

ATP a potent regulator of inositol phospholipids-phospholipase C and lipid mediators in brain cortex

Joanna Strosznajder and Robert P. Strosznajder

Department of Cellular Signalling and Department of Neurophysiology, Medical Research Centre, Polish Academy of Sciences, 3 Dworkowa St., 00-784 Warsaw, Poland

Abstract. Adenosine-5'-triphosphate (ATP) is stored and co-released with various neurotransmitters but it may also act as a fast excitatory neurotransmitter through the activation of purinoreceptor(s). In this study the effect of ATP on phospholipase C (PLC) degrading labelled PtdIns(4,5)P₂ and PtdIns in brain cortex slices, brain homogenate and subcellular fractions was investigated. It was found that ATP added into brain slices activated significantly and specifically PtdIns(4,5)P₂ degradation and this process was inhibited by theophylline. Moreover, ATP maintained a higher level of inositol(1,4,5)P₃ radioactivity in total water-soluble inositol metabolites. However, ATP added directly for the assay of PLC into brain homogenate or subcellular fractions inhibits phosphoinositide degradation in a receptor-independent manner and suppresses conversion of Ins(1,4,5)P₃ into Ins(1,4)P₂. Our results indicate that ATP acting extracellularly through a purinergic receptor(s) activates PtdIns(4,5)P₂ degradation and release of Ins(1,4,5)P₃. ATP acting directly on PLC inhibits in a receptor-independent manner phosphoinositide degradation, and protects against liberation of lipid-derived second messengers.

Key words: ATP, brain cortex, phospholipase C

INTRODUCTION

Adenosine-5'-triphosphate (ATP) has been detected in the extracellular environment of various tissues. Neurones, adrenomodulatory cells and platelets are known to store ATP within secretory vesicles and to release it by exocytosis during stimulation (Gordon 1986). ATP is a common constituent of synaptic vesicles and is released mainly as a co-transmitter with noradrenaline, acetylcholine or other substances in the peripheral nervous system (Zimmerman 1978, Castel et al. 1984) or with acetylcholine in the central nervous system (Richardson and Brown 1987). In contrast, ATP stimulates norepinephrine and dopamine uptake in PC12 pheochromocytoma cells (Eldefrawi and Shleman 1991). Cells injured by ischemia and dying cells release ATP in large amounts (Imai et al. 1964, Forrester and Williams 1977). Several recent studies have indicated that extracellular ATP may induce cell degeneration in various types of cells (Murgia et al. 1992, Cheng et al. 1994). The existence of purinergic nerves, which release ATP as a principal excitatory transmitter, has been reported (Burnstock 1975, Bean 1992). Extracellular ATP may act through P_2 (P_{2x} , P_{2y}) purinergic receptors. Two parts of the ATP molecule are crucial for the interactions with receptors: the vicinity of C_6 of the purine ring and the polyphosphate chain. Some modifications in these regions of the molecule result in the transformation of the agonist into the antagonist (Illes and Noremborg 1993). Activation of metabotropic P_2 receptor leads to an increase in the concentration of cytoplasmic free calcium $[Ca^{2+}]_i$. This effect is due to the receptor-dependent activation of phosphatidylinositol-4,5-bisphosphate [$PtdIns(4,5)P_2$] hydrolysis and release of inositol (1,4,5)trisphosphate [$Ins(1,4,5)P_3$]. The increase in $[Ca^{2+}]_i$ elicited by ATP may also be caused by the influx of extracellular Ca^{2+} through a P_2 receptor-activated calcium channel. The magnitude of the increase in $[Ca^{2+}]_i$ elicited by ATP is correlated with the fully ionized form of this nucleotide, ATP^{4-} , in the medium and not with the concentration of $MgATP^{2-}$ (Lin et al. 1993). Extracellular ATP has neuromodulatory ef-

fects; it can modulate Ca^{2+} uptake and transmitter release. ATP produces long-term potentiation (ATP-induced LTP) in CA_1 neurones of hippocampal slices, probably by activation of ecto-protein kinase and phosphorylation of extracellular domains of membrane protein in neurones (Lynch and Boundry 1984, Ehrlich et al. 1986, 1988). However, other mechanisms for ATP-induced LTP have also been suggested. There is a growing interest in the role of extracellular ATP in regulation of formation and action of $Ins(1,4,5)P_3$, diacylglycerol (DAG) and other messengers molecules in signal transduction processes in brain. Little is known about direct effects of ATP on membrane-bound and cytosolic phospholipases participating in regulation of lipid mediators in the brain.

