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QUARTERLY

RT-PCR and Northern blot analysis in search for a putative *Paramecium* beta-adrenergic receptor[°]

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RT-PCR and Northern blot analysis were performed in order to search for a putative beta-adrenergic receptor (β-AR) in *Paramecium* using several β_2-adrenergic-specific molecular probes. Under strictly defined RT-PCR conditions DNA species of expected molecular size about 360 bp were generated with the primers corresponding to the universal mammalian β_2-AR sequence tagged sites (located within the 4th and the 6th transmembrane regions of the receptor). This RT-PCR product hybridized in Southern blot analysis with the oligonucleotide probe designed to the highly conservative β_2-AR region involved in G-proteins interaction and located within the amplified region.

Northern hybridization was performed on *Paramecium* total RNA and mRNA with human β_2-AR cDNA and two oligonucleotide probes: the first included Phe 290 involved in agonist binding (Strader *et al.*, 1995) and the second was the backward RT-PCR primer. All these probes revealed the presence of about 2 kb mRNA which is consistent with the size of β_2-AR transcripts found in higher eukaryotes.

Since evolutionary lineage including unicellular eukaryotes diverged nearly simultaneously with animals one billion years ago (Sogin & Silberman, 1998), it is of interest which pathways of the signal transduction system evolved in these species. In fact, opiate receptor (O'Neil *et al.*, 1988; Zipser *et al.*, 1988), chemorepellent receptor (Kuruvilla & Hennessey, 1998) and insulin-like protein (Le Roith *et al.*, 1980; Christopher & Sundermann, 1995) have been identified in *Tetrahymena*; also the existence of pheromones – considered to be early growth factors and cytokines – has been reported in *Euplotes*

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Abbreviations: β-AR, β-adrenergic receptor; TM, transmembrane region.

(Luporini *et al.*, 1994; Vallesi *et al.*, 1995). In *Paramecium* two distinct signal transduction pathways sensitive to purine nucleotides (Mimikakis & Nelson, 1998), receptor guanylyl cyclase (Wong & Garbers, 1992), cAMP chemoreceptor (Van Houten *et al.*, 1991) and growth factor (Tanabe *et al.*, 1990) have been described.

We have previously observed a stereospecific and time- and dose-dependent effect of β-adrenergic antagonists and agonists on *Paramecium* (Wyroba, 1987; 1991; Wyroba & Płatek, 1999) and postulated that β-adrenergic receptor coupled to phagocytosis might be considered as a primitive hormone receptor (Wyroba, 1989). The DNA species of the same molecular size were revealed in Southern blot analysis of *Paramecium* genomic DNA with oligonucleotide probes designed to different transmembrane regions of the β_2-adrenergic receptor (Surmacz *et al.*, 1997; Wiejak *et al.*, 1998; Wyroba & Surmacz, 1997).

In continuation of those studies we report here the results of PCR, RT-PCR and Northern blot analysis with an oligonucleotide designed to the region including Phe 290 involved in agonist binding (Strader *et al.*, 1995), the set of universal mammalian β_2-AR primers (Venta *et al.*, 1996) and human cDNA for β_2-AR. These molecular probes are specific to the sequenced metazoan β_2-adrenergic receptors, exhibiting a high degree of homology between species (Clark *et al.*, 1989; Dixon *et al.*, 1986; Dohlman *et al.*, 1987; Kobilka *et al.*, 1987a, 1987b; Okamoto *et al.*, 1991; Strader *et al.*, 1989).

MATERIALS AND METHODS

Isolation of total RNA. *Paramecium aurelia* cells (3-day-old axenic cultures: strain 299s and 51s) were harvested and washed in sterile MSS solution (Wyroba, 1987). The cell pellet was used immediately or frozen at −70°C.

