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European Journal of Pharmacology 415 (2001) 151–156

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Characterisation of $P2Y_1$-like receptor in cultured rat pineal glands

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Received 15 June 2000; received in revised form 29 January 2001; accepted 2 February 2001

Abstract

The rat pineal gland possesses P2 receptors which potentiate the effect of noradrenaline-induced *N′*-acetyl-5-hydroxytryptamine (*N′*-acetyl-5-HT) production. In the current study, this receptor was characterised according to agonist selectivity and signal transduction mechanisms. 2-MethylthioATP (2MeSATP), 2-chloroATP (2-ClATP), adenosine 5′-*O*-2-thiodiphosphate, (ADPβS), ATP and ADP, but not UTP, potentiated noradrenaline-induced *N′*-acetyl-5-HT production in a concentration-dependent manner. 2MeSATP neither induced the production of adenosine 3′:5′-cyclic monophosphate (cyclic AMP), nor inhibited its formation when the glands were stimulated by forskolin. The phospholipase C inhibitor 1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122), but not the inactive analogue, 1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione (U73343), blocked the 2MeSATP effect. The P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-dissulphonic acid (PPADS), which inhibits phospholipase C-coupled $P2Y_1$ receptors, blocked the 2MeSATP effect. In conclusion, our data strongly suggest that the P2-like receptor that is present in rat pinealocytes and which is responsible for the potentiation of noradrenaline-induced *N′*-acetyl-5-HT production is a $P2Y_1$-like receptor, coupled to a G protein which stimulates phospholipase C.

Keywords: $P2Y_1$ receptor; Pineal gland; *N′*-acetyl-5-hydroxytryptamine production; Melatonin; Phospholipase C

1. Introduction

The pineal gland conveys information concerning the light–dark cycle to body centres for the organisation of seasonal and circadian rhythms. The pattern of secretion of the pineal hormone *N*-acetyl-5 methoxytryptamine, known as melatonin, forms the basis for this message. Melatonin is synthesised from 5-hydroxytryptamine (5-HT) via *N*-acetylation by arylalkylamine-*N*-acetyltransferase (E.C.2:3.1.87) to *N′*-acetyl-5-hydroxytryptamine (*N′*-acetyl5-HT) and *O*-methylation by hydroxyindole-*O*-methyltransferase (E.C.2.1.1.4) to melatonin (Klein et al., 1983). Both melatonin and *N′*-acetyl-5-HT are released in the blood stream (Niles et al., 1984). The gland is innervated primarily from the peripheral sympathetic tract (Kappers, 1960). Noradrenaline triggers the nocturnal peak of melatonin due to the induction of the synthesis and activation of the enzyme arylalkylamine-*N*-acetyltransferase, resulting in the formation of the precursor *N′*-acetyl-5-HT.

ATP is thought to be released along with noradrenaline during neurotransmission and has been suggested as a co-transmitter in postganglionic sympathetic neurones (Burnstock, 1976). It is now well established from functional and molecular biology studies that extracellular ATP exerts its effect directly, or through metabolites, such as ADP or adenosine, via specific receptors called purine receptors. There are two main families of purine receptors: adenosine or P1 receptors and P2 receptors, which recognise primarily ATP, ADP, UTP, and UDP. Adenosine/P1 receptors have been further subdivided into four subtypes, all of them coupled to G proteins. The P2 receptors are primarily subdivided into two main subtypes: ligand-gated ion channels (P2X receptors) and G protein-coupled receptors (P2Y receptors) (Abbracchio and Burnstock, 1994; Barnard et al., 1994; Fredholm et al., 1994). P2X receptors mediate the rapid (onset within 10 ms) nonselective passage of cations (Na^+, K^+ and Ca^{2+}) across the cell membrane, resulting in depolarisation and an increase in intracellular Ca^{2+} (Bean, 1992; Dubyak and El-Moatassim, 1993). Most P2Y receptors act via G protein coupling

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)00823-8

to activate phospholipase C, leading to the formation of inositol-1,4,5-triphosphate (IP_3) and to the mobilisation of intracellular Ca^{2+} (Boyer et al., 1989). Coupling to adenylyl cyclase by some P2Y receptors has also been described (Boyer et al., 1993). The response time of P2Y is longer than that of the rapid responses mediated by P2X receptors because it involves second-messenger systems and/or ionic conductances mediated by G protein coupling (Ralevic and Burnstock, 1998). To date, seven mammalian P2X receptors ($P2X_{1-7}$) and five P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$) have been cloned, pharmacologically characterised and accepted as valid members of the P2 family (Ralevic and Burnstock, 1998).

