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SHORT-TIME PHOSPHORYLATION OF PLASMA MEMBRANE PROTEINS BY ENDOGENOUS KINASES

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Summary: Endogenous protein phosphorylation in plasma membranes isolated from SV 40-transformed mouse fibroblasts was studied in the presence and absence of cyclic nucleotides. Using low concentrations of membrane protein the kinetics of ATP-dependent ^{32}P-incorporation showed a rapid phosphorylation reaction up to 2 sec of incubation which was stimulated by cAMP and inhibited by cGMP. This short-time phosphorylation reaction was followed by a rapid dephosphorylation and a slower rephosphorylation. This phenomenon was dependent on protein concentration.

The perspectives on cellular phosphoproteins have changed dramatically with the discovery of cAMP- and cGMP-dependent protein kinases (1,2). A general hypothesis is that protein phosphorylation and dephosphorylation represents a potential mechanism for the control of membrane events in eukaryotic organisms which might be mediated by cyclic nucleotides. Only very little information on the velocity of such regulatory reactions is available. Most investigations on the effects of cyclic nucleotides on endogenous phosphorylation of membrane proteins show linear kinetics up to an incubation time of 5-10 min (3-12), some up to 2 min (13-16). However, these kinetic data do not give detailed information on phosphorylation/dephosphorylation reactions in the time range of a few seconds. Since transmission or modulation of regulatory short-time signals might be of special biological importance we studied the short-time kinetics of endogenous protein phosphorylation with purified plasma membranes of SV 40-transformed mouse fibroblasts in the presence and absence of cyclic nucleotides.

MATERIALS AND METHODS

Chemicals: [γ-^{32}P]ATP (10-40 Ci/mmol), [γ-^{32}P]GTP (10-40 Ci/mmol) were purchased

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from NEN, Boston, USA. Aminophylline [(theophylline)$_2$.ethylenediamine] and ethyleneglycol-bis-(ß-aminoethyl ether)N'N-tetracetic acid (EGTA) were purchased from Sigma, St.Louis, USA; adenosine 3':5'-cyclic monophosphate (cAMP) and guanosine 3':5'-cyclic monophosphate (cGMP) from Boehringer, Mannheim, Germany. NCS tissue solubilizer was obtained from Amersham/Searle, Arlington, Ill., USA. All other chemicals were of analytical grade (Merck, Darmstadt, Germany). Kallikrein inactivator (17) was a gift from Bayer, Elberfeld, Germany.

Cells and Preparation of Membrane Fractions: All investigations were performed with STU-51A/232B mouse fibroblasts, a subline derived from SV 40-transformed embryonic cells of STU mice. The cells were grown in suspension culture, harvested in the exponential growth phase and washed 3 times with saline and once with 1mM $MgCl_2$. Thereafter, the cells were resuspended in 1mM $MgCl_2$ for 5 min and $5x10^7$ cells/ml were disrupted in a Dounce homogenizer. Nuclei and still intact cells were removed by centrifugation at 200xg and 4°C for 2 min. Thereafter, all membranous material was sedimented to a pellet at 30 000 rpm and 4°C for 30 min using a Spinco rotor Ti 30. The pellet was suspended in 5% sucrose, layered onto a continous gradient of 20-60% sucrose and centrifuged in a Spinco SW 40 rotor at 30 000 rpm and 4°C for 150 min. The membrane material of the upper band at about 30% sucrose, identified as plasma membrane fraction by marker enzyme assays, was sedimented to a pellet in a Spinco rotor Ti 30 at 30 000 rpm and 4°C for 60 min.The sucrose gradient centrifugation was repeated and the resulting plasma membrane fraction showed a 20-fold increase of the specific activity of phosphodiesterase and alkaline phosphatase. The final sediment was washed once with water and used for phosphorylation experiments without delay.

Phosphorylation of Membrane Fractions: Phosphorylation was carried out at 37°C in a total volume of 90µl using a glycine/phosphate buffer pH 7.8 (adjusted at 37°C) which contained EGTA and cyclic nucleotides. The individual incubation mixtures were prepared by mixing 10 µl of buffer solution, 1o µl of $MgCl_2$ solution, 20 µl of membrane suspension and 50 µl containing 50 µCi of [γ-^{32}P]ATP or [γ-^{32}P]GTP, respectively. The final concentrations were: 25 mM glycine, 25 mM phosphate, 0.3 mM EGTA, 10 mM $MgCl_2$, cAMP or cGMP of the given concentration between 10^{-4}M and 10^{-9}M and 1 mM aminophylline. In addition each sample contained 9µg of kallikrein inactivator to block endogenous proteases. The final content of membrane proteins ranged between 24 and 240 µg.

