The specificity of the protein kinase C α , β II and γ isoforms as assessed by an unnatural alcohol-appended peptide library

Xiongwei YAN¹, Kieran CURLEY¹ and David S. LAWRENCE²

Department of Biochemistry, The Albert Einstein College of Medicine of Yeshiva University, 1300 Morris Park Ave., Bronx, New York, NY 10461, U.S.A.

Previous studies using conventional peptide-based libraries have demonstrated that homologous protein-processing enzymes [e.g. the α , β II and γ isoforms of protein kinase (PKC)] typically display identical amino acid consensus sequences. These observations have hampered the acquisition of selective synthetic substrates for the individual members of these enzyme families. We describe here a parallel synthesis strategy, readily adaptable to the preparation of large libraries, that has led to the emergence of the first examples of selective substrates for the conventional PKC isoforms. In addition, we have found that a wide variety of

structurally diverse N-appended alcohol-containing residues, including tyrosine, serve as substrates for the PKC α , β II and γ isoforms. This broad active-site substrate specificity with respect to both natural and unnatural residues may prove to be especially applicable to the construction of transition-state analogues and suicide substrates, species that often require the presence of structurally elaborate functionality.

Key words: combinatorial library, protein kinase specificity, unnatural protein kinase substrates.

INTRODUCTION

Given the key role that protein kinases play in the general area of signal transduction, it is not at all surprising that members of this enzyme family have been the focus of a wide variety of structural and enzymological studies. The elucidation of structural motifs and substrate preferences sets the stage for the design of protein kinase inhibitors, species that may ultimately be elaborated into therapeutic agents. Initial protein kinase sequence-specificity studies utilized sequences identified from known protein substrates [1,2]. Peptides containing these sequences, as well as closely related single amino acid substitutions, were used to obtain consensus sequence motifs for members of the protein kinase family. More recently, protein kinase sequence selectivity has been examined via combinatorial peptide libraries [3–6]. These libraries, prepared by the split-and-pool approach, allow one to rapidly identify a consensus sequence, via protein kinase-catalysed phosphorylation of the peptide library and subsequent sequence analysis. Peptide-based protein kinase inhibitors have been identified in this fashion as well [6,7]. In general, the results obtained from combinatorial libraries are consistent with those of earlier studies that relied upon sequences obtained from confirmed protein substrates. Not surprisingly, subfamilies of structurally homologous protein kinases exhibit very similar sequence specificities toward conventional peptide substrates [3].

Libraries of compounds that target the ATP-binding site of protein kinases have also been described [8]. These small-molecule libraries are characterized by a high degree of structural diversity focused within a narrow spatial window. In contrast, although peptide-based libraries are often composed of a large number of different peptidic species, the 'local' diversity at each site along the peptide chain is typically limited to a comparatively few (i.e. about 20) amino acid residues.

We recently found that it is possible to distinguish between highly homologous proteins that exhibit identical affinities for the same peptide ligand by modifying the ligand with non-amino acid substituents. For example, peptide/non-peptide hybrids have been constructed that discriminate between the Src homology 2 (SH2) domains of the two closely related tyrosine-specific protein kinases, Lck and Fyn [9]. In an analogous vein, peptide-based species that are able to differentiate between the cAMP-dependent protein kinase (PKA), the cGMP-dependent protein kinase (PKG) and protein kinase C (PKC) have been constructed using non-naturally-occurring substituents [10,11]. The latter species were subsequently elaborated into both reversible [12] and irreversible [13] inhibitors that distinguish between PKA and PKG.

An analysis of the active-site substrate specificity of protein kinases serves as a probe of active-site structure and provides an assessment of which functionality can and cannot be accommodated within the active-site region. Such an analysis affords the necessary structural foundation required for the design of inhibitors containing elaborate functional groups, which are often present in transition-state analogues and suicide substrates. An active-site-specificity analysis for protein-processing enzymes (e.g. protein kinases) employs compounds that are typically synthesized via the methodical stepwise incorporation of active-site-directed amino acid analogues (e.g. serine) into an enzyme-targeted peptide. We describe here an N-terminally linked peptide alcohol library, synthesized in parallel, whose members serve as protein kinase substrates. This spatially focused active-site-directed library is composed of a diverse array of alcohol-containing functionality. Unlike conventional peptide libraries, which are typically limited to a comparatively few naturally occurring amino acid residues at each position, the parallel synthesis approach described herein can readily encompass all known and synthetically accessible hydroxy acids.

Abbreviations: t-BDMS, t-butyldimethylsilyl; DAG, diacylglycerol; DMF, dimethylformamide; Fmoc, fluorenylmethoxycarbonyl; PS, phosphatidylserine; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulphonyl; SH2, Src homology 2; Boc, t-butoxycarbonyl.

