Substrate-dependent activation requirements and kinetic properties of protein kinase C

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Received 14 April 1998

Abstract Protein kinase C (PKC) requires basic amino acids around the phosphorylated Ser or Thr. Previous studies of the effector requirements of PKCs α, β and γ with two commonly used substrates, MBP_{3-14} (AQKRPSQRSKYL) and peptide ε (ERMRPRKRQGSVRRRV), revealed that MBP_{3-14} phosphorylation required Ca^{2+}, phosphatidylserine and diacylglycerol, while peptide ε supported high levels of phosphatidylserine-dependent activity in the absence of Ca^{2+} or diacylglycerol. Since the Arg versus Lys content is much larger in peptide ε than in MBP_{3-14}, we examined the role of these amino acids in conferring substrate-dependent effector requirements for PKC activation. We substituted Lys for Arg in peptide ε (peptide ε[R → K]) and Arg for Lys in MBP_{3-14} (MBP_{3-14}[K → R]) and analyzed the effector requirements and kinetic properties of PKCs α, β and γ with the parent and modified peptides. In general, significant Ca^{2+} and diacylglycerol dependence was observed with peptide ε[R → K] as compared to peptide ε. On the other hand, the effector requirements with MBP_{3-14}[K → R] were the same as with MBP_{3-14}, presumably due to a subthreshold Arg content. Both K_m and V_{max} determined in the presence of Ca^{2+}, phosphatidylserine and diacylglycerol were increased by the peptide ε modification for all three isoenzymes, while the only effect of MBP_{3-14} modification was a decrease in K_m for PKCβ. K_m and V_{max} values for peptide ε and peptide ε[R → K] phosphorylation by PKCα were also determined in the absence of Ca^{2+} or diacylglycerol. While diacylglycerol had no effect, Ca^{2+} decreased the K_m for both substrates to a similar extent. Overall, the degree of effector dependence did not correlate with absolute K_m values. The mechanism of PKC activation by Arg-rich substrates, therefore, does not involve their ability to bind to the active site.

Key words: Protein kinase C; Phosphatidylserine

1. Introduction

Protein kinase C (PKC) is a family of Ser/Thr kinases involved in signal transduction pathways triggered by numerous extracellular stimuli [1]. Ten PKC isoenzymes have been identified which differ in subcellular localization, substrate specificity and regulation, allowing for the control of many physiological responses. Based on structural similarities and effector requirements, PKCs α, β, and γ constitute a subgroup known as conventional, classical or group A PKCs. While little is known about their physiological substrates, these isoenzymes require Ca^{2+}, phosphatidylserine (PS) and diacylglycerol (DG) for maximal activation using histone III-S or myelin basic protein (MBP) as model phosphate acceptors. In the absence of these effectors, PKC is maintained in an inactive state by an intramolecular autoinhibitory mechanism in which the C-terminal active site is bound by an N-terminal pseudosubstrate domain, preventing access of the substrate [2]. This pseudosubstrate resembles a PKC consensus phosphorylation site ($R/KX_{1-2}S/TXR/K$) (single-letter amino acid code; X indicates any amino acid) [3] except Ala replaces the phosphorylatable Ser or Thr. Activation of PKC involves binding of effectors to sites in the N-terminal regulatory region, recruitment of the enzyme to membranes and removal of the pseudosubstrate domain from the active site.

While group A PKCs are classified as Ca^{2+}- and lipid-dependent enzymes, their effector requirements actually vary with the substrate used, e.g. protamine sulfate is phosphorylated by PKC in the absence of effectors [4,5]. Unlike histone III-S and MBP, which are rich in Lys residues, protamine is Arg-rich, suggesting that the relative content of Arg and Lys of the substrate influences the activator requirements of PKC. In support of this idea, PKC requires Ca^{2+} in the presence of PS and DG to phosphorylate a random copolymer of poly-L-Lys-Ser (3:1), while phosphorylation of poly-L-Arg-Ser (3:1) is Ca^{2+}-independent [6]. In addition, group A PKCs bind protamine and poly-L-Arg more tightly than histone and poly-L-Lys in the absence of effectors and MgATP [7]. The role of Arg residues in substrates that confer effector-independent PKC activity was also demonstrated using synthetic peptides based on protamine [8]. While peptide R_4YGSR_6Y was phosphorylated by PKC in the absence of effectors, analysis of peptide fragments revealed that extended clusters of Arg residues must be present on both sides of the phosphorylation site to support effector-independent activity.

