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Pharmacological Attenuation of *Paramecium* Fluid-Phase Endocytosis

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Spectrophotometric quantification of fluid phase endocytosis in the presence of different pharmacological compounds was performed in the model unicellular eukaryote *Paramecium*. The kinetics of Lucifer Yellow Carbohydrazide (LY) uptake in cells exposed to forskolin and isoproterenol – known to stimulate phagocytosis in this cell – was analyzed. Reduction in both the rate of endocytosis and total accumulation of fluid phase marker was observed following the treatment. Forskolin diminished total LY accumulation by 11% and 21% after 5 min and 25 min of incubation, respectively, whereas the rate of uptake was lowered by 21% in comparison to control cells. The inhibitory effect of isoproterenol was less pronounced than that of forskolin. The total accumulation of LY was decreased by 11% in 5 min as compared to the untreated cells and this effect was persistent upon further exposition to this reagent up to 25 min. To better understand these observations, the effect of inhibitors of PKA and cAMP phosphodiesterase on fluid phase uptake was tested. 3-isobutyl-1-methyl xanthine (IBMX) caused 12% decrease in LY accumulation after 5 min of incubation. In combination with isoproterenol or forskolin, IBMX enhanced their inhibitory effect on fluid endocytosis, which was lowered by 25% and 29%, respectively. The strongest inhibitory effect on fluid endocytosis was exerted by the 10 μM PKA inhibitor, which diminished endocytosis by 35% in 5 min. These results suggest that *Paramecium* fluid phase uptake may be regulated through activation of PKA, although the precise mechanism of this process has not yet been elucidated.

Key words: Fluid endocytosis, *Paramecium*, isoproterenol, forskolin, IBMX, iPKA, Lucifer Yellow.

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Abbreviations: LY – Lucifer Yellow Carbohydrazide, iso – (-) isoproterenol, IBMX – 3-isobutyl-1-methyl xanthine, PKA – cAMP-dependent protein kinase, iPKA – inhibitor of PKA.

Fluid phase uptake and its regulation in different cell systems have focused attention (MANIAK 2001; KJEKEN *et al.* 2001) in relation to cell permeability (BRADBURY *et al.* 1992), motility (AYSCOUGH 2000; MICHALIK et al. 2003), secretion (HAZELTON *et al.* 2002) and other endocytic routes such as receptor-mediated endocytosis (MCKINLEY & WILEY 1988; PAGE *et al.* 1994; RAMOINO *et al.* 2001; WIEJAK *et al.* 2001) and phagocytosis (WYROBA 1991). It has been documented that the blocking of phagocytic activity leads to a decrease of fluid phase endocytosis in *Paramecium* cells (WYROBA 1991; RAMOINO *et al.* 2001), but it is not known how stimulation of phagocytosis affects the fluid phase uptake in this unicellular eukaryote. In studies on macrophages, it was observed that phagocytosis reduced fluid phase pinocytosis (WERB & COHN 1972). Major reduction seemed to be not in the rate of internalization but rather in the delivery of fluid to the lysosome in these cells (BUYS & KAPLAN 1987). Phagocytosis in *Paramecium* was significantly increased by catecholamines at physiological concentrations (WYROBA 1987, 1989). Norepinephrine and isoproterenol at 10^{-8}-10^{-3} M enhanced *Paramecium* phagocytosis after a few minutes of incubation with latex particles. Maximum stimulation was observed within 10 minutes after catecholamine administration and prolonged incubation caused a slow decrease in endocytic activity (WYROBA 1989; WYROBA & PŁATEK 1999). Forskolin exerted a 2-fold higher stimulating effect on *Paramecium* phagocytic activity than catecholamines (WYROBA 1987). Studies in other cell systems, however, have shown the opposite results. LACOSTE *et al.* (2001) reported the inhibitory effect of catecholamines on phagocytosis of oyster hemocytes. In human neutrophils, phagocy-

tosis was inhibited by elevating the cAMP level with forskolin (KURIYAMA *et al.* 1995; PRYZWANSKY *et al.* 1998).

