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Distinguishing between A Mitogenic and Two Closely Related Nonmitogenic Protein Kinases

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Protein kinases are key components in the signal transduction pathways that transmit external membrane-directed signals to the cell nucleus.[1] These enzymes share a strong primary sequence homology as well as a common substrate (i.e., ATP), yet they participate in a vast array of often unrelated signaling cascades. For example, one role of the cAMP-dependent protein kinase ("PKA")[2] is to regulate the enzymes of glycogen metabolism, whereas a function of the cGMP-dependent protein kinase ("PKG")[2] is to participate in nitric oxide-induced signaling. In addition, protein kinase C ("PKC")[3] serves as the receptor for the phorbol ester tumor promoters and has been strongly implicated in mitogenesis. In spite of their disparate biochemical roles, the primary sequences of PKA, PKC, and PKG are so similar that these enzymes have been positioned off the same secondary branch of the phylogenetic protein kinase tree.[4] Not surprisingly, PKA, PKC, and PKG share a number of enzymological traits, including a pronounced tendency to phosphorylate the same peptide substrates *in vitro* (*vide infra*). Unfortunately, this renders the acquisition of protein kinase-specific inhibitors problematical. Highly specific inhibitors are not only desirable as probes for elucidating the biochemical consequences of individual protein kinase action but also, as in the case of protein kinase C, may be of significant therapeutic value. Although protein kinases share a common ability to phosphorylate either L-serine/L-threonine or L-tyrosine amino acids in protein and peptide substrates, we have recently demonstrated that these enzymes will also phosphorylate a structurally diverse ensemble of alcohol-bearing residues not found in eukaryotic proteins.[5] Somewhat surprisingly, the active site preferences of protein kinases can differ, often in a dramatic fashion, toward these unnatural residues. For example, on the basis of differences in the active site substrate specificity of PKA and PKG,[6] we recently designed an inhibitor that is highly selective for the latter.[7] We now describe a structural motif that discriminates between PKC and its non-mitogenic counterparts, PKA and PKG.

PKA, PKC, and PKG catalyze the phosphorylation of L-serine positioned at the C-terminus of active site-directed peptides. In addition, all three enzymes phosphorylate the achiral ethanolamine residue contained within peptide **1**.[5,6,8] In addition to the phosphorylation of simple aliphatic alcohols, we recently found that PKA exhibits phenol kinase activity.[9] Unlike the hydroxyl moiety in **1**, the aromatic alcohol in **2** is rigidly positioned, both in terms of distance and orientation, relative to the adjacent peptide bond. We have now found that variations in both the distance and orientation parameters produce peptides that are only recognized by the active site of PKC.

Table 1. The K_m and k_{cat} Values for Peptides **2**–**4** with PKA, PKC, and PKG[a]

LR_4F-aminophenol	Kinetic Constants	PKA	PKC	PKG
(2)	K_m (μM)	64 ± 2	21 ± 6	52 ± 7
	k_{cat} (min^{-1})	0.20 ± 0.01	2.3 ± 0.3	0.38 ± 0.03
	k_{cat}/K_m (min^{-1} μM^{-1})	0.0031 ± 0.0001	0.11 ± 0.01	0.0073 ± 0.0003
(3)	K_m (μM)		0.69 ± 0.14	
	k_{cat} (min^{-1})	not a substrate[10]	2.3 ± 0.2	not a substrate[10]
	k_{cat}/K_m (min^{-1} μM^{-1})		3.3 ± 0.4	
(4)	K_m (μM)		17 ± 4	
	k_{cat} (min^{-1})	not a substrate[10]	3.4 ± 0.4	not a substrate[10]
	k_{cat}/K_m (min^{-1} μM^{-1})		0.20 ± 0.03	

[a] PKA was expressed and purified according to a previously described protocol.[13] Purified PKG (type 1α) was isolated from bovine lung.[14] PKC was purchased from Upstate Biotechnology, Inc. Assays were performed in triplicate at pH 7.1 and thermostatted in a water bath at 30 °C as described by Wood et al.[6]

Leu-Arg$_4$-Phe–NH (**1**, **2**, **3**, **4**)

Peptides **2**–**4** were prepared utilizing a previously described protocol.[9a] As is apparent from Table 1, peptide **2** is phosphorylated by all three protein kinases, a result which illustrates the overlapping substrate specificity exhibited by these enzymes. However, **2** is a significantly more efficient substrate for PKC than for the two cyclic nucleotide-dependent protein kinases. In particular, the k_{cat} term is approximately an order of magnitude greater with PKC than with either PKA or PKG. In contrast, the meta-substituted derivative contained within **3** fails to serve as a substrate for either PKA or PKG.[10] This result is not too surprising, given the fact that both the distance and

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(10) In addition to the standard assay conditions employed to generate the kinetic constants provided in Table 1, we incubated peptides **3** and **4**, individually, with PKA (500 nM) and PKG (180 nM) in the presence of 1 mM ATP for up to 6 h. Aliquots of the reaction mixture were removed at selected time intervals and the amount of phosphopeptide formed under these conditions assessed using a previously described protocol.[9] Even after 6 h of incubation, we detected less than 0.5% phosphorylation of **3** or **4** in the presence of either PKA or PKG.

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orientation of the alcohol in **3**, relative to the adjacent peptide bond, differs from what can be accessed by the hydroxyl moiety in **1**. The peptide backbone clearly plays a critical role in positioning the phosphorylatable residue within the active site region since, even a minor alteration in the structural relationship between the alcohol moiety and the adjacent peptide bond suppresses phosphoryl transfer. However, **3** not only is a PKC substrate, but it is 30-fold more efficiently phosphorylated than the ortho-substituted aromatic ring in **2**. These results indicate that the constraints responsible for positioning the phosphorylatable alcohol moiety, with respect to critical active site functional groups, are significantly more relaxed in the case of PKC than for either PKA or PKG. The corresponding para derivative (peptide **4**) is also phosphorylated by PKC. However, it fails to serve as a substrate for either PKA or PKG. Although **4** is less efficiently phosphorylated by PKC than **3**, the turnover rate as well as the Michaelis constant of the former are still impressive in light of the corresponding kinetic constants displayed by PKA and PKG toward peptide **2**. *The meta- and para-substituted derivatives are the first and only examples of alcohol-bearing residues that exhibit an absolute preference for PKC*. Furthermore, the aromatic nucleus is a particularly useful molecular scaffold since a variety of substituents can be readily appended that can potentially promote both enzyme affinity and specificity. Most importantly, however, the results described herein have unmistakable implications in terms of PKC inhibitor design. In particular, both transition state analogs[11] and suicide substrates[12] require interaction with the catalytic apparatus of the target enzyme. Clearly, only in the case of PKC is this possible with the structural motifs contained within peptides **3** and **4**.

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