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Three protein Kinases from calf uterus. Functional properties

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Patologia. — *Three protein Kinases from calf uterus. Functional properties.* Nota di GIOVANNI ALFREDO PUCA, ERNESTO NOLA, VINCENZO SICA e FRANCESCO BRESCIANI (*), presentata (**) dal Socio L. CALIFANO.

RIASSUNTO. — Sono state studiate le proprietà funzionali di 3 chinasi del citoplasma di utero di vitello. La chinasi *a* è cAMP dipendente e fosforila preferenzialmente gli istoni IIa; la chinasi *b* non è infuenzata dal cAMP e riconosce come substrato le protamine; la chinasi *c* è cAMP indipendente e fosforila l'α–caseina e gli istoni IIa. Altre differenze riguardano l'ottimo di pH, l'inibizione da fosfati e l'attivazione da ioni Mg^{2+}, Co^{2+} e Mn^{2+}.

In a previous communication [1] we have described the physical separation and molecular properties of three protein kinases of uterine cytosol. These kinases were labeled *a*, *b* and *c* and could be distinguished because of their preferential phosphorylation of either histone IIa, protamine or α–casein, respectively. Kinase *a* weighs 120,000, sediments at 6 S and shows an I.P. of 5.0. Kinase *b* weighs 65,000, sediments at 4.7 S and shows an I.P. of 5.5. Kinase *c* weighs 200,000, sediments at 7 S and shows an I.P. of 6.0. We now report on functional characteristics of these three kinases after their physical separation.

Partial purification and isolation of kinases *a*, *b* and *c* was achieved by the procedure described in the previous communication [1] and consisting of chromatography on DEAE–cellulose followed by gel filtration on Sephadex G–200 and electrofocusing. To improve separation of kinases *a* and *b*, the final electrofocusing has now been carried out on a pH 4.0 to 6.0 gradient, instead of the previous pH 3.0 to 10.0 gradient. After separation, the following studies were carried out. For each kinase, histones IIa, protamine and α-casein were used as substrate, both in the presence or absence of cyclic 3′,5′-AMP (fig. 1). Previous preliminary results [1] were confirmed and extended in that (i) kinase *a* is cyclic 3′,5′-AMP dependent and phosphorylates preferentially histones; (ii) kinase *b* is not affected by the presence of the cyclic nucleotide and shows a high preference for protamine: (iii) kinase *c* is also not affected by cyclic 3′,5′-AMP and phosphorylates α-casein and histones IIa at about the same rate, while phosphorylation of protamine proceeds at a comparatively much slower rate.

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(**) Nella seduta del 26 novembre 1973.

