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Dynamin-association with agonist-mediated sequestration of beta-adrenergic receptor in single-cell eukaryote *Paramecium*

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Summary

Evidence that dynamin is associated with the sequestration of the *Paramecium* β_2-adrenergic receptor (βAR) immunoanalogue is presented. We previously reported a dramatic change in the distribution of βAR analogue in the subcellular fractions upon isoproterenol treatment: it is redistributed from the membraneous to the cytosolic fraction, as revealed by quantitative image analysis of western blots. Here we confirm and extend this observation by laser scanning confocal and immunogold electron microscopy. In the presence of isoproterenol (10 μmol l^{-1}) βAR translocated from the cell surface into dynamin-positive vesicles in the cytoplasmic compartment, as observed by dual fluorochrome immunolabeling in a series of the confocal optical sections. Colocalization of βAR and dynamin in the tiny endocytic vesicles was detected by further electron microscopic studies.

Generally receptor sequestration follows its desensitization, which is initiated by receptor phosphorylation by G-protein-coupled receptor kinase. We cloned and sequenced the gene fragment of 407 nucleotides homologous to the β-adrenergic receptor kinase (βARK): its deduced amino acid sequence shows 51.6% homology in 126 amino acids that overlap with the human βARK2 (GRK3), and may participate in *Paramecium* βAR desensitization.

These results suggest that the molecular machinery for the desensitization/sequestration of the receptor immunorelated to vertebrate βAR exists in unicellular *Paramecium*.

Key words: dynamin, sequestration, desensitization, *Paramecium*, β_2-adrenergic receptor, GRK, isoproterenol, cloning, confocal imaging, immunological analysis.

Introduction

Using cell fractionation, SDS-PAGE, quantitative western blot, confocal immunolocalization and immunogold labeling techniques we identified the immunoanalogue of vertebrate β_2-adrenergic receptor in the unicellular eukaryote *Paramecium* (Wiejak et al., 2002). This provided a molecular basis for the previously reported physiological response of this cell to the beta-adrenergic ligands (Wyroba, 1989).

The 69 kDa polypeptide separated by SDS-PAGE in S2 and P2 *Paramecium* subcellular fractions cross-reacted with antibody directed against human β_2-adrenergic receptor (βAR). Quantitative image analysis of the western blots showed that the β-selective adrenergic agonist (–)-isoproterenol (Iso), previously shown to enhance phagocytic activity (Wyroba, 1989), evoked a redistribution of βAR analogue from the membranous (P2) to the cytosolic (S2) fractions. The relative increase in immunoreactive band intensity in the S2 fraction reached 80% and was paralleled by a 59% decrease in the P2 fraction. Confocal immunofluorescence studies revealed the βAR sites on the cell surface and at the ridge of the cytopharynx, where nascent phagosomes are formed, and the localization of the β-immunoanalogue was confirmed by immunoelectron microscopy. These results indicated that the 69 kDa *Paramecium* polypeptide immunorelated to vertebrate β_2AR appeared in this ciliate as a nutrient receptor. Pretreatment of the cells with 10 μmol l^{-1} Iso evoked a physiological response, together with a redistribution of immunoreactivity detected in the subcellular fractions, suggesting that a desensitization process had occurred.

Signaling by membrane receptors is terminated by endocytosis during the process of desensitization (Lefkowitz et al., 1983; Barak et al., 1994). One of the initial stages of desensitization is receptor phosphorylation by GRK kinases (G-protein-coupled receptor kinases), which act only on agonist-occupied receptor (Premont et al., 1995; Zhang et al., 1997).

We report here cloning of the gene fragment encoding the putative homologue of the βARK=GRK kinase, the enzyme involved in the beta-adrenergic receptor phosphorylation. The deduced amino acid sequence of this gene fragment showed 51.6% homology in 126 amino acids that overlapped with the human βARK2, including its catalytic and extension domain,

and 47.6% homology to the βARK1, the first cloned GRK kinase (Benovic et al., 1989) and the enzyme that specifically phosphorylates only the agonist-occupied form of the beta-adrenergic and closely related receptors. We also confirm that this enzyme is expressed in *Paramecium* by obtaining the mRNA sequence (deposited in GenBank, Accession no. AF346411).

In higher eukaryotes, receptor internalization/sequestration was found to occur *via* dynamin-dependent and clathrin-mediated endocytosis (Shetzline et al., 2002; Braun et al., 2003). We have recently cloned the N-terminal and middle domains (1091 nucleotides encoding 363 amino acids) of dynamin in *Paramecium*. This protein is essential in different endocytic processes (Damke et al., 1994; Schmid et al., 1998; Wiejak et al., 2003) and we showed the presence of the dynamin immunoanalogue localized to the transferrin-containing endosomes (Wiejak and Wyroba, 2002; Surmacz et al., 2003). Therefore, to elucidate whether receptor sequestration in *Paramecium* follows a pathway similar to that observed in mammalian cells, we performed experiments with antibodies directed against the C termini of human β_2AR and human dynamin 2. These antibodies were utilized for dual fluorochrome labeling of isoproterenol-pretreated cells, which were then examined by laser scanning microscopy and immunogold ultrastructural localization studies. The anti-dynamin antibody was also used in western blot analysis of *Paramecium* subcellular fractions to confirm its specificity.

Upon isoproterenol treatment the β-immunoanalogue was redistributed into intracellular vesicles, where its colocalization with dynamin was observed in a series of the confocal optical sections. Detailed immunogold detection by electron microscopy enabled us to identify the small intracellular vesicles, representing the early endosomal compartment, to which the β-adrenergic receptor and dynamin were localized. This suggests that β-adrenergic receptor was sequestered in a dynamin-dependent manner. To our knowledge, this represents the first case of dynamin-dependent receptor internalization in unicellular eukaryotes.

On the basis of the above mentioned observations we propose that the molecular machinery for the desensitization/sequestration of the G-protein-coupled receptors exists in this single-cell eukaryote.

Materials and methods

Cell cultivation

Paramecium aurelia strain 299s (5-day-old axenic cultures) were cultivated, collected and starved aseptically for 18 h as described previously (Wiejak et al., 2002).

