A potent and highly selective peptide substrate for protein kinase C assay

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Protein kinases exhibit substrate specificities that are often primarily determined by the amino acids around the phosphorylation sites. Peptides corresponding to protein kinase C phosphorylation sites in several different proteins were synthesized on SPOTs membrane which has recently been found to be applicable for studies of protein kinase specificity. After phosphorylation with protein kinase C, we chose the best phosphorylated peptides for the investigation of the importance of amino acids immediately adjacent to the phosphorylation site. The selectivity of the best protein kinase C substrates from this study was analysed with protein kinases A, CK1 and CK2. According to these tests, the most favourable characteristics of SPOTs-membrane-associated peptides were demonstrated by peptide KRAKRKTAKKR. Kinetic analysis of peptide phosphorylation

with protein kinase C revealed an apparent K_m of $0.49 \pm 0.13 \mu\text{M}$ and $V_{\max}$ of $10.0 \pm 0.5 \text{ nmol/min per mg}$ with soluble peptide KRAKRKTAKKR. In addition, we assayed several other soluble peptides commonly used as protein kinase C substrates. Peptide KRAKRKTAKKR showed the lowest K_m and the highest $V_{\max}/K_m$ value in comparison with peptides FKKSFKL, pEKRPQRSKYL and KRAKRKTAKKR. Furthermore, of the peptides tested, KRAKRKTAKKR was the most selective substrate for protein kinase C. The favourable kinetic parameters combined with the selectivity should make the KRAKRKTAKKR peptide useful as a substrate for protein kinase C in the assays of both purified enzyme and in crude cell extracts.

INTRODUCTION

Protein kinase C (PKC) belongs to a family of Ser/Thr kinases which has been shown to play a crucial role in signal transduction [1]. To date, 11 PKC isoenzymes have been identified and classified into three groups based on differences in their structure and cofactor requirements. Of these, the group of conventional PKC isoenzymes consisting of α , β (I and II) and γ is the best characterized and most abundant [2]. A number of proteins are known to be phosphorylated by PKC, and the amino acid sequences around their phosphorylation sites have been determined. Histone H1 is quite often used to assay PKC activity, but does not provide an accurate measure of PKC in crude tissue fractions, because it can be phosphorylated by many other kinases. A number of efforts have been made to prepare sensitive substrates specific to PKC [3–8], of which the ‘Nishizuka peptide’ pEKRPQRSKYL [9] is probably the most commonly used.

In general, protein kinases exhibit substrate specificities that are often primarily determined by the amino acids around the phosphorylation sites. Recently, a new and convenient method for peptide synthesis (SPOTs synthesis [10]), which allows easy preparation of multiple peptide substrates, has been tested for protein kinases both by others [11] and us [12]. The SPOTs method was found to be applicable for investigation of protein kinase specificity and enzyme–substrate interaction.

In this paper we describe the evaluation of synthetic peptide substrates derived from the protein sequences phosphorylated by PKC *in vivo* using sets of SPOTs-membrane-attached peptides. After qualitative scanning, the most favoured substrate was synthesized by conventional means, and its kinetic constants in solution were determined and compared with those for other good substrates for PKC. It was shown that this peptide is

superior to the others tested because of its sensitivity and selectivity for PKC. These features suggest its use in PKC assays in general and especially in the presence of other kinases.

MATERIALS AND METHODS

Materials

SPOTs™ kit was purchased from Cambridge Research Biochemicals. Fmoc amino acids, supplied as active esters, were included in the kit or purchased from Millipore. All reagents needed for peptide synthesis were from Sigma, except *N,N*-dimethylformamide and 20 % piperidine solution in *N,N*-dimethylformamide, which were from Millipore. Polyvinylpyrrolidone (M_r 25000) was a product of Serva. Peptide pGlu-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu was from Peninsula. [γ - ^{32}P]ATP was purchased from Amersham. Phosphorylase kinase and Ca^{2+} /calmodulin-dependent protein kinase II were products of Sigma. All other chemicals were of at least analytical grade.