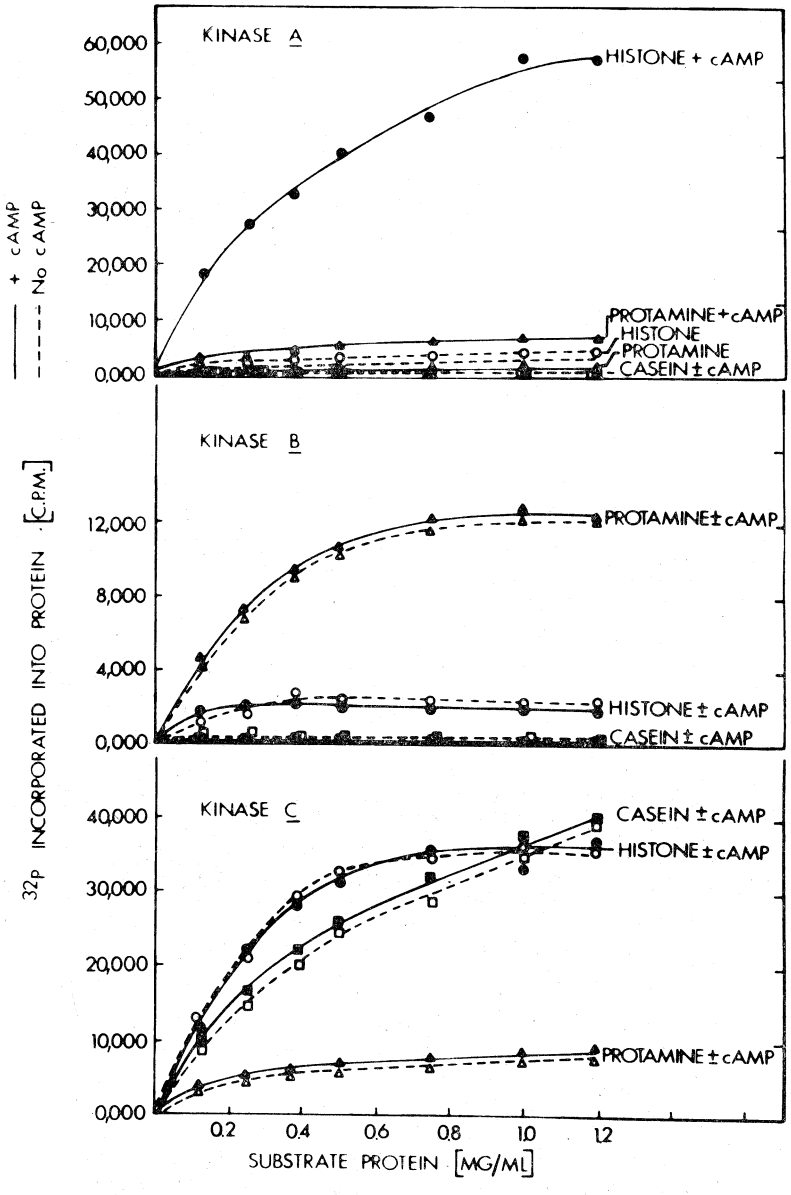

Fig. 1. – Effect of substrate concentration on activity rate of three purified protein kinases of calf uterus cytosol.

Enzyme activity was measured in a final volume of 0.2 ml, which included: 100 µl of purified enzyme solution after electrofocusing; 20 µM $^{32}P(\gamma)$–ATP (415,000 cpm); 15 mM $MgCl_2$; 50 mM Tris–HCl, pH 7.5, the required concentration of either histone–IIa (Sigma), or Protamine phosphate (from salmon sperm, essentially histone free. Sigma), or α–casein (Worthington); and when added, 2×10^{-6} M 3′,5′–cyclic AMP. Incubation at 36° C was started by adding the enzyme. Incubation time was 15 min for kinases *a* and *b*, and 30 min for kinase *c*. ^{32}P incorporation was measured as described earlier (1). Endogenous phosphorylation was measured incubating the above mixture without the exogenous substrate and was subtracted from phosphorylation occuring in the presence of exogenous substrate to give the data shown in this and in the other figures of this paper.

Enzyme activity at different pH in Tris (hydroxymethylamino-methane)-HCl buffer and in phosphate buffer was also studied and the results are shown in fig. 2. In Tris buffer, the one used throughout in this study, all three kinases