We have previously shown that, in denervated pineal glands, ATP and the less hydrolysable agonist 5′-adenylylimido-diphosphate (AMP-PNP), which have no effect alone, potentiate the production of *N*′-acetyl-5-HT induced by noradrenaline (Ferreira et al., 1994). The effect of ATP is inhibited by suramin and high doses of α,β-methylene ATP (α,β-mATP), suggesting that stimulation of P2-like receptors might also contribute to the production of melatonin.

The aim of the present paper was to investigate which subtype of P2-like receptors mediates the potentiation of the noradrenaline-induced production of *N*′-acetyl-5-HT in cultured rat pineal glands. Thus, in order to characterise this receptor, we investigated its selectivity for agonists, the second messengers responsible for the transduction signal and the effect of the antagonist pyridoxalphosphate-6-azophenyl-2′,4′-dissulphonic acid (PPADS), by measuring the product of arylalkylamine-*N*-acetyltransferase activity in cultured glands and in the incubation medium. The denomination P2-like receptor will be used since the characterisation is based on its pharmacological profile and signal transduction.

2. Materials and methods

2.1. Materials

2-MethylthioADP (2MeSADP), 2-methylthioATP (2MeSATP), 2-chloroATP (2-ClATP), PPADS, (1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino)hexyl]-1*H*-pyrrole-2,5-dione) (U73122) and forskolin were purchased from RBI, Natick, USA; ATP, *N*′-acetyl-5-HT, noradrenaline, UTP, adenosine 5′-*O*-2-thiodiphosphate (ADPβS), ADP, (1-[6 [[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino)hexyl]-2,5-pyrrolidinedione) (U73343), 3-isobutyl-1-methylxanthine (IBMX), BGJ_b medium—(Fitton–Jackson modification) and bovine albumin from Sigma, St. Louis, USA; ascorbic acid from Hoechst, Brazil; citric acid, EDTA, sodium acetate, sodium bisulphite, methanol, perchloric acid and acetic acid from Merck, Brazil; and cyclic AMP [^{125}I] radioimmunoassay kit from NEN™ Life Science Products–E.I. DuPont de Nemours, Boston, MA.

2.2. Pineal culture

Pineal glands were obtained from male or female Wistar rats (1–2 month-old) kept under a 12:12-h light–dark cycle and killed by decapitation between 0900 and 1100 h, as previously described (Ferreira et al., 1994). The glands were incubated (37°C; 95% O_2; 5% CO_2) in the BGJ_b medium, supplied with 2 mM glutamine, 0.1 mg/ml ascorbic acid, 100 units/ml penicillin, 100 μg/ml streptomycin and 1 mg/ml bovine serum albumin (fraction V), in a 24-multiwell plate (1 gland per well, 200 μl per well) for 48 h prior to treatment. Presynaptic elements degenerate during this period, resulting in a completely denervated preparation (Parfitt et al., 1976).

2.3. N′-Acetyl-5-HT production measurements

After 48 h, the cultured pineal glands were placed in fresh medium for 1 h and then incubated with the agonists for 5 h. Each gland was incubated with one concentration of the following agonists: 2MeSATP (30 μM–1 mM), 2-ClATP (30 μM–1 mM), ADPβS (10 μM–1 mM), ATP (10 μM–1 mM), ADP (10 μM–1 mM) and UTP (1 mM), simultaneously with noradrenaline (10 nM). PPADS (10, 30 and 60 μM), when present, was added 15 min prior to other treatments. At the end of the incubation period, glands and medium were placed in different microtubes and stored at −70°C. The *N*′-acetyl-5-HT content in the gland and incubation medium was determined by high-performance liquid chromatography and measured by electrochemical detection based on a previously described method (Ferreira et al., 1994). Briefly, each gland was homogenized (5 s) in ice-cold 0.1 M perchloric acid (120 μl) containing 0.02% EDTA and 0.02% sodium bisulphite. Protein and cell debris were removed by centrifugation ($13{,}000 \times g$, 5 min, 4°C). Twenty microlitres of the clear supernatant or the incubation medium was injected into the chromatographic system (Shimadzu), which was isocratically operated. The mobile phase (0.1 M sodium acetate, 0.1 M citric acid, 0.15 mM EDTA, 10% methanol, pH 3.7) flowed at a rate of 1.0 ml/min through a 5-μm Resolve C_{18} reversed-phase column (150×3.9 mm i.d., Waters). The detector potential was adjusted to +0.90 V (vs. Ag/AgCl reference electrode).