Lipid vesicles were prepared as described by Papanikolaou et al. [13].

Protein kinases

The catalytic subunit of protein kinase A (PKA) was prepared from pig heart essentially as described by Zoller et al. [14]. The specific activity was 960 units/mg, where 1 unit is defined as the amount of enzyme transferring 1 nmol of phosphate from ATP to mixed histone (1 mg/ml incubation) in 1 min.

PKC was prepared from pig spleen by the method originally described by Parker et al. [15] and modified by Ferrari et al. [16]. The specific activity was 4000 units/mg (1 unit defined as above

Abbreviations used: PKA, protein kinase A; PKC, protein kinase C; PK-CK1, protein kinase CK1 (formerly termed casein kinase I); PK-CK2, protein kinase CK2 (formerly casein kinase II).

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with histone H1 as phosphate acceptor). The enzyme consisted mainly of β -isoform with traces of PKC- α , as assessed by Western blot.

Protein kinases CK1 and CK2 (PK-CK1 and PK-CK2) were prepared from rat liver as described by Meggio et al. [17]. The specific activity was 60 units/mg for PK-CK1 and 115 units/mg for PK-CK2. One unit is defined as the amount of enzyme transferring 1 nmol of phosphate from ATP to casein (2 mg/ml incubation) in 1 min.

The enzymes were not purified to homogeneity but contained no other detectable phosphotransferase activity.

Synthesis of SPOTs peptides

SPOTs-membrane-attached peptides were synthesized utilizing Fmoc chemistry essentially as described in [10] according to the directions of the manufacturer of the SPOTs kit. After completion of the synthesis of the peptides, the membrane was coated with polyvinylpyrrolidone (2 mg/ml in PBS) overnight at room temperature, followed by three washes, 10 min each, with the same buffer. The structures of the peptides were in some cases confirmed by amino acid analysis.

Synthesis of soluble peptides

N-Acetylated C-amidated peptides, Ac-Lys-Arg-Ala-Lys-Arg-Lys-Thr-Thr-Lys-Lys-Arg-NH₂, Ac-Lys-Arg-Ala-Lys-Arg-Lys-Thr-Ala-Lys-Lys-Arg-NH₂, Ac-Phe-Lys-Lys-Ser-Phe-Lys-Leu-NH₂, were synthesized at the Peptide Synthesis Unit, Institute of Medical and Physiological Chemistry, Biomedical Centre, Uppsala University, Uppsala, Sweden using Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry. The product was purified by HPLC (Vydac C₁₈) using an acetonitrile gradient in 0.1 % trifluoroacetic acid/water. The purity of the peptides was confirmed by MS and amino acid analysis.

Phosphorylation of SPOTs peptides

All phosphorylation reactions were carried out at 30 °C. The specific radioactivity of [γ -³²P]ATP was 50–150 c.p.m./pmol. The phosphorylation reactions were initiated by immersing the SPOTs™ membrane pieces into the incubation mixtures, using 100–150 μ l of solution for each peptide spot. Horizontally placed test tubes were rotated around the longitudinal axis at 6 rev./min to ensure appropriate mixing. The reaction mixtures and times were as follows.

For PKA, 55 mM Tris/HCl buffer, pH 7.5, 0.005 % Triton X-100, 5 mM Mes buffer, pH 7.5, 25 μ M EDTA, 0.5 mg/ml BSA, 5 mM MgCl₂, PKA (2 units/peptide spot) and 0.1 mM [γ -³²P]ATP, for 30 min.

For PKC, 45 mM Tris/HCl buffer, pH 7.5, 0.005 % Triton X-100, 0.65 mM calcium acetate, 60 μ g/ml phosphatidylserine, 1 μ g/ml diolein, 0.1 mM EDTA, 0.5 mg/ml BSA, 5 mM MgCl₂, PKC (1.5 units/peptide spot) and 0.1 mM [γ -³²P]ATP, for 1 h.