Total RNA was isolated by the acid guanidinium thiocyanate/phenol/chloroform extraction method as described by Chomczynski & Sacchi (1987), except that chloroform/isoamyl alcohol mixture (24:1) was used.

mRNA isolation and cDNA synthesis. *Paramecium aurelia* cells were used for mRNA isolation with the Micro-Fast Track mRNA Isolation Kit (Invitrogen). The obtained mRNA sample was further used either for Northern blot analysis or to synthesize cDNA by reverse transcription with the cDNA Cycle Kit (Invitrogen). cDNA synthesis was performed using 180 ng of mRNA isolated from *Paramecium aurelia* strain 299s and 320 ng of mRNA extracted from the strain 51s.

Northern-blot analysis. Both the total RNA and mRNA were size fractionated in 1 × Mops solution (5 × Mops: 0.1 M Mops, pH 7.0, 40 mM sodium acetate, 5 mM EDTA, pH 8.0) on 1% agarose/formaldehyde gels and transferred to a nylon membrane (Hybond™-N, Amersham) (Sambrook *et al.*, 1989). The size of β_2-adrenergic mRNA was determined from the position of relative molecular mass marker (G 319, Promega).

Blots were prehybridized at 65°C for 2 h in a commercial hybridization solution (Sigma) or in the hybridization solution containing: 5 × Denhardt's, 5 × SSC, 50% formamide, 100 μg/ml fish sperm DNA (Amersham). Fish sperm DNA was sonicated and denaturated for 10 min at 95°C before usage. Next, overnight hybridization was carried out at 42–44°C in the same solution with the digoxigenin-labeled probes: oligonucleotide No. 5, No. 9, β-actin RNA (Boehringer-Mannheim) or human β_2-adrenergic receptor cDNA. Digoxigenin-labeled human β-actin RNA probe and β_2-AR cDNA were denaturated for 10 min at 95°C and then cooled on ice before addition to the hybridization mixture.

Probe No. 5 (5′ – TTAACA(T)ATGAAGAATGGT(C)AACCA, 23 mer) was constructed to the 6th transmembrane region (TM 6) of

β_2-AR according to *Paramecium* codon usage (Caron & Meyer, 1985; Kink *et al.*, 1990; Martindale, 1989; Preer *et al.*, 1985; Preer *et al.*, 1987). This region includes Phe 290 involved in agonists binding (Strader *et al.*, 1995).

Probe No. 9 was a 24-mer oligonucleotide of the sequence reported in Wiejak *et al.* (1998). These probes were 3'-end labeled with DIG-11-ddUTP (Martin *et al.*, 1990; Subramanian *et al.*, 1994; Surmacz *et al.*, 1997).

cDNA for human β_2-adrenergic receptor in pGEM vector (kindly donated by Dr. B. Kobilka, Howard Hughes Medical Institute, Stanford University Medical Center) was isolated by *Eco*RV (Promega)/*Nco*I (Boehringer Mannheim) digestion and extracted from the gel (after electrophoresis) using the High Pure PCR Product Purification Kit (Boehringer Mannheim). Purified cDNA (1.1 kb) was labeled by nick translation (Sambrook *et al.*, 1989) with DIG-11-dUTP (Surmacz *et al.*, 1997).

After hybridization membranes were washed in 2 × SSC/0.1% SDS at room temperature (2 × 5 min), 0.1 × SSC/0.1% SDS at hybridization temperature (2 × 15 min), 150 mM NaCl/50 mM Tris/0.05% Tween, pH 7.5 (2 × 5 min) followed by incubation (40 min) in 1% blocking reagent (Boehringer Mannheim) and then with anti-digoxigenin-alkaline phosphatase Fab fragments (Boehringer Mannheim) at 1:5000 (in the same blocking reagent) for 60 min (Subramanian *et al.* 1994). Then the blots were washed in the blocking reagent (15 min), 150 mM NaCl/50 mM Tris, pH 7.5 (3 × 5 min) and rinsed in detection buffer (0.1 M Tris, 0.1 M NaCl, pH 9.5). Signal detection was achieved using CDP-Star (Boehringer Mannheim) and exposure on Hyperfilm ECL (Amersham). To perform the same blot analysis the filters were washed free of the probes: 10 min in hot 0.1% SDS, 5 min in 1 × SSC. The efficacy of stripping was checked by exposure on Hyperfilm.