Chemicals

Taq polymerase, deoxynucleotides and agarose were from GibcoBRL (Gaithersburg, USA), pGEM-T vector, *E. coli* strain JM 109 and *Eco*RI from Promega (Madison, WI, USA), goat anti-dynamin 2 antibody (Ab) from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA) and all the other reagents were from Sigma (Steinheim, Germany).

Confocal imaging

Untreated and isoproterenol-treated (10 μmol l^{-1} for 10 min) cells were fixed and processed for confocal imaging as previously described (Wiejak et al., 2002). The primary antibodies were: rabbit polyclonal Ab against the C-terminal 15-amino acid residues of human β_2-adrenergic receptor (β_2AR: kindly donated by Dr M. Von Zastrow, University of California, San Francisco, USA; Von Zastrow and Kobilka, 1992) diluted 1:500 in blocking reagent and goat anti-dynamin 2 Ab (1:500). These were followed by the secondary FITC-conjugated anti-rabbit Ab (1:150) and TRITC-conjugated anti-goat Ab (1:500), respectively. In the control samples the primary antibodies were omitted.

The confocal laser scanning system Leica DM IRE2 (oil immersion objectives 63×) was used. Images were collected and processed using Leica confocal software 2.0 and Adobe Photoshop 6.0.

Electron microscopy

Immunoelectron microscopy was performed as described previously (Wiejak et al., 2002) using the same set of the primary antibodies (1:250) as in the confocal imaging, i.e. anti-β_2AR and anti-dynamin. In control experiments the primary Abs were omitted. The secondary Abs were: anti-rabbit IgG (1:20) conjugated with colloidal 10 nm gold particles to visualize βAR and anti-goat conjugated with colloidal 5 nm gold to visualize dynamin. Ultrathin sections were observed in a JEM 1200 EX electron microscope.

Immunodetection by confocal and electron microscopy was performed in triplicate.

Western blot analysis

Protein fractionation, SDS-PAGE and western blotting were performed as described previously (Surmacz et al., 2001). Blots were stained in 0.5% Ponceau Red in 3% trichloroacetic acid before immunodetection. The recombinant rat dynamin 2 (kindly donated by Dr D. D. Binns from Department of Pharmacology, U.T. Southwestern Medical Center, Dallas, USA) was used as a positive control for immunoblot analysis.

Immunodetection was performed using primary antibody against the C-terminal region of human dynamin 2 (Santa Cruz, CA, USA) at 1:500 (overnight at 4°C) followed by incubation with the horseradish peroxidase-conjugated anti-goat IgG (1:1000) for 1 h and processing for chemiluminescent detection using West Pico (Pierce, USA).

PCR and cloning

Polymerase chain reaction (PCR) was performed on the genomic DNA isolated from *Paramecium* (Subramanian et al., 1994). PCR settings were: denaturation at 94°C (30 s), annealing at 52°C (30 s) and extension at 72°C (1 min) for 29 cycles using the PTC-200 DNA Engine from MJ Research. Additional extension at 72°C for 10 min was applied after the

last cycle. PCR reactions were performed in a total volume of 25 μl and the reaction mixture contained 0.75 μg of genomic DNA as a template, 0.4 μmol l^{-1} of each primer, 100 μmol l^{-1} each of deoxynucleotidyl triphosphates, 2 mmol l^{-1} of $MgCl_2$, 1× PCR buffer and 2 units of Taq Polymerase (Amersham, Little Chalfont, UK).

Degenerate primers were synthesized according to *Paramecium* codon usage and were originally based on the amino acid sequences of *Paramecium* endocytic proteins cloned by us, and public databases of mammalian species. Forward: 5′-TAATT/CTGT/CTGGAAAATT/CATT/CAA and backward: 5′-TAATCA/TGCA/TGGAAAATCA/TTC. The control samples not containing the template did not yield any PCR products. PCR products were separated by gel electrophoresis (60 V for 2 h) on 1.5% agarose followed by ethidium bromide staining and a brief rinse in double-distilled water to visualize DNA under UV light. The band of interest was transferred from the 1.5% agarose gel to DEAE-cellulose, eluted as described in Sambrook et al. (1989) and subcloned into pGEM-T Easy vector (according to the manufacturer's instructions; Promega). Transformation of bacteria (*E. coli* strain JM 109), selection of positive clones (white colonies) and isolation of plasmids with insert by alkaline lysis were performed using standard procedures (Sambrook et al., 1989). A restriction enzyme digest with *Eco*RI (2 U 1^{-1} μg plasmid at 37°C for 90 min) confirmed the presence of insert of the correct size.

Sequencing

The isolated plasmid DNA was labeled with Dig-Taq DNA Sequencing Kit (Boehringer-Manheim, Germany) and sequenced in Sequi-Gen GT Sequencing Cell (Bio-Rad, Hercules, USA).

10 μg of plasmid DNA was denatured at 95°C for 3 min. The completed reactions were resolved on a denaturing gel (6% polyacrylamide, 7 mol l^{-1} urea, 1× TBE) and run at a constant voltage of 1500 V for various lengths of time. Gel was blotted onto positively charged nylon membranes (Boehringer-Manheim, Germany) for capillary transfer (45 min). Chemiluminescent detection was performed with CDP-Star (Boehringer-Manheim, Germany) at 1:1000 followed by exposure to Hyperfilm ECL (Amersham, Little Chalfont, UK). Additionally, the isolated plasmid DNA was automatically sequenced using the standard procedures. Sequences searches were performed by the BLAST algorithm on the NCBI databases. Sequence alignment was performed using Clustal W version 1.6 (Thompson et al., 1994).

Results

The β_2-adrenergic receptor immunoanalogue in *Paramecium* cells was demonstrated using immunodetection in confocal laser scanning and electron microscopy to be localized mainly on the cell surface, as reported previously (Wiejak et al., 2002). In the presence of the β-selective adrenergic agonist isoproterenol (10 μmol l^{-1}), the β-adrenergic sites were translocated from the cell surface (Fig. 1A) to the cytoplasmic compartment (Fig. 1B). Such a pattern of localization suggested that the beta-immunoanalogue had been internalized, so anti-dynamin Ab was applied to investigate whether the dynamin-dependent process had occurred.