¹ These authors contributed equally to this work.

² To whom correspondence should be addressed (e-mail dlawrenc@aecom.yu.edu).

The initial focus of our library analysis was the conventional isoforms of protein kinase C [14,15]. The PKC family has attracted considerable interest due to the apparent key roles played by these enzymes in a wide variety of biological phenomena, including memory [16], fertilization [17], carcinogenesis [18] and various cardiovascular, central-nervous-system, immune and infectious disease states [19]. At least 12 isoforms of PKC have been reported, and these have been partitioned into separate subfamilies (conventional, novel and atypical) based upon their mode of regulation. We describe here the ability of members of the conventional subfamily (α , β II and γ) to catalyse the phosphorylation of an array of structurally diverse alcohol-containing residues.

EXPERIMENTAL

General

All chemicals were obtained from Aldrich, except for [γ - 32 P]ATP (DuPont NEN), BSA (Sigma), protected amino acid derivatives and Rink resin (Advanced ChemTech, Louisville, KY, U.S.A.) and Liquiscint (National Diagnostics, Atlanta, GA, U.S.A.). The α , β II and γ isoforms of PKC were purchased from Panvera (Madison, WI, U.S.A.).

Synthesis

Preparation of NH_2 -Phe-[Arg(Mtr)]₄-Lys(Boc)-Rink resin (where Mtr is 4-methoxy-2,3,6-trimethylbenzenesulphonyl and Boc is t-butoxycarbonyl)

A standard fluoren-9-ylmethoxycarbonyl (Fmoc)/benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate peptide synthesis protocol was employed to synthesize this peptide on the Rink resin via an automated peptide synthesizer. The attachment for each amino acid was performed according to the following program (on a 2 g-of-resin scale): (a) 3×30 ml of dichloromethane; (b) 1×20 ml of 30% (v/v) piperidine in dichloromethane (1 min); (c) 1×30 ml of 30% piperidine in dichloromethane (20 min); (d) 2×30 ml of dichloromethane; (e) 1×30 ml of propan-2-ol; (f) 3×30 ml of dichloromethane; (g) 1×30 ml of 4 equiv. of *N*-methylmorpholine in dichloromethane; (h) 3 equiv. of Fmoc-protected amino acid, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate, hydroxybenzotriazole and 6 equiv. of *N*-methylmorpholine in 30 ml of dichloromethane/dimethylformamide (DMF) (1:1, v/v) (60–90 min) (the coupling time was 90 min for all the arginine residues, and 60 min for the other residues); (i) 3×30 ml of dichloromethane; (j) 3×30 ml of 33% (v/v) ethanol in dichloromethane; (k) 2×30 ml of dichloromethane. After completion of the desired sequence, the Fmoc group was removed with 30 ml of 30% piperidine (30 min). The resin was then transferred to a fritted-glass funnel with DMF/dichloromethane, washed with DMF, dichloromethane and methanol, and then dried *in vacuo*. The resultant NH_2 -Phe-[Arg(Mtr)]₄-Lys(Boc)-Rink resin was then subsequently condensed, at the N-terminus, with a variety of hydroxycarboxylic acids.

Preparation of peptides $\text{CH}_3\text{CONH-SFRRRRK-amide}$ (**1**), $\text{CH}_3\text{CONH-(D-)SFRRRRK-amide}$ (**2**), $\text{CH}_3\text{CONH-TFRRRRK-amide}$ (**3**), $\text{CH}_3\text{CONH-(D-)TFRRRRK-amide}$ (**4**) and $\text{CH}_3\text{CONH-YFRRRRK-amide}$ (**24**)

A 100 mg portion of $\text{H}_2\text{N-Phe-[Arg(Mtr)]}_4\text{-Lys(Boc)-Rink resin}$ (0.03 mmol substitution level) was suspended in 1 ml of dichloromethane/DMF (1:1, v/v). A 0.3 mmol portion of Fmoc-Ser-OH [Fmoc-(D-)Ser-OH, Fmoc-Thr-OH, Fmoc-(D-)Thr-OH or Fmoc-

Tyr-OH], benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate, hydroxybenzotriazole and *N*-methylmorpholine were added. The suspension was shaken at room temperature for 2 h. The resin was filtered and the solid residue washed with excess DMF and dichloromethane. The Fmoc group was then removed with 10 ml of 30% piperidine in dichloromethane (20 min). The resin was washed with excess DMF and dichloromethane, and was acetylated with acetic anhydride. The resin was washed with excess DMF, dichloromethane and methanol, and dried *in vacuo*. The peptides were then cleaved from the resin and subsequently purified (see below) to give compounds that furnished satisfactory fast-atom-bombardment mass-spectral analyses.

