ATP GENERATION BY HUMAN PLACENTAL CELL PLASMA MEMBRANES INDUCED BY INSULIN AND EPIDERMAL GROWTH FACTOR

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Insulin and epidermal growth factor (EGF) possess definite similarity in the mechanisms of their action and influence on cell metabolism, and it is on this basis that, together with certain other hormones and hormone-like factors, they have been included in the group of peptide growth factors [3].

EGF and insulin, on binding with their specific receptors, induce their phosphorylation, catalyzed by the receptors themselves, which possess protein kinase activity [5, 7, 12].

It was demonstrated previously that short-living ATP accumulates in the course of 45-60 sec, in a quantity of about 10^{-9} mole per milligram membrane protein [1] under the influence of insulin in the plasma membranes of adipocytes and of certain other insulin target cells.

At the same time, it has been shown that an increase in the ATP concentration leads to autophosphorylation of EGF receptors even independently of the presence of this hormonal factor [6].

The aim of the present investigation was to discover whether the action of insulin and EGF causes accumulation of membrane-bound ATP in the plasmalemma of the human placenta and to determine the possible pathways of its utilization.

EXPERIMENTAL METHOD

The following reagents were used: human insulin (Institute of Clinical Physiology, C.N.R., Pisa, Italy), EGF (from Sigma, USA), phenylmethylsulfonyl fluoride — PMSF (from Calbiochem, USA) adenosine-5'-triphosphate, adenosine-5'-diphosphate, and NADH (from Reanal, Hungary), cytochrome c from horse heart, β,γ-methyleneadenosine-5'-triphosphate, adenylylimidodiphosphate, 5'-fluorosulfonylbenzoyladenosine, antimycin A, NADP, glycylglycine, D-glucose, tris-hydroxymethylaminomethane, bovine serum albumin (BSA), naphthalene, Dowex 1 × 8 resin (100-200 mesh), glucose-6-phosphate dehydrogenase (from Serva, West Germany), β-hydroxybutyric acid (from Loba-Chemie, Austria), rotenone (from British Drug Houses, England), hexokinase, diphenyloxazole (PPO, from Fluka, Switzerland), ^{14}C-ATP (Czechoslovakia, specific radioactivity 17.02 GBq/mmole), diphenyloxazolylbenzene (POPOP), sucrose, methanol, ethylene glycol, and salts were from Soyuzreaktiv (USSR), and were not below the chemically pure grade.

The TsLR-1 centrifuge was used for centrifugation, fluorescence was measured on an MFP-4a spectrofluorometer (Hitachi, Japan), and for radiometry of the samples an SL-4000 scintillation counter (Roche Bioelectronique, France) was used.

Fresh human placentas (29), obtained from a maternity home on the day of the experiments, in a vacuum flask with ice, were used in the experiments.

The initial stage of preparation of the coarse membrane fraction was done by the method in [8]. During washing of the fraction with 0.05 M Tris-HCl buffer, pH 7.6, containing 0.25 M sucrose, the serine protease activity PMSF was added to it in a final concentration of 1 mM. The subsequent procedures of preparation of plasmalemma-enriched articles (PEP) were carried out by the method in [15]. The degree of enrichment was monitored in relation to the marker enzyme 5'-mononucleotidase. PEP was suspended in cold 0.25 M sucrose solution and the suspen-

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sion was poured in volumes of 1.5 ml into Erlenmeyer flasks, to which the remaining components of the reaction mixture had been added beforehand.

In each Erlenmeyer flask with a capacity of 20 ml, the reaction mixture in a total volume of 3.6 ml, including 1.5 ml of membrane suspension, contained, in a final concentration: 40 mM Tris-HCl (pH 7.5), 2.5 mM $MgSO_4$, 2 mM ADP, 5 mM KH_2PO_4, 20 mM NaF, 0.01 mM NADH, 10 mM β-hydroxybutyric acid, 277 μg/ml of BSA, 0.1 mM cytochrome C, 1.5 μM antimycin A, 1 mM KCN, 7.61 μM rotenone, and 76 mM sucrose. One of the inhibitors, namely adenylylimidodiphosphate, β,γ-methyleneadenosine-5'-triphosphate, or 5'-fluorosulfonylbenzoyladenosine, was added to the reaction mixture up to a final concentration of 2 μM. The reaction was started by addition of insulin or EGF in final concentrations of 4 and 1 μg/ml, respectively, dissolved in 20 μl of 2 mM glycylglycine buffer. The samples were incubated for 1 min at 30°C in an atmosphere of 100% oxygen with continuous shaking.

The subsequent procedures — stopping the reaction by freezing in liquid nitrogen, heating to 100°C, fractionation of the supernatant on columns with Dowex resin 1 × 8, and freeze drying, were carried out as described previously [2]. Completeness of elution of ATP from the column was tested by adding ^{14}C-ATP to the supernatants obtained (20 μl, 500 cpm)

The ATP concentration in the freeze-dried products was determined by Kornberg's method, and reduction of NADP was recorded fluorometrically [2]. The protein concentration was determined by Lowry's method.