PCR and RT-PCR. PCR was performed using either isolated *Paramecium* genomic DNA or first strand cDNA (in RT-PCR) as a template. The reagents were: Taq polymerase (Perkin-Elmer, Gibco BRL, Fisher Biotech), deoxynucleotides (Promega, Gibco BRL) and $MgCl_2$ (Promega, Gibco BRL). PCR was performed with 34 cycles of denaturation (95°C, 30 s), annealing (55°C, 30 s) and extension (72°C, 1 min) with additional extension at 72°C for 10 min after the last cycle (PTC-150 HB Mini Cycler, PTC-200 DNA Engine, MJ Research).

The primers were characteristic for the β_2-adrenergic receptor structure: *Forward primer* (Probe No. 8): 21 mer, 4th hydrophobic region (TM 4) of the sequence given in Surmacz *et al.* (1997).

Backward primer (Probe No. 9): 24 mer, 6th hydrophobic region (TM 6) (Wiejak *et al.*, 1998). These primers correspond to the mammalian primers No. 6 and No. 7, respectively, and were synthesized according to the sequence of *Canis familiaris* ADRB2 STS DNA (5'-primers: GenBank, Accession #L77384), which belong to the set of "gene-specific universal mammalian sequence-tagged sites" for β_2-AR (Venta *et al.*, 1996).

PCR and RT-PCR products were analyzed on ethidium bromide-stained agarose (1.8%) gels in the presence of molecular marker pBR 322 DNA/*Msp*I (BioLabs, New England) and then capillary transferred to a nylon membrane (Zeta-Probe, BioRad). The blots were further examined by Southern (1975) hybridization analysis as described previously (Surmacz *et al.*, 1997; Wyroba *et al.*, 1995) with the oligonucleotide probe No. 1 (Wiejak *et al.*, 1998; Wyroba & Surmacz, 1997) which is located within the amplified region.

The PCR product of about 360 bp, hybridizing to the molecular probe No. 1 in Southern blot analysis, was extracted from 1.5% standard agarose gel using the QIAEX II Agarose Gel Extraction Kit (Qiagen). The eluted DNA (about 600 ng/sample) – the primary PCR product – was diluted 5- to 25-fold and served as a template for reamplification performed under the same conditions.

RESULTS

The set of primers corresponding to the "gene-specific universal mammalian sequence-tagged sites" for the β_2-adrenergic receptor (Venta *et al.*, 1996) was used for PCR and RT-PCR analysis of *Paramecium* DNA and cDNA. The codon sequences of these primers – No. 8 (located in 4th hydrophobic region, TM 4) and No. 9 (6th hydrophobic region, TM 6) – were biased according to the *Paramecium* codon preferences (Caron & Meyer, 1985; Kink *et al.*, 1990; Martindale, 1989; Preer *et al.*, 1985; Preer *et al.*, 1987). In PCR analysis using the isolated *Paramecium* DNA as a template with primers No. 8 and No. 9 the major product formed was about 360 bp. A very faint band about 430 bp was also visible in ethidium bromide-stained agarose gel (Fig. 1 A).

As a control, the PCR product generated from human genomic DNA template with primers No. 6 and No. 7 was analyzed on the same gel. This set of primers was synthesized according to mammalian codon usage (Venta *et al.*, 1996) as described in Materials and Methods. The only reaction product observed was that of the predicted about 360 bp (Fig. 1A, lane 3). It should be noted that the mammalian codon primers also generated the PCR product from *Paramecium* genomic DNA template (not shown).