General protocol for the preparation of the alcohol-appended library (peptides **5** and **7–24**)

A 100 mg portion of NH_2 -Phe-[Arg(Mtr)]₄-Lys(Boc)-Rink resin (0.03 mmol substitution level) was suspended in 1 ml of dichloromethane/DMF (1:1, v/v) and then 0.3 mmol of the hydroxycarboxylic acid (the aliphatic alcohols were first protected as the butyldimethylsilyl derivatives; see below), *N*-methylmorpholine, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate and hydroxybenzotriazole were added. The mixture was shaken at room temperature until the peptide was fully modified (typically 2 h). The peptide-resin conjugates were then separately filtered through a fritted-glass funnel and the peptide-Rink resin was sequentially washed with excess DMF, dichloromethane, methanol and anhydrous diethyl ether, and then dried *in vacuo*. The substituted peptides were subsequently cleaved from the resin and purified (see below) to provide compounds that gave satisfactory fast-atom-bombardment mass-spectral analysis.

Preparation of $\text{HOCH}_2\text{CH}_2\text{CONH-FRRRRK-amide}$ (**6**)

This was prepared as described in the general protocol above, except that β -propiolactone was employed in place of the corresponding hydroxy acid.

General procedure for the preparation of t-butyldimethylsilyl-*O*-(CH_2)_{*n*}COOH

The alcohol moieties of the hydroxyalkane carboxylic acids were protected as a t-butyldimethylsilyl ether prior to condensation with the N-terminus of NH_2 -Phe-[Arg(Mtr)]₄-Lys(Boc)-Rink resin. A 5.6 mmol portion of $\text{HO-CH}_2(\text{CH}_2)_{n-1}\text{CH}_2\text{-OH}$ ($n = 4-6$) was dissolved in 11 ml of tetrahydrofuran, followed by addition of 0.27 g of NaH. The reaction mixture was stirred at room temperature for 45 min, and then 0.84 g of t-butyldimethylsilyl chloride (t-BDMS-Cl, 5.6 mmol) was introduced and stirred at room temperature for another 45 min. The reaction mixture was then poured into 100 ml of diethyl ether, and washed twice with aq. 10% K_2CO_3 and NaCl (30 ml each), and finally dried with anhydrous Na_2SO_4 . The solvent was evaporated and the mono-protected $\text{tBu}(\text{CH}_3)_2\text{Si-O-CH}_2(\text{CH}_2)_{n-1}\text{CH}_2\text{-OH}$ (where tBu is t-butyl) dissolved in 13 ml of DMF and directly oxidized with 6.6 g of pyridinium dichromate (17.5 mmol) at room temperature for 3 h. The mixture was poured into 100 ml of water and extracted with chloroform, and dried over anhydrous Na_2SO_4 . The $\text{tBu}(\text{CH}_3)_2\text{Si-O-CH}_2(\text{CH}_2)_{n-1}\text{COOH}$ was then purified via silica-gel chromatography to provide the desired compounds in yields generally higher than 60%. All compounds gave satisfactory NMR and mass-spectral analyses.

Cleavage and deprotection of peptide-Rink resins

The peptide-Rink resin (≈ 100 mg) was transferred to a small reaction vessel containing 1 ml of a trifluoroacetic acid/thioanisole (9:1, v/v) mixture. The reaction vessel was then shaken at room temperature for 10 h. The resin was filtered, under reduced pressure, and then washed twice with trifluoroacetic acid. The filtrates were then combined (≈ 5 ml) and an 8–10-fold volume of cold anhydrous diethyl ether (≈ 50 ml) was added in a dropwise fashion. The mixture was kept at 4 °C for at least 1 h. The precipitated peptide was then collected via filtration through a fine sintered-glass filter funnel under a light vacuum. The precipitate was then washed with cold anhydrous diethyl ether (2 $\times$ 5 ml), dissolved in 10 ml of water, and finally freeze-dried to provide the crude peptide.

General protocol for peptide purification

Ion-exchange chromatography was performed on sulphopropyl-Sephadex CM C-25 (1.5 cm $\times$ 25 cm bed) equilibrated in 50 mM sodium acetate, pH 3.5. Peptides were eluted by applying a 400 ml KCl gradient (0.6–1.5 M KCl). The peptides were then partially desalted with methanol and further purified on a preparative HPLC apparatus using three Waters radial compression modules (25 cm $\times$ 10 cm) connected in series [gradient A, 0.1% trifluoroacetic acid in water; solvent B, 0.1% trifluoroacetic acid in acetonitrile, 0–3 min (100% A); a linear gradient from 3 to 20 min (75% A and 25% B); a steep final linear gradient to 90% B for cleaning purposes].

