THREE PROTEIN KINASES FROM CALF UTERUS. SUBCELLULAR DISTRIBUTION AND PHYSICAL PROPERTIES.

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SUMMARY

Three protein-kinases which phosphorylate preferentially either histones (kinase a), protamine (kinase b) or casein (kinase c) were separated from calf uterus cytosol. Kinase a weights 120,000, sediments at 6 S and shows an isoelectric point (I.P.) of 5.0. Kinase b weights 65,000, sediments at 4.7 S and shows an I.P. of 5.5. Kinase c weights 200,000, sediments at 7.0 S and shows an I.P. of 6.0. Only kinase a binds, and is stimulated by, 3',5'-cyclic AMP.

Phosphorylation of nuclear proteins occurs at a substantially increased rate in tissues stimulated to grow (1, 2, 3). This phenomenon occurs very early after application of the growth stimulus and is likely to be connected in an unknown way with induction of gene expression in the responsive cells (4, 5, 6). With the aim of assessing the possible significance of protein phosphorylation in the mechanism of estrogen-induced growth, we have started a study of protein phosphokinase activity in the uterus using as substrate both, acidic (casein) and basic (histones and protamine) proteins. We have found that there are separate protein-kinases which preferentially use either one of the three substrate tested. Hereafter we report on their subcellular distribution and partial purification and characterization. It should be mentioned that protein phosphokinase activity of uterus has already received some attention (7, 8).

Protein kinase activity in the different subfractions was tested as described in legend to table 1, without substrate (endogenous activity) as

TABLE I

DISTRIBUTION OF PROTEIN KINASE ACTIVITY IN SUBCELLULAR FRACTIONS[a] OF CALF UTERUS

Fraction	nmole ^{32}P incorporated in 30 min at 36°C by the total fractions of 10gm calf uterus[b]							
	No substrate		Increase in ^{32}P incorporation due to: α-casein		Histone IIa		Protamine	
	− cAMP	+ cAMP	− cAMP	+ cAMP	− cAMP	+ cAMP	− cAMP	+ cAMP
1,500g sediment (nuclear)	64	67	160	150	43	70	23	21
20,000g sediment (mithocondrial)	6	7	9	8	20	20	7	4
105,000g sediment (microsomal)	8	8	10	10	10	10	3	4
105,000g supernatant (cytosol)	93	99	525	508	1545	2240	422	440

a: homogenization was carried out with a Ultraturrax, PT/32, in 10 mM Tris-HCl pH 7.4, 1 mM dithioerithrite (DTE), 1mM EDTA and 0.25 M sucrose. The sediment at 1,500g was washed three more times with the same homogenization buffer. The supernatant of the 1,500g sediment was further fractionated. 1,500g, 20,000g and 105,000g sediments were resuspended by gentle homogenization in 10 mM Tris-HCl pH 7.4, 1 mM EDTA and 1 mM DTE.

b: Assay of protein-kinase activity. Final sample volume was 350 µl containing 50 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 15 µM γ^{32}P-ATP (6.5×10^5 c.p.m.); 50 µl of the different subcellular fractions; and, when indicated, 250 µg of either Histone IIa (Sigma), protamine-phosphate (from salmon sperm, Sigma), or α-casein (Worthington) with or without addition of 1 µM of 3',5'-cyclic AMP. After 30 min at 36°C, the reaction was stopped by addition of 350 µl of 20% TCA containing 2 mM cold ATP and 20 mM disodium phosphate. After 10 min at 4°C, samples were filtered through Whatman GF/A glass fiber discs and washed with 50 ml of 5% TCA containing 20 mM disodium phosphate. Discs were counted in a liquid scintillation spectrometer using 10 ml of a toluene-phosphor solution containing 3.92 gm ‰ of 2,5-diphenyl-oxazole, 0.18 g‰ p-bis (o-methylstiryl) benzene and 330 ml ‰ of Triton x-100 in Toluene. Zero time values were subtracted from experimental values in order to obtain actual incorporation data.

well as with either casein, histones or protamine as substrate and both, in the absence or presence of cyclic 3',5'-AMP. The results of these tests are summarized in table 1 and show that (i) whatever the substrate used,