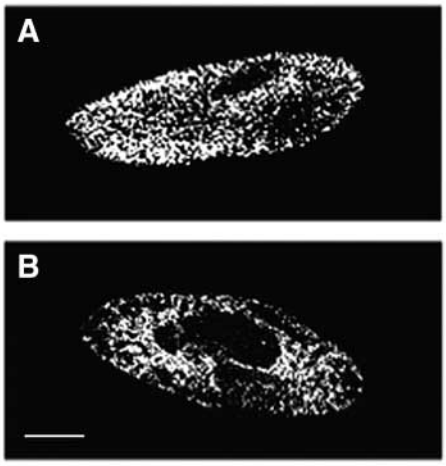

Fig. 1. Redistribution of β-adrenergic receptor immunoanalogue in isoproterenol-treated *Paramecium* cells, viewed by confocal microscopy. (A) Control cells, (B) isoproterenol-treated cells (10 μmol l^{-1}) immunolabeled with antibodies against human β_2-adrenoreceptor and processed for confocal microscopy as described in Materials and methods. β-adrenergic sites undergo translocation from the cell membrane (A) to the cytoplasmic compartment upon isoproterenol treatment (B). Bar, 15 μm.

Dual fluorochrome immunolocalization was performed in isoproterenol-treated and untreated *Paramecium* cells and was displayed by superimposing FITC staining (green) representing β-adrenergic receptor immunoanalogue and TRITC staining representing dynamin distribution (red) (Fig. 2). In isoproterenol-treated cells, colocalization of βAR and dynamin (yielding a yellow orange image) was observed in consecutive confocal optical sections performed at a vertical resolution of 0.6 μm. Small yellow-orange-fluorescing punctate accumulations were seen (Fig. 2A–D, arrows). Such a pattern of localization was not observed in the untreated cells (Fig. 2E). The immunostaining was not detected in the negative control in which primary antibodies were omitted (data not shown).

Ultrastructural detection by immunogold electron microscopy revealed that in the isoproterenol-treated cells βAR and dynamin colocalize in a population of intracellular vesicles approx. 40–55·nm in diameter (Fig. 3A–D). Almost no βAR was detected on the membrane, only a single, scarce gold particles may be found (Fig. 3A, arrow). In untreated cells, colocalization of βAR and dynamin was detected on the surface (Fig. 3E). When βAR and dynamin were localized separately using anti-βAR and anti-dynamin antibody, respectively, a significant presence of βAR on the plasma membrane was observed (Fig. 3F), whereas dynamin was localized on/beneath the membrane (Fig. 3H) and in the coated pits (Fig. 3G).