PKC assay

Assays were performed in triplicate at pH 7.5 and thermostatically controlled in a water bath maintained at 30 °C. The final assay volume was 40 μ l and contained 62.5 mM Hepes, 0.75 mM CaCl_2 , 12.5 mM MgCl_2 , 0.5 mM EGTA, 7.5 μ g/ml phosphatidylserine (PS), 1.6 μ g/ml diacylglycerol (DAG) and between 0.10 and 30 ng of PKC, depending upon the efficacy of the substrate. For the determination of the kinetic constants the following concentrations were employed: 50 μ M [γ - ^{32}P]ATP (750–4000 c.p.m./pmol) and a substrate concentration that varied over a 10-fold range around the apparent K_m . Phosphorylation reactions were initiated by the addition of 8 μ l of PKC from a stock solution (20 mM Tris/HCl/1 mM dithiothreitol/1 mM EDTA/0.75 mg/ml BSA, pH 7.5). Reactions were terminated after 5–20 min, depending upon the efficacy of the substrate, by spotting 25 μ l aliquots on to 2.1-cm-diameter phosphocellulose paper discs. After 10 s, the discs were immersed in 10% (v/v) acetic acid and allowed to soak with occasional stirring for at least 1 h. The acetic acid was decanted off, and the discs were collectively washed with 4 vol. of 0.5% H_3PO_4 , 1 vol. of water, followed by a final acetone rinse. The disks were air-dried and then placed in plastic scintillation vials containing 6 ml of Liquiscint prior to counting for radioactivity.

RESULTS AND DISCUSSION

A peptide library containing a diverse structural array of serine mimetics can be rapidly prepared via the N-terminal acylation of an active-site-directed peptide (Figure 1). This approach has four attributes. First, a wide variety of alcohol-bearing carboxylic acids are commercially available, and an even wider variety are synthetically accessible in a few steps. Secondly, an almost

unlimited array of carboxylic acids containing non-phosphorylatable, and therefore potentially inhibitory, functionality, are available as well. Thirdly, the vast majority of carboxylate moieties can be activated with standard coupling reagents and therefore efficiently coupled to the nucleophilic N-terminus of a peptide. Finally, the carboxylic acid functionality is compatible with a large assortment of other functional groups.

The conventional PKC isoforms recognize and phosphorylate peptides containing serine either upstream or downstream from positively charged residues [20]. On the basis of the general consensus sequence of Lys/Arg_{1–3}Xaa_{0–2}Ser/ThrXaa_{0–2}Arg/Lys_{1–3}, in combination with the observation that a P + 1 phenylalanine is beneficial in terms of substrate efficacy [21], we designed the parent peptide acetyl-Ser-Phe-Arg-Arg-Arg-Arg-Lys-amide (**1**). The C-terminal lysine residue was incorporated to allow ready determination of peptide concentration via the quantitative ninhydrin method [22]. As is evident from Table 1, peptide **1** is an outstanding substrate for the α -, β II- and γ -isoforms of PKC. The side-chain protected peptide, Fmoc-Phe-Arg-Arg-Arg-Lys-amide-resin (**1**), was prepared via conventional solid-phase peptide synthesis and then separately condensed with an assortment of alcohol-containing carboxylates using a standard coupling methodology. For the purposes of this study, we decided to cleave the peptides from the resin support in order to investigate, in solution, their ability to serve as PKC substrates. Clearly, an alternative possibility is to simply employ the peptide library in its resin-bound form. The latter would merely require the use of a robust linkage between the peptide and the resin (e.g. the standard Merrifield resin), so that the side-chain protecting groups can be removed without cleaving the peptide-resin bond.

PKC catalyses the phosphorylation of D-serine as well as configurationally related analogues in conventional and C-terminally derivatized peptides [11,23]. This pattern holds for N-terminus-appended serine residue as well. The V_{max} for the D-serine-containing peptide (**2**) is close to that of its L-serine-based counterpart **1** for all three PKC isoforms (Table 1). In addition, the K_m associated with the phosphorylation of **2** is only modestly larger than that exhibited with **1**. In short, the α -, β II- and γ -isoforms of PKC are excellent D-serine kinases. PKC also phosphorylates threonine at the N-terminus of an active-site-directed peptide. The L-threonine-containing peptide (**3**) is phosphorylated by all three PKC isoforms virtually as efficiently as its L-serine-based counterpart (**1**). However, inversion of both the α - and β -configurations produces a markedly weaker substrate (i.e. **4**), in terms of both K_m and V_{max} .