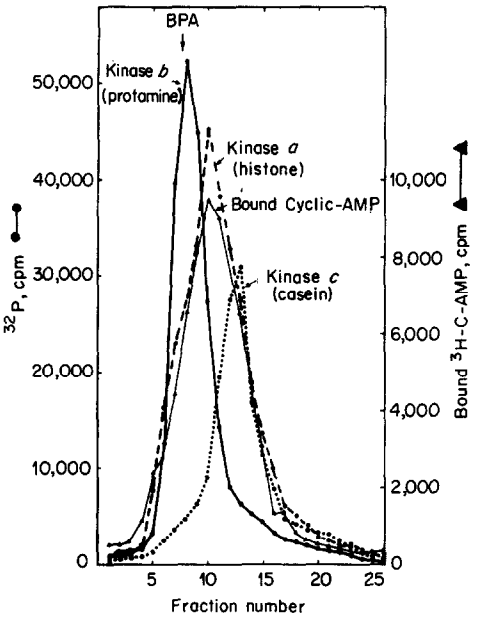

Fig. 1 - Sucrose density gradient analysis of protein phosphokinase activity of calf uterus cytosol. 0.7 ml of calf uterus cytosol and 0.1ml of 1% Bovine Plasma Albumin (BPA, 4.4l S) were layered over a 6-28% linear sucrose gradient in 10mM Tris-HCl buffer, pH 7.4, containing 1.5mM EDTA. After 21 hr of centrifugation at 40,000 rpm, + 2° C, in the SW41 rotor (Beckman-Spinco), fractions of 25 drops were collected from the bottom of the tube. Enzyme activity was measured on 50 µl aliquots of each fraction as described in legend to table I, except that histones were 300 µg of the Arginin-rich fraction (Worthington) and c.p.m. of γ^{32}P--ATP were 4.5×10^5. Sedimentation of BPA was determined by O.D. at 280 mµ. The binding of 3',5'-cyclic AMP-^{3}H was assayed as follows: to 0.1 ml of each sucrose gradient fraction, 0.2 ml of a water solution containing 25 mM Tris-HCl, pH 7.0, 15mM $MgCl_2$ and 4.5×10^5 c.p.m. of 3',5'--cyclic AMP-^{3}H (spec. act. = 20.7C/mmole) were added and the mixture kept at +4°C for 90 min before filtering through Millipore discs (HA, 0.45 mµ). Discs were washed with 30 ml of the same Tris-$MgCl_2$ buffer, dried and counted in toluene Triton-X100-phosphor solution.

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most part of the activity is found in the soluble cytoplasmic fraction (cytosol); (ii) virtually all phosphorylating activity towards histones and protamine is found in cytosol; 2-5% of this activity is found in the nuclear fraction but it could well derive from cytoplasmic contamination; (iii) a substantial fraction of activity towards casein is present in the nuclear fraction: 17-25% of total; (iiii) cyclic 3',5'-AMP stimulates only the activity towards histones ($\Delta\%$ = +45).

Sucrose gradient centrifugation of uterine cytosol furnishes undisputable proof that there are separate protein-kinases which preferentially use either one of the three tested substrates. After 21 hours of centrifugation on a 6-28% sucrose gradient at 40,000 rpm in a SW 41 rotor at + 2° C, the gradient was fractionated and the phosphorylating activity in fractions assayed using as substrate either protamine, casein or arginine-rich histones. The results are presented in fig. 1 and show three well defined discrete peaks, one for each substrate: arginine-rich histone-kinase activity (kinase a) shows a peak at about 5.9 S; protamine-kinase activity (kinase b) shows a peak sedimenting at about 4.7 S; and, casein-activity (kinase c) shows a peak at about 7.2 S. Arginine-rich histone activity also shows a definite shoulder in the protamine region, while the other two activity peaks are rather symmetric. We may thus conclude that the three activities correspond to physical entities which sediments at different rate on a sucrose gradient.