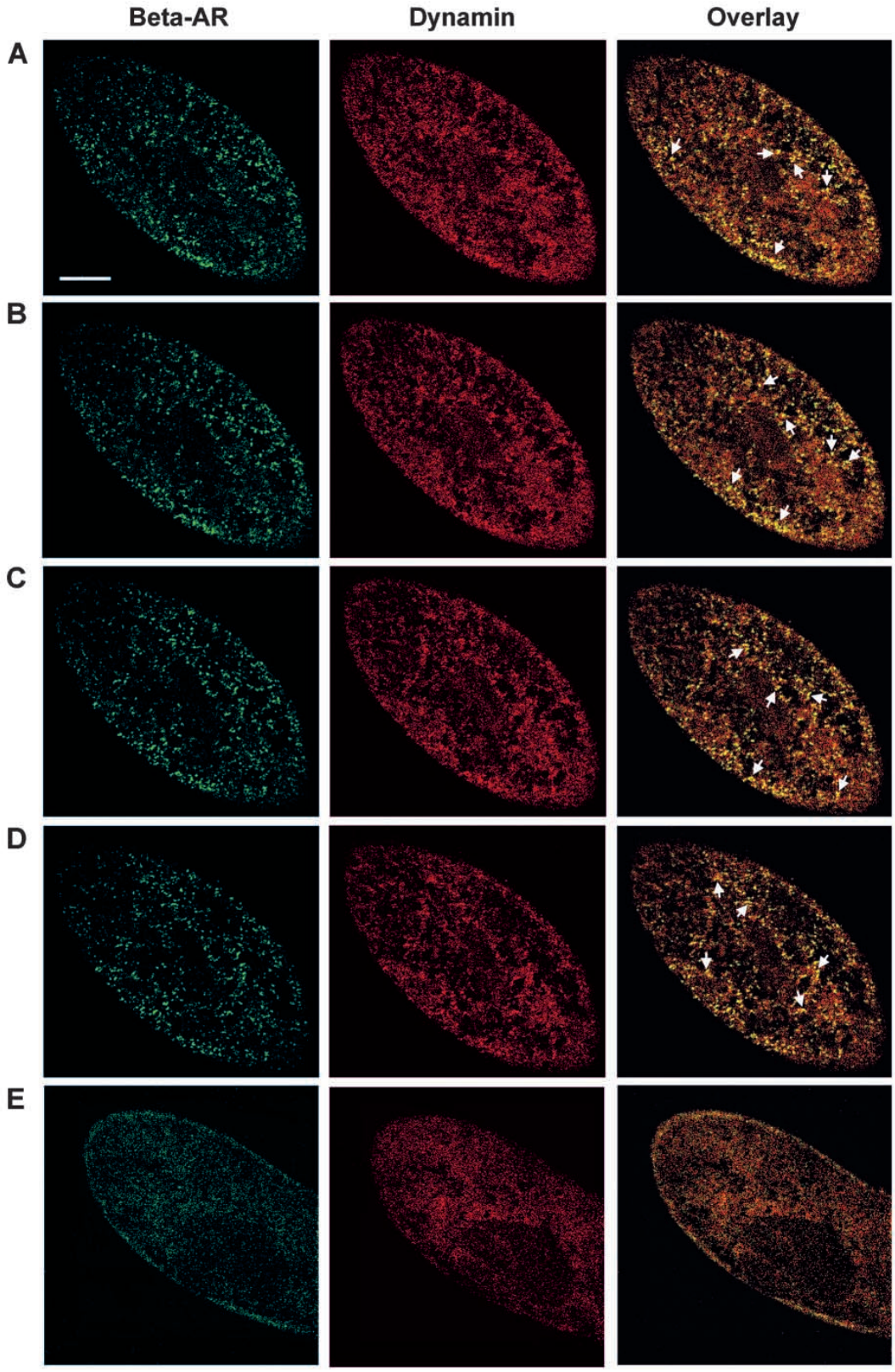

Fig. 2. Localization of dynamin and β-adrenergic receptor (βAR) immunoanalogue in isoproterenol-treated and untreated *Paramecium* cells using confocal laser scanning microscopy. Dual fluorochrome immunolabeling was performed as described in Materials and methods. Colocalization (yielding a yellow orange image) of βAR (green) and dynamin (red) inside the cell in small punctate accumulations (arrows) was observed in the overlay of the series of confocal sections performed at 0.6 μm intervals (A–D). Such a pattern of colocalization was not observed in the untreated cells (E). Bar, 16 μm.

The anti-dynamin antibody (directed against the C-terminal region of human dynamin 2) that was used for confocal and electron microscopic studies was further used for western blot analysis. It revealed one immunoreactive band of approx. 105 kDa (Fig. 4B, lane 1) in the S2 fraction isolated from *Paramecium* cells and separated by SDS-PAGE (Fig. 4A, lane 1). This result is consistent with the migration pattern of recombinant rat dynamin 2 obtained in the same blot under our experimental conditions (Fig. 4A,B, lane 2).

The molecular basis for the initiation of desensitization/sequestration process in *Paramecium* seems to be the putative βAR kinase. Its gene fragment was identified serendipitously by us during PCR cloning of genes involved in endocytic processes.

The PCR product of ~400 bp (Fig. 5A) was excised from the gel, eluted and reamplified, yielding a band of the same molecular size (Fig. 5B). These DNA species were purified (as described in Materials and methods), subcloned into pGEM-T vector and used for transformation of bacteria. Plasmids isolated from the positive clones were digested with *Eco*RI and revealed the presence of the insert of the correct size of ~420 bp (Fig. 5C). The identified gene fragment of 407 nucleotides contained one short intron of 25 bp (data not shown). The computer-assisted alignment of the deduced amino acid sequence revealed a high homology to the human βARK2 – the enzyme involved in the phosphorylation of the agonist-occupied beta-adrenergic receptor. This sequence displayed a 23.8% identity, 51.6% homology and 62.7% similarity to this enzyme including its catalytic and extension domains in a 126-amino-acid overlap (Fig. 6). Alignment with βARK1, the first identified beta-adrenergic receptor kinase (Benovic et al., 1989), revealed 24.6% identity, 47.6% homology and 61.9% similarity.