PKC catalyses the phosphorylation of N-linked achiral alcohols. A wide variety of aliphatic hydroxy acids are commercially available, which provides the opportunity to explore whether differences exist in the ability of the α -, β II- and γ -isoforms of PKC to recognize unnatural serine/threonine mimetics. Peptide **5** contains an alcohol functionality positioned one carbon atom closer to the adjacent peptide backbone than its counterparts in serine and threonine. However, **5** is notable for a reason other than its ability to act as a PKC substrate. The starting carboxylic acid (HOCH_2COOH) is readily available from commercial sources. In contrast, the precursor for the corresponding serine derivative, namely α -hydroxyglycine, is an unstable hemiaminal. In short, the structural diversity accessible with N-terminally appended hydroxy acids is significantly greater than that possible with conventional amino acids positioned within the peptide chain.

The hydroxypropionic acid in **6** is the achiral analogue of serine. Since the alcohol in this residue is positioned, relative to the adjacent amide bond, in a fashion identical with that in serine, it is reassuring that **6** is a more efficient substrate than **5**

Figure 1 Synthesis and structure of the N-terminally derivatized peptide library

N-terminally derivatized peptides are synthesized on a solid support via standard methodology, and, while still on the solid support, condensed with an array of activated aliphatic and aromatic carboxylic acids. Peptides were then cleaved from the resin and the side-chain protecting groups removed.

for all three PKC isoforms. Residues containing side chains longer than the hydroxyethyl moiety in **6** also serve as PKC substrates. Although peptides **7** and **8** are substrates for all three PKC isoforms, PKC γ appears to be the most promiscuous in that it phosphorylates **6**, **7** and **8** with equal efficacy. In contrast, the α and β isoforms exhibit a definite preference for peptide **6**. In terms of $V_{\max}/K_m$, peptide **8** is a 15-fold better substrate for PKC γ than for PKC α and nearly 90-fold better than for PKC β , an unexpected observation given the presumed close structural similarity of these isoforms. Peptides **9** and **10** are also phosphorylated by the three PKC isoforms, but less efficiently than their shorter-chain-length counterparts contained within peptides **6–8**.

In addition to threonine (peptide **3**) and its doubly configurationally inverted analogue (peptide **4**), PKC α , PKC β and PKC γ catalyse the phosphorylation of other secondary alcohols, including **11** and **12** (Table 1). The stereochemistry of the former corresponds to that found at the β -centre in L-threonine. This peptide exhibits a $V_{\max}$, with all three PKC isoforms, similar to those displayed by peptide **6**, which contains the achiral hydroxyethyl side chain. The major difference between the secondary alcohol contained in **11** and the primary alcohol housed in **6** is the K_m term, which is an order of magnitude higher for **11**. The

corresponding L-serine- and L-threonine-containing peptides, **1** and **3**, do not exhibit any significant differences in K_m as PKC substrates. Inversion of the β -configuration in **11** generates a somewhat weaker PKC substrate (i.e. **12**) that, although it exhibits a lower K_m , displays a substantially reduced $V_{\max}$ compared with that of **11**. The lower K_m is particularly pronounced with the β and γ isoforms. Finally, the α , β and γ isoforms of PKC also phosphorylate the tertiary alcohol contained within **13**.

We have previously reported that serine/threonine-specific protein kinases will recognize appropriately substituted phenols as substrates [24,25]. For example, both PKA and PKG catalyse the phosphorylation of a C-appended *ortho*-derivatized phenol, but not that of the corresponding *meta* and *para* derivatives [25]. In contrast, PKC not only recognizes all three N-terminus substituted phenols as substrates, but the α and γ isoforms actually prefer the *para* substitution pattern (Table 1). In the case of PKC γ , $V_{\max}/K_m$ for **16** is 30-fold greater than that of **14** and 50-fold greater than that of **15**. The preference for the *para*-substitution pattern is surprising, given the fact that the hydroxyl moiety in the *ortho* position exhibits a greater structural similarity (in terms of both distance and orientation to the amide backbone) to that found in serine [26].