In this paper, the results of studies on fluid-phase endocytosis in *Paramecium* cells treated with isoproterenol and forskolin under conditions stimulating phagocytic activity are presented. In order to elucidate the mechanism underlying the effect of these two compounds, we subsequently analyzed the influence of inhibitors of PKA and cAMP phosphodiesterase on fluid uptake.

Lucifer Yellow was used as the fluid-phase marker. This reagent was found to be a useful tool for monitoring this type of endocytosis both in the higher eukaryotes (WOLKERS *et al*. 2003) and *Paramecium* (WIEJAK *et al*. 2001

Material and Methods

Cell and culture method

Paramecium aurelia species complex strain 299s axenic cultures (SOLDO *et al.* 1966) were cultivated (5-day-old cells), collected and starved aseptically for 18 h as described previously (WYROBA 1987) so as to be devoid of autofluorescence (WYROBA *et al.* 1981).

Modulation of fluid phase uptake

The following reagents, stimulating the process of phagocytosis, were used:

1. (-)-isoproterenol – β-selective adrenergic agonist (Sigma, Steinheim, Germany);

2. forskolin – adenylate cyclase activator (Sigma, Steinheim, Germany).

The cells were treated with these reagents at the final concentration reported to effectively stimulate phagocytosis in *Paramecium* cells: iso at 10 μM and forskolin at 1 μM (WYROBA 1987, 1989). Cells were treated with forskolin or iso in the absence or presence of either IBMX (3'-isobutyl-1-methylxanthine, 100 μM, Sigma, Steinheim, Germany), that is the cAMP phosphodiesterase inhibitor or PKA inhibitor (CHENG *et al*. 1986) at 5 μM or 10 μM for up to 25 min. Forskolin and IBMX were dissolved in DMSO (Sigma, Steinheim, Germany), in a concentration not higher than 0.9% in the reaction mixture in order to exclude its effect on cell metabolism. Control samples (not treated) contained an identical amount of DMSO without the reagents. Prior to each experiment an additional control was performed by testing the phagocytosis of the dyed polystyrene monodispersed latex (0.95μ, Polysciences, Inc., Warrington, USA) as described elsewhere (WYROBA 1987).

Results

Fluid phase spectrofluorimetric assay

The accumulation of Lucifer Yellow Carbohydrazide (25 μg/ml, Sigma, Steinheim, Germany), a fluid phase marker, was quantified at 20±1°C by estimation of fluorescence in the Perkin-Elmer Luminescence Spectrometer LS-5B at excitation 430 nm and emission at 540 nm as described elsewhere (WIEJAK *et al.* 2001). After various times of incubation aliquots of the cells were withdrawn from the medium and immediately placed in a 150 excess of ice-cold phosphate-saline buffer (WYROBA 1987) followed by centrifugation (600 × g) for 30 sec. The cell pellet was subjected subsequently to an identical washing procedure in order to remove the free label. The supernatant and the cell pellet from the final washing were collected for assay of LY. Spectrofluorimetric estimation of LY uptake and protein determination was performed as described in WIEJAK *et al.* (2001) from 3-4 independent series of experiments. The standard curve of LY fluorescence was estimated at each series of experiments. The cell fluorescence was expressed per mg of cell protein, estimated by the Lowry method and assayed spectrophotometrically at 750 nm using albumin fraction V (Calbiochem, Nottingham, UK) as a standard and related to cell density and volume.

Fluid-phase endocytosis was quantified spectrophotometrically as an uptake of LY – an anionic fluorochrome – that was proved to be a useful marker of this process in *Paramecium.* Isoproterenol and forskolin caused a decrease in the LY accumulation compared to the control cells. Forskolin diminished LY uptake by 11% and 21% after 5 min and 25 min of incubation, respectively (Fig. 1). The inhibitory effect of iso was less pronounced than that of forskolin and total accumulation of LY was decreased by 11% in 5 min as compared to the untreated cells and this effect was persistent upon further exposition to this reagent up to 25 min (Fig. 1).