An additional information concerning binding of cyclic 3',5'-AMP has also been gained from gradient in fig. 1: aliquot parts of each fraction were incubated with the tritiated nucleotide and separation of free from protein-bound cyclic 3',5'-AMP was achieved by filtration through Millipore filters. The profile of bound nucleotide is shown in fig. 1 and coincides quite well with the histone-kinase peak. This finding is in agreement with data in table 1 showing that only the histone-kinase activity is stimulated by cyclic 3',5'-AMP.

We have succeded in further improving separation of the three physical entities with different kinase activity by the procedure which may be briefly described as follows. The crude uterine cytosol was filtered by gravity through a column of DEAE-cellulose saturated with the same buffer (pH 7.4) used for homogenization; all protein-kinase activity of cytosol remains adsorbed on the cellulose at this pH and molarity. A KCl gradient was then applied and the eluate divided into two fractions: fraction I which is eluted between 0.03 and 0.12 M KCl and contains most histone-and prctamine-kinase activities; fraction II which is eluted between 0.12 and 0.18 M KCl and contains most of the casein-kinase activity. Electrofocusing of fraction I (fig. 2) and of fraction II (fig. 3) show that the three different kinase activities focus at significantly different pH: kinase a

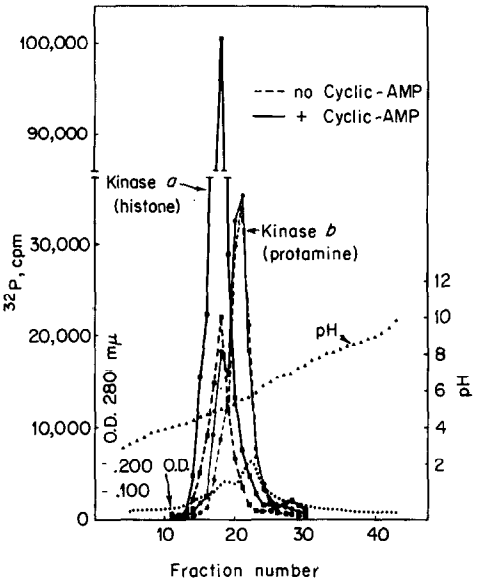

Fig. 2.

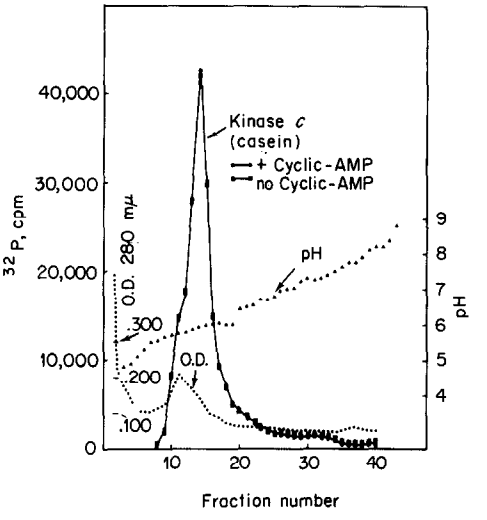

Fig. 3.

Fig. 2 - Isoelectric fractionation of DEAE-cellulose Fraction I, i.e. the 0.03M to 0.12M KCl eluate from a DEAE-cellulose column (pH 7.4) through which calf uterus cytosol had been previously filtered by gravity. A 5-50% sucrose gradient containing 1% ampholine pH 3 to pH 10 was focused in a LKB 110 ml electrofocusing column until the milliamperage fell to a constant 1.4 mA at 800V and at+3°C. Fraction I, which had been previously concentrated under reduced pressure, was made to replace an equal volume of the sucrose-pH gradient in the middle of the column. Voltage was applied again until milliamperage decreased again to 1.5 mA, at 800V and +4°C. After discarding the initial 10 ml, about 2 ml fractions were collected at +4°C and their pH measured soon after. Enzyme activity of fractions was assayed as described in legend to table I. Continous lines show activity in the presence of 3',5'-cyclic AMP using either histone IIa or protamine as substrate.

Fig. 3 - Isoelectric fractionation of DEAE-cellulose Fraction II, i.e. the 0.12M to 0.18M KCl eluate from a DEAE-cellulose column (pH 7.4) through which calf uterus cytosol had been previously filtered by gravity. Experimental conditions were as described in legend to fig. 2, except that 1% ampholine pH 5 to pH 8 was used and that at the end of the run milliamperage was 0.8, at 800V and +3°C. Protein phosphokinase activity was assayed as described in legend to table I, with (circles) or without (squares) 3',5'--cyclic AMP.