The present study was undertaken to characterize the role of ATP acting extracellularly and in a cell free system in regulation of inositol phosphates and arachidonic acid (AA) release by phospholipases C and A_2 in the brain.

METHODS

Chemicals

[2- 3H inositol] phosphatidylinositol s.a. 17 Ci/mmol, [2- 3H inositol] phosphatidylinositol-(4,5)-bisphosphate s.a. 1.5 Ci/mmol, [1- ^{14}C] arachidonylo-phosphatidylinositol s.a. 57 mCi/mmol were obtained from Amersham, UK.

Adenosine 5'-triphosphate (ATP), $ATP\gamma S$, guanosine 5'-0-(3-thio)triphosphate ($GTP\gamma S$), EGTA, theophylline, were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Dowex X8 was obtained from Serva Feinbiochemica GmbH, Germany.

Preparation of brain slices

Wistar rats (200-220 g) were decapitated and the brains were rapidly removed. The cerebral cortex slices 0.35 x 0.35 mm were prepared using McIlwain tissue chopper with cooled razor blade. The brain slices before stimulation with agonist were preincu-

bated for 15 min in Krebs-Henseleit (KRBS) buffer pH 7.4 containing 10 mM glucose and equilibrated with O₂/CO₂ (95:5). The slices were then centrifuged at 1,000 x g for 15 min, resuspended in KRBS and used for experiments. ATP in concentration range 0.25-1.0 mM or 100 μ M ATP γ S or 100 μ M GTP γ S or theophylline 10 μ M was added for 10 min preincubation time. Then the slices were quickly homogenized and used as a source of enzymes (PLC, PLA₂) for the determination of inositol phospholipids degradation ([³H]inositol-PtdIns, [³H]inositol-PtdIns(4,5)P₂, [¹⁴C]arachidonoylo-PtdIns).

Preparation of synaptic plasma membrane and cytosol

These fractions were isolated from dissected brain hemispheres homogenized in a Dounce-type glass homogenizer in ice-cold isolation medium, containing 0.32 M sucrose and 10 mM Tris-HCl buffer pH 7.4. The homogenate (10 %, w/v) was centrifuged for 3 min at 1,100 x g. The resulting supernatant was centrifuged for 10 min at 17,000 x g to yield a crude mitochondrial fraction (P₂). Subsequently, the pellet was twice shocked in 1 mM Tris-HCl buffer pH 7.0 by homogenization and subsequent centrifugation at 10,000 x g for 10 min. Finally, the combined supernatants, after adjusting to 0.32 M sucrose, were centrifuged at 48,000 x g for 20 min to obtain the synaptic plasma membranes (SPM). The supernatant resulting from 17,000 x g centrifugation was then centrifuged at 104,000 x g for 60 min to obtain the cellular cytosol. These subcellular fractions were obtained as described previously by Strosznajder and Strosznajder (1989).

Assay for phospholipase C activity, acting against [³H] phosphatidylinositol [PtdIns] and [³H] phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P₂]

The enzyme activity was assayed by measuring the formation of radioactive inositol phosphate(s) from labelled exogenous substrate, 10 nmol

PtdIns(4,5)P₂ (25,000-28,000 cpm) or 20 nmol PtdIns (40,000 cpm) was added per incubation vial and organic solvent was evaporated under nitrogen. After addition of 20 mM Tris-HCl buffer, pH 6.6, for determination of phospholipase C acting on PtdIns(4,5)P₂ and 20 mM Tris-HCl buffer, pH 7.8 in the case of PtdIns and 0.1% sodium deoxycholate, each tube was vigorously vortexed for 1 min. Moreover, the assay system contained 10 mM LiCl, 10 mM NaF, 0-2.0 mM CaCl₂ or 10 mM EGTA and about 50 μ g protein in a final volume of 200 μ l. Mixtures were incubated for 15 min [PtdIns(4,5)P₂] and for 30 min [PtdIns] at 37°C and the reaction was stopped with 1 ml chloroform/methanol/HCl 100:100:0.6 (v/v/v) and then 0.3 ml of H₂O was added. After shaking, phases were separated by centrifugation at 3,000 x g for 10 min. A 0.5 ml portion of the aqueous phase was mixed with 10 ml of Bray's scintillation fluid for determination of radioactivity in Wallach 1409 LKB scintillation counter. The rest of the aqueous phase was used for the separation of [³H] inositol phosphates according to the method described by Berridge et al. (1983). Under the above conditions, the hydrolyzed products increased linearly with incubation time and the amounts of protein.