LY accumulation was characterized by a very high initial uptake reaching 580.8 ng LY/mg protein/hr, which decreased by almost 80% to 124.4 ng LY/mg protein/hr during the next 20 min of incubation of untreated cells (Table 1), similar to previous observations (WIEJAK *et al*. 2001). Such a pattern of fluid phase uptake was also observed in the treated cells in spite of the lower amount of LY accumulated. In the forskolin-treated ciliates

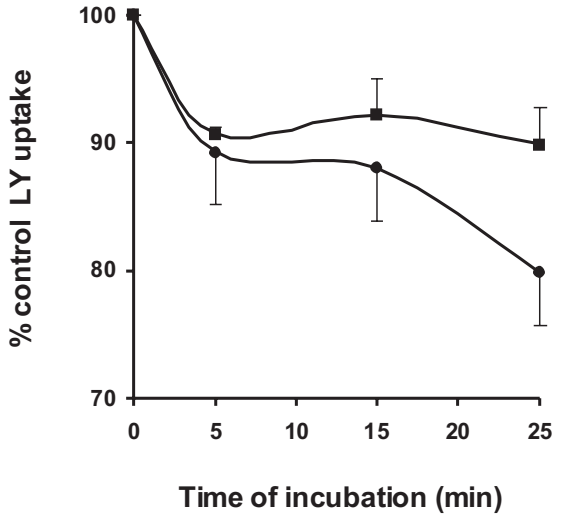

Fig. 1. Kinetics of Lucifer Yellow accumulation by *Paramecium* cells in the presence of forskolin (●) and isoproterenol (■) in comparison to the control. Results based on the spectrofluorimetric assay. Mean values of n=4±SD. Statistical differences were calculated by Student's *t*-test. Forskolin: P<0.05; Isoproterenol: P<0.05

accumulation of the marker was diminished by 81% (from 518.4 to 99.1 ng LY/mg protein/hr), whereas in the iso-treated cells by 79% (from 531.6 to 111.6 ng LY/mg protein/hr) when the amount of LY was assessed after 5 min and 25 min of incubation, respectively.

Both forskolin and iso diminished the rate of LY uptake calculated per cell (Table 1). Initially it ranged from 0.444 pl/cell/hr in control cells to 0.396 pl/cell/hr and 0.408 pl/cell/hr in forskolin- and isoproterenol-treated cells, respectively, after 5 min of incubation (Table 1). Forskolin exerted a stronger effect than iso on the rate of LY uptake that was lowered by 21% (0.076 pl/cell/hr) in comparison to the control (0.096 pl/cell/hr) after 25 min of treatment.

The putative mechanism of isoproterenol effect would be to act via beta-adrenergic receptor/adenylate cyclase/cAMP mechanisms leading to activation of PKA (COLLINS & SURWIT 2001), whereas forskolin acts as an activator of adenylate cyclase (SEAMON *et al.* 1981).

To better understand this regulation, effect of IBMX and PKA inhibitor was investigated. These reagents attenuated the fluid phase uptake to a different degree. IBMX caused a 12% decrease in LY accumulation and this effect persisted during the next 20 min of incubation (Fig. 2). This inhibitor caused a further reduction in LY accumulation when administrated together with forskolin or isoproterenol that was reduced by 29% and 25%, respectively, of the control value after 5 min of incubation. Upon prolonged incubation, this inhibitory effect started to wear off and after 25 min amounted to 77% for forskolin- and 81% for isoproterenol-treated cells in comparison to the untreated cells (Fig. 2).