Table 1 K_m and V_{max} for the PKC α -, PKC β - and PKC γ -catalysed phosphorylation of substrates 1–24

Peptide no.	PKC ... Parameter ...	PKC α		PKC β		PKC γ	
		K_m (μ M)	V_{max} (μ mol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$)	K_m (μ M)	V_{max} (μ mol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$)	K_m (μ M)	V_{max} (μ mol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$)
1		10 $\pm$ 2	24 $\pm$ 2	8.0 $\pm$ 0.9	8.0 $\pm$ 0.3	2.1 $\pm$ 0.3	8.0 $\pm$ 0.3
2		49 $\pm$ 5	17 $\pm$ 1	37 $\pm$ 4	4.9 $\pm$ 0.2	7.6 $\pm$ 0.4	11 $\pm$ 1
3		11 $\pm$ 3	15 $\pm$ 1	23 $\pm$ 2	5.0 $\pm$ 0.1	4.7 $\pm$ 0.6	5.5 $\pm$ 0.2
4		113 $\pm$ 20	(14 $\pm$ 1) $\times 10^{-3}$	110 $\pm$ 30	(9.9 $\pm$ 0.8) $\times 10^{-3}$	83 $\pm$ 10	(10 $\pm$ 1) $\times 10^{-3}$
5		114 $\pm$ 3	(47 $\pm$ 1) $\times 10^{-3}$	64 $\pm$ 10	(15 $\pm$ 1) $\times 10^{-3}$	73 $\pm$ 6	(20 $\pm$ 1) $\times 10^{-3}$
6		33 $\pm$ 1	1.3 $\pm$ 0.1	23 $\pm$ 1	0.32 $\pm$ 0.01	17 $\pm$ 1	1.6 $\pm$ 0.1
7		61 $\pm$ 4	1.9 $\pm$ 0.1	110 $\pm$ 5	0.30 $\pm$ 0.01	20 $\pm$ 3	1.6 $\pm$ 0.1
8		43 $\pm$ 2	0.32 $\pm$ 0.01	78 $\pm$ 12	0.10 $\pm$ 0.01	19 $\pm$ 2	2.2 $\pm$ 0.1
9		77 $\pm$ 15	(35 $\pm$ 3) $\times 10^{-3}$	65 $\pm$ 3	(19 $\pm$ 1) $\times 10^{-3}$	97 $\pm$ 13	(55 $\pm$ 3) $\times 10^{-3}$
10		24 $\pm$ 2	(10 $\pm$ 1) $\times 10^{-3}$	27 $\pm$ 2	(6.7 $\pm$ 0.1) $\times 10^{-3}$	7.7 $\pm$ 0.9	(16 $\pm$ 1) $\times 10^{-3}$
11		342 $\pm$ 46	0.89 $\pm$ 0.04	272 $\pm$ 23	1.1 $\pm$ 0.1	312 $\pm$ 5	1.6 $\pm$ 0.1
12		146 $\pm$ 9	(40 $\pm$ 2) $\times 10^{-3}$	17 $\pm$ 1	(4.9 $\pm$ 0.1) $\times 10^{-3}$	18 $\pm$ 2	(36 $\pm$ 1) $\times 10^{-3}$
13		128 $\pm$ 7	(8.7 $\pm$ 0.1) $\times 10^{-3}$	539 $\pm$ 57	(47 $\pm$ 5) $\times 10^{-3}$	516 $\pm$ 23	(22 $\pm$ 1) $\times 10^{-3}$
14		147 $\pm$ 10	(1.5 $\pm$ 0.1) $\times 10^{-3}$	32 $\pm$ 7	(3.7 $\pm$ 0.1) $\times 10^{-3}$	88 $\pm$ 10	(5.2 $\pm$ 0.1) $\times 10^{-3}$
15		296 $\pm$ 61	(11 $\pm$ 1) $\times 10^{-3}$	57 $\pm$ 4	(5.9 $\pm$ 0.1) $\times 10^{-3}$	218 $\pm$ 20	(8.3 $\pm$ 0.2) $\times 10^{-3}$
16		71 $\pm$ 7	(32 $\pm$ 1) $\times 10^{-3}$	56 $\pm$ 5	(7.8 $\pm$ 0.3) $\times 10^{-3}$	7.6 $\pm$ 0.2	(15 $\pm$ 1) $\times 10^{-3}$
17		68 $\pm$ 2	(6.6 $\pm$ 0.1) $\times 10^{-3}$	225 $\pm$ 38	(7.5 $\pm$ 0.1) $\times 10^{-3}$	35 $\pm$ 5	(6.9 $\pm$ 0.4) $\times 10^{-3}$
18		84 $\pm$ 15	(2.4 $\pm$ 0.2) $\times 10^{-3}$	68 $\pm$ 11	(1.8 $\pm$ 0.1) $\times 10^{-3}$	524 $\pm$ 63	(8.0 $\pm$ 0.4) $\times 10^{-3}$
19		122 $\pm$ 25	(5.5 $\pm$ 0.1) $\times 10^{-3}$	158 $\pm$ 22	(4.8 $\pm$ 0.2) $\times 10^{-3}$	477 $\pm$ 40	(8.1 $\pm$ 0.6) $\times 10^{-3}$
20		97 $\pm$ 15	(9.1 $\pm$ 0.5) $\times 10^{-3}$	517 $\pm$ 65	(14 $\pm$ 1) $\times 10^{-3}$	65 $\pm$ 10	(4.6 $\pm$ 0.1) $\times 10^{-3}$
21		175 $\pm$ 7	(2.4 $\pm$ 0.1) $\times 10^{-3}$	47 $\pm$ 6	(2.6 $\pm$ 0.3) $\times 10^{-3}$	107 $\pm$ 8	(22 $\pm$ 1) $\times 10^{-3}$
22		98 $\pm$ 13	(24 $\pm$ 1) $\times 10^{-3}$	50 $\pm$ 6	(6.6 $\pm$ 0.1) $\times 10^{-3}$	23 $\pm$ 4	(19 $\pm$ 1) $\times 10^{-3}$
23		130 $\pm$ 11	(6.7 $\pm$ 0.1) $\times 10^{-3}$	150 $\pm$ 19	(4.8 $\pm$ 0.2) $\times 10^{-3}$	75 $\pm$ 4	(5.0 $\pm$ 0.1) $\times 10^{-3}$
24		426 $\pm$ 65	(12 $\pm$ 1) $\times 10^{-3}$	78 $\pm$ 14	(3.9 $\pm$ 0.1) $\times 10^{-3}$	406 $\pm$ 43	(7.3 $\pm$ 0.3) $\times 10^{-3}$