Assay of arachidonic acid release from [1-¹⁴C]arachidonoylo-phosphatidylinositol

The whole procedure was carried out in the presence of 200 μ g of protein using 25 nmol of [1-¹⁴C] AA-PtdIns (30,000 cpm), 0.1% sodium deoxycholate, 20 mM Tris-HCl pH 7.8, 1-2 mM CaCl₂ in a final volume of 200 μ l of incubation mixture. The labelled arachidonic acid (AA) and the other lipids were extracted according to the method of Bligh and Dyer (1959) and AA was separated on Thin Layer Chromatography (TLC), using solvent system chloroform/acetone (96:4, v/v).

Separation of water soluble inositol metabolites

The water soluble inositol metabolites were applied to a small column (0.5 x 7.0 cm) containing

0.4 g Dowex AG 1 x 8 (200-400 mesh). Free inositol and inositol phosphates (InsP₁, InsP₂, InsP₃) were eluted sequentially according to the procedure described by Berridge et al. (1983); between 16 and 20 ml were collected for each fraction. Aliquots of each fraction (0.5 ml) were taken for radioactivity measurements, in a LKB Wallach 1409 scintillation counter.

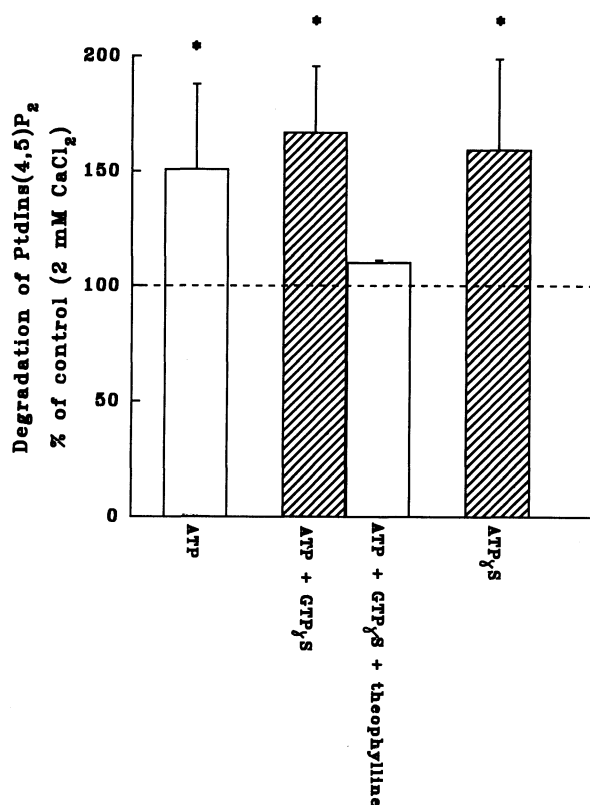


Fig. 1. Effect of extracellular ATP on PtdIns(4,5)P₂ degradation in brain cortex slices. Brain cortex slices were preincubated in KRBS buffer pH 7.4 for 10 min at 37°C with 250 μM ATP, in some cases in the presence of GTP_γS (100 μM), theophylline (10 μM) or ATP_γS (100 μM). Then slices were homogenized and used as a source of enzyme -PLC. Labelled phosphatidyl-4,5-bisphosphate was used as an exogenous substrate in the assay condition, described in Methods. Degradation of PtdIns(4,5)P₂ was assessed from the measured formation of radioactive IP_(n) in aliquots of water phase after extraction. The data are means ± SD from 3-4 experiments. Statistical significance was evaluated by Student *t*-test, **P* < 0.05 (compared with the control value).

