# Distinct structural requirements of $Ca^{2+}$ / phospholipid-dependent protein kinase (protein kinase C) and cAMP-dependent protein kinase as evidenced by synthetic peptide substrates

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Received 4 February 1985; revised version received 15 March 1985

Protein kinase C, purified to near homogeneity from the brain, has been tested toward a variety of synthetic peptide substrates including different phosphorylatable residues. While it proved totally inactive toward the tyrosyl peptide Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Arg-Arg-Gly, as well as toward several more or less acidic seryl peptides, it phosphorylates with a $Ca^{2+}$/phospholipid-dependent mechanism, at seryl and/or threonyl residues, many basic peptides, some of which are also good substrates for cAMP-dependent protein kinase (A-kinase). Among the peptides tested, however, the best substrate for protein kinase C, with kinetic constants comparable to those of histones, is the nonapeptide Gly-Ser-$Arg_6$-Tyr, which is not a substrate for A-kinase. Moreover, although the peptide Pro-$Arg_5$-Ser-Ser-Arg-Pro-Val-Arg is a good substrate for both kinases, its derivative with ornitines replacing arginines is phosphorylated only by protein kinase C. Some typical substrates of A-kinase on the other hand, like the peptides Phe-$Arg_2$-Leu-Ser-Ile-Ser-Thr-Glu-Ser and $Arg_2$-Ala-Ser-Val-Ala, are phosphorylated by protein kinase C rather slowly and with unfavourable kinetic constants. It is concluded that, while both protein kinase C and A-kinase need basic groups close to the phosphorylatable residues, their primary structure determinants are quite distinct.

*Protein kinase C* *cAMP-dependent protein kinase* *Phosphorylation site* *Synthetic peptide* *Phosphopeptide*

## 1. INTRODUCTION

The site recognition of several protein kinases has been shown to largely depend on the amino acid sequence around the target residues. Substantial evidence supporting such a conclusion has been provided by synthetic peptide substrates reproducing with suitable modifications the sites phosphorylated in the intact proteins. In particular, cAMP-dependent protein kinase (A-kinase) readily phosphorylates only peptides having at least two basic residues (either Arg-Arg or Arg-Lys), at one to two residues from the N-terminal side of the phosphorylatable amino acid [1–3], whereas type-2 casein kinases exhibit an opposite structural requirement consisting of a cluster of acidic residues adjacent to the C-terminal side of the phosphorylatable residue [4,5]. Several other classes of protein kinases, on the other hand, including phosphorylase kinase, calcium and calmodulin-dependent myosin light chain kinase, cGMP-dependent protein kinase, and H4 protein kinase, can phosphorylate peptides which are also more or less suitable for A-kinase (e.g., [6–10]), suggesting that their structural requirements may be similar, though not identical, to that of A-kinase. This could be true also of the $Ca^{2+}$-activated, phospholipid-dependent protein kinase (protein kinase C) which phosphorylates residues of histones and myelin basic protein that are also affected by A-kinase, though with different efficiencies [11–14]. Furthermore, to our



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00145793/85/$3.30 

knowledge, the only report using synthetic peptides as suitable substrates for protein kinase C [15] describes the phosphorylation of a single nonapeptide reproducing a fragment of histone H1 which is also affected by A-kinase for having a basic triplet on its N-terminal side. Here we unambiguously show, with the aid of a wide variety of synthetic peptide substrates, that the structural determinants of protein kinase C and A-kinase are quite distinct. In particular, we show that peptides which are not phosphorylated at all by A-kinase for having basic clusters on their carboxyl rather than their amino terminal side or for having Orn replacing Arg, can be excellent substrates for protein kinase C.

## 2. MATERIALS AND METHODS

Protein kinase C was purified to near homogeneity from bovine brain according to [16]. To improve the recovery in some preparations the last step (phenyl-Sepharose chromatography) was omitted. Comparable results were obtained with these preparations and with the purer enzyme. The catalytic subunit of cAMP-dependent protein kinase, purified to homogeneity from skeletal muscle [17], was kindly provided by Dr D.G. Hardie (Dundee). Histones (either type IIA or type IIIS as indicated in the tables) were from Sigma.