For PK-CK1 and PK-CK2, 130 mM Tris/HCl buffer, pH 7.5, 0.005 % Triton X-100, 140 mM NaCl, 5.7 % glycerol, 5 mM MgCl₂, PK-CK1 (1 unit/peptide spot) or PK-CK2 (0.5 unit/peptide spot) and 1 vol of 0.1 mM [γ -³²P]ATP, for 2 h. Under these conditions, peptide phosphorylation did not reach a plateau with any of the kinases used.

After incubation, the SPOTs membrane pieces were washed five times with 75 mM H₃PO₄ for 10 min at 4 °C, dried at room temperature and subjected to autoradiography. Exposure times were less than 1 h in all cases and were adjusted according to the specific radioactivity of [γ -³²P]ATP to obtain sufficient resolution in each case.

Phosphorylation of soluble peptides

For PKA, the reactions were carried out in a final volume of 220 μ l of buffer containing substrate peptide at various concentrations, 25 mM Tris/HCl, pH 7.5, 0.0025 % Triton X-100, 5 mM Mes buffer, pH 7.5, 0.025 mM EDTA, 0.5 mg/ml BSA, 9.4 mM MgCl₂, PKA and 0.19 mM [γ -³²P]ATP.

For PKC, the reactions were carried out in a final volume of 225 μ l of buffer containing substrate peptide at various concentrations, 50 mM Tris/HCl, pH 7.5, 0.0025 % Triton X-100, 0.7 mM calcium acetate, 60 μ g/ml phosphatidylserine, 1 μ g/ml diolein, 0.35 mM EDTA, 0.13 mg/ml BSA, 5 mM MgCl₂, PKC and 0.1 mM [γ -³²P]ATP.

For PK-CKI and PK-CKII, the reactions were carried out in a final volume of 280 μ l of buffer containing substrate peptide at various concentrations, 130 mM Tris/HCl, pH 7.5, 0.0015 % Triton X-100, 140 mM NaCl, 6.4 % glycerol, 5.4 mM MgCl₂, PK-CKI or PK-CK2 and 0.11 mM [γ -³²P]ATP.

For phosphorylase kinase, the reactions were carried out in a final volume of 225 μ l of buffer containing substrate peptide at various concentrations, 40 mM Tris/HCl buffer, pH 7.4, 0.001 % Triton X-100, 80 μ M CaCl₂, 0.6 mM dithiothreitol, 1 mg/ml BSA, 9 mM MgCl₂, phosphorylase kinase and 0.15 mM [γ -³²P]ATP.

For Ca²⁺/calmodulin-dependent protein kinase II, the reactions were carried out in a final volume of 110 μ l of buffer containing substrate peptide at various concentrations, 40 mM Tris/HCl, pH 7.5, 0.002 % Triton X-100, 0.32 mM calcium acetate, 12 μ g/ml calmodulin, 4.7 % glycerol, 0.02 mM dithiothreitol, 0.01 mM EDTA, 0.2 mM 2-mercaptoethanol, 0.2 μ M 4-(2-aminoethyl)benzenesulphonyl fluoride, 0.0002 % leupeptin, 5 mM MgCl₂, Ca²⁺/CaM-dependent protein kinase and 0.1 mM [γ -³²P]ATP.