PKA inhibitor was used at two concentrations: 5 μM (Fig. 3A) and 10 μM (Fig. 3B). Incubation of cells with 5 μM PKA inhibitor initially attenuated fluid phase uptake by 11% and this effect was diminished to 8% by increasing incubation time to 25 min (Fig. 3A). PKA inhibitor at 5 μM enhanced the attenuating effect of forskolin and isoproterenol on fluid phase uptake (Fig. 3A). The inhibitory effect on LY uptake was much stronger when the higher concentration (10 μM) of PKA inhibitor was used (Fig. 3B): a 35% and 26% decrease was observed after 5 and 25 min of incubation, respectively. In this case, however, no further reduction in uptake of LY was observed when this compound was combined with iso or forskolin (Fig. 3B).

Table 1

Quantitation of total accumulation of Lucifer Yellow and its rate of uptake in control, forskolin- and isoproterenol-treated *Paramecium* cells. Mean value of four experiments. Data expressed: for the rate of uptake in ng per mg of protein/hr, whereas for the accumulation of LY in pl per cell/hr

Time of incubation (min)	LY accumulation (ng/mg protein/hr)			Rate of LY uptake (pl/cell/hr)		
	Control	Forskolin	Iso	Control	Forskolin	Iso
5	580.8	518.4	531.6	0.444	0.396	0.408
15	209.2	184.0	192.8	0.161	0.142	0.148
25	124.4	99.1	111.6	0.096	0.076	0.086

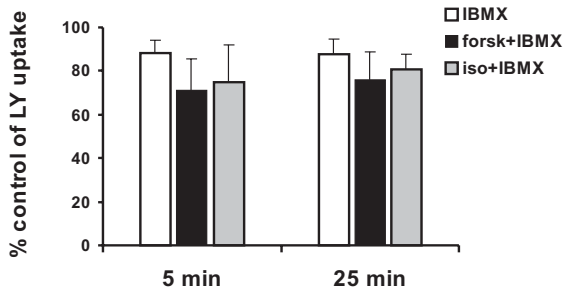

Fig. 2. Effect of IBMX on accumulation of Lucifer Yellow in *Paramecium* cells. The inhibitor (100 μM) was added to the incubation medium alone or in combination with isoproterenol (iso) or forskolin (forsk). Mean values ±SD of three independent experiments are shown. Statistical differences were calculated by Student's *t*-test, P<0.05.

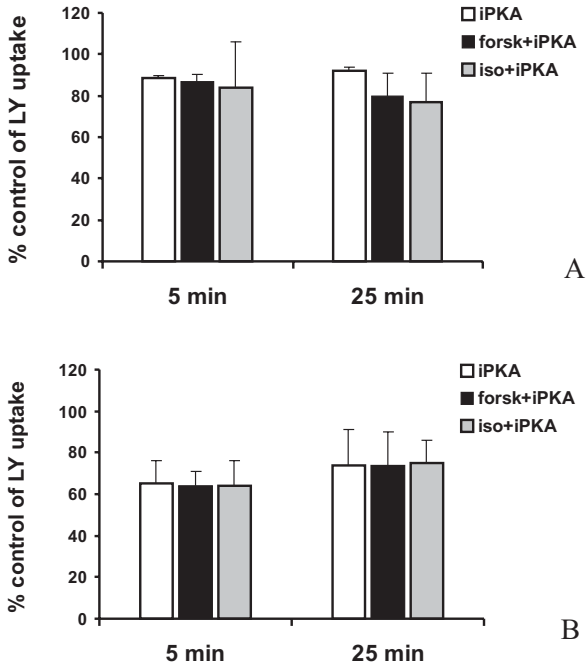

Fig. 3. Action of PKA inhibitor (iPKA) on Lucifer Yellow uptake in *Paramecium* as quantified in the presence or absence of isoproterenol (iso) or forskolin (forsk). iPKA effect studied at two concentrations: 5 μM (A) and 10 μM (B) in comparison to the control. Data are mean values ±SD of 3 independent experiments. Statistical differences were calculated by Student's *t*-test, P<0.05.