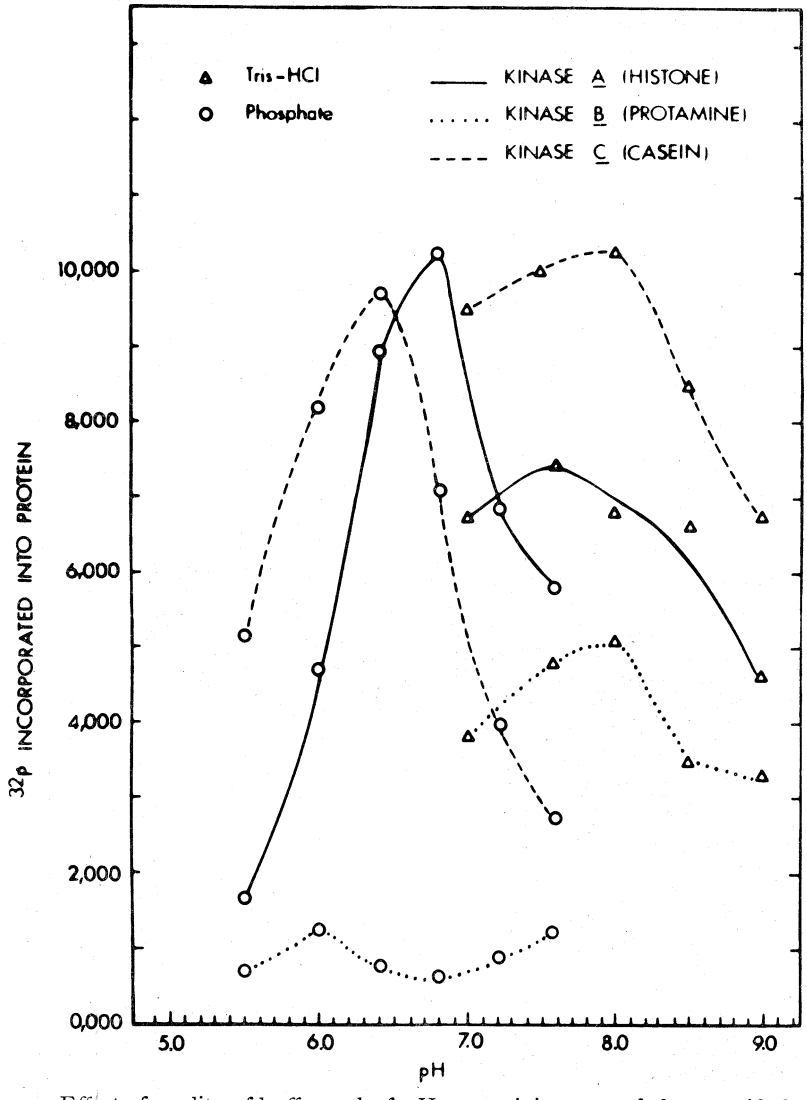

Fig. 2. – Effect of quality of buffer and of pH on activity rate of three purified protein kinases of calf uterus cytosol.

Assay conditions were similar to those described in legend to fig 1, except that 200 μg of the specified substrate and 20 μl of either kinase *a* or *c* or 40 μl of kinase *b* were used in each test. Incubation (36° C) was 20 min for kinases *a* and *b* and 40 min for kinase *c*. Kinase *a* was analyzed only in the presence of 2×10^{-6} M 3′,5′-cyclic-AMP. (△—△) 100 mM Tris-HCl-buffer; (○—○) 70 mM Sodium Phosphate buffer.

show a rather vague pH dependency within the range possible with this buffer; the optimum is at about pH 7.5–8.0. On the contrary, in phosphate buffer there is a sharp pH dependency of activity of kinase *a* (optimum at pH 6.8) and kinase *c* (optimum at pH 6.4); activity of kinase *b* is inhibited by phosphate

in the pH range of this buffer. In addition to the slight difference in pH optimum, kinases *a* and *c* differ because kinase *a* is more active in phosphate, while kinase *c* is more active in Tris-buffer.

Further differences among the kinases under study appear from an investigation of the influence of Mg^{2+}, Co^{2+} and Mn^{2+}. The results of this investigation are presented in figs. 3 and 4. Mg^{2+} produces the most marked activa-