We characterized the effector dependencies of purified PKCs α, β and γ with the commonly used PKC substrates, peptide ε (ERMRPRKRQGSVRRRV), which corresponds to the pseudosubstrate domain of PKCε [9] with a Ser for Ala substitution, and MBP_{3-14} (AQKRPSQRSKYL) (the phosphorylated serine is underlined). In general, each isoenzyme displayed a high degree of effector dependence with MBP_{3-14}, but phosphorylated peptide ε with dramatically reduced effector requirements [10]. The relative amount of Arg versus Lys in peptide ε (43.8% versus 6.6%) is much higher than in MBP_{3-14} (16.6% versus 16.6%). Furthermore, peptide ε has a higher density of Arg on both sides of the phosphorylation site. To test the hypothesis that the substrate-dependent differences in effector requirements relate to the Arg and Lys content of these substrates, we examined modified peptides in which Arg was replaced by Lys in peptide ε (peptide ε[R → K]) and Lys by Arg in MBP_{3-14} (MBP_{3-14}[K → R]). Unlike other studies of this nature which examined a limited

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Abbreviations: DG, diacylglycerol; MBP, myelin basic protein; PKC, protein kinase C; PS, phosphatidylserine

PII S0014-5793(98)00567-5

Table 1
Kinetic parameters of PKCs α, β and γ with histone, peptide ε, peptide ε[R→K], MBP_{3-14} or MBP_{3-14}[K→R] as substrate

		Histone	MBP_{3-14}	MBP_{3-14}[K→R]	Peptide ε	Peptide ε[R→K]
PKCα	K_m	2.4±0.2	9.4±0.4	8.7±0.6	3.5±0.3	12.6±1.1
	V_{max}	2.8±0.1	13.1±0.2	14.4±0.3	6.0±0.1	20.2±0.6
	V_{max}/K_m	1.2	1.4	1.7	1.7	1.6
PKCβ	K_m	4.7±0.2	33.5±1.2	16.2±0.8	7.4±0.5	20.7±1.4
	V_{max}	3.4±0.1	13.8±0.2	15.5±0.3	8.3±0.2	21.7±0.6
	V_{max}/K_m	0.7	0.4	1.0	1.1	1.0
PKCγ	K_m	3.8±0.5	7.3±0.8	6.0±0.8	6.3±1.0	10.6±1.1
	V_{max}	1.9±0.1	7.1±0.3	6.6±0.3	5.8±0.3	13.1±0.5
	V_{max}/K_m	0.5	1.0	1.1	0.9	1.2

Kinase activity was determined in the presence of Ca^{2+}, PS and DG. Histone concentrations used were 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.5, 5, 10, 25 and 50 μM. Peptide concentrations used were 1, 2, 4, 6, 8, 10, 15, 25, 50 and 100 μM. K_m values are in μM and V_{max} values are in μmol P_i mg^{-1} min^{-1}.

set of effector conditions, used an undefined mixture of PKC isoenzymes, or used only a single isoenzyme, we have conducted a systematic analysis of the effector requirements of the parent and modified peptides with purified α, β and γ isoenzymes. In addition, we provide a comparative analysis of the kinetic constants (K_m and V_{max}) for phosphorylation of these substrates.