All phosphorylation reactions were carried out at 30 °C. For each peptide, reaction times and enzyme amounts were adjusted so that the initial phosphorylation rates could be measured. The phosphorylation reactions were initiated by the addition of the [γ -³²P]ATP solution (specific radioactivity 50–150 c.p.m./pmol) and monitored by transferring aliquots (30 μ l for PKA, PKC, phosphorylase kinase and Ca²⁺/calmodulin-dependent protein kinase, 40 μ l for PK-CK1 and PK-CK2) of the reaction mixture on to 2 cm $\times$ 2 cm pieces of phosphocellulose paper. The papers were immediately immersed in ice-cold 75 mM phosphoric acid, washed with H₃PO₄ five times for 10 min, dried at 80 °C and the radioactivity was measured as Čerenkov radiation. The reactions with protein substrates were terminated by applying aliquots to Whatman paper and precipitating the proteins with trichloroacetic acid followed by washing in the same solution. The kinetic parameters of the phosphorylation reaction were calculated by non-linear regression analysis using the Graphpad Prism program.

RESULTS

SPOTs peptide design and phosphorylation

Previous studies with peptides derived from different proteins phosphorylated by PKC have resulted in a long list of potential peptide substrates for PKC. On the basis of these data, we chose several peptides with favourable kinetic constants (mostly according to their low K_m values) to be synthesized on SPOTs membrane and qualitatively tested under identical reaction conditions. The sequences and origins of these peptides are shown in Table 1.

All chosen peptide sequences were synthesized using the SPOTs method. For some of them, a corresponding non-phos-

Table 1 Sequences of phosphorylatable sites of selected mammalian PKC substrate proteins

Amino acids not included in SPOTs-membrane-attached peptides are shown in italics. ND, not determined; EGF, epidermal growth factor.

Peptide	Original protein	K_m (μ M)	Reference
GSRRRRRRY	Galline-1 (protamine)	11.6	16
KRPSQRSKYL	Myelin basic protein	15	18
PRRRRRSSRPVR	Thyminine (protamine)	31.5	16
GAGRGLSLRFSWGA	Myelin basic protein	22	19
RTPPPSQGGKR	Myelin basic protein	ND	19
PLSRTLVAACK	Glycogen synthase	4.1	20
AKRRRLSSLRA	Ribosomal protein S6	0.51	20
VRKRTLRL	EGF receptor	1.40	20
KRAKRKTTKKR	Myosin light chain	2	8
RFARKGSLRQKNV	PKC pseudosubstrate	0.2	21
EPHAKKSKISASRKLQKLT	Troponin I	12	22
FDLRGKFKRPTLRRVRIASADA	Troponin I	1	22
MHFGGYIQKAQTERKSGKRQ	Troponin T	10	22
ERKSGKRQTEREKKKILA	Troponin T	45	22
QKVSCTRKGAKVTGRWK	Troponin T	15	22
AYHRSRKRVA	T-cell antigen CD8	38	23
GGRLRRALSVRRG	Glycogen synthase	5.4	24
FKKSFKL-NH ₂	Myristolated alanine-rich C kinase substrate	18	4

Figure 1 Phosphorylation of SPOTs-membrane-attached peptides by PKC

Peptide sequences: 1, GSRRRRRRY; 2, GARRRRRRY; 3, KRPSQRSKYL; 4, KRPAQRAKYL; 5, RRASVA; 6, PRRRRSSRPVR; 7, KRAKRKTTKKR; 8, FARKGSLRQKN; 9, KKKSKISASRKL; 10, RGKFKRPTLRRV; 11, IQKAQTERKSGK; 12, RKGKQTEREK; 13, RGLSLRFSWGA; 14, RTPPPSGRR; 15, RRAPPAGRR; 16, PLSRTLVAACK; 17, AKRRRLSSLRA; 18, VRKRTLRL; 19, TRGKAKVTGRWK; 20, AYHRSRKRVA; 21, VRKTLRL; 22, PLRRALSVRRG; 23, FKKSFKL.

phosphorylatable peptide with alanine(s) replacing serine/threonine was synthesized. In addition, a well-established substrate for PKA (RRASVA) was also synthesized. The SPOTs membrane was phosphorylated with PKC and subjected to autoradiography. The results obtained from this initial screening are shown in Figure 1. It can be seen that the peptides are phosphorylated to different degrees, negative controls (no serine/threonine peptides) clearly showing lower radioactivity than their phosphorylatable counterparts (compare no. 2 with no. 1, no. 4 with no. 3, no. 15 with no. 14). PKC could even phosphorylate RRASVA (minimal peptide phosphorylated by PKA), which is widely used as a specific substrate for PKA, as also are its slightly modified variants LRRASVA and LRRASLG.