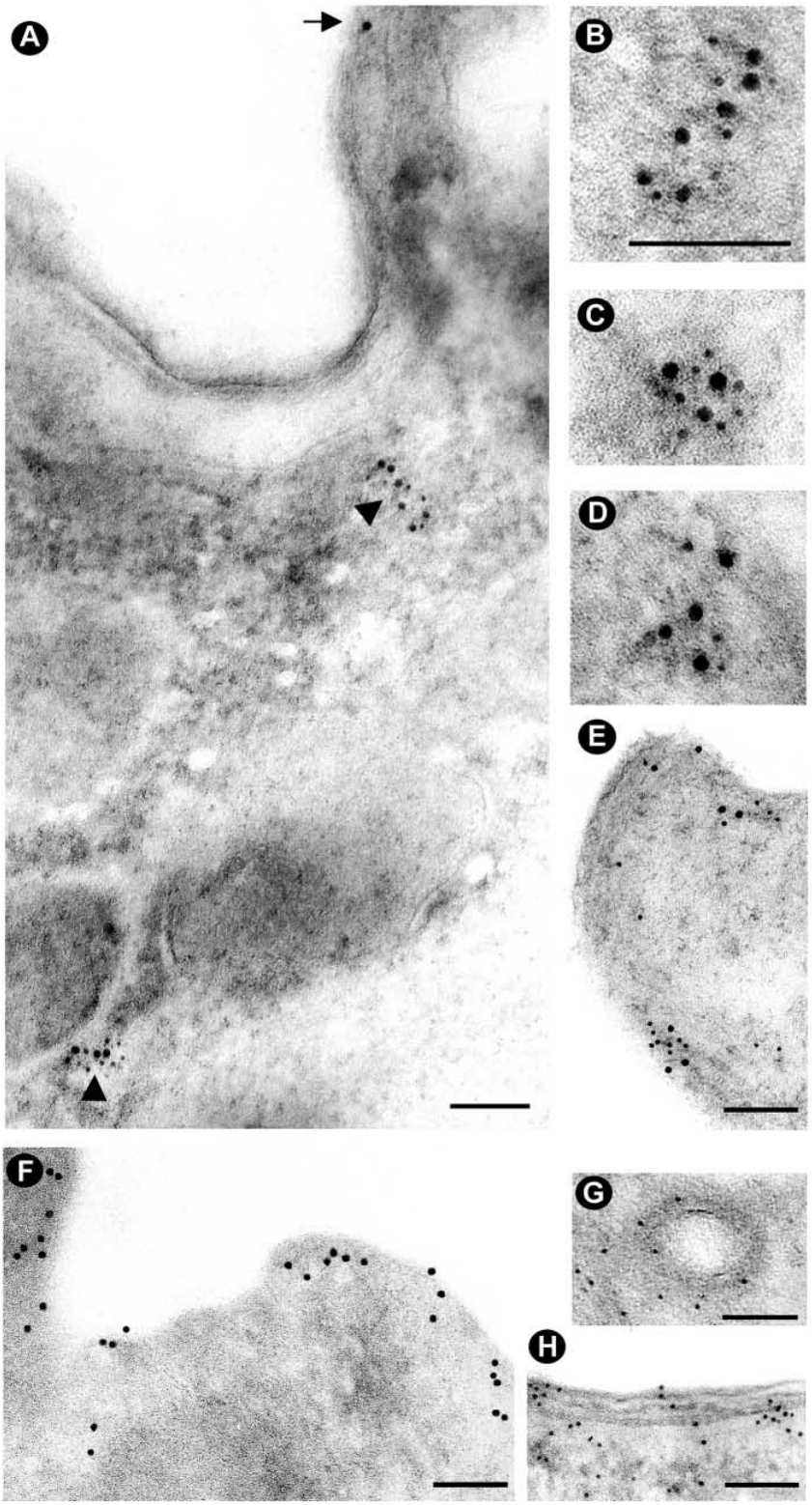

Fig. 3. (A–D) Dynamin-dependent sequestration of β-adrenergic receptor in isoproterenol-treated *Paramecium* cells, visualized by post-embedding immunogold electron microscopy. Immunolocalization of βAR (10 nm gold particles) and dynamin (5 nm gold particles), was performed as described in Materials and methods. Colocalization of βAR and dynamin in the small endocytic vesicles is observed (A, arrowheads; B–D). No or only scarce βAR on the surface membrane is observed (A, arrow). (E) In untreated cells βAR and dynamin colocalized on the plasma membrane. (F–H) Immunolocalization of βAR alone (F) and dynamin alone (G,H), detected by anti-βAR and anti-dynamin antibodies, respectively. A significant presence of βAR on the cell surface (F) occurs, whereas dynamin was localized both on the membrane (H) and in the coated pits (G). Bars, 100 nm.

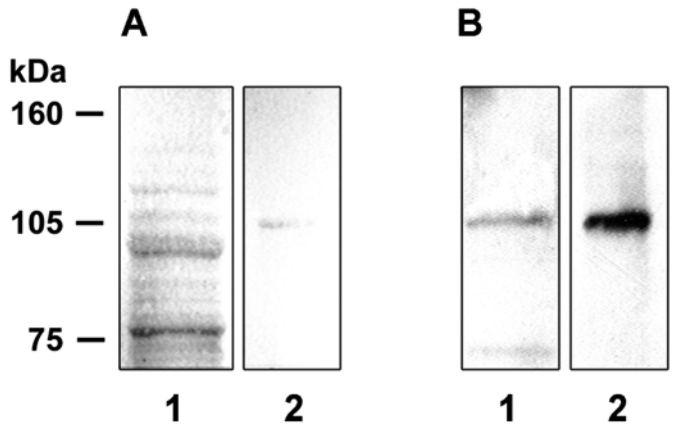

Fig. 4. Identification of dynamin in *Paramecium* cells using antibodies against the C-terminal region of human dynamin 2. (A) SDS-PAGE (Ponceau Red stained) and (B) western blot analysis of protein fraction S2 (lane 1). The recombinant rat dynamin 2 (lane 2) was used as a positive control for immunoblot analysis. One immunoreactive band of ~105 kDa was detected. Positions of the molecular marker are shown on the left. The western blots shown are representative of three independent experiments.

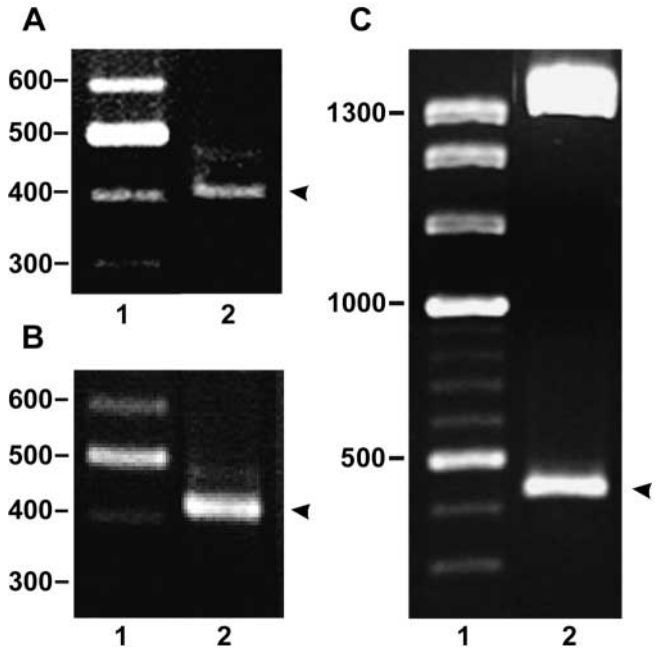

Fig. 5. PCR amplification and cloning of the putative βARK homologue in *Paramecium*. (A) PCR product of ~400 bp (lane 2, arrowhead) was electrophoretically transferred onto DEAE-cellulose. DNA was eluted and used as a template for PCR reamplification resulting in the product of predicted molecular size of ~400 bp (B; lane 2, arrowhead). Following subcloning into pGEM-T vector and transfection, the plasmid DNA was isolated from the positive colonies (as described in Materials and methods) and subsequently digested with *Eco*RI. The presence of an insert of the correct size was detected (C; lane 2, arrowhead). Lanes 1 in A–C are molecular mass standards.

Discussion

Dual fluorochrome immunostaining in confocal laser microscopy and immunogold detection in electron microscopy suggest the existence of the dynamin-dependent agonist-mediated sequestration of the beta-adrenergic receptor immunoanalogue in *Paramecium*. Upon isoproterenol treatment βAR redistributed from the cell surface to the cytoplasmic compartment, undergoing internalization. Its partial colocalization with dynamin was detected in the small punctate accumulations in the series of the consecutive optical confocal sections from cells labeled simultaneously with antibodies against the human βAR and dynamin. This receptor distribution was further examined at higher resolution by immunogold technique in transmission electron microscopy: tiny endocytic vesicles were revealed in which βAR colocalized with dynamin. The diameter of the vesicles (40–55 nm) to which βAR homologue is translocated corresponds to the early endosomal compartment observed in transferrin-internalizing *Paramecium* cells by immunoelectron microscopy (J. Wiejak et al., unpublished observations). We have also identified dynamin in *Paramecium* by western blot analysis using the same antibody as for confocal and electron microscopic studies. Its apparent molecular mass in migration in SDS gel analysis and localization confirm that the protein is dynamin or an immunologically related protein.

