REVIEWS

Plasma Membrane Signal-Transducing ATP

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 128, No. 7, pp. 4-12, July, 1999
Original article submitted April 8, 1999

Plasma membranes of target cells generate considerable amounts of ATP in response to binding of growth factors, cytokines, and oncoproteins. Plasma membrane ATP is formed at the stage of ligand-receptor signal transduction by the anaerobic pathway with the involvement of plasma membrane redox systems and Na^+ (but not adenylate cyclase). The assumption on the involvement of transitory reversed Na^+,K^+-ATPase in the synthesis of plasma membrane ATP is confirmed by inhibitory analysis. ATP-producing activity of plasma membrane-enriched particles isolated from various target cells in response to various growth factors was studied. The formation of plasma membrane ATP is stimulated by growth factors and cytokines interacting with integral tyrosine kinase receptors or soluble tyrosine kinases in the cytosol. Various tyrosine kinase inhibitors act by utilizing plasma membrane ATP. Plasma membrane ATP is assumed to be a messenger and amplifier of ligand-receptor signals in plasma membranes of animal cells.

Key Words: *plasma membrane; ATP; growth factors; transmembrane signaling; oncogenesis*

Phosphorylation is the main mechanism responsible for induction or suppression of cell activity and realization of the major biological processes. Disturbances in the regulation of this mechanism cause a number of diseases [6,13,22,38,39].

A new class of hormone-like compounds, polypeptide growth factors and cytokines involved in proliferation, differentiation, and transformation of many types of cells, including immunocompetent cells, was discovered over the past decades [19,34]. More than 40 years ago, discoveries of cAMP [59] and GTP-binding proteins [50] produced a great success in studying of the molecular mechanisms of hormone action. Studies of E. G. Krebs [37] and D. A. Walsh *et al.* [61] revealed the role of cAMP in activation of cAMP-dependent protein kinases as regulatory enzymes.

In the early 1980s, M. J. Berridge described 2 second messengers, inositol triphosphate and diacylglycerol [15-17]. Y. Nishizuka reported the role of protein kinase C in intracellular signal transduction [43-48]. Studies of A. G. Gilman revealed the role of G-proteins in transmission of hormone-receptor signals [26,27]. A general concept of the main mechanism of transmission and amplification of extracellular signals for cell growth, proliferation, mitogenesis, differentiation, and chemotaxis perceived by membrane receptors was derived from these data.

Tyrosine kinases play the major role in the transduction of receptor-mediated extracellular signals for cell growth, proliferation, differentiation, and oncogenesis [31-33,54]. Extracellular signals are transduced by intracellular messengers coupled with the corresponding protein kinases. However, the central mechanism responsible for activation of tyrosine kinases remains unclear.

History

In 1980, it was shown that the preparation of plasma membrane-enriched particles (PMEP) from rat skeletal muscles in a medium containing Tris-HCl (pH 7.5),

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0007-4888/99/0007-0661$22.00

ADP, Mg^{2+}, creatine, inorganic phosphate, NaF, NADH-dependent oxidation system (NADH, cytochrome C, and molecular oxygen) rapidly (less than 1 min) utilized inorganic phosphate from the incubation medium in the presence of 4 μg/ml insulin. An insulin-stimulated rise of ATP after the addition of creatine to the medium was accompanied by accumulation of free and total creatine in PMEP. This effect was not observed during incubation without insulin [3,4,8].

Studies of total creatine accumulation were followed by direct measurements of ATP in the incubation medium. Various methods for terminating this reaction, including freezing of the incubation medium in liquid nitrogen followed by short-term heating to the boiling point, were used. ATP was separated from other nucleotides by ion-exchange chromatography and measured spectrofluorometrically by the method of Kornberg. Recently DMSO extraction and luminometric luciferin-luciferase method have been used for isolation and measurement of plasma membrane ATP. Experiments showed that ATP was accumulated within 0.5-2 min in a PMEP fraction incubated with the corresponding growth factor, insulin, or cytokine in a medium containing Tris-HCl (pH 7.5), ADP, Mg^{2+}, inorganic phosphate, and NaF during NADH oxidation in the presence of cytochrome C, molecular oxygen, nonhydrolyzed ATP analogues (5'-p-fluorosulfonylbenzoyladenosine (FSBA), App(CH_2)p, AppNHp), and Na^+. During the first minute of incubation, 1-100 nmol/mg protein ATP was synthesized [5,9]. Bioenergetically, ATP synthesis from ADP and inorganic phosphate is associated with oxidase activity of membranes.