Discussion

The aim of this study was to analyze the kinetics of the fluid phase uptake in *Paramecium* cells exposed to the pharmacological modulators of endocytic routes – under the conditions of increased phagocytic activity. Experiments were performed under strict conditions to ensure intactness of the cells and their viability since it is known that optimal LY internalization requires an intact cytoskeleton (WOLKERS *et al*. 2003).

It has been reported that *Paramecium* cells internalized LY in a time-dependent manner and the rate of accumulation was not linear: it decreased 5-fold after prolonged incubation time in comparison to the initial rate of uptake (WIEJAK *et al.* 2001). Now we demonstrate that forskolin and isoproterenol evoked a reduction in both the rate of uptake and the total accumulation of fluid phase marker Lucifer Yellow.

Both forskolin and isoproterenol initially decreased fluid phase uptake by ~11%. After prolonged incubation time, the total LY uptake in forskolin-treated cells was gradually decreased to 79% of that observed in the control, untreated ciliates but was almost unchanged in isoproterenol-treated cells after 25 min of treatment.

In mammalian cells isoproterenol evoked retardation of fluid-phase endocytosis of FITC-dextran as reported by SOJAKKA *et al.* (1999) in studies on rat cardiac myocytes. The inhibitory effect of forskolin on fluid phase uptake was also detected in other cells. PATAKI *et al.* (1995) reported that forskolin decreased fluid phase by 29% in rabbit alveolar macrophages whereas BRADBURY & BRIDGES (1992) observed a 63% reduction in the uptake of the horseradish peroxidase (HRP) in the secretory cell line T84. Both the amount and rate of the Lucifer Yellow transported by malpighian tubules of the house cricket as the result of db-cAMP (dibutyryl-cAMP) stimulation was decreased by 40% and 70%, respectively, compared to the unstimulated transport (HAZELTON *et al.* 2002). However, it should be noted that db-cAMP had a very slight stimulating effect on *Paramecium* phagocytosis (WYROBA 1987) and almost negligible on fluid phase uptake (WYROBA *et al*. – unpublished). In goldfish intestinal epithelium (KILIAAN *et al.* 1996) and in isolated ileum of rats (BIJLSMA *et al.* 1996), forskolin decreased the amount of accumulated HRP. Interestingly, fluid phase endocytosis was either unchanged or slightly enhanced only in the myeloid cell line HL-60, if increased cAMP levels were elicited by forskolin (FOTI *et al.* 1997). Combination of IBMX or PKA inhibitor with iso or forskolin in *Paramecium* enhanced the inhibitory effect of isoproterenol and forskolin. IBMX – cAMP phosphodiesterase inhibitor – decreased LY accumulation by 12%. This is in agreement with the results of PATAKI *et al*. (1995).

Isoproterenol is a strong, synthetic catecholamine that transduces its signal from the cell membrane via β-adrenergic receptor coupled to Gs (*stimulatory heterotrimeric G protein*), and in a consequence the cAMP level is increased. In studies of the effect of iso on phagocytosis, maximum stimulation was observed within 10 minutes after catecholamine addition and prolonged incubation caused a slow decrease in endocytic activity (WYROBA 1989), presumably as a result of receptor desensitization (LEFKOWITZ *et al.* 1983). The

isoproterenol effect may be correlated with receptor desensitization that is initiated by phosphorylation of agonist-occupied receptor (BARAK *et al.* 1994; PREMONT *et al.* 1995). Interestingly, a similar relationship was observed in the present study: the effect of isoproterenol was less pronounced than that of forskolin, especially after prolonged incubation when administered alone or in combination with IBMX.