The peptides found to be most highly phosphorylated in this first series of substrates were chosen as parent peptides for further modification: no. 3, KRPSQRAKYL, no. 7, KRAKRKTTKKR and no. 21, VRKTLRL. Peptide no. 23 and corresponding background peptide were also included. As Ser-7 in peptide 3 is not phosphorylated [18], it was considered appropriate to replace it with Ala.

Effect of amino acid replacement

In this series, the amino acid adjacent to Ser/Thr was replaced by Pro, Lys, Ala, Glu or Leu, which introduces both positively and negatively charged and large non-polar and small aliphatic amino acid residues, and Pro can also induce steric hindrances. The results of this experiment are shown in Figure 2. The most striking result was that any of these quite radical changes in the immediate vicinity of the phosphorylation centre caused only subtle changes in substrate efficiency. Even if PKC is known to recognize basic amino acids in the substrate peptide as specificity determinants, it tolerated the replacement of Lys with Glu remarkably well, as peptide KRESQRAKYL (no. 5) is only slightly less phosphorylated than KRKSQRAKYL (no. 2) or KRPSQRAKYL (no. 1, the parent peptide in this group). In the third group, VRKTERRL (no. 21) and VRKTKRRL (no. 22) are phosphorylated to approximately the same level. The largest effect observed in this experiment was the decrease in phosphorylation of peptide VRKTPRRL (no. 19). The introduction of Pro at the adjacent position C-terminal of Thr changed the peptide to a very poor substrate, lowering the phosphorylation level to only slightly above background (peptide VRKALRRL, no. 18).

Specificity of peptides for PKC

As the substitutions did not significantly alter the degree of phosphate incorporation into the peptides derived from the original protein structures, these parent peptides were chosen for further investigation. The next study was undertaken to establish the specificity of these peptides as PKC substrates. The next peptide array on SPOTs membrane therefore carried one peptide from each structurally related group of substrates, all accompanied by its respective background peptide where Ala was substituted for phosphorylatable amino acids. When Thr-8 was replaced with an alanine residue (Figure 2, compare no. 8 with no. 10), phosphate incorporation seemed not to be affected, which suggested that Thr-7 was the phosphorylation site. Therefore an analogue of peptide KRAKRKTTKKR containing only one Thr at position 7 was also synthesized. The resulting set of nine SPOTs peptides was tested with both PKC and PKA (Figure 3). The phosphorylation data obtained with PKC (Figure 3a) confirmed the results obtained in previous experiments.

The least favourable of the five substrates tested was VRKTKRRL, which showed markedly lower reactivity than the others, as suggested also by the previous experiment. Peptides devoid of Ser/Thr residues showed no phosphate incorporation. No significant difference in the degree of phosphorylation was observed for KRAKRKTAKKR and KRAKRKTTKKR which confirmed the hypothesis of only one phosphorylation site in this peptide.

It was observed that several of the PKC substrates tested were weakly phosphorylated even by PKA (Figure 3b). In fact, the only substrate that was not even marginally phosphorylated by this kinase was KRAKRKTAKKR. No non-specifically bound [³²P]phosphate or [γ -³²P]ATP was attached to any of the background peptides after the incubation of PKA.

Figure 2 Phosphorylation of selected PKC substrate variants on SPOTs membrane

(a) Autoradiograph of the membrane; (b) peptide sequences.