Plasma Membrane as ATP Generator

Localization of ATP biosynthesis on the outer plasma membrane of target cell was confirmed in experiments on human erythrocyte ghosts. Human erythrocytes have no intracellular membranes, which always contaminate highly purified plasma membrane fraction from many types of cells.

Insulin-stimulated formation of short-lived signal-transducing ATP was confirmed by ^{32}P incorporation into ^{32}P-ATP in plasma membrane fragments isolated from human erythrocytes and incubated in the previously described medium under anaerobic conditions [11,36].

Experiments with adenylate cyclase inhibitor P^1,P^5-di(adenosine-5')pentaphosphate and removal of all components except ADP and FSBA from the incubation medium showed that plasma membrane adenylate cyclase is not involved in the synthesis of signal ATP. These experiments were carried out on PMEP isolated from human T lymphocytes. It was shown that the presence of only ADP (the substrate for adenylate cyclase) in the medium is insufficient for ATP biosynthesis on the plasma membrane, on the other hand, the presence of P^1,P^5-di(adenosine-5')pentaphosphate in the incubation medium in concentrations suppressing adenylate cyclase did not inhibit ATP biosynthesis on the plasma membrane stimulated by tumor necrosis factors (TNF) [28].

Insulin and other growth factors stimulated plasma membrane ATP formation only in oxygen atmosphere. When oxygen was replaced with 100% nitrogen or argon, insulin and growth factors had no effects on ATP synthesis [6]. Therefore, the plasma membrane redox system is involved in the production of signal ATP [24].

Arsenate, an inhibitor of ATP synthesis in the reaction of aerobic phosphorylation, inhibited the insulin-stimulated accumulation of ATP by plasma membrane fragments isolated from human erythrocytes. This also confirms the aerobic pathway of plasma membrane ATP biosynthesis [6].

Electron acceptors are essential for ATP synthesis, and ATP is not formed without electron carriers, while substitution of cytochrome C for iron-containing acceptor (ferritin transferrin) typical of the outer plasma membrane [42] had no effects on ATP biosynthesis [28].

Stimulation of the amiloride-sensitive Na^+/H^+ exchange is an essential component of mitogenic response to various growth factors. Studies of TNF-α showed that ATP synthesis induced in plasma membranes of mouse splenocytes and rat hepatocytes was completely inhibited with amiloride (Na^+/H^+ exchange inhibitor and blocker of Na^+ influx) and the antitumor antibiotic adriamycin [28].

Under the effects of insulin, erythrocyte ghosts isolated in a Na^+-free choline-chloride medium accumulated ATP in concentrations directly proportional to the concentration of Na^+ in the medium [1].

Proton secretion to the external medium can be associated with Na^+ inward current (antiport) and ATP synthesis. Therefore, this is proton-activated Na^+-translocating phosphorylation. NADH oxidation induced by insulin and growth factors under the effects of proton-pumping flavoprotein NADH oxidase leads to generation of a proton gradient ($\Delta\Psi$), which modulated the functioning of Na^+,K^+-ATPase, whose catalytic activity becomes reversible. Antitumor antibiotic adriamycin blocks proton-pumping NADH oxidase (Fig. 1).

Biological Role of Plasma Membrane ATP

Further experiments showed that signal ATP is synthesized in PMEP isolated from various target tissues containing high-affinity receptors for the correspon-

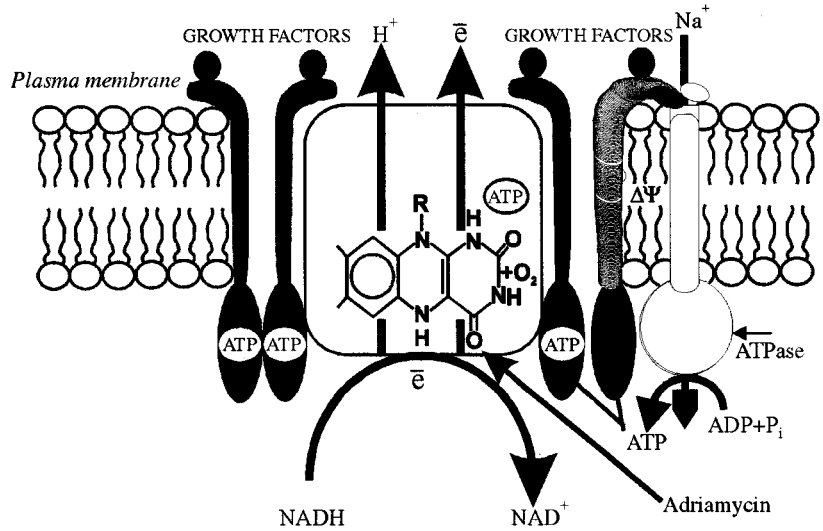

Fig. 1. Possible pathway of plasma membrane ATP synthesis. P_i: inorganic phosphate.