This may be due to the desensitization process of the beta-adrenoreceptor (β-AR) immunoanalogue reported by us previously in this cell (WIEJAK *et al*. 2002; WIEJAK *et al*. 2004b) in which GRK (*G protein-coupled receptor kinase*) is indispensable. In fact, the deduced amino acid sequence of a cloned gene fragment homologous to the β-adrenergic receptor kinase (βARK=GRK2) exhibited 51.6% homology in a 126 amino acid overlap to the human βARK2 (WIEJAK *et al*. 2004b). By SDS-PAGE, cell fractionation and Western blot it was shown that isoproterenol evoked redistribution of β-AR analogue in *Paramecium* from membranous (P2) to cytosolic (S2) fraction (WIEJAK *et al*. 2002). However, up to now the beta-adrenoreceptor in *Paramecium* has not yet been cloned. Therefore other possibilities of regulation should also be taken into consideration. Adenylate cyclase catalytic activity can be regulated through PKA-mediated phosphorylation (IWAMI *et al*. 1995; CHEN *et al*. 1997). In fact, CHEN *et al*. (1997) reported that beta-adrenergic stimulation in lung epithelial cells activates PKA.

We therefore attempted to investigate the influence of PKA inhibitor on fluid phase uptake. 10 μM PKA inhibitor exerted the greatest inhibitory effect on fluid endocytosis and diminished it by 35% after 5 min of incubation. This is consistent with the results of PAGE *et al.* (1994), who have shown that LY uptake in cultured atrial myocytes and isolated intact atria from adult rats was inhibited by the protein kinase inhibitors.

Both PKA and adenylate cyclase in *Paramecium* display special features in comparison to the mammalian enzymes. PKA lacks a dimerization domain and may have a unique autophosphorylation site sequence (CARLSON & NELSON 1996). It is known that this enzyme is also involved in endocytosis (GRIFFITHS *et al.* 1990). Adenylate cyclases (AC) from *Plasmodium, Paramecium* and *Tetrahymena* are novel ion channel/enzyme fusion proteins. The occurrence of this highly unique subtype of adenylate cyclase appears to be restricted to ciliates and apicomplexa (WEBER *et al.* 2004). The detailed mechanism of *Paramecium* AC regulation is not yet known. The purified AC of 96 kDa, i.e. comparable in size with the prototypical mammalian versions, displayed AC activity, no forskolin stimulation and cation conductance. Control of cAMP levels in *Paramecium* is linked to a K+ outward current and cAMP formation is enhanced upon hyperpolarization. Therefore it was suggested that this AC operates as an ion channel (SCHULTZ *et al.* 1992; WEBER *et al*. 2004). Interestingly, it was shown that in mammalian cells the beta-adrenoceptor agonists might exert their effect by K+ channel opening in bronchial asthma (SMALL *et al.* 1993). Therefore, such a possibility should also be taken into account when trying to understand the results obtained in this study.

It was previously observed that PMA (4beta-phorbol-12-myristate-13-acetate) – protein kinase C activator – decreased fluid phase uptake in *Paramecium*, as measured by LY accumulation, and that the protein kinase C inhibitor – GF 109203 X did not affect this process. These results on the effect of PKC modulators suggested that PMA action on fluid phase uptake is not mediated by PKC signalling (WIEJAK *et al.* 2001). A similar effect was obtained by BRADBURY & BRIDGES (1992) on T84 cells, where PMA caused no significant reduction in the level of endocytosis compared to the control, nor did it reverse the inhibitory effect of PKA activation. Our present results may suggest that fluid phase uptake in *Paramecium* may be regulated by PKA via a yet unidentified factor. It should be pointed out that such a new pathway involving the direct activation of PKA by Smad proteins was recently discovered in mammalian cells (ZHANG *et al.* 2004). Since the regulator and effector molecules involved in uptake and transit are largely conserved between higher and lower eukaryotes (SUBRAMANIAN *et al*. 1994; MANIAK 2001; SURMACZ *et al*. 2003; WIEJAK *et al.* 2003; WIEJAK *et al*. 2004a) further studies may unravel the mechanism underlying the observed effects.

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