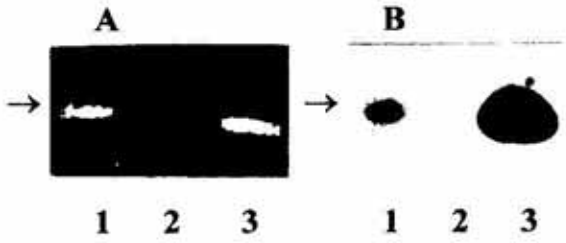

Figure 1. Polymerase chain reaction (PCR) amplification of *Paramecium aurelia* genomic DNA using beta-adrenergic-specific primers (No. 8 and No. 9).

A) Electrophoresis of PCR products: lane 1, *Paramecium* (strain 299s) genomic DNA template; lane 2, control without template; lane 3, human genomic DNA, used as a control with the set of primers No. 6 and No. 7 as described in Materials and Methods. B) Southern blot analysis of PCR products (lanes 1-3), using the β-adrenergic-specific probe No. 1 which is located within the amplified region. The major band revealed is of about 360 bp (arrow), which is the predicted molecular size.

To characterize the PCR products, Southern blot analysis was performed with oligonucleotide probe No. 1 – located within the amplified region – which hybridized only to the DNA species of about 360 bp, i.e. to the PCR product of the expected molecular size (Fig. 1B). This major PCR product was excised from the gel, purified and next used as a template – upon dilution recommended for better outcome of the polymerase chain reaction (Hengen, 1995) – for secondary PCR. The reamplification reaction gave rise to a unique band of the same molecular size about 360 bp (Fig. 2A) which – like the primary PCR product – also hybridized to the molecular probe No. 1 (Fig. 2B).

In order to check whether the putative *Paramecium* β-adrenoreceptor gene is expressed, isolation of mRNA from 3-day-old log-phase cells was carried out. The generated cDNA was then used as a template for PCR amplifi-

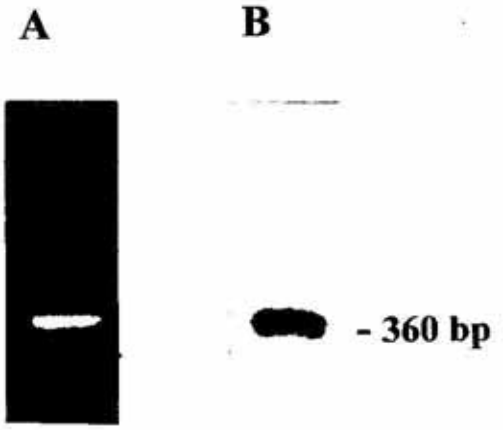

Figure 2. Reamplification of about 360 bp PCR product shown in Fig. 1.

A) Electrophoresis of PCR reamplification product. B) Southern hybridization with oligonucleotide probe No. 1. Only one band at about 360 bp was hybridizing to the molecular probe No. 1.

cation with the same set of primers (No. 8 and No. 9) as in the PCR from genomic DNA.

Under strictly defined RT-PCR conditions (with 2 mM $MgCl_2$) – when cDNA synthesis was performed with random primers – the only product observed was that of about 360 bp (Fig. 3A, lane 3) which hybridized to molecular probe No. 1 when analyzed by Southern blotting (Fig. 3B). These PCR/RT-PCR-generated DNA species will be further analyzed.

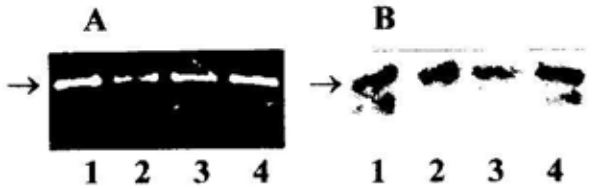

Figure 3. Polymerase chain reaction (PCR) amplification of *Paramecium* cDNA using β-adrenergic-specific primers (No. 8 and No. 9).

