Pyrimidinoceptor potentiation by ATP in NG108-15 cells

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Abstract Regulation of inositol phospholipid hydrolysis by UTP and UDP in neuroblastoma×glioma hybrid cell line NG108-15 was potentiated in the presence of ATP. The effect of ATP was dose dependent and shifted the EC_{50} value for these uracil nucleotides up to three powers of magnitude, having no influence on the maximal value of the response. Adenine nucleotides (ADP, AMP, adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), β,γ-methyleneadenosine 5′-triphosphate (βγMeATP), 3′-*O*-(4-benzoyl)benzoyl ATP (BzATP) and 3′-deoxyadenosine 5′-*O*-(1-thio)triphosphate (dATPαS)) as well as adenosine, had no influence on the pyrimidinoceptor response. The potentiation effect was abolished by excess of EDTA. The results were in agreement with the hypothesis of pyrimidinoceptor affinity regulation via extracellular phosphorylation of the receptor protein, initiated by ATP. This mechanism may have physiological implication for functioning of uracil nucleotides as endogenous signaling molecules.

Key words: UTP; UDP; ATP; ATP analog; Pyrimidinoceptor; Inositol phospholipid hydrolysis; Extracellular receptor phosphorylation

1. Introduction

Extracellular nucleotides regulate physiological responses in various tissues by activating appropriate receptors [1]. Several of these receptors possess clear selectivity against uracil nucleotides, pointing to the possibility that these compounds may function as endogenous signaling molecules [2–5]. The pyrimidinoceptor concept can be supported by the possibilities for recycling of the uracil nucleotides and formation of their pools in cells [2]. However, the amount of the stored UTP in granules of chromaffin cells and platelets forms only about 10% of the appropriate ATP level [2]. Therefore the question whether the uracil nucleotides can be released in quantities sufficient to stimulate pyrimidinoceptors, has still no answer.

Here we present evidence that the effectiveness of UTP as well as UDP to activate phosphoinositol lipid breakdown in neuroblastoma×glioma hybrid cell line NG108-15 can be dramatically increased in the presence of ATP, probably initiating phosphorylation of the receptor by ectokinases. As the shift of the effective concentration interval for UTP and UDP exceeded several powers of magnitude, far enough to compensate for the low abundance of uracil nucleotides in tissues, the effect discovered may have a clear physiological implication in cell regulation by these compounds.

2. Materials and methods

2.1. Cell culture

Neuroblastoma×glioma hybrid NG108-15 cells were obtained from the European Collection of Cell Cultures and were grown in high-glucose Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) of fetal calf serum, 0.1 mM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine. Cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in 75-cm^2 tissue culture dishes.

2.2. Chemicals

UTP, UDP, UMP, ATP, ADP, AMP, adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), β,γ-methyleneadenosine 5′-triphosphate (βγMeATP), 3′-*O*-(4-benzoyl)benzoyl ATP (BzATP) and 3′-deoxyadenosine 5′-*O*-(1-thio)triphosphate (dATPαS) (Boehringer Mannheim, Sigma, Amersham) were analyzed and purified by HPLC (Gilson) on an anion exchange column Mono Q HR5/5 (Pharmacia Biotech) by monitoring absorbance at 258 nm for adenine nucleotides and 270 nm for uracil nucleotides. Linear gradients from 0 to 0.8 M NaCl in 50 mM phosphate buffer (pH 7.0) and from 0.1 to 1.2 M ammonium carbonate in water at the flow rate 1 ml/min were used. The latter gradient was used for preparative purification of nucleotides. Adenosine and other chemicals were of the highest available grade of purity.