ding growth factors, mitogens, cytokines, and stimulators of chemotaxis and adhesion. Formation of signal ATP was induced by growth factors, whose receptors were associated with integral tyrosine kinases of plasma membranes (receptors for insulin, EGF, PDGF, HGF, NGF, and VEGF [7]).

In the case of cytokines, formation of signal ATP by plasma membranes of target cells is essential for activation of intracellular nonreceptor tyrosine kinases.

When growth factors interact with the cell surface, the signal pathway originating from the plasma membrane initiates a multistage protein kinase phosphorylation cascade from initial activation catalyzed by ATP receptor tyrosine kinase (RTK) to expression of transcription factors in the nucleus. Signals from growth factor receptors are transmitted by RTK into the cell (through protein-protein interactions involving 4-6 various kinases) and cause expression of nuclear oncogenes. This multistage cascade and the signal pathway of apoptosis have common side reactions [58].

Many mitogens, cytokines, and tumor promoters are known to induce short-term expression of nuclear oncogenes (*c-mys*, *c-fos*, and *c-jun*) [31]. These primarily responding [62] or immediate early genes [40] are involved in cell growth and differentiation, programmed cell death, and *in vitro* cell development and survival. It is now proved that these genes are mainly associated with cell proliferation and differentiation.

We found that the rise in aerobic ATP synthesis in plasma membranes is associated with expression of the *c-myc* nuclear antigen in rat hepatocytes activated with mouse and human TNF-α. In our experiments, ATP biosynthesis on the plasma membrane lasting for 45-60 sec was followed by expression of the *c-myc* nuclear antigen. Blockade of ATP synthesis with the antitumor antibiotic adriamycin was accompanied by 0.5-2-h blockade of *c-myc* expression. Therefore, ATP synthesized on the plasma membrane can participate in the transduction of the proliferative signal into the cell nucleus [28].

When studying the effects of growth factors, cytokines, and oncoproteins we hypothesized that short-lived plasma membrane signal ATP acts as a mediator and amplifier of transmembrane transduction of mitogenic signals for cell growth.

This short-lived intermediate signal molecule activates tyrosine kinases and triggers the phosphorylation cascade that begins from the plasma membrane.

Membrane receptors for growth factors and transformed proteins of many retroviral oncogenes represent tyrosine kinases [18,20,33,53,63].

RTK have extracellular ligand-binding, common transmembrane, near-membrane, and catalytic tyrosine kinase domains containing the ATP-binding center, protein substrate-binding center, and carboxy-terminal region [63].

Ligand-induced oligomerization (dimerization) or oligoheterodimerization of growth factors RTK is essential for binding of growth factors [60], autophosphorylation of tyrosine residues in the tyrosine kinase molecule, and intrinsic tyrosine kinase activity characteristic of this receptor.

The binding of epidermal, fibroblast, endothelium, and hepatocyte growth factors leads to rapid autophosphorylation of receptors on the cytoplasmic surface of the cell membrane. The insulin receptor is a dimer consisting of α,β-chains linked by the disulfide bond. The insulin-receptor binding activate tyrosine kinase via rapid phosphorylation [52].

Fibroblast growth factor (FGF) activates RTK due to incorporation of ATP γ-phosphate and heparin sulfate proteoglycan potentiates the this process. Inhibi-