The desensitization process is defined as attenuation of the receptor responsiveness upon agonist stimulation and it is a consequence of combination of different mechanisms (January et al., 1997; Ferguson, 2001). It is initiated by receptor phosphorylation including by G-protein-coupled receptor kinase (GRK) that acts only on agonist-occupied receptor (Premont et al., 1995; Claing et al., 2002; Sorkin and Von

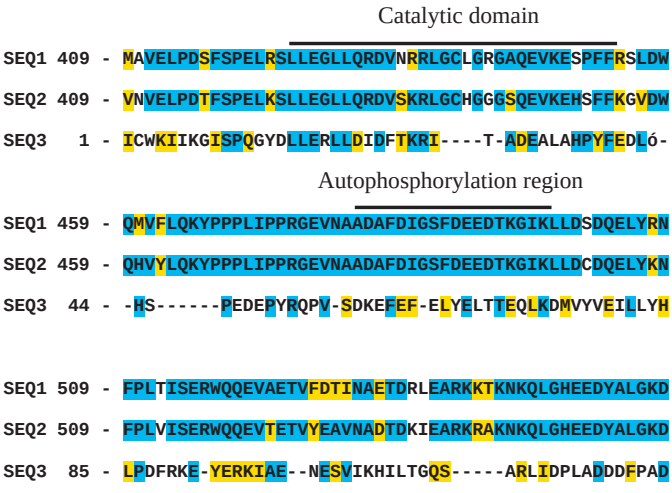

Fig. 6. Multiple alignment of βARK genes from different species. The amino acid sequence of bovine βARK1 (SEQ 1, Accession no. P21146), human βARK2 (SEQ 2, Accession no. P35626) and deduced amino acid sequence of *Paramecium* gene fragment (SEQ 3, Accession no. AF346410) were aligned using the CLUSTAL W program. Identical residues are marked in blue and homologous residues in yellow. Conservative regions, i.e. the catalytic domain and autophosphorylation region, are indicated.

Zastrow, 2002). We reported here the partial cloning of putative βAR kinase in *Paramecium* cell. Its deduced amino acid sequence shows 51.6% homology to the human βARK2 in 126 amino acid overlap including its catalytic and extension domains, and 47.6% homology to the first cloned βARK1 from the bovine brain (Benovic et al., 1989). We also proved that this putative enzyme is expressed in *Paramecium* by obtaining the corresponding mRNA sequence (GenBank: accession no. AF346411; data not shown).

We performed immunocytochemical localization of the beta-immunoanalogue in Isopreterol-treated *Paramecium* cells using rabbit polyclonal antibody (Ab), raised by Von Zastrow and Kobilka (1992), against the C-terminal region of human β_2-adrenergic receptor. Such an antibody is suitable for examining the effect of agonists since receptor phosphorylation by βAR kinase occurs at the C-terminal Ser/Thr residues of the agonist-occupied receptor (Barak et al., 1994; Premont et al., 1995; Krupnick and Benovic, 1998).

Agonist treatment leads to the redistribution of the receptors away from the cell surface by a process of endocytosis, also known as internalization or sequestration (Chuang and Costa, 1979a,b; January et al., 1997; Pierce et al., 2002).

Our findings of βAR redistribution in *Paramecium* upon isoproterenol treatment were consistent with those reported by Barak et al. (1997) and Waldo et al. (1983). The translocation of β_2AR from the plasma membrane to an intracellular compartment occurred very rapidly, exhibiting a $t_{1/2}$ of ~2 min in 1321N1 human astrocytoma cells (Waldo et al., 1983). Barak et al. (1997) reported that, upon desensitization, a functionally intact β_2AR–green fluorescent protein conjugate was localized on endosomal membranes within minutes of agonist treatment.

There is evidence that the clathrin- and dynamin-dependent machinery of internalization is involved in the response to the β_2AR agonist isoproterenol (Zhang et al., 1996, 1997; Gagnon et al., 1998; Laporte et al., 1999; Walker et al., 1999; Seachrist et al., 2000; Ferguson, 2001; Paing et al., 2002; Pierce et al., 2002). Walker et al. (1999) reported that some G-protein-coupled receptors, such as the β_2-adrenergic receptor, internalized in clathrin-coated vesicles and this process was mediated by G-protein-coupled receptor kinases (GRKs), beta-arrestin and dynamin. Zhang et al. (1996) demonstrated that dynamin, a GTPase that regulates the formation and internalization of clathrin-coated vesicles, is essential for the agonist-promoted sequestration of the β_2AR. They reported that expression of dynamin K44A, a dominant-negative mutant of dynamin that inhibits clathrin-mediated endocytosis, prevented endocytosis of the β_2AR. In HEK293 cells this dynamin mutant profoundly inhibited agonist-induced internalization and downregulation of the β_2AR (Zhang et al., 1996; Gagnon et al., 1998) whereas in COS-1 and HeLa cells it attenuated these processes (Gagnon et al., 1998). Von Zastrow and Kobilka (1992) observed isoproterenol-regulated internalization and recycling of human β_2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. Kallal et al. (1998) reported that β_2AR-GFP in HeLa cells following a short agonist exposure distributed into early endosomes and colocalized with rhodamine-labeled transferrin. These results seem to be consistent with our data that some βAR was sequestered into vesicles corresponding to the early endosomes. Dynamin was localized to such endosomes in *Paramecium* cells during transferrin internalization (Surmacz et al., 2003).

As far as we know, there is no proof of the existence of dynamin–dependent sequestration in the unicellular eukaryotes, though protozoa have been found to be sensitive to a variety of neurotransmitters and neuropeptides (Le Roith et al., 1980; O'Neill et al., 1988; Wyroba, 1989; Renaud et al., 1995; Yang et al., 1997; Christensen et al., 1998; Vallesi et al., 1998; Csaba and Kovacs, 2000; Iwamoto et al., 2000; Delmonte Corrado et al., 2001). Some cases of receptor desensitization have been reported in protozoa but not the presence of G-protein-coupled kinase (Ayala and Kierszenbaum, 1990; Van Haastert et al., 1992; Xiao et al., 1999).