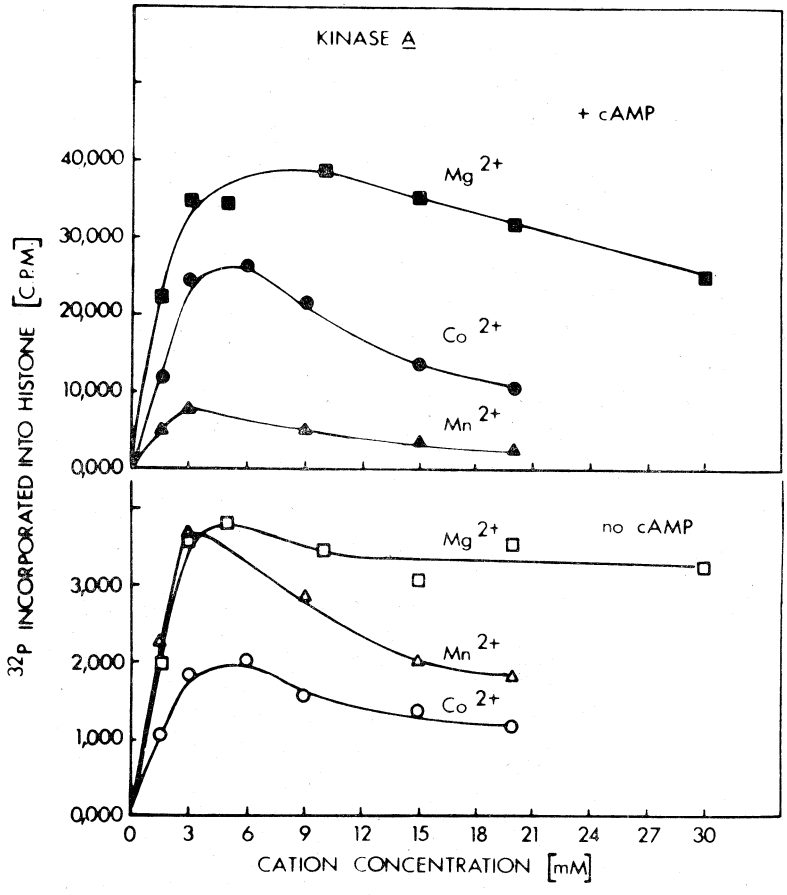

Fig. 3. – Effect of cation concentration on activity rate of purified protein kinase *a*. Assay conditions were as described in legend to fig. 1, except that 250 μg of histone IIa, 100 μl of enzyme solution and 0.2 M Tris–HCl, pH 7.5 were used in each test. Incubation time was 20 min at 36° C. 3′,5′–cyclic–AMP concentration was 2.5×10^{-6} M.

tion effect on all three kinases. However, the Mg^{2+} concentration at which there is half maximal activation is different: for kinase *a* it is about 1.2 mM; for kinase *b* about 2.0 mM; and for kinase *c* about 3.0 mM. Furthermore, a clear inhibition by higher Mg^{2+} concentration in the range tested is clearly in evidence for kinases *a* and *c*, while there is no such effect for kinase *b*. With regard to Co^{2+} and Mn^{2+}, the lower concentrations at which there is half maximal activation of kinases *a*, *b* and *c*, respectively, are 1.6 mM, 1.2 mM and 5 mM for Co^{2+} and 1.2 mM, 1.0 mM and 2.4 mM for Mn^{2+}. As one can see