2. Materials and methods

2.1. Materials

Bovine brain L-α-phosphatidylserine (PS) was purchased from Avanti Polar Lipids, Inc., and other materials from sources previously identified [10]. Peptides ε[R→K] (EKMKPKKKQGSVKKKV) and MBP_{3-14}[K→R] (AQRRPSQRSRYL) (residues substituted in the parent peptide are underlined) were synthesized in the University of Calgary Peptide Synthesis Core Facility and purified as previously described [10]. Concentrations of peptide stock solutions were determined by quantitative amino acid analysis in the University of Calgary Protein Sequencing Core Facility. Rat brain PKCs α, β and γ were purified to homogeneity by sequential chromatography on DEAE-Sephacel, phenyl-Sepharose and hydroxylapatite [10]. Reduced Triton X-100 was used during hydroxylapatite chromatography to prevent irreversible oxidative modification of PKCα [10].

2.2. PKC assays

PKC activity was determined by quantification of the incorporation of ^{32}P from [γ-^{32}P]ATP into histone III-S, peptide ε, peptide ε[R→K], MBP_{3-14}, or MBP_{3-14}[K→R]. For determination of K_m and V_{max}, assays were performed at 30°C in a volume of 30 μl containing 20 mM HEPES-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM $CaCl_2$, 0.3 μg/ml PKC, 0.1 mM [γ-^{32}P]ATP (~300 cpm/pmol), variable substrate, and 0.3 mg/ml PS, 62 μg/ml DG and 0.03% Triton X-100 as mixed lipid:detergent micelles. After a 2 min pre-incubation at 30°C, reactions were initiated by the addition of ATP. Reactions were terminated after 5 min by spotting 20 μl onto 1×2 cm squares of Whatman P81 phosphocellulose paper and immersing in 0.5% H_3PO_4. Papers were washed 3×5 min in 0.5% H_3PO_4, dried, and ^{32}P incorporation was quantified by Čerenkov counting. K_m and V_{max} values were calculated by linear regression analysis of Lineweaver-Burk plots and are expressed as the mean±S.E.M. of three independent experiments, each carried out in triplicate. For determination of effector dependence, assays were performed as above in a volume of 180 μl containing either 0.2 mM $CaCl_2$ or 10 mM EGTA, in the absence or presence of PS/DG or PS Triton X-100 mixed micelles or Triton X-100 micelles alone. Substrate concentrations were ~5×K_m. Reactions were terminated by spotting 20 μl at selected times onto P81 paper squares which were washed as described above. Activities were reported as the mean±S.E.M. of three independent experiments, each carried out in triplicate.

3. Results and discussion

The enzymatic properties of PKCs α, β and γ were initially characterized using the standard group A PKC substrate, Lys-rich histone III-S. The kinetic constants (K_m and V_{max}) for phosphorylation were determined in the presence of Ca^{2+}, PS and DG (Table 1). The K_m values for this substrate were in the low micromolar range (2.4–4.7 μM) and the maximal rates of phosphorylation were similar for all three isoenzymes, in general agreement with values previously reported [11–13]. Also in agreement with previous reports [10,11,13–15], PKCs α, β and γ were absolutely dependent on PS and required both Ca^{2+} and DG for maximal activity, while varying sub-maximal activities were observed in the absence of Ca^{2+} or DG (Fig. 1). In our hands, high rates of PS-dependent phosphorylation were observed with PKCγ in the absence of Ca^{2+} and/or DG (53–74% of maximum) while PKCα activity was near basal levels under these conditions. PKCβ displayed an intermediate level of regulation with a high degree of Ca^{2+}-