RESULTS

ATP (250 μM) added to brain cortex slices preincubated in KRBS buffer pH 7.4 containing 2.0 mM CaCl₂ induces statistically significant increase of PtdIns(4,5)P₂ degradation and higher level of Ins(1,4,5)P₃ radioactivity. This effect of ATP was only slightly stimulated by 100 μM GTP_γS but it was eliminated by theophylline (10 μM). The nonhydrolyzable analog of ATP, ATP_γS (100 μM) exerts a similar effect to ATP itself (Fig. 1). ATP increased in a time-dependent manner the level of Ins(1,4,5)P₃ radioactivity in total water-soluble inositol metabolites (Fig. 2).

Analysis of the percentage distribution of labelled inositol(1,4,5)P₃ in total radioactivity of water-soluble inositol metabolites indicated a significantly higher Ins(1,4,5)P₃ contribution in the presence of ATP comparing to the control condition (without ATP), (Fig. 3).

To determine whether the ATP-evoked increase in Ins(1,4,5)P₃ was associated with the activation of PtdIns(4,5)P₂-specific phospholipase C, similar experiments were carried out on the effect of ATP on PtdIns degradation. It was found that ATP had no

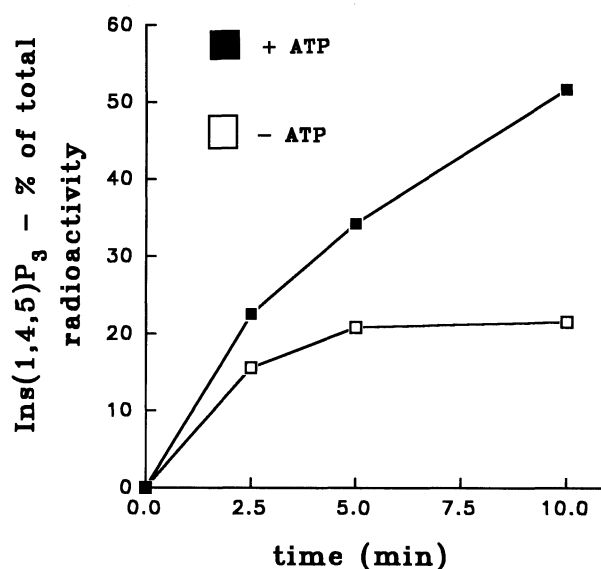


Fig. 2. Time course of Ins(1,4,5)P₃ accumulation during ATP action in brain cortex slices. The results are means of triplicate determination from a typical experiment.

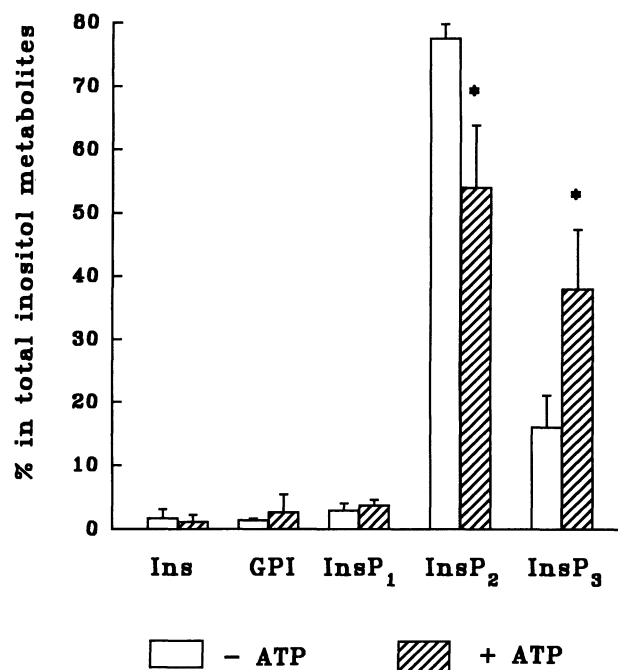


Fig. 3. Effect of ATP on the radioactivity level of Ins(1,4,5)P₃ and other inositol water-soluble metabolites in total radioactivity. The experiments were carried out as described in the legend to Fig. 1. The aliquots of water phase after lipid extraction was separated using ion-exchange column chromatography. Ins-inositol, GPI-glycerophosphoinositol, InsP₁-inositol(1)monophosphate, InsP₂-inositol(1,4)bisphosphate, InsP₃-inositol(1,4,5)trisphosphate. Statistical significance was evaluated by Student *t*-test, **P*<0.05.

effect on PtdIns degradation. The level of InsP₁ was not changed in the presence of ATP (Fig. 4). GTPγS had no effect on PtdIns degradation (data not shown).