The synthesis, purification and chemical characterization of most peptides used in this study were described in [18–20]. The synthesis of the peptides termed S6-1 and X6 will be described elsewhere. The nomenclature of peptides is given in tables 1 and 2.

The phosphorylation experiments were performed by incubating either histones or peptides with [$\gamma$-$^{32}$P]ATP in the presence of either protein kinase C or A-kinase (1–2 units, one unit being defined as the amount of enzyme that catalyzes the transfer of 1 pmol phosphate from ATP to histones IIA in 1 min under standard conditions). Phosphorylation by protein kinase C was assayed by 10 min incubation at 30°C in 50 $\mu$l of a medium containing 5 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$ and 20 $\mu$M [$\gamma$-$^{32}$P]ATP (spec. act. 400–600 cpm/pmol) either in the presence or absence of 0.5 mM $CaCl_2$ plus phosphatidylserine (200 $\mu$g/ml). In some experiments diolein from Sigma (1.25 $\mu$g/ml) was used as an activator, in the presence of low concentrations of $CaCl_2$ (10 $\mu$M) and phosphatidylserine (2.5 $\mu$g/ml). Whenever $CaCl_2$ was omitted, 0.5 mM EGTA was present. In control experiments either the protein kinase or the substrates were omitted. Conditions for phosphorylation by A-kinase were identical, except for the omission of $CaCl_2$ and phosphatidylserine. The concentration of histones was 80 $\mu$M and that of peptide substrates is detailed in the figures and tables. The reaction was terminated and [$^{32}$P]ATP was removed by addition of acetic acid and the application of an aliquot of the sample onto phosphocellulose paper [21].

Kinetic constants were calculated from double reciprocal plots constructed from initial rate measurements fitted to the Michaelis-Menten equation by the least squares method.

The isolation and detection of radiolabeled phosphoamino acids from the phosphorylated derivatives of peptides Th-1 and Th-2 (including both Ser and Thr) and Ga-1 (including both Ser and Tyr) were performed by 4 h hydrolysis in 6 N HCl at 105°C followed by high-voltage electrophoresis at pH 1.9 [22]. Under these conditions phosphoserine is well separated from both phosphothreonine and phosphotyrosine.

## 3. RESULTS AND DISCUSSION

Preliminary experiments had shown that protein kinase C is fully inactive toward the tyrosyl peptide Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Arg-Arg-Gly, which is suitable for tyrosine protein kinases [23], as well as toward the very acidic peptides which are readily affected by casein kinase-TS (type-2) [5] and the slightly acidic peptide Leu-Ser-Ile-Ser-Thr-Glu-Ser (not shown).

On the other hand, protein kinase C proved more or less active toward several basic peptides which are also phosphorylated by A-kinase for having two or more arginines close to the N-terminal side of their seryl residue(s).

However, a first remarkable difference between these two protein kinases was disclosed by replacing the guanido groups of Arg with amino groups, to give the corresponding ornityl derivatives. As shown in fig.1, such a substitution is deleterious for A-kinase, whereas it is tolerated by protein kinase C.