2.3. Assay of inositol phosphates

NG108-15 cells were seeded in a 24-well culture plate at density $\sim 5\times10^4$ cells/well, and used for assay after 3 days when grown to subconfluence. Inositol lipids were radiolabeled by overnight incubation of cells with *myo*-[2-^{3}H]inositol (2 μCi/ml, Amersham) in 200 μl of inositol-free DMEM. The medium was not changed after [^{3}H]inositol addition. Nucleotide effects were initiated by addition of 50 μl of ligand solution in 50 mM LiCl and the assay mixture was incubated 10 min at 37°C. The reaction was stopped by aspirating the medium and adding 500 μl of ice-cold 5% TCA. The TCA-containing supernatant was extracted three times with 500 μl of diethyl ether and inositol phosphates were isolated by using Dowex AG1-X8 gel (Bio-Rad, 100–200 mesh, formate form). The columns (bed volume 0.8 ml) were washed with water (2×5 ml) and 50 mM ammonium formate (10 ml) and inositol phosphates were eluted with 1 M ammonium formate in 0.1 mM formic acid (2×5 ml). The amount of [^{3}H]inositol phosphates formed was determined by counting radioactivity of the eluate (scintillation cocktail EcoLume, ICN). Under the experimental conditions used the basal radioactivity of samples remained between 800 and 1000 cpm/sample and the maximal radioactivity achieved was 6000–7000 cpm/sample. The results were calculated as means ± S.E.M. from three independent experiments and normalized versus the results of the control experiments at 10 μM UTP concentration. The dose-response curves were processed by a non-linear regression analysis program Prism (Version 2.00).

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Abbreviations: ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); βγMeATP, β,γ-methyleneadenosine 5′-triphosphate; BzATP, 3′-*O*-(4-benzoyl)benzoyl ATP; dATPαS, 3′-deoxyadenosine 5′-*O*-(1-thio)triphosphate; EDTA, ethylenediamine tetraacetate; DMEM, Dulbecco's modified Eagle's medium; TCA, trichloroacetic acid; HPLC, high performance liquid chromatography

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3. Results

3.1. Activation of phospholipase C by nucleotides in NG108-15 cells

The synthesis of inositol phosphates in NG108-15 cells was activated by UTP and UDP in a dose-dependent manner, yielding the EC_{50} values $(3.9 \pm 0.6) \times 10^{-7}$ M and $(1.3 \pm 0.6) \times 10^{-6}$ M, respectively (Fig. 1). The potency of UMP was considerably weaker ($EC_{50} = (7.5 \pm 0.6) \times 10^{-4}$ M). The same biochemical response was also activated at high concentrations of ATP, ADP, ATPγS and dATPαS, characterized by the EC_{50} values $(3.0 \pm 0.7) \times 10^{-4}$ M, $(2.4 \pm 0.7) \times 10^{-4}$ M, $(7.0 \pm 0.8) \times 10^{-4}$ M and $(4.4 \pm 0.9) \times 10^{-4}$ M, respectively. In the presence of βγMeATP, AMP and adenosine, there was no statistically significant increase in formation of inositol phosphates if compared with the basal level. Thus, the receptors revealed clear specificity for UTP and UDP.

At nucleotide concentrations above 1 mM a decrease of the ligand-induced effect was observed in the case of UTP and UDP, as well as in the case of adenine nucleotides, resulting in a bell-shaped form of the dose-response curves (data not shown). However, as not relevant to the present study, these effects of high ligand concentration will be discussed elsewhere.

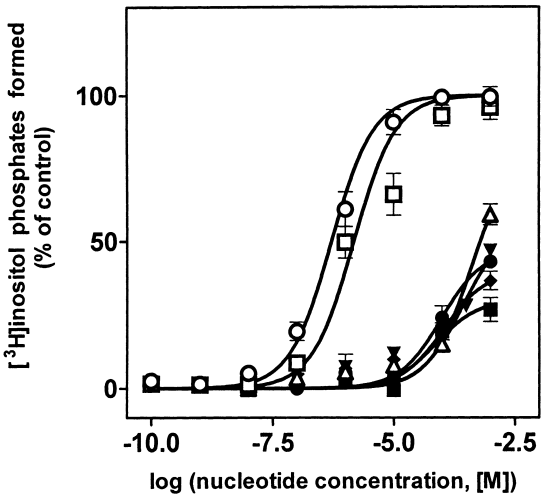

Fig. 1. Dose-dependent stimulation of synthesis of inositol phosphates in neuroblastoma×glioma hybrid NG108-15 cells by uracil and adenine nucleotides (○, UTP; □, UDP; △, UMP; ●, ATP; ■, ADP; ▼, ATPγS; ♦, dATPαS).