Paramecium emerged early in evolution, about 1.5 billion years ago, prior to the divergence of plants, animals and yeast (Sogin and Elwood, 1986), and βAR appeared in this evolutionary ancient cell as a nutrient receptor. Taking into account that the beta-adrenergic system began to diverge about 0.6 billion years ago (Fryxell, 1995), it seems that the desensitization/sequestration mechanism developed early in evolution – almost parallel to the appearance of this receptor in unicellular eukaryotes.

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References

Ayala, A. and Kierszenbaum, F. (1990). Regulation of *Trypanosoma cruzi* infectivity by alpha- and beta-adrenergic agonists: desensitization produced by prolonged treatments or increasing agonist concentrations. *Parasitol.* **100**, 429-434.

Barak, L. S., Tiberi, M, Freedman, N. J., Kwatra, M. M., Lefkowitz, R. J. and Caron, M. G. (1994). A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated beta2-adrenergic receptor sequestration. *J. Biol. Chem.* **269**, 2790-2795.

Barak, L. S., Ferguson, S. S. G., Zhang, J., Martenson, C., Meyer, T. and Caron, M. G. (1997). Internal trafficking and surface mobility of a functionally intact beta2-adrenergic receptor-green fluorescent protein conjugate. *Mol. Pharmacol.* **51**, 177-184.

Benovic, J. L., DeBlasi, A., Stone, W. C., Caron, M. G. and Lefkowitz, R. J. (1989). Beta-adrenergic receptor kinase: primary structure delineates a multigene family. *Science* **246**, 235-240.

Braun, L., Christophe, T. and Boulay, F. (2003). Phosphorylation of key serine residues is required for internalization of the complement 5a (C5a) anaphylatoxin receptor via a beta-arrestin, dynamin, and clathrin-dependent pathway. *J. Biol. Chem.* **278**, 4277-4285.

Christensen, S. T., Leick, V., Rasmussen, L. and Wheatley, D. N. (1998). Signaling in unicellular eukaryotes. *Int. Rev. Cytol.* **177**, 181-253.

Chuang, D. M. and Costa, E. (1979a). Evidence for internalization of the recognition site of beta-adrenergic receptors during receptor subsensitivity induced by (–)-isoproterenol. *Proc. Natl. Acad. Sci. USA* **76**, 3024-3028.

Chuang, D. M. and Costa, E. (1979b). Beta-adrenergic receptors of frog

erythrocytes. Biochemical sequelae following stimulation with isoproterenol. *Neurochem. Res.* **4**, 777-793.

Claing, A., Laporte, S. A., Caron, M. G. and Lefkowitz, R. J. (2002). Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. *Prog. Neurobiol.* **66**, 61-79.

Csaba, G. and Kovacs, P. (2000). Insulin uptake, localization and production in previously insulin treated and untreated *Tetrahymena*. Data on the mechanism of hormonal imprinting. *Cell Biochem. Funct.* **18**, 161-167.

Damke, H., Baba, T., Warnock, D. E. and Schmid, S. L. (1994). Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J. Cell Biol.* **127**, 915-934.

Delmonte Corrado, M. U., Politi, H., Ognibene, M., Angelini, C., Trielli, F., Ballarini, P. and Falugi, C. (2001). Synthesis of the signal molecule acetylcholine during the developmental cycle of *Paramecium primaurelia* (Protista, Ciliophora) and its possible function in conjugation. *J. Exp. Biol.* **204**, 1901-1907.

Ferguson, S. S. (2001). Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol. Rev.* **53**, 1-24.

Fryxell, K. J. (1995). The evolutionary divergence of neurotransmitter receptors and second-messenger pathways. *J. Mol. Evol.* **41**, 85-97.

Gagnon, A. W., Kallal, L. and Benovic, J. L. (1998). Role of clathrin-mediated endocytosis in agonist-induced down-regulation of the beta2-adrenergic receptor. *J. Biol. Chem.* **273**, 6976-6981.

Iwamoto, M., Murata, T., Morii, H., Watanabe, Y. and Nakaoka, Y. (2000). Activation of prostacyclin synthesis by mechanical stimulation in the ciliated protozoan *Tetrahymena thermophila*. *Biochem. Biophys. Res. Commun.* **279**, 635-638.

January, B., Seibold, A., Whaley, B., Hipkin, R. W., Lin, D., Schonbrunn, A., Barber, R. and Clark, R. B. (1997). Beta2-adrenergic receptor desensitization, internalization and phosphorylation in response to full and partial agonists. *J. Biol. Chem.* **272**, 23871-23879.

Kallal, L., Gagnon, A. W., Penn, R. B. and Benovic, J. L. (1998). Visualization of agonist-induced sequestration and down-regulation of a green fluorescent protein-tagged beta2-adrenergic receptor. *J. Biol. Chem.* **273**, 322-328.

Krupnick, J. G. and Benovic, J. L. (1998). The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu. Rev. Pharmacol. Toxicol.* **38**, 289-319.

Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S., Caron, M. G. and Barak L. S. (1999). The beta2-adrenergic receptor/beta-arrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc. Natl. Acad. Sci. USA* **96**, 3712-3717.

Lefkowitz, R. J., Stadel, J. M. and Caron, M. G. (1983). Adenylate cyclase-coupled beta-adrenergic receptors: structure and mechanisms of activation and desensitization. *Annu. Rev. Biochem.* **52**, 159-186.

Le Roith, D., Shiloach, J., Roth, J. and Lesniak, M. A. (1980). Evolutionary origins of vertebrate hormones: substances similar to mammalian insulins are native to unicellular eukaryotes. *Proc. Natl. Acad. Sci. USA* **77**, 6184-6188.

O'Neill, J. B., Pert, C. B., Ruff, M. R., Smith, C. C., Higgins, W. J. and Zipser, B. (1988). Identification and characterization of the opiate receptor in the ciliated protozoan, *Tetrahymena*. *Brain Res.* **450**, 303-315.