2.4. Signaling pathways

Adenylyl cyclase pathway was evaluated by measuring the ability of 2MeSATP to induce the production of cyclic AMP or to inhibit the production of this second messenger induced by forskolin. Glands treated for 20 min with IBMX (0.1 mM) were incubated for 15 min more with

2MeSATP (0, 1 or 3 mM) in the presence or absence of forskolin (0.1 mM). The glands were homogenised, lysed (acetic acid 5 mM, 5 min, boiling) and stored at −70°C. Samples were assayed in duplicate for cyclic AMP using a radioimmunoassay (RIA) kit with [^{125}I]cyclic AMP as a tracer (NEN™). Protein was measured by a dye-binding method using bovine serum albumin as a standard (Bradford, 1976).

The involvement of phospholipase C was evaluated by inhibiting the potentiation of noradrenaline-induced *N′*-acetyl-5-HT production by 2MeSATP with the phospholipase C inhibitor, U73122. This inhibitor was added to the cultured pineal glands 1 h prior to addition of noradrenaline (10 nM) plus 2MeSATP (0.1 mM). Control experiments were performed with U73343 (10 μM), a similar compound that does not inhibit phospholipase C. At the end of the incubation period, glands and medium were placed in different microtubes, stored at −70°C, and the *N′*-acetyl-5-HT content in the gland and incubation medium was determined by high-performance liquid chromatography.

2.5. Data analysis

Results of *N′*-acetyl-5-HT content are expressed as nanogram/pineal or nanogram/well and cyclic AMP levels as picomol/microgram protein. All data are presented as means ± S.E.M. Statistical comparisons were made by Student's *t* test or analysis of variance (ANOVA) followed by Newman–Keuls test, when appropriate. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of agonists on N′-acetyl-5-HT production

Glands stimulated with noradrenaline (10 nM) had a basal production of *N′*-acetyl-5-HT of 2.5 ± 0.4 ng/pineal and 15.6 ± 1.8 ng/well ($n = 23$). In pineals stimulated with noradrenaline (10 nM), the P2-agonists, 2-ClATP, 2MeSATP, ADPβS, ADP and ATP increased *N′*-acetyl-5-HT production in a concentration-dependent manner (Fig. 1). 2MeSATP induced the greatest increase in *N′*-acetyl-5-HT production (about 4.4 times over the basal noradrenaline-induced response). UTP (1 mM) failed to potentiate noradrenaline-induced *N′*-acetyl-5-HT production as measured in the gland and in the medium (2.05 ± 0.6 ng/pineal; 17.3 ± 3.4 ng/well, $n = 6$).

3.2. Signaling pathways

Most $P2Y_1$-like receptors act via G protein coupling to activate phospholipase C, leading to the formation of IP_3 and mobilization of intracellular Ca^{2+}. Coupling to adenylyl cyclase by some $P2Y_1$-like receptors has also been described. The next step was to evaluate the participation