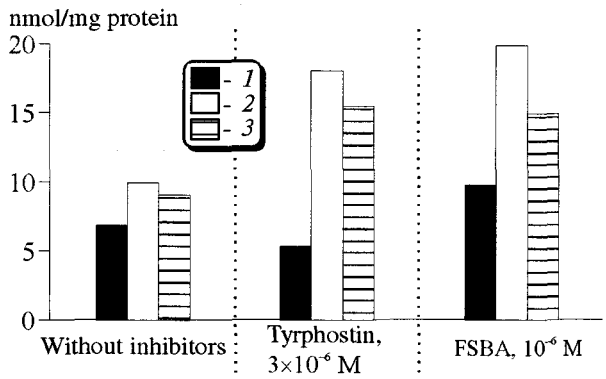

Fig. 2. Effects of tyrphostin and 5'-p-fluorosulfonylbenzoyladenosine (FSBA) on ATP accumulation in plasma membrane-enriched particles from rat liver during the first minute of incubation in the control (*1*) and in the presence of 5 μM human tumor necrosis factor-α (*2*) and 100 μg/ml α-fetoprotein (*3*).

tory analysis and phosphoamino acid analysis showed that γ-phosphate most likely originates from signal ATP molecule formed on the plasma membrane in response to stimulation with growth factors and cytokines.

Dimerization of receptors is a necessary but insufficient factor for activation of growth factor RTK. Another essential activator is ATP that triggers the phosphorylation cascade toward the nucleus.

Practically all studied growth factors added to the incubation medium induced the formation of plasma membrane signal ATP in the presence of the ATP analogue FSBA. Hence, all RTK use common activation mechanism. Autophosphorylation enhances the ability of growth factor receptor to phosphorylate other signal target proteins.

FSBA is structurally similar to ATP. Tyrosine kinase attacks FSBA instead of ATP, and therefore FSBA, but not ATP binds to the ATP-binding center of the enzyme, while ATP is accumulated in the medium [35]. Signal ATP was discovered due to the use of this inhibitor [10].

The role of plasma membrane ATP in growth signal transduction was confirmed in experiments with inhibitors of RTK, the first and major factor in the cascade of γ-phosphate transfer toward the cell nucleus. The following RTK inhibitors were used: FSBA (structural analogue of ATP), quercetin, and tyrphostin-25.

Various concentrations of quercetin (0.3, 1.5, 3, and 30 μg/ml) decreased not only EGF-stimulated, but also basal ATP in PMEP isolated from human intestinal tumor. This was probably due to the fact that quercetin inhibits not only RTK, but also ATPase activity responsible for the synthesis of plasma membrane ATP [12]. Higher concentrations of quercetin inhibited oxidation-reduction reactions on the plasma membrane [12], which are also involved in the biosynthesis of plasma membrane ATP.

Tyrphostin-25 binds to RTK tyrosine residues and blocks ATP γ-phosphate-binding sites, thus preventing RTK autophosphorylation [41,56]. Tyrphostin-25 (3 μM) increased the level of ATP in PMEP isolated from rat hepatocytes. EGF and human TNF-α were used as growth factors (Fig. 2).

What is the role of RTK autophosphorylation and activation with signal ATP? Short-term phosphorylation of tyrosine residues yields phosphotyrosines, which