3.2. Influence of ATP on UTP and UDP induced synthesis of inositol phosphates

Simultaneous application of ATP and UTP or ATP and UDP led to a non-additive effect, i.e. the total amount of inositol phosphates formed did not exceed the level observed at optimal concentration of UTP alone (Fig. 2). At the same time the dose-response curves for UTP and UDP, measured in the presence of ATP, revealed a significant potentiation of the effect of the uracil nucleotides. This shift of the dose-response curves was clearly dependent on ATP concentration and in the presence of 1 mM ATP the effective UTP and UDP concentrations were lowered into the picomolar range, yielding the EC_{50} values $(1.1 \pm 0.2) \times 10^{-12}$ M and $(1.2 \pm 0.2) \times 10^{-11}$ M, respectively (Fig. 2).

3.3. Influence of other nucleotides on UTP induced synthesis of inositol phosphates

The experiments described above were repeated in the presence of ADP, AMP and adenosine (10 μM–1 mM), as well as βγMeATP and ATPγS (0.1 mM). It was found that none of these ligands potentiated the UTP effect on synthesis of inositol phosphates. Moreover, these ligands had no influence on the maximal level of the UTP response.

3.4. Effect of EDTA on UTP induced synthesis of inositol phosphates

In separate experiments 4 mM EDTA was added into the assay medium before nucleotides to bind the divalent cations present. It was found that the excess of this chelating ligand had no effect on the EC_{50} value for UTP, equal to $(2.0 \pm 0.5) \times 10^{-7}$ M in the Mg^{2+} and Ca^{2+} free medium, as

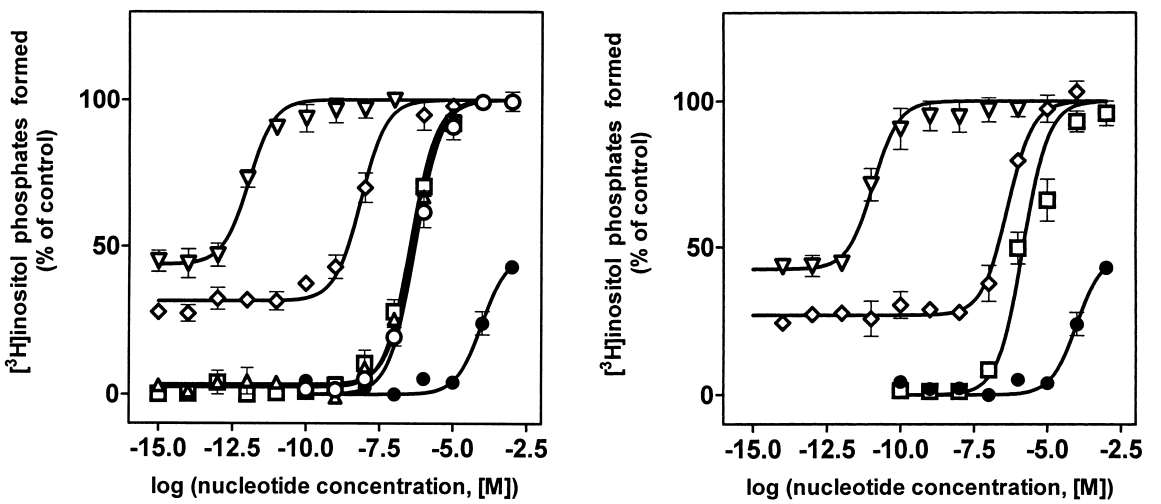

Fig. 2. Potentiation of the effect of UTP (left) and UDP (right) on pyrimidinoceptor regulated synthesis of inositol phosphates in the presence of various concentrations of ATP (△, 1 μM; □, 10 μM; ◇, 100 μM; ▽, 1 mM). The dose-response curves for UTP (○), UDP (□) and ATP (●) alone were shown for comparison.

well as on the range of the response of cells observed. At the same time the potentiating effect of ATP on the UTP induced inositol phosphate formation was completely abolished in the presence of EDTA.