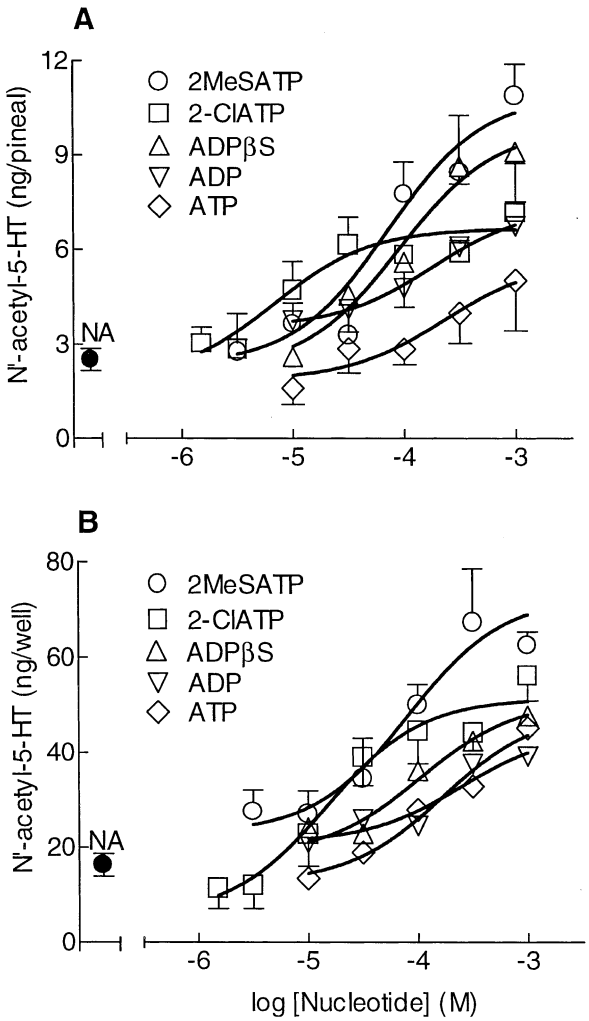

Fig. 1. Concentration–effect curves for 2MeSATP, 2-ClATP, ADPβS, ADP and ATP on *N′*-acetyl-5-HT production induced by noradrenaline (NA, 10 nM, $n = 23$) as measured in pineal tissue (A) and in the incubation medium (B). Glands were incubated for 5 h. Each point on the concentration–effect curves is the mean ± S.E.M. for each agonist concentration ($n = 4$–10 glands tested independently). When no error bar is shown, the error was smaller than the symbol.

of adenylyl cyclase or phospholipase C in the signal pathway.

3.2.1. Cyclic AMP involvement after stimulation of P2 receptors in the pineal gland

2MeSATP neither induced the production of cyclic AMP, nor inhibited its formation when the glands were stimulated by forskolin (0.1 mM) (Fig. 2). Therefore, the P2Y receptor present in the rat pineal gland does not act via G protein coupled to adenylyl cyclase.

3.2.2. Effect of phospholipase C inhibitor and PPADS on the potentiating effect of 2MeSATP

The phospholipase C inhibitor U73122, but not the inactive analogue U73343, blocked the potentiating effect of 2MeSATP in noradrenaline-stimulated pineal glands (Fig. 3A,B). Neither analogue had an effect on the action

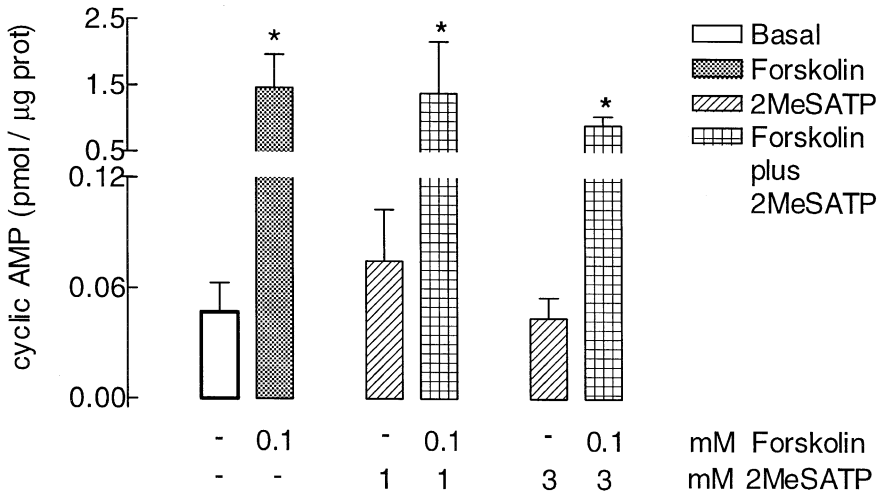

Fig. 2. Effect of 2MeSATP on cyclic AMP production in rat pineal gland. Glands were incubated in the presence of IBMX (0.1 mM, 20 min). Subsequently, 2MeSATP (1 mM or 3 mM) in the presence or absence of forskolin (0.1 mM) was added for 15 min. Values are means ± S.E.M.; $n = 3$ per treatment. $^{*}P < 0.05$ compared to glands not stimulated with forskolin.

of the threshold concentration of noradrenaline (10 nM), which suggests that this agonist acted through β_1-adrenoceptors.

PPADS, a putative inhibitor of $P2Y_1$ receptors which activate phospholipase C but not adenylyl cyclase (Boyer et al., 1994), was able to block the 2MeSATP-induced increase in *N′*-acetyl-5-HT production (Fig. 3C,D). Thus, we concluded that in the rat pineal gland the P2 receptors are coupled to G protein, which stimulates phospholipase C and promotes an increase in intracellular Ca^{2+}.