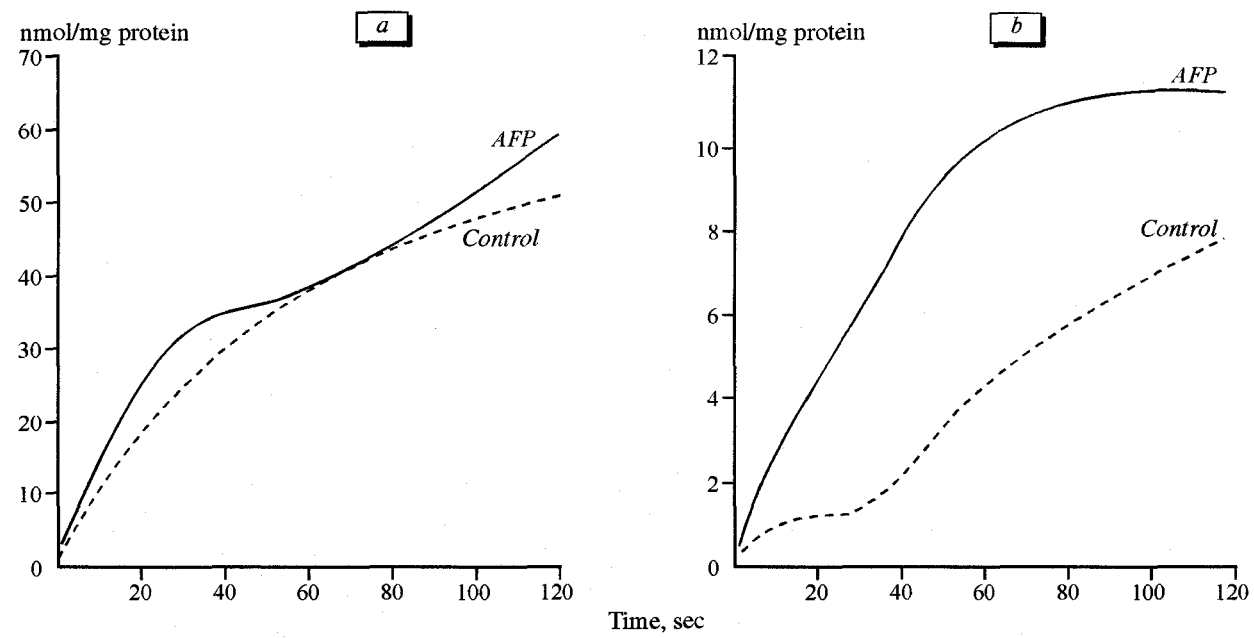

Fig. 3. Stimulatory effect of 100 μg/ml α-fetoprotein (AFP) on biosynthesis of plasma membrane ATP in human T lymphocytes in the absence (*a*) and presence of 17.6 mM diadenosine pentaphosphate (*b*).

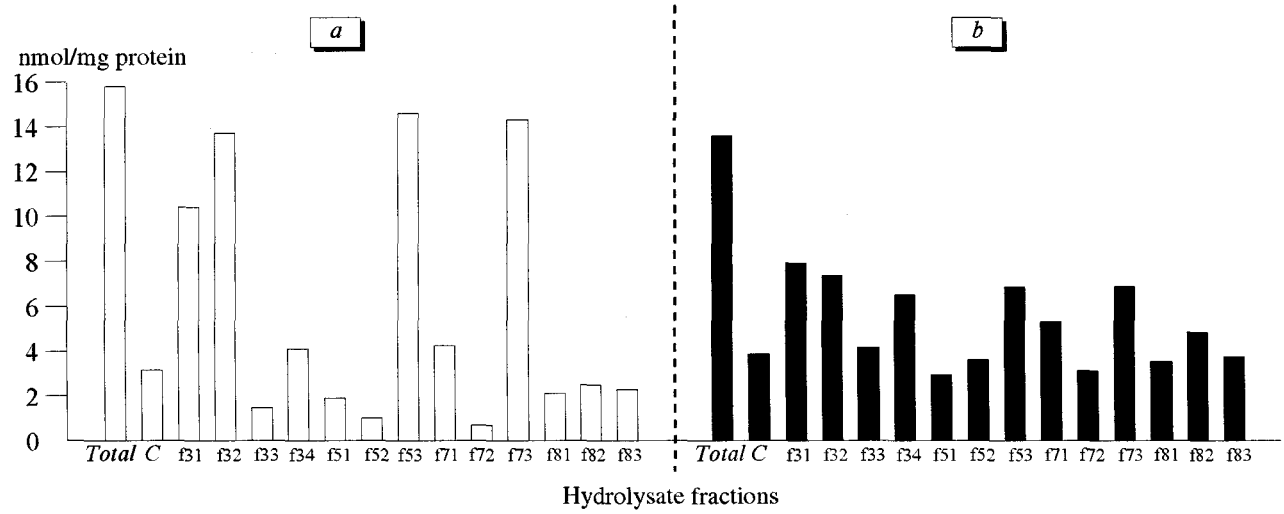

Fig. 4. Effect of cattle brain hydrolysate fractions on ATP-forming activity in plasma membrane-enriched particles from rat liver (*a*) and rat brain synaptosomes (*b*) during 2-min incubation. *Total:* nonfractionated hydrolysate; and *C:* control (without peptides).

serve as "docking centers" for other target proteins by forming recognition and binding sites for Scr-homologous domains (SH_2 domains) of intracellular proteins. This series of signal molecules includes phospholipase C-γ, phosphatidylinositol-3-kinase (PI-3-kinase), GAP (GTPase-activating protein), $p60^{c\text{-}Src}$, $p21^{ras}$, Raf-1-kinase, MAP-kinase, and S6-kinase [55]. This was also demonstrated for Src-kinases (nonreceptor tyrosine kinases), products of Src-oncogene in virus-induced carcinogenesis [21,30].