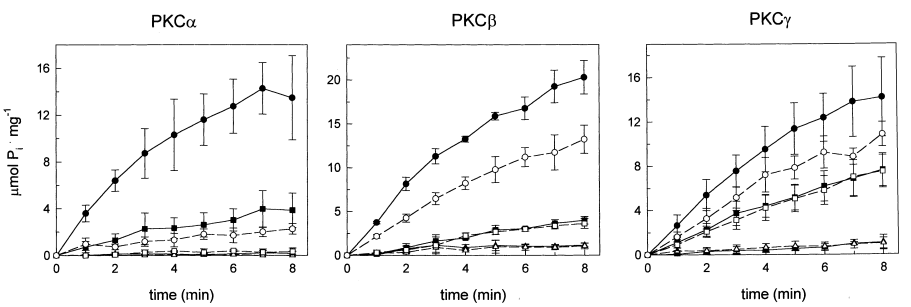

Fig. 1. Effector dependence of PKCs α, β and γ with histone III-S as substrate. Reactions contained histone III-S concentrations of 19.5 μM (PKCα), 23.7 μM (PKCβ), or 19.1 μM (PKCγ). Kinase activity was determined in the presence of Ca^{2+}, PS and DG (●), Ca^{2+} and PS (■), Ca^{2+} alone (▲), EGTA, PS and DG (○), EGTA and PS (□), or EGTA alone (△).

independent activity (62% of maximum). These results emphasize the complex regulation of group A PKCs.

While the kinetic constants of peptide ε and MBP_{3-14} phosphorylation have only been reported in separate studies using PKCs α, β_{II} and γ [13] and PKCβ [16], respectively, we determined the K_m and V_{max} values of both substrates, as well as peptide ε[R→K] and MBP_{3-14}[K→R], with PKCs α, β and γ (Table 1). These values may then be compared with the substrate-dependent effector requirements of these isoenzymes in order to gain insight into how Arg-rich and Lys-rich substrates differentially affect PKC activity. As with histone III-S, these kinetic studies were conducted in the presence of Ca^{2+}, PS and DG. With MBP_{3-14} substrate, both K_m and V_{max} were higher than with histone III-S; however, the phosphorylation efficiencies (V_{max}/K_m) of these substrates were similar. When Lys residues were substituted with Arg in MBP_{3-14} (MBP_{3-14}[K→R]), the only appreciable effect was to decrease the K_m of PKCβ by half. With peptide ε substrate, both the K_m and V_{max} values were lower than with MBP_{3-14} for PKCα (2.7 and 2.2 times, respectively) and PKCβ (4.7 and 1.7 times, respectively). In contrast, the kinetic constants of PKCγ with peptide ε were almost identical to those with MBP_{3-14}. The lack of substrate preference with PKCγ, in terms of K_m and/or V_{max}/K_m, is somewhat surprising since peptide ε most closely resembles the optimal substrate sequence motif of each of the group A PKCs [17]. Furthermore, the optimal substrate sequence of PKCγ is almost identical to that of PKCα [17]. On the other hand, another study demonstrated that PKCγ has distinct substrate specificity as compared to PKCs α and β [18].

Substitution of the Arg residues in peptide ε with Lys (peptide ε[R→K]) resulted in a significant increase in both kinetic constants for all three isoenzymes. In fact, these values exceeded those observed with MBP_{3-14} and MBP_{3-14}[K→R], with the exception of the K_m of MBP_{3-14} with PKCβ. The change in K_m and V_{max} from the parent peptide was most