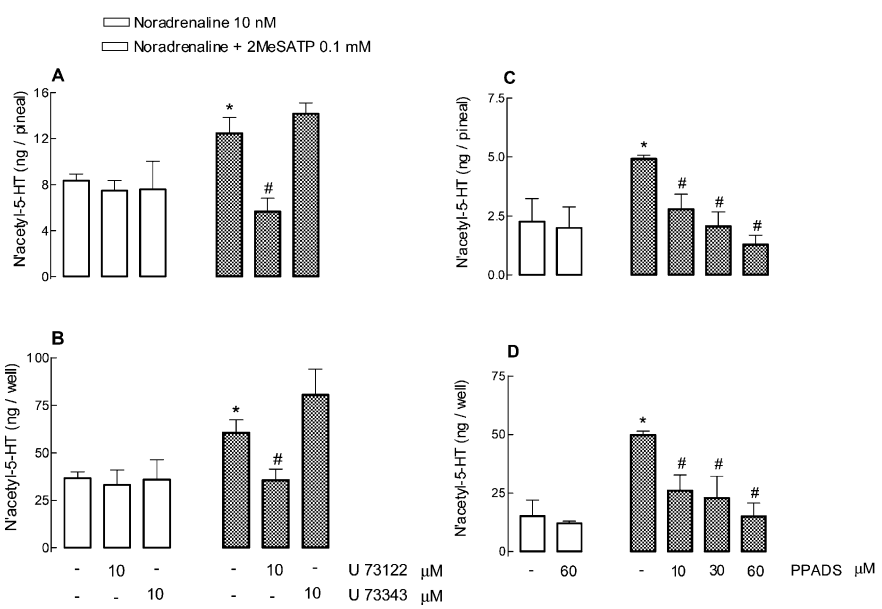

Fig. 3. Inhibition of the effect of 2MeSATP on noradrenaline-induced *N′*-acetyl-5-HT production by phospholipase C inhibitor and PPADS. (A,B): U73122 (10 µM) and U73343 (10 µM) were added 1 h before addition of noradrenaline (10 nM) or noradrenaline plus 2MeSATP (0.1 mM). *N′*-acetyl-5-HT was measured in pineal tissue (A) and in the incubation medium (B). Values are means ± S.E.M., ($n = 4$ per treatment). (C,D): PPADS (10–60 µM) was added 15 min prior to noradrenaline (10 nM) and noradrenaline plus 2MeSATP (0.1 mM). *N′*-acetyl-5-HT was measured in pineal tissue (C) and in the incubation medium (D). Values are means ± S.E.M. ($n = 4$ per treatment). $^{*}P < 0.05$ compared to noradrenaline; $^{\#\#}P < 0.05$ compared to noradrenaline plus 2MeSATP.

4. Discussion

The aim of this study was to characterise, in terms of pharmacological properties and signal transduction mechanisms, the subtype of P2-like receptor which mediates the potentiation of noradrenaline-induced *N*′-acetyl-5-HT (melatonin precursor) in the rat pineal gland. In a previous study, we have shown that the rat pineal gland has a functional P2-like receptor (Ferreira et al., 1994). ATP and the less hydrolysable agonist, AMP-PNP, were able to potentiate the production of *N*′-acetyl-5-HT induced by noradrenaline, while suramin and high doses of α,β-mATP blocked the adenine nucleotide-induced effect. $P2X_1$, $P2X_3$, $P2X_4$ and $P2X_6$ involvement was excluded since α,β-mATP activates the first two subtypes and the two last are insensitive to suramin (Ralevic and Burnstock, 1998). In the present paper, we show that ADP and ADPβS, which act at some P2Y receptors, but are weak or inactive at P2X receptors, also potentiate noradrenaline-induced *N*′-acetyl-5-HT production, suggesting that this response is probably mediated by a subtype of the P2Y-like receptor.