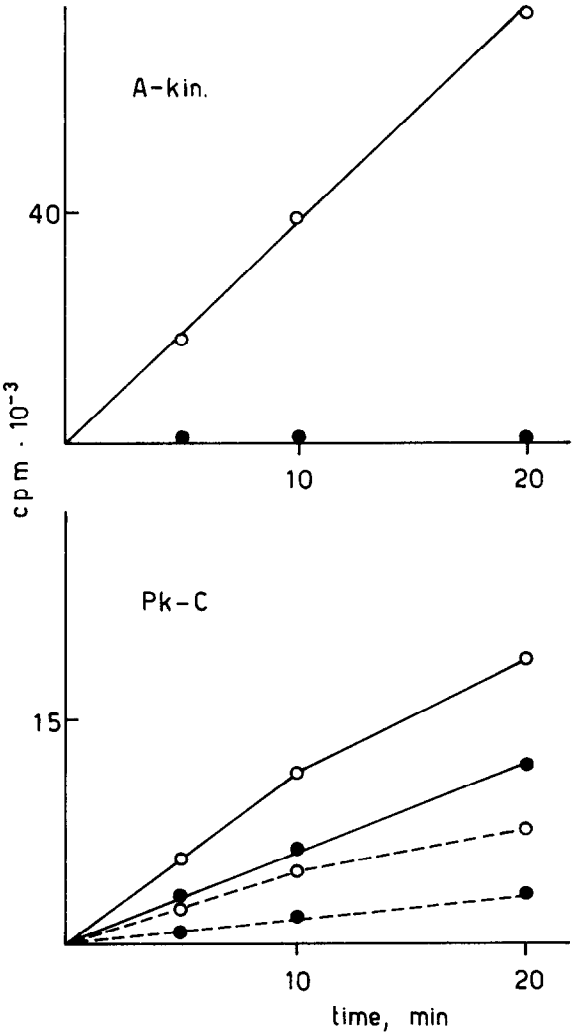


Fig.1. Phosphorylation of the tetradecapeptide Pro-Arg-Arg-Arg-Arg-Arg-Ser-Ser-Arg-Pro-Val-Arg (○) and its ornityl derivative (●) by either A-kinase (A) or protein kinase C (B). In (B) solid and broken lines denote phosphorylation in the presence and absence of $Ca^{2+}$ and phosphatidylserine, respectively. General conditions are detailed in section 2. The concentration of peptides was 0.8 mM.

Additional criteria supporting different structural requirements of protein kinase C and A-kinase were provided by comparing the phosphorylation of several basic peptides differing for the number and position of arginines relative to the phosphorylatable residue(s). The structures of these peptides and their phosphorylation rates by protein kinase C and A-kinase are reported in table 1. No parallelism can be observed between the phosphorylations by the two enzymes, the most striking discrepancy consisting in the very fast phosphorylation by protein kinase C of the nonapeptide Gly-Ser-$Arg_6$-Tyr (Ga-1) which is devoid of any basic residue on the N-terminal side of serine and consequently is fully unaffected by A-kinase. It should be noted that this peptide, which is phosphorylated only at its seryl and not at its tyrosyl residue (not shown), is a better substrate than histones, and even the pentapeptide corresponding to its N-terminal fragment Gly-Ser-Arg-Arg-Arg is still appreciably phosphorylated by protein kinase C. The phosphorylation of these peptides, as well as that of the others listed in table 1, by protein kinase C, is dependent on calcium and phospholipids. (The sometimes significant basal phosphorylation of histones observed in the absence of any activator was probably due to the presence of traces of the proteolytically activated form of protein kinase C as it varied significantly from one preparation to another. It was also observed, however, that with some peptide substrates the basal activity of protein kinase C was especially remarkable (unpublished) suggesting that the phosphorylation in the absence of activators could be promoted by structural features of the substrate. This would be in agreement with the observation that the phosphorylation of protamines by protein kinase C is already maximal in the absence of calcium and phospholipids [24].)

On the other hand, the typical peptide substrates of A-kinase, characterized by just a couple of Arg on the N-terminal side of serine and corresponding to the phosphorylation sites of the $\alpha$-subunit of phosphorylase kinase (Ph-1) and liver pyruvate kinase (Py-1) are not as good as histones for protein kinase-C. If the enzyme was activated by diolein in the presence of limiting amounts of calcium and phosphatidylserine the phosphorylation rates of Ga-1, Ga-2 and Ga-3 were increased, but the order of the phosphorylation rates for the other peptides remained roughly the same.