Data analysis of water-soluble inositol metabolites presented in Table I indicated a slightly higher percentage contribution of the level of glycerophosphoinositol (GPI) radioactivity in total water-soluble inositol metabolites.

This result may suggest that ATP activates fatty acid release by the action of phospholipase(s) A (A₂ and A₁).

Consequently the extracellular action of ATP on AA release was investigated using radioactive [1-¹⁴C]arachidonoyl PtdIns. There was an insignificant effect of ATP on AA release (Fig. 4). During degradation of [1-¹⁴C] AA PtdIns an insignificant increase of the radioactivity level in DAG was observed (Fig. 4). In the other experiments the effect

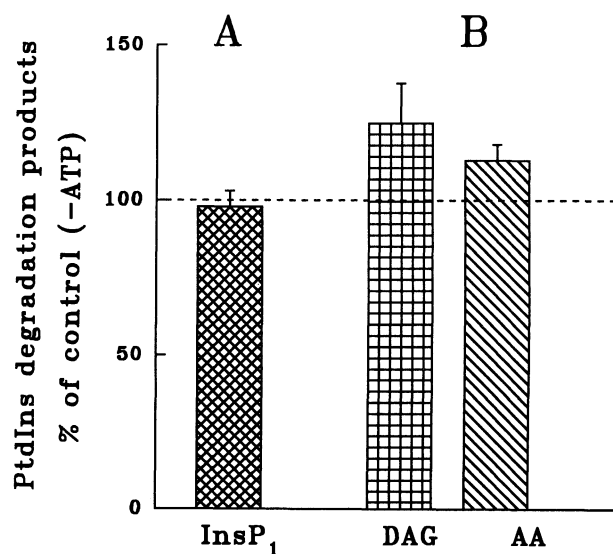


Fig. 4. [2-³H] inositol-PtdIns and [1-¹⁴C] AA-PtdIns degradation during ATP action in brain cortex slices. A, the experiments were carried out using [2-³H]inositol-PtdIns as a exogenous substrate. The InsP₁ radioactivity was determined in water phase after extraction. B, the experiments were performed using [1-¹⁴C]arachidonoyl-PtdIns. The radioactivity was determined in [1-¹⁴C]AA - DAG and [1-¹⁴C]AA after lipid extract separation on TLC. The results are means ± SD from 3-4 experiments.

of ATP was investigated in a cell free system. Brain cortex homogenate, synaptic plasma membrane and cytosol were used as source of PLC. Labelled exogenous inositol phospholipids PtdIns(4,5)P₂ and PtdIns were used as substrates. ATP added into brain homogenate significantly inhibited PtdIns(4,5)P₂ and also PtdIns hydrolysis (Fig 5).

TABLE I

Effect of ATP on product formation of PtdIns hydrolysis in brain cortex slices

Inositol metabolites	% of total radioactivity	
	-ATP	+ATP
Inositol	5.0±2.6	5.1±5.08
GPI	3.43±1.74	4.85±1.59
IP ₁	91.13±9.55	89.96±4.48

The value are means ± SD from 6 experiments; GPI, glycerophosphoinositol; IP₁, inositol monophosphate.