Impaired regulation of proliferative processes is one of the major mechanisms of pathogenesis of malignant tumors. Production of proliferation-regulating polypeptides, growth factors, by tumor cells and stromal environment is responsible for unlimited growth of tumor cells. The presence of functionally active receptors for growth factors on tumor cells triggers the mechanism of auto- or paracrine growth stimulation.

Such growth factors as EGF, FGF, PDGF, TNF-α, and IGF were demonstrated to induce their own expression [25,29]. EGF and TNF-α not only stimulate the growth and proliferation of normal cells in the liver, intestine, and skin, but also induce the development of human osteosarcoma and melanoma [21, 23,51].

We investigated the mechanism of ATP biosynthesis in PMEP isolated from malignant tumors of various origins (stomach, intestine, mammary gland, nerves, lungs, and pancreas). Effects of some growth factors acting via tyrosine kinase receptors, in particular, EGF, FGF, TNF-α, interleukin-2, NGF, α-fetoprotein, and insulin were studied.

It was found that the intensity of signal-transducing ATP synthesis in plasma membranes isolated from malignant tumors is higher than in normal tissues. This is probably due to the use of all reserve capacities of signal transduction in tumor cells directed to competitive survival.

α-Fetoprotein is a large plasma glycoprotein synthesized in fetal liver and embryonic yolk sac and in patients with primary hepatocellular carcinoma.

Blood concentration of α-fetoprotein increases during repair processes and carcinogenesis in the liver [14,57]. Certain types of T cells can recognize and destroy tumor cells and their products (antigens). T cells expressing α-fetoprotein receptors perform receptor-mediated synthesis of signal ATP. Human T lymphocytes stimulate ATP biosynthesis in PMEP in the presence of oncoprotein, and adenylate cyclase is not involved in this process (Fig. 3).

Some Applications of Plasma Membrane ATP Assay

We studied the effects of therapeutic plasmapheresis on the synthesis of signal ATP in human erythrocyte membranes in the presence of insulin. Therapeutic plasmapheresis is a promising tool for removal of toxicants and immune complexes. ATP synthesis in erythrocyte membranes in patients with various diseases was much lower than in healthy donors. After plasmapheresis, the insulin-stimulated synthesis of ATP tended to increase. This confirms the efficiency of toxicant removal [2].

Massive resection and extensive irreversible alterations in the liver (hepatitis, cirrhosis) accompanied by a critical decrease in its weight reduces its repair capacity. The search for new hepatotropic growth-stimulating factors is of considerable importance, since the maintenance of hepatocyte regeneration capacity is a prerequisite for extensive liver resections. Cell growth capacity is usually evaluated by ^{3}H-thymidine incor-