4. Discussion

Synthesis of inositol phosphates in NG108-15 cells was regulated by nucleotide receptors, which revealed clear preference for UTP and UDP over ATP and its analogs. This result agreed well with the rank order of the effect of uracil and adenine nucleotides, published by Lin for the same cell line [6]. Therefore the uracil nucleotides may well act as endogenous signaling molecules for these cells and the appropriate receptors may belong to the $P2Y_4$ and/or $P2Y_6$ subtypes [7–9].

Although the cell responses were evoked by UTP and ATP at very different concentrations, Lin has suggested that both these nucleotides interact with the same receptor on NG108-15 cells [6]. This suggestion was based on the absence of additivity between the effects of UTP and ATP, as well as on the possibility of desensitization of the cells to the effects of both these nucleotides by their extended pretreatment with UTP. Although the same phenomena were observed in the present study, a more significant difference between the behavior of uracil and adenine nucleotides pointed to the involvement of distinct regulatory mechanisms for uracil and adenine nucleotides.

Firstly, in the presence of ATP a dramatic dose-dependent increase in potency of uracil nucleotides was observed, while no increase in potency of ATP analogs was found under the same conditions. This means that most probably the uracil and adenine nucleotides act at distinct target sites.

Secondly, among all the investigated adenine nucleotides only ATP was able to enhance potency of UTP (and UDP), although the synthesis of inositol phosphates was similarly initiated by several adenine-containing ligands. Thus, the activation of phospholipase C and potentiation of the effect of uracil nucleotides should occur via different mechanisms.

Although the former phenomenon may be related to some still unspecified influence of the externally applied adenine nucleotides on synthesis of inositol phosphates, the ligand specificity of this effect seems to support the idea of involvement of some receptors. These receptors may well belong to the channel-coupled subtypes, also governed by ATP and its analogs. For example, the $P2X_7$ receptor subtype, activated by micromolar ATP concentrations and operating a non-selective ion channel was found in this cell line [10]. Therefore, it can be supposed that ATP may cause 'leaking' of intracellular compounds, including uracil nucleotides, into the extracellular medium. Although the amount of uracil nucleotides released through these 'pores' should be small, this may still be sufficient to activate the pyrimidinoceptors, simultaneously potentiated by ATP. Following this hypothetical scheme the non-additive nature of the maximal response of UTP and ATP, as well as the desensitization phenomena observed by Lin [6], can be easily understood.

The results of the present study point to the possibility that the observed potentiation of pyrimidinoceptors on NG108-15 cells, occurring in the presence of ATP, can be related to extracellular phosphorylation of these proteins. As the γ-phosphate of ATP is transferred to the target protein in this reaction, the ATP analogs cannot participate in this process and therefore were unable to potentiate pyrimidinoceptors. Moreover, as the actual substrate for protein kinases is MgATP, the abolishment of the potentiation effect in the presence of EDTA also agrees with the phosphorylation hypothesis.

If the intracellular reversible phosphorylation of proteins has been recognized as a key regulatory mechanism in numerous cellular functions, much less is known about the regulatory role of the extracellular protein phosphorylation [11]. The results of the present study point to the possibility that the latter process may be implied in regulation of the responsiveness of NG108-15 cells to extracellular UTP. Until recently the possibilities of cell regulation by uracil nucleotides have been thought to be limited by low physiological concentration of these compounds, not corresponding to the experimentally detectable effectiveness of binding of these ligands on the appropriate receptors. Therefore the possibility of significant increase in affinity of pyrimidinoceptors through extracellular phosphorylation of these proteins may have a clear physiological significance for intracellular communication by means of uracil nucleotides.

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