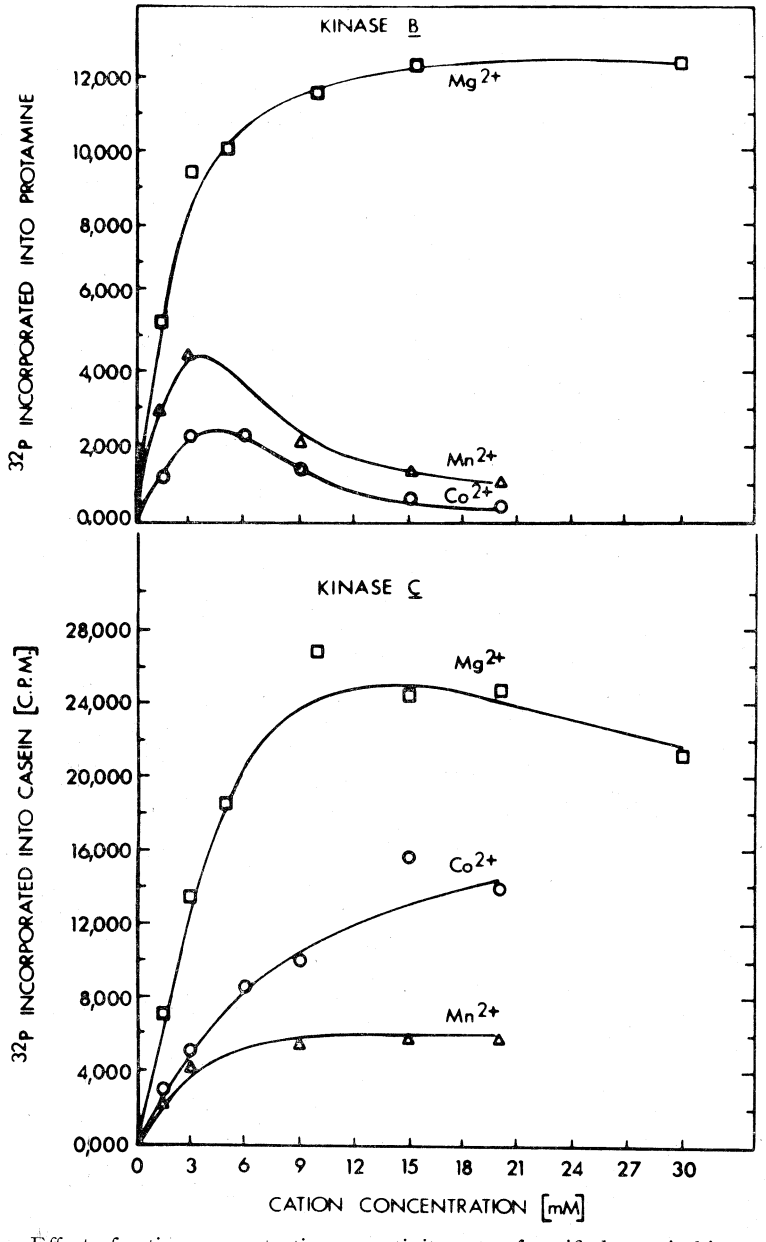

Fig. 4. – Effect of cation concentration on activity rate of purified protein kinases *b* and *c*.

Assay conditions were as described in legend to fig. 1 except that 250 μg of either protamine (kinase *b*) or α-casein (kinase *c*), 100 μl of the purified kinases, and 0.2 M Tris–HCl, pH 7.5 were used. Incubation time (36° C) was 20 min for kinase *b* and 40 min for kinase *a*.

from inspection of curves in figs. 3 and 4, many other differences appear to exist with regard to (i) relative effectiveness of these three ions, (ii) the concentrations at which inhibition of activity appears, and (iii) the specific ion requirement for the activation effect of cyclic 3′,5′-AMP to appear in full (kinase *a*).

A summary of results is presented in Table I. In conclusion, we were able to isolate three separate protein phosphokinases of uterus by exploiting their differential affinity for three different proteins, at least two of which (α–casein and protamine) are not normal uterine components. Our results also show that the three phosphokinases which were identified have a high degree of specificity and show quite different ionic requirements, pH optimum, cyclic 3′,5′-AMP dependence, and so on.

TABLE I

Some properties of three purified protein kinases of calf uterus.

	Preferential substrate	Activation [a] by cAMP (15 mM Mg^{2+}, 20 μM ATP) %	pH optimum		Cation concentration (mM) at which activity rate is half maximal		
			Tris-HCl	Phosphate	Mg^{2+}	Co^{2+}	Mn^{2+}
Kinase *a*....	Histone IIa	1,000–1,300	7.5 (more active in Phosphate than in Tris-HCl)	6.8	1.2 (all cations inhibit at high concentration)	1.6	1.2
Kinase *b*....	Protamine	100	8.0	inactive	2.0 (Co^{2+} and Mn^{2+} inhibit at high concentration)	1.2	1.0
Kinase *c*....	α–casein [b]	100	8.0 (more active in Tris-HCl than in Phosphate)	6.4	3.0 (Mg^{2+} inhibits at high concentration)	5.0	2.4

(*a*) (Activity in the presence of cAMP/activity in the absence of cAMP) × 100.
(*b*) Kinase *c* also phosphorylates Histone IIa at about the same rate as α–casein.

The above findings have a few implications worth mentioning explicitly. (i) If by the use of three generic substrates one is able to detect three different kinases, then one must expect that many more kinases exist in the uterus. (ii) Our results do not corroborate previous claims that this class of enzymes has low substrate specificity [2–5]. We suggest that the apparent low specificity found in previous studies was a consequence of using rough preparations or even whole subcellular fractions actually containing a multiplicity of specific kinase. Corollary implications of practical value are the following: (iii) When using a generic kinase substrate one must have highly purified enzyme preparations in order to obtain meaningful results. (iv) Finally, and more important, if there is high substrate specificity then one may not be able to demonstrate a particular kinase activity unless the right substrate is available.

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