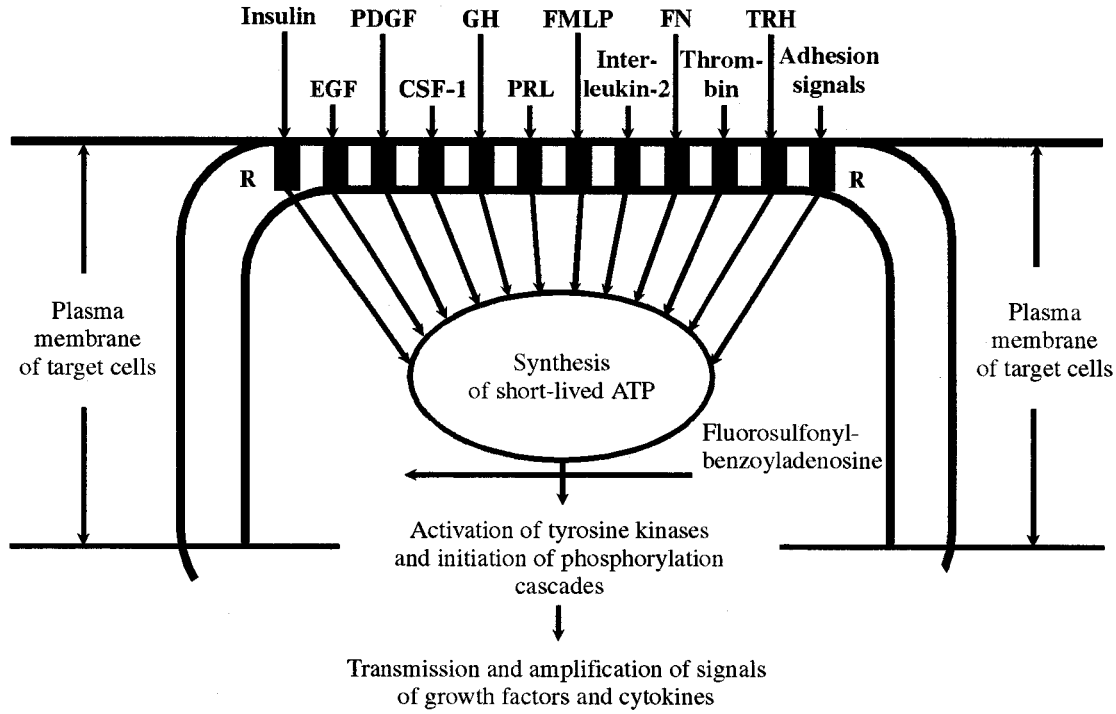

Fig. 5. Hypothetical scheme of regulation of transmission and amplification of growth factor and cytokine signals. Signal ATP serves as a transducer and amplifier of signals for cell growth and proliferation. R, growth factor receptors.

poration into DNA, ^{3}H-uridine incorporation into RNA, and by the intensity of protein synthesis. However, these methods cannot be applied for express screening of natural or newly synthesized peptides displaying growth-stimulating activities. We elaborated a rapid luminometric method for detecting signal ATP in PMEP isolated from rat liver or cultured cells.

Cerebrolysin, a product of enzymatic hydrolysis of animal (primarily, cattle) cerebral protein is widely used for the therapy of vascular senile dementia, brain injuries (traumas, concussions, and contusions), strokes, epilepsy, and Parkinson's disease. The hydrolysis yields a fraction of peptides and free amino acids. Peptides cross the blood-brain barrier and exert a growth-stimulating effect on nervous cells (similarly to nerve growth factors). Intense accumulation of plasma membrane ATP in rat hepatocytes and brain synaptosomes in the presence of various fractions of cattle brain

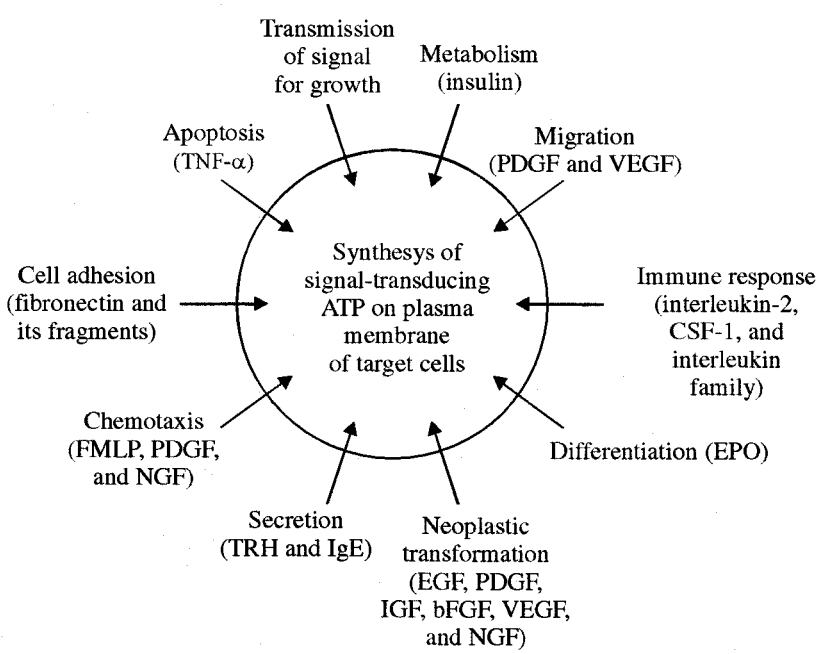

Fig. 6. Role of signal-transducing ATP in the regulation of vital cell processes.

hydrolysate allowed us to identify peptides with maximum biological activity (Fig. 4).

Thus, ATP synthesis from ADP and inorganic phosphate in response to receptor-mediated signals for cell growth in PMEP isolated from various target cells (muscle and secretory cells, hepatocytes, blood cells, immune system and connective tissue cells) attests to universal nature of this phenomenon. Receptors of many biologically active regulators and drugs probably act through similar signal amplification systems (Fig. 5).

Thus, apart from mechanical and osmotic functions and role in biosynthetic processes, the ATP-ADP cycle functions as a transducer and amplifier of signals. ATP is a universal cell amplifier of signals of growth factors, cytokines, and oncoproteins (Fig. 6).

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