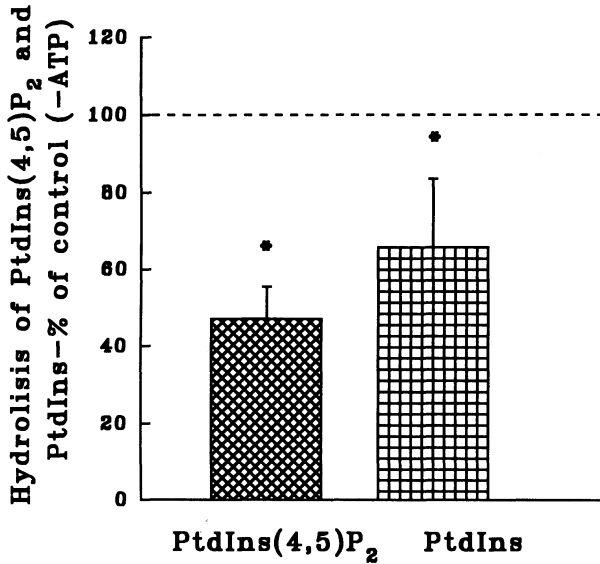


Fig. 5. Effect of ATP on PtdIns(4,5)P₂ and PtdIns degradation by enzyme of brain cortex homogenate. The results are means \pm SD from 3-5 experiments carried out in triplicate. Statistical significance was evaluated by Student *t*-test, **P*<0.05.

However, in spite of this inhibitory action ATP maintained a higher percentage contribution of Ins(1,4,5)P₃ in total inositol metabolites (Fig. 6). ATP also inhibited significantly PtdIns degradation by membrane-bound enzyme (by about 30-40%), however, this effect of ATP was observed exclusively in the presence of high CaCl₂ concentration (1 mM or 2 mM). In lower CaCl₂ concentration between 50 μ M and 500 μ M ATP had no significant effect on PtdIns degradation (data not shown).

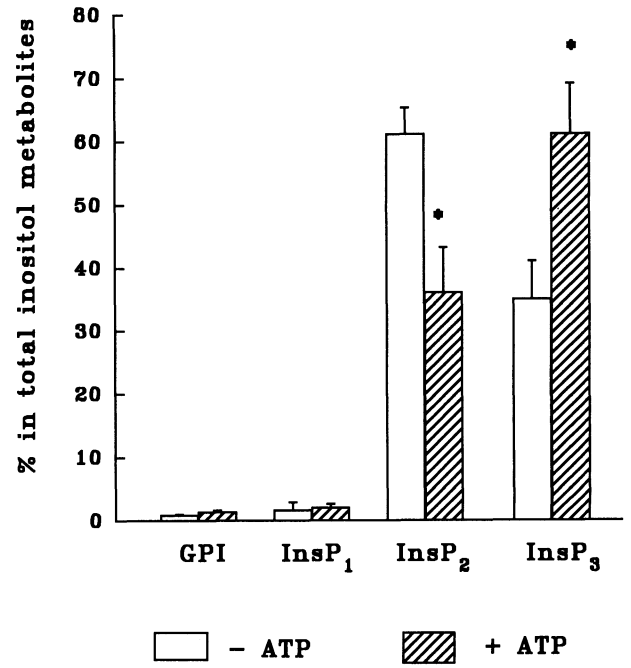


Fig. 6. Effect of ATP on the percentage distribution of inositol metabolites radioactivity in total inositol metabolites. The result are means \pm SD from 3-4 experiments. Statistical significance was evaluated by Student *t*-test, **P*<0.05

The action of ATP depends on the presence and concentration of calcium and magnesium ions.

Our further studies demonstrate that ATP is a potent modulator of inositol phosphate release by membrane-bound and cytosolic phospholipase C acting on PtdIns(4,5)P₂. ATP in the presence of 1 mM MgCl₂ significantly protects Ins(1,4,5)P₃ against its conversion to Ins(1,4)P₂ (Table II).

TABLE II

ATP action on inositol(1,4,5) trisphosphate and other inositol derivatives distribution in synaptic plasma membrane (SPM) and cytosolic fractions expressed in % of total inositol metabolites

Inositol metabolites	SPM		Cytosol	
	MgCl ₂	MgCl ₂ + ATP	MgCl ₂	MgCl ₂ + ATP
Ins	0.00	0.00	0.44 \pm 0.35	0.00
GPI	0.00	0.00	0.40 \pm 0.32	0.00
InsP ₁	8.20 \pm 6.69	0.00	9.93 \pm 2.75	3.46 \pm 1.16
InsP ₂	51.65 \pm 6.35	16.41 \pm 10.79	89.22 \pm 3.35	49.31 \pm 3.21
InsP ₃	40.14 \pm 7.86	83.56 \pm 8.81	0.00	47.23 \pm 5.09

The value are means \pm SD from 3-4 experiments carried out in triplicate.