A) Electrophoresis of RT-PCR products: lanes 1 and 4 – the template was cDNA obtained from *Paramecium aurelia*, strain 51 (lane 1 – cDNA reverse transcribed from mRNA using random primers, lane 4 – using oligo dT primers); lanes 2 and 3 – the template was cDNA obtained from *Paramecium aurelia* mRNA, strain 299s (lane 2 – using oligo dT primers, lane 3 – using random primers). The final concentration of $MgCl_2$ was 2 mM in lanes 1–3 and 4 mM in lane 4. B) Southern blot analysis of RT-PCR products with the oligonucleotide probe (No. 1) specific to β-adrenoreceptor. Arrows indicate the product of the expected molecular size i.e. of about 360 bp.

Next, Northern blot analysis of *Paramecium* mRNA and total RNA was performed looking for a β-adrenergic receptor transcript. Four different digoxigenin-labeled probes were used. Three of them were β-adrenergic-specific:

- human β_2-adrenergic receptor cDNA,
- probe No. 5 – located in TM 6 including Phe 290 involved in binding of agonists (Strader *et al.*, 1995),
- probe No. 9 – used as the backward primer in PCR reaction.

The fourth probe was human β-actin RNA (Boehringer Manheim) used as a control.

When total RNA was analyzed with human β_2-AR cDNA probe only one hybridizing band of approx. 2 kb was observed (Fig. 4B) and an overexposure did not reveal any secondary bands. A transcript of the same molecular size was detected in *Paramecium* mRNA with the oligonucleotide probe No. 9 (Fig. 4A).

β-Actin RNA probe revealed a transcript (Fig. 4C) of the expected size (about 1.8 kb) and when the same blot was subsequently reprobed with the β-adrenergic-specific oligonucleotide No. 5, only one band about 2 kb was detected (Fig. 4D). The position of 17 S rRNA – typical for *Paramecium* rRNA (Findly & Gall, 1978; 1980) – as revealed in the ethidium bromide-stained agarose-formaldehyde gel is shown in Fig. 4E.

DISCUSSION

A PCR product of the predicted molecular size was obtained from both the *Paramecium* genomic DNA and cDNA templates with the set of primers corresponding to the "gene-specific universal mammalian sequence-tagged sites" for the β_2-adrenergic receptor (Venta *et al.*, 1996). This product hybridized with the molecular probe comprising the essential region of the β-adrenergic receptor involved in G-protein interaction, including Ser 262 – the phosphorylation site by protein kinase A in the process of receptor desensitization (Clark *et al.*, 1989; Okamoto *et al.*, 1991).

The PCR reaction product of about 360 bp corresponded in size to that expected for DNA amplified from β-adrenoreceptor gene with no intron between DNA encoding the region TM 4 and TM 6 of the receptor. Indeed, the metazoan β-adrenergic receptors sequenced up to now have been found to be intronless (Amend & Guan, 1995; Chung *et al.*, 1987; Dixon *et al.*, 1986; Jasper *et al.*, 1993; Kobilka *et al.*, 1987b).

Taken together with our previous Southern hybridization analyses performed on *Parame-*

cium genomic DNA using several β_2-AR-specific molecular probes (Surmacz *et al.*, 1997; Wyroba & Surmacz, 1997), the current PCR and RT-PCR results suggest that the region homologous to that in between TM 4 and TM 6 of metazoan β-receptor was amplified. Backward primer No. 9 was previously used in the set with the forward primer constructed to the TM 3 – including Asp 113 involved in agonist and antagonist binding – and generated the product of the predicted molecular size (530 bp) which also hybridized to the probe No. 1 localized within the amplified region (Wiejak *et al.*, 1998).