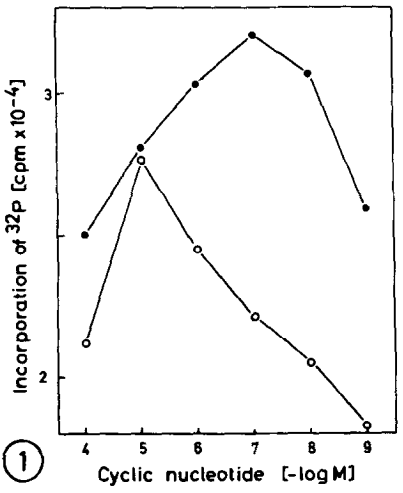
All solutions were mixed and preincubated at 37°C for 1 min except the solutions of the labeled trinucleotides which were added thereafter thus marking

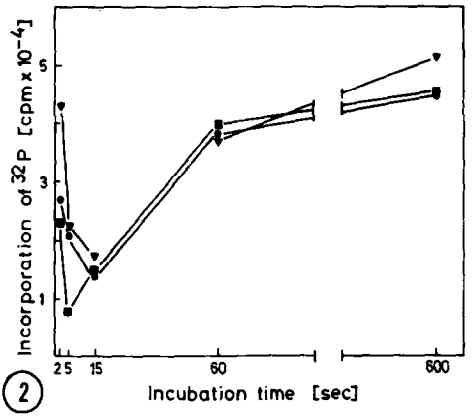
zerotime of the phosphorylation reaction. The endpoint of the reaction was marked by addition of 10 µl of 0.1 M phosphate buffer/0.1 M EDTA pH 7.2 containing 10% SDS and 10% ß-mercaptoethanol, followed at once by boiling at 100°C for 5 min. Thereafter the samples were brought to 10% of glycerol and used directly for SDS polyacrylamide gel electrophoresis. SDS-gel electrophoresis was performed in 5% polyacrylamide gels (5x90 mm). Gels were cut into 1.5 mm slices and extracted with NCS solubilizer.

RESULTS

Effects of concentration of cyclic nucleotides on protein phosphorylation: Endogenous phosphorylation of the plasma membrane fraction with [γ-^{32}P]ATP was allowed to proceed for 1 min at pH 7.8 and 37°C in the presence of cyclic AMP or cyclic GMP, 10 mM $MgCl_2$ and 1mM aminophylline. Under these conditions overall phosphorylation exhibited maxima at concentrations of 10^{-7}M cyclic AMP (Fig.1) and 10^{-5}M cyclic GMP, respectively. An increase of the aminophylline concentration to 10 mM resulted in a 40-50% reduction of membrane-bound [^{32}P]phosphate at the conditions of maximum incorporation. Experiments performed with [γ-^{32}P]GTP showed that the GTP-dependent overall phosphorylation of the plasma membrane was not significantly influenced by addition of cyclic GMP or cyclic AMP.

Short-time kinetics and the effect of protein concentration: The kinetic experiments were performed under standard conditions using concentrations of cyclic nucleotides which had shown optimal stimulation of overall phosphorylation after 1 min incubation. The shortest incubation time which could be achieved under the experimental conditions described was 2 ± 0.5 sec. Further incubation intervals were 5, 15, 60 and 600 sec. In Fig.2 the data of protein-bound [^{32}P]phosphate are given for a low concentration of membrane proteins (24µg per sample). The time courses of phosphorylation in the presence or absence of cyclic nucleotides indicate a rapid phosphorylation up to 2 sec followed by a rapid dephosphorylation and a slow rephosphorylation which started between 5 and 15 sec. The stimulation of short-time phosphorylation in the presence of cyclic AMP after 2 sec incubation ranged between 70-100%