Table 2 Velocity of the PKC γ -catalysed phosphorylation of peptides 7 and 24

The PKC γ concentration employed for peptide **7** was 10 pg/ μ l and for peptide **24** was 3.2 ng/ μ l. Reactions were performed in the presence of (a) 'buffer' (pH 7.5, 62.5 mM Hepes/12.5 mM MgCl₂/0.5 mM EGTA), (b) 'Ca²⁺' (buffer/750 μ M Ca²⁺), (c) 'PS/DAG' (buffer/7.5 μ g/ml PS/1.6 μ g/ml DAG) and (d) 'Ca²⁺/PS/DAG' (buffer/750 μ M Ca²⁺/7.5 μ g/ml PS/1.6 μ g/ml DAG). In the experiment listed in row (e), PKC was incubated with buffer containing Ca²⁺, PS, DAG and 125 μ M ATP at 30 °C. After 20 min, a 2 mM EGTA solution was added to complex the 750 μ M Ca²⁺ that was present, and the reaction mixture was subsequently assayed with peptide **24** according to the standard protocol.

Conditions	Velocity (nmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$)	
	Peptide ... 7	24
(a) Buffer	0.16 $\pm$ 0.02	0.47 $\pm$ 0.08
(b) Buffer/Ca ²⁺	0.23 $\pm$ 0.02	0.55 $\pm$ 0.07
(c) Buffer/PS/DAG	1.33 $\pm$ 0.09	1.1 $\pm$ 0.1
(d) Buffer/Ca ²⁺ /PS/DAG	1.35 $\pm$ 0.08	2.3 $\pm$ 0.3
(e) Buffer/Ca ²⁺ /PS/DAG + EGTA	—	0.69 $\pm$ 0.08

In addition to simple hydroxybenzoic acids (Table 1), the α , β and γ isoforms of PKC phosphorylate a host of aromatic alcohols (Table 1). Of particular note is **21**, which exhibits a V_{max}/K_m with PKC γ that is 15-fold greater than that displayed with PKC α . In addition to primary, secondary, tertiary and aromatic alcohols, the α , β and γ isoforms of PKC utilize a benzylic alcohol (**23**) as a substrate as well (Table 1). The K_m and V_{max} values are similar to those obtained for the aromatic alcohols surveyed in the present study.

Protein kinases are commonly categorized by their ability to phosphorylate the aliphatic alcohols of serine and threonine or the aromatic alcohol of tyrosine. Given the diverse array of

aromatic alcohols described above that serve as substrates for PKC, we prepared the tyrosine-containing peptide **24**. In general, the kinetic constants associated with the PKC-catalysed phosphorylation of **24** compares favorably with those reported for various protein tyrosine kinases with synthetic tyrosine-containing peptide substrates [27,28].