The oligonucleotides constructed for *Paramecium* studies comprised the essential motifs of β_2-adrenoreceptor. The active sites for binding β-adrenergic ligands lie within the transmembrane spanning domains (TM) of the receptor and oligonucleotide probe No. 5 was designed to the TM 5 of the β_2-adrenergic receptor including Phe 290 which is involved in agonist binding (Strader *et al.*, 1989; 1995). This probe as well as the PCR backward primer used in this study and a human β_2-AR cDNA were applied to detect the putative β-adrenergic receptor transcript in *Paramecium* mRNA and total RNA. Northern blot analysis with these three different probes revealed the presence of mRNA of approx. 2 kb which is consistent with the size of β_2-adrenergic mRNA found in other species. The transcripts for β_2-adrenergic receptor have been reported to be in the range of 1.95–2.3 kb for hamster (Dixon *et al.*, 1986), human (Kobilka *et al.*, 1987a), rat (Collins *et al.*, 1988; Frielle *et al.*, 1987; Gocayne *et al.*, 1987; Marchetti *et al.*, 1994) and dog (Emala *et al.*, 1996).

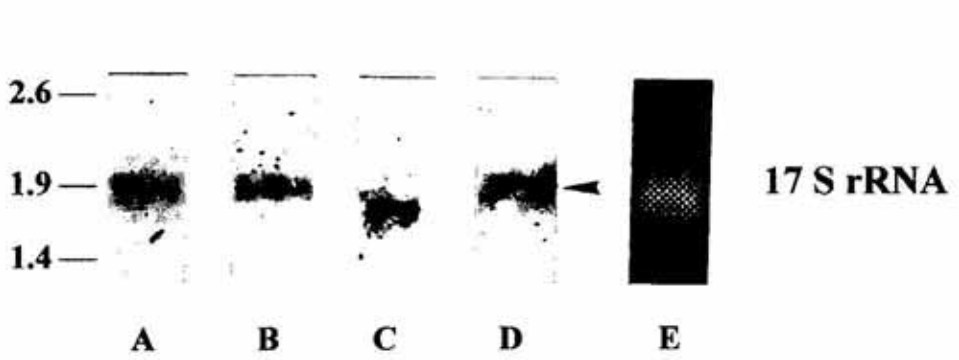

Figure 4. Northern blot analysis of *Paramecium aurelia* RNA with three different β-adrenergic-specific probes and β-actin as a control.

A, mRNA (about 5 μg); B–E, total RNA (40 μg). The probes were: A) the oligonucleotide probe No. 9 (used as a primer in RT-PCR and PCR analysis shown in Figs. 1–3); B) human β_2-adrenergic cDNA; C) human β-actin RNA; D) β-adrenergic-specific probe No. 5. C and D is the same blot analysis: after exposure, the filter was washed free of the β-actin probe (C) and rehybridized successively with probe No. 5 (D). The arrowhead indicates the position of about 2 kb β_2-AR mRNA transcript. E) Position of 17 S rRNA shown on an ethidium bromide-stained formaldehyde agarose gel. RNA isolation from *Paramecium* cells (A and B, strain 51s; C–E, strain 299s), size-fractionation on a 1% agarose/formaldehyde gel, Northern blotting and digoxigenin-labeling of the probes were carried out as described in Materials and Methods.

β-Adrenergic receptor is an evolutionarily ancient molecule and belongs to the rhodopsin family (Dixon *et al.*, 1986). The rhodopsin family of proteins arose before the division of prokaryotes from eukaryotes and later gave rise to muscarinic acetylcholine receptor and β-adrenergic receptor among others (Ohno, 1987). Cytophysiological and biochemical observations carried out on *Paramecium aurelia* indicated that β-adrenergic system exists in this organism (Croce *et al.*, 1990; Wyroba, 1987; 1989; 1991; Wyroba & Płatek, 1999). We have suggested that hormone receptors in

Paramecium – although formed early in evolution – seem not to be different from those of vertebrates in their ability to discriminate between agonists and antagonists (Wyroba, 1989). In fact, Blum (1967) postulated that most of the major components of the catecholamine system originated before metazoa developed and presumably acted as a metabolic control system at the intracellular level before they were adapted for endocrine and neurotransmitter functions in the metazoa.

Since the emergence of *Paramecium* is placed early in evolution (Sogin & Elwood, 1986; Sogin & Silberman, 1998), it is of interest to look for further evidence of the existence of β-adrenergic system in this cell and its function in this unicellular eukaryote.

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