The kinetic constants reported in table 2 show that the excellent phosphorylation efficiency of the nonapeptide Gly-Ser-$Arg_6$-Tyr (Ga-1) observed with protein kinase-C is accounted for by both high $V_{max}$ and low $K_m$ values, of the same order as those obtained with histones (table 2) and with

Table 1

Phosphorylation rates of synthetic peptides by either protein kinase C (Pk-C) or the catalytic subunit of cAMP-dependent protein kinase (A-kin)

| | | Pk-C | | A-kin |
|---|---|---|---|---|
| | | +Ca/PS | −Ca/PS | |
| | Histones (HIIA Sigma) | 100 | 40 | 100 |
| Ph-1 | Arg-Arg-Leu-Ser-Ile-Ser-Thr-Glu-Ser | 25 | 15 | 84 |
| Py-1 | Arg-Arg-Ala-Ser-Val-Ala | 84 | 33 | 362 |
| Py-2 | Arg-Arg-Ala-Thr-Val-Ala | 11 | 3 | 4 |
| Th-1 | Arg-Arg-Arg-Arg-Tyr-Arg-Arg-Ser-Thr-Val-Ala | 203 | 145 | 150 |
| Th-2 | Arg-Arg-Ser-Thr-Val-Ala | 76 | 25 | 75 |
| S6-1 | Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala | 345 | 172 | 77 |
| Ga-1 | Gly-Ser-Arg-Arg-Arg-Arg-Arg-Arg-Tyr | 364 | 121 | 0 |
| Ga-2 | Gly-Ser-Arg-Arg-Arg | 111 | 32 | 0 |
| Ga-3 | Gly-Ser-Orn-Orn-Orn | 89 | 25 | 0 |
| $X_6$ | Ser-Gly-Orn-Orn-Orn-Orn | 83 | 26 | 0 |

The phosphorylation rates are relative to that of histones (100%). The concentration of peptides was 0.8 mM and that of histones 1.3 mg/ml. The nomenclature of peptides is reminiscent of the protein substrate. They are derived from $\alpha$-subunit of phosphorylase kinase (Ph), liver pyruvate kinase (Py), the protamines thynnine Z1 (Th) and Galline (Ga) and the ribosomal protein S6 (S6). The longest peptide exactly reproducing the protein segment is indicated with number 1. The higher numbers indicate shorter and/or modified derivatives. X6 indicates a hexapeptide having no known relationship with any protein fragment. Basic residues are underlined

Table 2

Kinetic constants for protein kinase C with synthetic peptides

| | Peptides | Apparent $K_m$ ($\mu$M) | $V_{max}$ (pmol·min$^{-1}$) | $\frac{V_{max}}{K_m} \times 10^3$ |
|---|---|---|---|---|
| Ga-1 | Gly-Ser-Arg-Arg-Arg-Arg-Arg-Arg-Tyr | 11.6 (10.2) | 4.87 (9.60) | 420 |
| Ga-2 | Gly-Ser-Arg-Arg-Arg | 412.0 (397) | 1.78 (3.02) | 4 |
| Th-1 | Arg-Arg-Arg-Arg-Tyr-Arg-Arg-Ser-Thr-Val-Ala | 128 (102) | 1.63 (1.25) | 12 |
| Th-2 | Arg-Arg-Ser-Thr-Val-Ala | 231 | 0.70 | 3 |
| Py-1 | Arg-Arg-Ala-Ser-Val-Ala | 455 (510) | 2.04 (2.00) | 4 |
| Py-2 | Arg-Arg-Ala-Thr-Val-Ala | 276 (260) | 0.35 (0.35) | 1 |
| S6-1 | Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala | 230 | 3.40 | 14 |
| Th-B | Pro-Arg-Arg-Arg-Arg-Arg-Ser-Ser-Arg-Pro-Val-Arg | 31.5 | 3.12 | 99 |
| | Histones HIIA | 4.8 | 2.17 | 450 |
| | Histones HIIIS (lysine rich) | 15.9 (15.7) | 3.24 (1.3) | 203 |