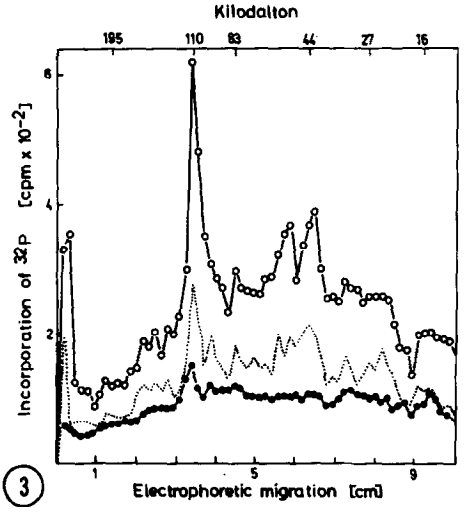


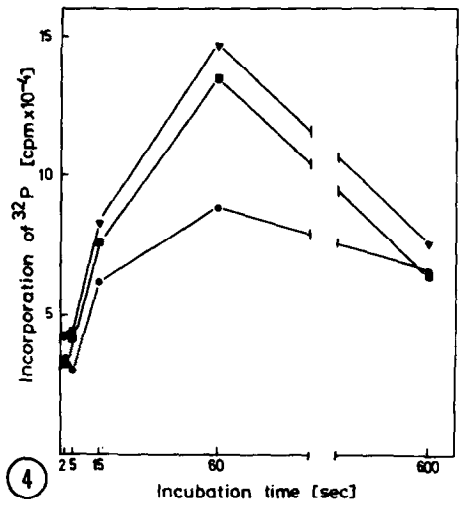
1. Effects of cyclic nucleotide concentration on [^{32}P]incorporation into plasma membrane proteins. Phosphorylation reactions were carried out under standard conditions using [γ-^{32}P]ATP as radioactive precursor. Incubation time was 1 min, the content of membrane protein was 108 µg/sample. Effect of cAMP (●); effect of cGMP (o).

2. Effect of incubation time on phosphorylation/dephosphorylation kinetics in the absence and presence of cyclic nucleotides using a low plasma membrane protein concentration (24 µg/sample) and [γ-^{32}P]ATP as precursor. (●) without cyclic nucleotides; (▼) 10^{-7}M cAMP; (■) 10^{-5}M cGMP.

while cyclic GMP showed a 20-30% inhibition under these conditions. After 5 sec incubation it appeared that the presence of cyclic GMP stimulated dephosphorylation yielding only 10-40% of protein bound [^{32}P]phosphate when compared to the control or to incubation with cyclic AMP. In the phase of rephosphorylation, after 5 to 15 sec incubation, the presence of cyclic nucleotides did not have a very specific effect on the time course of phosphorylation.

The pattern of phosphoproteins obtained after SDS poly-acrylamide gel electrophoresis was not significantly influenced by cyclic nucleotides. As can be seen in Fig.3, the relative distribution of phosphoproteins after 2 sec incubation is very similar in the presence and absence of cyclic nucleotides





3. SDS polyacrylamide gel (5%) electrophoresis of the plasma membrane fraction, after 2 sec of incubation of a low concentration of plasma membrane proteins (42µg/sample) under standard conditions. Dotted line: Without cyclic nucleotides; (o) 10^{-7}M cAMP; (●) 10^{-5}M cGMP.

4. Effect of incubation time on phosphorylation/dephosphorylation kinetics in the absence and presence of cyclic nucleotides using a high plasma membrane protein concentration (240µg/sample) and [γ-^{32}P]ATP as precursor. (●) without cyclic nucleotides; (▼) 10^{-7}M cAMP; (■) 10^{-5}M cGMP.

and differs essentially only in the absolute amount of [^{32}P]phosphate incorporated. The pattern also resembled that obtained after 1 min incubation.

Somewhat different time courses of phosphorylation were obtained when the concentration of membrane proteins was increased by a factor 10 (240µg per sample). The phase of short-time phosphorylation and dephosphorylation could be no more recognized but late-phase phosphorylation was now found to be stimulated by cyclic AMP as well as by cyclic GMP (Fig.4). After 60 sec maximum stimulation was observed ranging between 20-70% in the presence of cyclic AMP and between 10-50% in the presence of cyclic GMP. Thereafter, all time courses show a phase where dephosphorylation predominatly occurred, more rapid in the presence of cyclic nucleotides than without.

DISCUSSION

The phenomenon of short-time phosphorylation/dephosphorylation of plasma membrane proteins might represent a biologically significant regulatory system. The phosphorylation data obtained after 2 sec incubation with low protein concentrations do not necessarily correspond to maximum short-time phosphorylation. These data are most likely already part of the rapid dephosphorylation time course and the maximum of short-time phosphorylation is possibly achieved in less than one second. The phase of relatively slow rephosphorylation correlates most likely with the linear phosphorylation kinetics described by a number of other investigators (3-16).

Short time phosphorylation of membrane proteins, stimulated by cyclic nucleotides or not, appears to be not selective. As far as it can be judged from the phosphoprotein patterns no specific preference of one or the other membrane protein during short-time phosphorylation is evident.

The influence of high concentrations of membrane proteins on the time course of phosphorylation is difficult to interpret. Our data do not necessarily indicate that short-time phosphorylation/dephosphorylation might not have occurred in this case since the phase of rapid dephosphorylation could have already come to the end after 2 sec incubation. On the other hand, late-phase phosphorylation of high concentrations of membrane proteins was found to be stimulated in the presence of cyclic nucleotides. At low protein concentrations late-phase stimulation by cyclic nucleotides attained significant values only after 10 min incubation. A possible explanation of this phenomenon is offered by the assumption that increasing concentrations of membrane protein are speeding up the time course of short-time phosphorylation/dephosphorylation and rephosphorylation.

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