$P2Y_4$ receptor mRNA is found in different portions of the rat brain and its expression is high in the pineal gland of neonatal rats (Webb et al., 1998). However, the response studied here is probably not mediated by $P2Y_4$ receptors because 2-ClATP, which is inactive at $P2Y_4$ receptors (Harden et al., 1998), but not UTP, which is active at this receptor subtype (Ralevic and Burnstock, 1998), potentiated noradrenaline-induced *N*′-acetyl-5-HT production. It is interesting to note that in other tissues, such as rat myocytes, $P2Y_4$ mRNA is found only in neonatal animals (Webb et al., 1996). The lack of response to UTP also suggests that $P2Y_2$-like receptors do not mediate the action of P2 agonists in pinealocytes, because the $P2Y_2$ receptor is activated by ATP and UTP with similar potency. Characteristically, among all P2Y receptors, the $P2Y_1$ subtype is activated by 2MeSATP, ADP, ADPβS, 2-ClATP and ATP, but not by UTP (Ralevic and Burnstock, 1998; Harden et al., 1998). Therefore, the agonistic effect for potentiating noradrenaline-induced *N*′-acetyl-5-HT production points to the presence of a $P2Y_1$-like receptor.

Stimulation of $P2Y_1$-like receptors does not promote the production of *N*′-acetyl-5-HT by itself. Similar to other agents which elevate pinealocyte cytosolic Ca^{2+} (K^+, ouabain, ionomycin, ionophore A23187 or α_1-adrenoceptor agonists, Sugden et al., 1986, 1987), P2 receptor agonists only act after prior stimulation of noradrenoceptors. Therefore, the potentiation of noradrenaline-induced *N*′-acetyl-5-HT production mediated by P2 agonists is probably due to an elevation of intracellular Ca^{2+}.

In order to evaluate the signal transduction pathway which couples the agonist–receptor interaction and the potentiation of noradrenaline-induced *N*′-acetyl-5-HT production, the 2MeSATP effect on cyclic AMP levels, in the absence or in the presence of forskolin, and the ability of an inhibitor of phospholipase C to block the 2MeSATP-induced potentiation of *N*′-acetyl-5-HT production were tested. Coupling to adenylyl cyclase was excluded because 2MeSATP neither induced the production of cyclic AMP nor reduced the amount of cyclic AMP produced by forskolin stimulation. This result makes it possible to discard the presence of $P2Y_{11}$-like receptors which use both phospholipase C and adenylyl cyclase pathways (Communi et al., 1997).

The phospholipase C inhibitor U73122 was not able to modify the effect of noradrenaline (10 nM), confirming that at this concentration noradrenaline is not able to mobilize Ca^{2+} (Sugden et al., 1987).

The 2MeSATP potentiation of noradrenaline-induced *N*′-acetyl-5-HT production was inhibited by the phospholipase C inhibitor U73122, but not by the inactive analogue U73343, suggesting a coupling to phospholipase C. This was confirmed by testing the effect of PPADS. This compound was originally put forward as a P2X-selective antagonist but is now accepted as a non-selective (but non-universal) P2 receptor antagonist (Ralevic and Burnstock, 1998). In systems where the P2X receptor is not present or does not participate in the functional response, PPADS can be used to discriminate $P2Y_1$ from $P2Y_2$ receptors, and $P2Y_1$ receptors coupled to phospholipase C from $P2Y_1$ receptors coupled to inhibition of adenylyl cyclase (Boyer et al., 1994; Ralevic and Burnstock, 1996, 1998). PPADS blocked the effect of 2MeSATP, thereby corroborating the hypothesis that $P2Y_1$-like receptors in the pineal gland are coupled to G protein which activates phospholipase C. Therefore, taking together the effect of U73122 and PPADS, the hypothesis that the effect of 2MeSATP on the potentiation of *N*′-acetyl-5-HT production is dependent on phospholipase C activation is reinforced.

In conclusion, in rat pineal gland, the potentiation of noradrenaline-induced *N*′-acetyl-5-HT production is mediated by $P2Y_1$-like receptors, which are coupled to a G protein that stimulates phospholipase C. Thus, ATP might contribute to the production of melatonin by stimulating $P2Y_1$-like receptors. The presence of a sympathetic innervation and $P2Y_1$ receptors suggests that ATP may be a co-transmitter of noradrenaline in the rat pineal gland. In fact, it was shown recently that electrical stimulation of pineal nerve terminals releases noradrenaline and ATP (Barbosa et al., 2000).

Acknowledgements

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant No. 96/04497-0). R.P.M. is a research fellow of a Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Z.S.F. is a graduate fellow of FAPESP. The

technical assistance of Débora Aparecida de Moura is gratefully acknowledged.

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