The nomenclature of the peptides is the same adopted in table 1 except for the new peptide Th-B corresponding to the fragment 1–12 of thynnine [20]. The $K_m$ of histones was calculated assuming an average molecular mass of 16 kDa. The data in parentheses were extrapolated from experiments made using diolein (1.25 $\mu$g/ml) as an activator, in the presence of limiting amounts of $CaCl_2$ (10 $\mu$M) and phosphatidylserine (2.5 $\mu$g/ml)

some putative protein substrates of protein kinase C [25,26]. On the other hand, the pentapeptide Gly-Ser-Arg-Arg-Arg and the hexapeptides Arg-Arg-Ala-Ser-Val-Ala and Arg-Arg-Ser-Thr-Val-Ala exhibit $K_m$ values 20- to 40-fold higher, and significantly lower $V_{max}$ values than the nonapep-

tide Ga-1. Activation of protein kinase C by diolein in the presence of limiting amounts of calcium and phosphatidylserine resulted in $K_m$ values very similar to those obtained by activation with high concentrations of $CaCl_2$ and phosphatidylserine. The $V_{max}$ values with the peptides Ga-1 and Ga-2, however, were notably higher in the presence of diolein while those with histones and with peptide substrates having the basic residues on their N-terminal side were either unchanged or even lower (table 2, in parentheses).

A comparison between the kinetic constants of the couples of more or less related peptides Ga-1/Ga-2, Th-1/Th-2 and S6-1/Ga-B (see table 2) suggests that the phosphorylation efficiency is improved by increasing the number of basic residues close to the phosphorylatable one(s), either on their C- or N-terminal side, or on both. Seemingly however, the C-terminal basic residues are more effective than the N-terminal ones in lowering the $K_m$. Such a concept would also be in agreement with the relatively high $K_m$ value (130 $\mu$M) calculated for a synthetic nonapeptide whose three basic residues are all located on its N-terminal side [15].

Collectively our data support different site recognition determinants for protein kinase C and for A-kinase; though both enzymes require basic groups close to the phosphorylatable residue(s), protein kinase C neither needs their obligatory location on the N-terminal side nor shows any preference for the guanido over the amino radicals. On the other hand, while the N terminal Arg-Arg doublet allows optimal efficiency for A-kinase, a larger number of basic residues (possibly more than three) seems to be required to optimize the phosphorylation by protein kinase C. Also, the location of at least some such residues on the C-terminal side is apparently required to reach optimal efficiencies, whereas in the case of A-kinase such a structural feature seemingly represents a negative factor [2,19]. It should be reminded on this matter that A-kinase also needs hydrophobic group(s) on the C-terminal side of serine [1], a requirement which is evidently not shared by protein kinase C.

The concept disclosed by our data that protein kinase C preferentially affects residues surrounded by several basic groups fits very well with the structure of the EGF receptor site which is phosphorylated by protein kinase C, consisting of a threonyl residue comprised in a very basic sequence which includes as many as 7 arginines, one lysine and one histidine concentrated within a stretch of 13 residues [27]. Although the replacement of Thr for Ser in a moderately basic hexapeptide decreases the phosphorylation efficiency by protein kinase C, such an impairment is less drastic than with A-kinase (see table 1). Furthermore, phosphothreonine accounts for about 30% of the phosphorylation occurring when the hexapeptide $Arg_2$-Ser-Thr-Val-Ala is the substrate (not shown). It is conceivable on the other hand that the unfavourable factor arising from the presence of Thr instead of Ser can be overcome by the unusually high number of basic residues close to it in the EGF receptor site affected by protein kinase C. The powerful inhibition of protein kinase C by the very basic peptide $Arg_4$-Ala-Gly-$Arg_4$ (unpublished), also supports the importance of multiple basic residues in determining the binding of the protein substrate to protein kinase C.

## ACKNOWLEDGEMENTS

This work was supported by a grant from the Consiglio Nazionale delle Ricerche (CT 82.00194.04). The skilful technical assistance of Mr Giuseppe Tasinato and the excellent secretarial aid of Miss Monica Vettore are gratefully acknowledged.

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