Paing, M. M., Stutts, A. B., Kohout, T. A., Lefkowitz, R. J. and Trejo, J. (2002). Beta-arrestins regulate protease-activated receptor-1 desensitization but not internalization or down-regulation. *J. Biol. Chem.* **277**, 1292-1300.

Pierce, K. L., Premont, R. T. and Lefkowitz, R. J. (2002). Seven-transmembrane receptors. *Nat. Rev. Mol. Cell. Biol.* **3**, 639-650.

Premont, R. T., Inglese, J. and Lefkowitz, R. J. (1995). Protein kinases that phosphorylate activated G protein-coupled receptors. *FASEB J.* **9**, 175-182.

Renaud, F. L., Colon, I., Lebron, J., Ortiz, N., Rodriguez, F. and Cadilla, C. (1995). A novel opioid mechanism seems to modulate phagocytosis in *Tetrahymena*. *J. Eukaryot. Microbiol.* **42**, 205-207.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning. A Laboratory Manual*, 2nd edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Schmid, S. L., McNiven, M. A. and De Camili, P. (1998). Dynamin and its partners: a progress report. *Curr. Opin. Cell Biol.* **10**, 504-512.

Seachrist, J. L., Anborgh, P. H. and Ferguson, S. S. (2000). Beta2-adrenergic receptor internalization, endosomal sorting, and plasma membrane recycling are regulated by Rab GTPases. *J. Biol. Chem.* **275**, 27221-27228.

Shetzline, M. A., Walker, J. K., Valenzano, K. J. and Premont, R. T. (2002). Vasoactive intestinal polypeptide type-1 receptor regulation. Desensitization, phosphorylation, and sequestration. *J. Biol. Chem.* **277**, 25519-25526.

Sogin, M. L. and Elwood, H. J. (1986). Primary structure of the *Paramecium tetraurelia* small-subunit rRNA coding region: phylogenetic relationships within the Ciliophora. *J. Mol. Evol.* **23**, 53-60.

Sorkin, A. and Von Zastrow, M. (2002). Signal transduction and endocytosis: close encounters of many kinds. *Nat. Rev. Mol. Cell. Biol.* **3**, 600-614.

Subramanian, S. V., Wyroba, E., Andersen, A. P. and Satir, B. H. (1994). Cloning and sequencing of parafusin, a calcium-dependent exocytosis-related phosphoglycoprotein. *Proc. Natl. Acad. Sci. USA* **91**, 9832-9836.

Surmacz, L., Wiejak, J. and Wyroba, E. (2003). Evolutionary conservancy of the endocytic machinery in the unicellular eukaryote *Paramecium*. *Biol. Cell* **95**, 69-74.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680.

Vallesi, A., Giuli, G., Ghiara, P., Scapigliati, G. and Luporini, P. (1998). Structure-function relationships of pheromones of the ciliate *Euplotes raikovi* with mammalian growth factors: cross-reactivity between Er-1 and interleukin-2 systems. *Exp. Cell Res.* **241**, 253-259.

Van Haastert, P. J., Wang, M., Bominaar, A. A., Devreotes, P. N. and Schaap, P. (1992). cAMP-induced desensitization of surface cAMP receptors in *Dictyostelium*: different second messengers mediate receptor phosphorylation, loss of ligand binding, degradation of receptor, and reduction of receptor mRNA levels. *Mol. Biol. Cell* **3**, 603-612.

Von Zastrow, M. and Kobilka, B. K. (1992). Ligand-regulated internalization and recycling of human β_2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. *J. Biol. Chem.* **267**, 3530-3538.

Waldo, G. L., Northup, J. K., Perkins, J. P. and Harden, T. K. (1983). Characterization of an altered membrane form of the beta-adrenergic receptor produced during agonist-induced desensitization. *J. Biol. Chem.* **258**, 13900-13908.

Walker, J. K., Premont, R. T., Barak, L. S., Caron, M. G. and Shetzline, M. A. (1999). Properties of secretin receptor internalization differ from those of the beta2-adrenergic receptor. *J. Biol. Chem.* **274**, 31515-31523.

Wiejak, J., Surmacz, L. and Wyroba, E. (2002). Immunoanalogue of vertebrate beta-adrenergic receptor in the unicellular eukaryote *Paramecium*. *Histochem. J.* **34**, 51-56.

Wiejak, J., Surmacz, L. and Wyroba, E. (2003). Dynamin involvement in *Paramecium* phagocytosis. *Eur. J. Protistol.* **39**, 416-422.

Wiejak, J. and Wyroba, E. (2002). Dynamin: characteristics, mechanism of action and function. *Cell. Mol. Biol. Lett.* **7**, 1073-1080.

Wyroba, E. (1989). Beta-adrenergic stimulation of phagocytosis in the unicellular eukaryote *Paramecium aurelia*. *Cell Biol. Int. Rep.* **13**, 667-678.

Xiao, Z., Yao, Y., Long, Y. and Devreotes, P. (1999). Desensitization of G-protein-coupled receptors. Agonist-induced phosphorylation of the chemoattractant receptor cAR1 lowers its intrinsic affinity for cAMP. *J. Biol. Chem.* **274**, 1440-1448.

Yang, W. Q., Braun, C., Plattner, H., Purvee, J. and Van Houten, J. L. (1997). Cyclic nucleotides in glutamate chemosensory signal transduction of *Paramecium*. *J. Cell Sci.* **110**, 2567-2572.

Zhang, J., Ferguson, S. S. G., Barak, L. S., Menard, L. and Caron, M. G. (1996). Dynamin and beta-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. *J. Biol. Chem.* **271**, 18302–18305.

Zhang, J., Ferguson, S. S., Barak, L. S., Aber, M. J., Giros, B., Lefkowitz, R. J. and Caron, M. G. (1997). Molecular mechanisms of G protein-coupled receptor signaling: role of G protein-coupled receptor kinases and arrestins in receptor desensitization and resensitization. *Receptor Channels* **5**, 193-199.