Figure 3 Phosphorylation of PKC substrates and respective background peptides

Two identical arrays of 3 × 3 spots were phosphorylated with PKC (a) and PKA (b). Amino acid sequences of the peptides: A1, KRKSQRAKYL; A2, KRKAQRAKYL; A3, KRAKRKTAKKR; B1, VRKTKRRL; B2, VRKAQRRL; B3, KRAKRKT TTKKR; C1, FKKSFKL; C2, FKKAFKL; C3, KRAKRKA AKKR.

Figure 4 Phosphorylation of PKC substrates

Two identical arrays were phosphorylated with PK-CK1 (a) and PK-CK2 (b). Amino acid sequences: A1, KRKSQRAKYL; A2, KRKAQRAKYL; B1, FKKSFKL; B2, FKKAFKL; C1, KRAKRKTAKKR; C2, KRAKRKA AKKR.

A similar study was carried out with PK-CK1 and PK-CK2 (formerly known as casein kinases I and II) which generally display requirements for acidic amino acids in the vicinity of Ser/Thr. As shown in Figure 4, no phosphorylation of any of the peptides occurred. After prolonged exposure of the SPOT's membrane on the autoradiography film, non-specific binding of

Table 2 Kinetic constants for PKC-catalysed phosphorylation of synthetic peptides

Results are means ± S.E.

Peptide	K_m (μ M)	V_{max} (nmol/min per mg)	$10^3 \times V_{max}/K_m$
Ac-FKKSFKL-NH ₂	28.6 ± 15.2	0.24 ± 0.04	8.4
pEKRPSQRSKYL	29.3 ± 6.5	15.6 ± 1.3	532
Ac-KRAKRKT TTKKR-NH ₂	1.8 ± 0.6	16.3 ± 1.9	9056
Ac-KRAKRKTAKKR-NH ₂	0.49 ± 0.13	10.0 ± 0.5	20408

[³²P]ATP to the spots was registered, which was at the same level for both the background peptide and the corresponding substrate in all cases. This result indicates that none of these peptides is a substrate for either PK-CK1 or PK-CK2.

KRAKRKTAKKR therefore appears to be both the most reactive and the most specific peptide substrate for PKC, according to the qualitative results obtained by the SPOTs method.

Kinetic constants

On the basis of the results from the SPOTs peptide experiments, we decided to synthesize soluble peptides KRAKRKTAKKR, KRAKRKT TTKKR and FKKSFKL and compare them with the widely used PKC substrate pEKRPSQRSKYL (Nishizuka peptide) which is commercially available from several companies. Peptide KRKSQRAKYL, tested by the SPOTs method and found to be an effective substrate (see Figure 3), possesses a considerable degree of similarity to the Nishizuka peptide. Replacement of Ser-8 with Ala and truncation at the N-terminus of the Nishizuka peptide are shown to influence the kinetic constants of the soluble peptide only marginally [17]. The synthesis of soluble KRKSQRAKYL was therefore considered to be unnecessary, as it is highly analogous to the Nishizuka peptide.

The peptides were analysed in solution as substrates for PKC over a wide range of concentrations. Table 2 summarizes the kinetic constants calculated for these four peptides measured with PKC. All the peptides followed Michaelis–

Table 3 Activity of various protein kinases towards synthetic soluble peptides

Peptide concentrations were at least $4 \times K_m$. Standard substrates were histone H1 for PKC, mixed histone for PKA, casein for PK-CK1 and PK-CK2, phosphorylase *b* for phosphorylase kinase and myelin basic protein for Ca^{2+} /calmodulin-dependent protein kinase. n.d., not detectable.

	Amount of attached phosphate (pmol/min per ml)				
	KRAKRKTAKKR	KRAKRKTTKKR	pEKRPSQRSKYL	FKKSFKL	Standard substrate
PKC	221	397	403	5.4	208
PKA	n.d.	n.d.	31.7	0.7	332
PK-CK1	n.d.	n.d.	n.d.	n.d.	710
PK-CK2	n.d.	n.d.	n.d.	n.d.	603
Phosphorylase kinase	1.2	3.4	4.4	1.5	267
Ca^{2+} /calmodulin-dependent protein kinase	n.d.	—*	—*	—*	120

* These peptides were not tested with Ca^{2+} /calmodulin-dependent protein kinase.