EXPERIMENTAL RESULTS

Data showing the effect of insulin and EGF on accumulation of ATP in PEP from human placenta in the presence of the ATPase inhibitor, adenylylimidodiphosphate [14], are given in Figs. 1 and 2. As was stated above, the incubation medium contained rotenone, antimycin A, and potassium cyanide. This was done to produce maximal inhibition of the mitochondrial electron-transport oxidation chain, in order to prevent any possible action of mitochondria. Stimulation of ATP accumulation in the plasmalemma of human placenta, both by insulin and by EGF, was observed in all experiments.

ATP generation by plasmalemma of the target cells is thus evidently a characteristic initial mechanism of the action of several peptide growth factors.

ATP accumulation in the plasmalemma induced by peptide growth factors, is probably due to transient reversal of the ATPase reaction in response to a signal due to transmembrane movement of ions: protons into the external medium, and a counterflow of Na^+ ions, caused by accumulation of protons inside the cell, due to the activity of plasmalemmal NADH-dehydrogenase, which is set in motion by a flow of electrons (a transmembrane flow, possibly). In this way, an electric field is created on the membrane [2].

Data on ATP accumulation in PEP from human placenta in the presence of inhibitors of various processes of ATP consumption are given in Fig. 3: adenylylimidodiphosphate [14], β,γ-methylene-ATP [4], and 5'-fluorosulfonylbenzoyladenosine [10]. The greatest ATP accumulation was observed in the presence of the protein kinase inhibitor 5'-fluorosulfonylbenzoyladenosine, probably evidence of the consumption mainly of membrane-bound ATP, synthesized under the influence of peptide growth factors (insulin and EGF), for phosphorylation of membrane proteins, more especially specific receptors of insulin and EGF.

The protein kinase similar to RSVpp 60^{vsrc}-tyrosine kinase, bound with the insulin receptor in the human placenta, possesses phosphatidylinosine kinase activity [11]. Regulation of phosphoinositide metabolism, mediated through the receptor, may thus play an important role in conduction of the signal of insulin and other growth factors. The order of events can be described approximately as follows: 1) formation of the insulin-receptor complex; 2) autophosphorylation and activation of receptor-bound protein (tyrosine) kinase; 3) phosphorylation of phosphatidylinositol; 4) hydrolysis of phosphatidylinositol-4',5'-diphosphate by insulin-activated phospholipase C to inositol triphosphate and diacylglycerol. Inositol triphosphate induces Ca^{++} mobilization, and diacylglycerol activates several protein kinases, including protein kinase C. Diacylglycerol and Ca^{++} are secondary messengers for protein phosphorylation (at serine) by protein kinase C [11]. There is evidence that, after activation of protein kinase C by phorbol ester, a diacylglycerol analog, it is rapidly incorporated into the membrane [13].

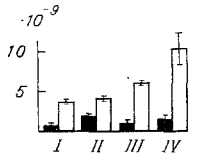

Fig. 1

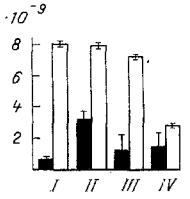

Fig. 2

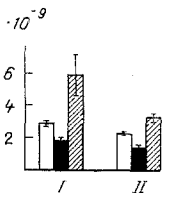

Fig. 3

Fig. 1. Accumulation of membrane-bound ATP in PEP preparations from human placenta induced by insulin. Ordinate, ATP concentration in PEP after incubation for 1 min (in moles/mg protein of PEP/min). Black columns denote incubation in absence of insulin, white columns — in presence of insulin. Each determination was done in a pooled sample from four Erlenmeyer flasks. Height of column corresponds to mean value of two determinations. I, II, III, IV) Nos. of experiment.

Fig. 2. Accumulation of membrane-bound ATP in PEP preparations from human placenta induced by EGF. Black columns denote incubation in absence of EGF, white columns — in presence of EGF. Remainder of legend as to Fig. 1.

Fig. 3. Effect of inhibitors of ATP utilization on accumulation of membrane-bound ATP in PEP from human placenta induced by insulin and EGF. Ordinate, difference in concentrations of ATP in PEP incubated (1 min) in presence and absence of insulin (I) and EGF (II) (ATP). Black columns denote ATP on incubation in presence of adenylyl-imidodiphosphate, white columns — in presence of β,γ-methylene-ATP, obliquely shaded columns — in presence of 5-fluorosulfonylbenzoyladenosine. Remainder of legend as to Fig. 1.

It can be tentatively suggested that ATP, accumulated by PEP from human placenta, may be utilized in several processes: 1) for autophosphorylation of β-subunits of insulin receptors possessing protein kinase activity; 2) for phosphorylation of diacylglycerol to phosphatidic acid and of phosphatidylinositol to phosphatidylinositol-4',5'diphosphate by diacylglycerol kinase and phosphatidylinositol kinase, and also by phosphatidylinositol-4-phosphate kinase respectively; 3) for phosphorylation of various membrane proteins by protein kinase C, including the α-subunits of G-proteins [9].

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