP^{4-}) is the only active agonist. According to this classification, the inhibition of adenylate cyclase activity by a P_2 -purinoceptor agonist in immortalized Schwann cells is mediated by a P_{2y} receptor subtype. The nucleotide-dependent stimulation of *InsP* release is more complex and appears to be mediated by a different receptor subtype, probably a P_{2u} . In addition, the two effects showed different sensitivity to preincubation with pertussis toxin, indicative of coupling through more than one G protein.

The more puzzling result of the present study is that low levels of Ca^{2+} and Mg^{2+} (approx. 10 μM each) enhanced *InsP* release by ATP or UTP. We thought of, and tested, two possible explanations for this observation. (1) The effects of ATP detected in media containing low Ca^{2+} and Mg^{2+} may be triggered by the interaction of ATP^{4-} with a P_{2z} subtype. The presence of Ca^{2+} and Mg^{2+} (1.2 mM and 0.9 mM respectively) results predominantly in the formation of MgATP^{2-} and CaATP^{2-} , co-existing with low amounts of ATP^{4-} (approx. 4 μM ATP^{4-} in HBSS⁺, compared with approx. 40 μM in HBSS⁻). To test this

possibility, we carried out experiments in which Schwann cells were assayed in HBSS⁺ in the presence of up to 2 mM ATP to generate a concentration of ATP^{4-} equivalent to that in HBSS⁻ containing 100 μM ATP (40 μM ATP^{4-}). However, under these experimental conditions, we did not observe an increase in *InsP* formation compared with that in HBSS⁺ with 100 μM ATP (results not shown). (2) Cultured Schwann cells exhibit a high ecto-nucleotidase activity, which decreases the effective concentration of ATP during the assay (Figure 4). Ca^{2+} - and Mg^{2+} -activated ecto-ATPases, which hydrolyse ATP and other nucleotides, have been described on the surface of many cell types [24]. As described above (Figure 4), the similar rates of ATP hydrolysis in the presence or absence of cations cannot explain the differential effects of ATP. Furthermore, the results obtained in the presence of Gd^{3+} or Co^{2+} indicate that $\text{Ca}^{2+}/\text{Mg}^{2+}$ influx in high Ca^{2+} and Mg^{2+} -containing media is not responsible for the decreased response to nucleotides. An alternative possibility is that these cations may interfere with the binding of nucleotide to the receptor subtype that triggers *InsP* release. This is suggested by the fact that the ATP-mediated inhibition of cAMP accumulation was not modified when the experiments were carried out in either HBSS⁻ or HBSS⁺. In this context, Azula et al. [25] have suggested that Ni^{2+} impairs thrombin-induced signal transduction by acting on the agonist and/or its receptor in human platelets. Other plausible explanations for the decreased effect of ATP on *InsP* release in the presence of bivalent cations include the eventual activation of other signalling pathways, which may lead to inhibition of phospholipase C activation (such as increased phospholipase D activity, resulting in diacylglycerol production and protein kinase C activation). Further studies are needed to test this hypothesis.

Treize et al. [26] have recently reported that the potency of ATP in triggering depolarization of rat vagus nerve is significantly increased when Ca^{2+} and Mg^{2+} are omitted from the incubation media. In addition, these authors observed changes in the rank order of potency of several ATP analogues in the presence or absence of these bivalent cations. A P_{2x} receptor subtype appeared to be involved when the experiments were carried out in the presence of Ca^{2+} and Mg^{2+} ; a P_{2y} subtype was suggested when the studies were carried out in their absence. The explanation proposed by these authors (although not tested) was that their results reflected differential metabolism of nucleotides involving $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent ecto-nucleotidases.

In peripheral nervous system, ATP released at adrenergic synaptic terminals [27] controls the release of sympathetic transmitter [28], induces depolarization of isolated vagus nerve [29] and increases the release of inositol phosphates in isolated sciatic nerves [30]. Our results, together with the recent report by Lyons et al. [31], represent the first observations of nucleotide effects mediated by expression of P_2 purinergic receptors in Schwann cells.

In conclusion, immortalized Schwann cells express P_{2u} and P_{2y} purinoceptors which are coupled to stimulation of phospholipase C and inhibition of adenylate cyclase. As mentioned above, these two signalling systems play a key role in the mechanisms regulating Schwann-cell proliferation and differentiation. We are currently defining the G-proteins linked to these effects and the role of these signal-transduction pathways in Schwann-cell function.

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