Menten kinetics. Peptide KRAKRKTAKKR showed the highest affinity for PKC, exhibiting a K_m approximately 60-fold lower than that of the commercially available pEKRPSQRSKYL. In spite of a 1.6-fold higher $V_{\max}$ for the latter peptide compared with KRAKRKTAKKR, the $V_{\max}/K_m$ ratio for the KRAKRKTAKKR is 38-fold higher than for pEKRPSQRSKYL, which makes it the best peptide substrate for PKC in this study. Although the substitution of Ala-8 by Thr-8 in KRAKRKTAKKR increases the $V_{\max}$ 1.6-fold, the $V_{\max}/K_m$ ratio decreases as a result of the increase in K_m , making it a less effective substrate.

To verify the specificity of the peptides as substrates for PKC, all the conventionally synthesized soluble peptides were tested with PKA, PK-CK1, PK-CK2, phosphorylase kinase and Ca^{2+} /calmodulin-dependent protein kinase II (Table 3). PK-CK1 and PK-CK2 did not phosphorylate any of these peptides whereas the standard substrate, casein, was phosphorylated under the same conditions. pEKRPSQRSKYL and FKKSFKL were phosphorylated by PKA at a level considerably higher than background (pEKRPSQRSKYL to 5% and FKKSFKL to 8% of the respective phosphate incorporation achieved with PKC under the same conditions). As for KRAKRKTTKKR and KRAKRKTAKKR, no phosphorylation by PKA of either was observed under the same reaction conditions at peptide concentrations 7.6–10-fold higher than the respective K_m values for PKC. However, when reactions were carried out at still higher peptide concentrations, there was significantly higher [^{32}P]phosphate incorporation into KRAKRKTTKKR than into KRAKRKTAKKR (Figure 5). It should also be mentioned that KRAKRKTTKKR showed approximately twice as high a background level as KRAKRKTAKKR irrespective of the kinase used for the test (even for PK-CK1 and PK-CK2) (results not shown).

The peptides were also tested with two other protein kinases with broad substrate specificity which require basic amino acids as substrate determinants, Ca^{2+} /calmodulin-dependent protein kinase II and phosphorylase kinase. The former did not phosphorylate peptide KRAKRKTAKKR under the conditions used (Table 3). The only protein kinase that exhibited some activity with the peptides was phosphorylase kinase, the phosphorylation level being low with all the peptides tested. The lowest activity was shown against KRAKRKTAKKR which amounted to 0.4% of the activity obtained with PKC under the same conditions.

Figure 5 Time course of phosphorylation of PKC substrates by PKA

□, ■, 123 μM KRAKRKTAKKR; ○, ●, 119 μM KRAKRKTTKKR. Open symbols, in the presence of the enzyme; closed symbols, in the absence of the enzyme.

DISCUSSION

A considerable number of proteins have been shown to be phosphorylated by PKC *in vivo*. Synthetic peptides derived from the primary sequence around the phosphorylation sites of these proteins are considered to be high-affinity substrates for PKC. In addition, cationic/basic proteins, such as histone H1, are routinely used to assay conventional PKC isoenzymes. However, there exist several other protein kinases that recognize basic residues as specificity determinants, therefore one needs to ensure that the observed phosphorylation actually originates from PKC activity. Several synthetic peptides that have been shown to be favourable substrates for PKC are phosphorylated by multiple protein kinases [6,7], although less effectively than by PKC.