(histones) focuses at pH 5.0; kinase b (protamine) focuses at pH 5.5; and kinase c (casein) focuses at pH 6.0.

One can see from fig. 2 and 3 that the different kinase activities

TABLE II

SUMMARY OH PHYSICAL CHARACTERISTICS OF THREE PROTEIN KINASES OF CALF UTERUS

	$S_{w,20}$	Stokes radius	M.W.	f/f_o	I.P.
Kinase a	5.9	48.5	118.000	1.5	5.0
Kinase b	4.7	33.5	65.000	1.3	5.5
Kinase c	7.2	66.0	196.000	1.72	6.0

$S_{w,20}$: by sucrose gradient centrifugation; Stokes radius: by gel filtration; M.W.: from M.W. = $4\pi\eta NaS/(1 - \bar{v}\rho)$, assuming $\bar{v}$ = 0.725; f/f_o: from $f/f_o = a/(3\bar{v}\ M.W./4\pi N)^{1/3}$; I.P.: from electrofocusing experiments.

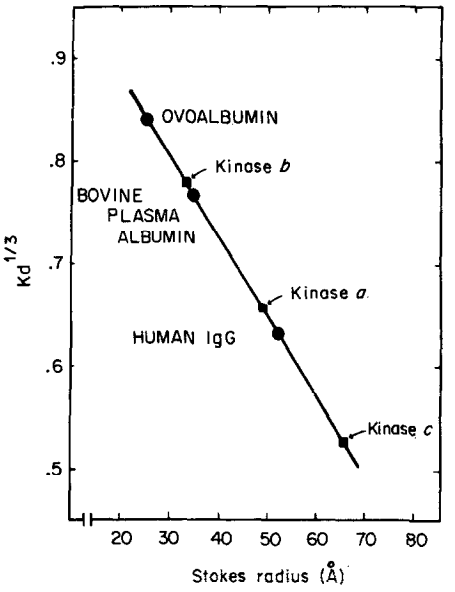

Fig. 4 - Gel filtration of calf uterus protein-kinase activities. A 207 ml column of Sephadex G-200 was equilibrated at +4°C with a water solution containing 10mM Tris-HCl, pH 7.4, 100mM KCl, 1mM EDTA and 1mM DTE. The column was then calibrated with ovoalbumin, bovine plasma albumin and H-γ-globulin using an upward flow of 12 ml/hr. Purified kinase a, b and c were analyzed in subsequent runs on the same column and under identical experimental conditions of standard proteins. Elution volumes were plotted according to Porath ($K_d^{1/3} = \alpha - \beta a$) where a = Stokes radius. Distribution coefficients, k_ds, were derived from $k_d = (V_e - V_o)/(V_t - V_o)$, where V_e (elution volume) was the volume corresponding to peak elution of proteins V_o (void volume = 67 ml) was measured with blue-dextran, V_t (total) volume) was 207 ml. Stokes radiuses of standard proteins were from the literature (9).

were tested in the presence or absence of cyclic 3',5'-AMP and again the results confirm that only kinase a (histones) is stimulated by the cyclic nucleotide.

In order to have an indication of Stokes radius and molecular weight, we have also carried out 'gel filtration' of kinases a, b and c on a calibrated Sephadex G-200 column. The results are given in fig. 4 according to Porath ($K_d^{1/3} = \alpha - \beta a$) where a is the Stokes radius. The computed radiuses and molecular weights are given in the summary table 2.

In conclusion, we have identified three protein-kinases of calf uterus which are different (i) in their physical properties (ii) in their substrate affinity and (iii) in their response to cyclic 3',5'-AMP. In general, the finding of one separate protein-kinase for each one of the three substrates which were tested supports the view, on simply statistical grounds, that a multiplicity of protein-kinases exist in calf uterus. This complex situation warns against simple studies of protein-kinase activity in crude cellular fractions or even in partially purified preparations, using more or less generic substrates.

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