DISCUSSION

The present study indicates that extracellular ATP interacts with a cell surface purinergic P₂ receptor that is coupled to phospholipase C acting on PtdIns(4,5)P₂ releasing of Ins(1,4,5)P₃ and DAG in equimolar amounts. ATP maintains a higher percentage contribution of Ins(1,4,5)P₃ among water-soluble inositol metabolites.

These results suggest that ATP protects the Ins(1,4,5)P₃ degradation by regulation of inositol phosphate kinase(s) and phosphatases. It is known from previous studies of Ragan et al. (1988) that Ins(1,4,5)P₃ is degraded preferentially by 5-phosphatase to inositol 1,4-bisphosphate and then by a 1-phosphatase to Ins(4)P. Our results indicate that ATP inhibits the conversion of Ins(1,4,5)P₃ to Ins(1,4)P₂ and consequently stimulates Ins(1,4,5)P₃ accumulation in a time-dependent manner during receptor stimulation.

Extracellular ATP has no effect on labelled PtdIns degradation. Analysis of percentage contribution of water-soluble inositol metabolite radioactivity indicates that inositolmonophosphate (IP₁) represents about 90% of total inositol metabolites. A small pool of radioactivity of about 3.4%, which increases to over 4.0% during ATP stimulation, was found in glycerophosphoinositol.

The higher radioactivity level of GPI suggests that ATP may be involved in liberation of fatty acids. Our further studies with [1-¹⁴C] arachidonylo-PtdIns did not lead to significant increase of labelled AA. Extracellular ATP causes the AA generation and the subsequent release of eicosanoids in endothelial cells (Boeynaems and Pearson 1990). ATP exerted a similar effect in astrocytes (Bruner and Murphy 1990), however, ATP did not lead to the activation of phospholipase A₂ and to the release of AA in neuroblastoma X glioma (NG 108-15) cells (Lin et al. 1993).

On the basis of our results it is impossible to exclude the activation of prostaglandin (PG) formation during ATP stimulation of purinergic receptor(s) in brain cortex slices. On the other hand, it is impossible to exclude that ATP significantly

stimulated reestrification of the released AA into glycerolipids.

The physiological role of ATP in neuronal processes has not been well understood and not firmly established. Our preliminary study has shown that ATP may differently regulate phosphoinositide degradation and liberation of lipid-derived second messengers acting through receptors or directly on PLC.

ATP stored in synaptic vesicles is co-released with neurotransmitters upon nerve stimulation (Unsworth and Johnson 1990), and it can itself mediate fast synaptic transmission in mammalian neurones (Evans et al. 1992). ATP through P₂ receptor(s) activates PLC acting on inositol phospholipids. However, acting in a cell free system ATP can inhibit phosphoinositide degradation through PLC, but this ATP effect depends on many factors, for example on Ca²⁺ or Mg²⁺ concentrations.

The magnitude of ATP effect on phosphoinositides degradation is correlated with the concentration of the fully ionized form of ATP⁴⁻ in the medium and not with the concentration of MgATP²⁻ as it was presented by Lin et al. (1993). We have observed in our study an inhibitory effect of ATP on phosphoinositides degradation in a cell free system.

In spite of lower InsP_n formation, ATP maintains a higher percentage contribution of Ins(1,4,5)P₃ among water-soluble inositol metabolites by inhibition of Ins(1,4,5)P₃ conversion to Ins(4,5)P₂. It seems that ATP in a cell free system through allosteric modification of PLC and through its role on kinase-phosphatase of IP(n) regulates the level of DAG and Ins(1,4,5)P₃ independently from P₂ receptor(s). This action of ATP on PLC may protect the cells against excessive liberation of lipid-derived second messengers.

We will continue this study to better understand the mechanism of ATP action on lipid mediators release and intracellular calcium signalling in the brain under different experimental conditions.

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This paper is dedicated to Professor Stella Niemierko on the occasion of her 90th birthday, with esteem and admiration