Both we [12] and other groups [11] have shown that peptides that are bound covalently to cellulose membranes can serve

directly as kinase substrates and may be used for investigating substrate specificity of protein kinases. We therefore used this method (SPOTs synthesis) to compare several peptides that have previously been reported to be good substrates for PKC (Table 1). In spite of similar low K_m values reported for these peptides, they were phosphorylated to different degrees under identical reaction conditions (Figure 1). Modifications in the immediate vicinity of the phosphorylation centre caused only marginal changes in the reactivity of the peptides (Figure 2). The most sensitive substrate within a structurally related group of peptides was then resynthesized to examine the specificity of these peptides with PKA, PK-CK1 and PK-CK2. A certain amount of cross-reactivity with PKA was observed for some (Figure 3).

The most favourable characteristics on SPOTs membrane were demonstrated by KRAKRKTAKKR, which was both sensitive and the most selective of the peptides. Kinetic data from the experiments with the corresponding soluble peptides (Table 2) confirmed the results obtained by the SPOTs method. Compared with other peptides tested in the present study, KRAKRKTAKKR, which is derived from the N-terminal sequence of the smooth-muscle myosin light chain (residues 3–13, where Ala-7 is replaced by Arg and Thr-10 by Ala), was the most potent and selective substrate for PKC.

Peptide FKKSFKL has been used with good results for direct measurement of membrane-bound PKC activity [4], for following the partial purification of PKC from A7r5 VSMC cells [5] and in PKC assays [25,26]. The K_m value reported for this peptide (26 μ M [5]) is very similar to that obtained by us (28.6 μ M; Table 2). However, because of the low V_{max} value (Table 2), it is inferior to KRAKRKTAKKR.

Peptide pEKRPSQRSKYL (Nishizuka peptide) is often used for measuring PKC activity, as it was reported to be a very specific substrate for the conventional PKC isozymes (PKC- α , - β , - γ) [9]. It exhibited a K_m value quite similar to that of FKKSFKL in our hands, but exhibited a considerably higher V_{max} value (Table 2).

Compared with these quite widely used PKC substrate peptides, KRAKRKTAKKR demonstrated more favourable substrate characteristics. None of the other protein kinases that recognize basic residues as specificity determinants in the phosphorylation sequence demonstrated significant reactivity with this peptide. Only Thr-7 is phosphorylated in the intact myosin light chain [27] (amino acid no. 9 in the complete intact protein chain) by PKC, which seems also to be the case for the synthetic peptide. The presence of Thr-8 in the peptide was not required for optimum kinetics, as demonstrated by the more than 2-fold increase in V_{max}/K_m value when Thr-8 was replaced by Ala. We cannot explain the discrepancy between our data and those of Hassell et al. [8], where the presence of Thr-8 was found to promote the phosphorylation of Thr-7, as a slightly modified peptide compared with the one described here (KRAKAKTAKKR) with only one Thr became a relatively poor substrate for PKC compared with a Thr-Thr counterpart. The explanation might, of course, lie in the different origins of the enzyme as well as in different isoenzyme compositions.

Furthermore, even if KRAKRKTAKKR was a poorer substrate for PKA than both FKKSFKL and pEKRPSQRSKYL, the substitution Thr-8 $\rightarrow$ Ala decreased the cross-reactivity with

PKA even further (Figure 3). Together with the absence of substrate inhibition for PKC at concentrations up to $300 \times K_m$, this feature allows KRAKRKTAKKR to be used at high substrate concentrations when necessary. Interpretation of data obtained using KRAKRKTAKKR should be easier, since this peptide contains only one potential phosphorylation site, which is also an advantage for analysis of cell extracts (e.g. during PKC purification).

Moreover, this peptide contains eight positively charged amino acids, which ensures efficient binding to phosphocellulose paper, which is still the most widely used direct method for PKC assays.

In conclusion, the favourable kinetic parameters combined with the selectivity should facilitate the use of peptide KRAKRKTAKKR as a substrate for PKC in the assays of both the purified enzyme and in crude cell extracts.

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