A hallmark of the activity of the α , β and γ isoforms of PKC is their ability to be activated by Ca²⁺ and DAG. We chose two different substrates to examine whether an analogous effect is observed for the phosphorylation of non-serine-containing peptide substrates. Peptide **7** is one of the best substrates that we have identified, yet it possesses a phosphorylatable residue with no analogy to any naturally occurring alcohol-containing residue. Although peptide **24** contains a naturally occurring tyrosine moiety, this residue had not previously been shown to serve as a substrate for 'classic' serine/threonine- 'specific' protein kinases. As is evident from Table 2, (row b) Ca²⁺ provides a modest rate enhancement for the PKC γ -catalysed phosphorylation of peptides **7** and **24** versus (row a) buffer alone. Furthermore, this rate enhancement is even more substantial in the presence of (row c) PS/diacylglycerol. Curiously, the addition of (row d) Ca²⁺ to PS/DAG does not appear to dramatically improve the phosphorylation rate for peptide **7**. In contrast, the PKC γ -catalysed phosphorylation of **24** is twice as rapid with Ca²⁺/PS/DAG than with PS/DAG. Most noteworthy, however, is the fact that the phosphorylation of both peptide substrates is dramatically accelerated in the presence (versus the absence) of Ca²⁺/PS/DAG.

The tyrosine kinase activity displayed by the α , β and γ PKC isoforms could be due to an unidentified protein kinase that was inadvertently co-purified with each of the PKCs. The results illustrated in Table 2 appear to rule out this possibility, since the existence of a tyrosine kinase that displays the same molecular mass, as well as the same catalytic properties (i.e. Ca²⁺-, DAG-

Figure 2 Semi-logarithmic plot of $V_{\max}/K_m$ for the PKC α (○), PKC β (△) and PKC γ (×)-catalysed phosphorylation of peptide substrates 1–24

and PS-dependent activity) of PKC, is exceedingly remote. However, it is conceivable that a PKC-dependent tyrosine kinase co-purified with PKC (i.e. a tyrosine kinase whose activity is switched on by PKC-catalysed phosphorylation). Consequently, we preincubated PKC with Ca^{2+} , DAG, PS and ATP, for 20 min. Since these conditions are virtually identical with those of our standard assay, any PKC-dependent enzyme present in the reaction media should be phosphorylated. After 20 min the PKC incubation mixture was treated with a 2 mM EGTA buffer solution, which is sufficient to complex the Ca^{2+} that PKC requires for activation. A control experiment was also performed, one in which the added buffer solution did not contain EGTA. If a PKC-dependent tyrosine kinase impurity is responsible for the observed phosphorylation of **24**, then the addition of EGTA should have no effect on the rate of tyrosine phosphorylation. However, addition of EGTA dramatically lowers this activity by 65% (Figure 2), an observation that effectively rules out the presence of any PKC-dependent tyrosine kinase that may have inadvertently co-purified with PKC. These results are consistent with the notion that PKC is directly responsible for the phosphorylation of the tyrosine residue in peptide **24**.

Substrate efficiency, as defined by $V_{\max}/K_m$, is summarized in Figure 2 for the 24 peptide/non-peptide conjugates examined in the present study. As one might predict, given the strong sequence similarity between the α , β and γ isoforms of PKC, the majority of substrates are processed with nearly identical efficacy by all three isoforms. Nevertheless, it is evident that these PKC isoforms do not exhibit similar $V_{\max}/K_m$ values for all substrates examined – an intriguing observation given the small size of the library constructed in this preliminary survey. Peptide **8** is clearly favoured by PKC γ over that of the β (88-fold) and α (15-fold) isoforms. By contrast, a previously reported PKC γ consensus-sequence peptide (identified from a combinatorial peptide-based library) fails to display any selectivity amongst the conventional PKC isoforms [3]. Peptides **12** and **21** likewise display PKC γ selectivity. These promising initial results suggest that it may be possible to ultimately acquire selective PKC substrates via several rounds of sub-library synthesis and analysis.

In summary, a library of alcohol-appended peptides has been prepared and used to assess the substrate specificity of the three closely related α , β and γ isoforms of PKC. This library contains a structural attribute typically associated only with small molecule libraries, namely a high structural diversity focused within a spatially restricted window. PKC α , β and γ phosphorylate an array of peptide-appended chiral and achiral amino alcohols, as well as tertiary, benzylic and aromatic alcohols. Unexpectedly,

these conventional serine/threonine-specific protein kinases catalyse the phosphorylation of a tyrosine-bearing member of the peptide library, a 'tyrosine kinase activity' that is both Ca^{2+} - and DAG-dependent. These observations suggest that the library's unique peptide-alcohol structural motif extends the array of alcohol-bearing residues recognized as PKC substrates. Finally, we have identified the first example of a synthetic substrate that displays a marked preference for one member of the conventional PKC subfamily.

This work was supported by research grant GM45989 (to D.S.L.) from the National Institutes of Health. K. C. is a recipient of a postdoctoral fellowship from the Ladies Auxiliary of the Veterans of Foreign Wars.

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Received 21 February 2000/25 April 2000; accepted 17 May 2000