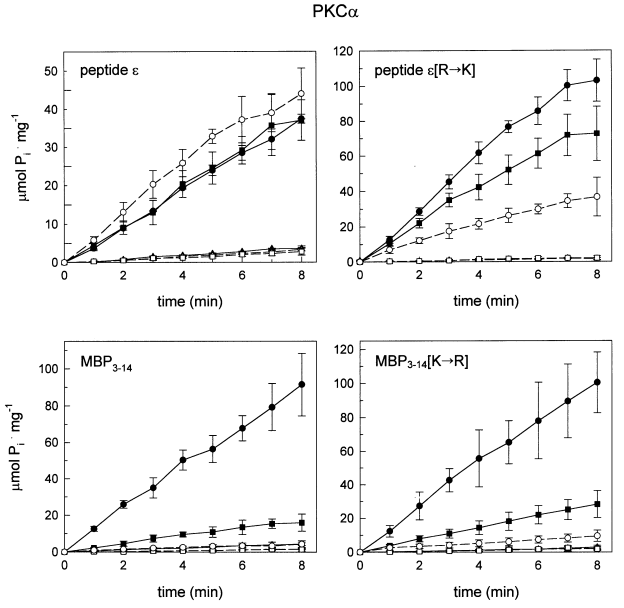

Fig. 2. Effector dependence of PKCα with various peptide substrates. Reactions contained substrate concentrations of 19.5 μM (peptide ε), 73.0 μM (peptide ε[R→K]), 51.5 μM (MBP_{3-14}), or 46.5 μM (MBP_{3-14}[K→R]). Symbols as in Fig. 1.

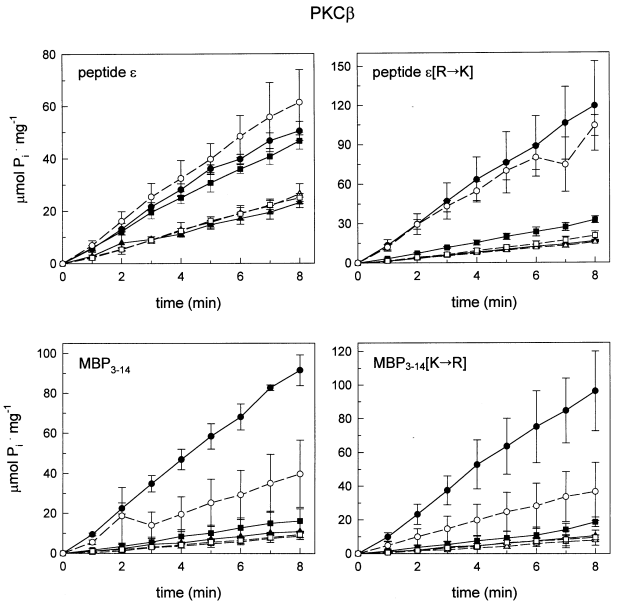

Fig. 3. Effector dependence of PKCβ with various peptide substrates. Reactions contained substrate concentrations of 37.5 μM (peptide ε), 103.5 μM (peptide ε[R→K]), 167.3 μM (MBP_{3-14}), or 81.2 μM (MBP_{3-14}[K→R]). Symbols as in Fig. 1.

dramatic for PKCα (3.6 and 3.4 times, respectively), while PKCγ was affected the least (1.7 and 2.4 times, respectively). Each group A PKC, therefore, exhibits unique sensitivity to the Arg and Lys contents of these substrates. The inability of the Arg-rich MBP_{3-14}[K→R] to elicit an effect in PKCs α and γ, as discussed above, may be explained by the lower density of Arg in this substrate (33.3%) as compared to peptide ε (50%). While a decrease in K_m was seen with PKCβ, MBP_{3-14} is a relatively poor substrate for this isoenzyme, as indicated by its relatively high K_m and low V_{max}/K_m ratio, such that it may be more sensitive to the substrate modification.

Overall, the decreased affinity of PKC for peptide ε[R→K] substrate as compared to peptide ε agrees with the observation that Lys-rich polypeptides bind group A PKCs less tightly than Arg-rich polypeptides [7]. While those studies were performed using a microtiter binding assay in the absence of effectors and MgATP, our results provide additional information with respect to substrate affinity in the presence of effectors and in a catalytically active setting. Consequently, we also demonstrate that the decreased affinity of Lys-rich peptide ε[R→K] allows for a higher turnover rate, resulting in similar catalytic efficiency as compared to peptide ε.

The effector dependencies of PKCs α, β and γ were examined with each peptide substrate by measuring initial rates of phosphorylation (Figs. 2–4). For each peptide, the substrate concentration used corresponded to five times the K_m to prevent possible substrate-limiting effects. Maximal activity with MBP_{3-14} required the presence of Ca^{2+}, PS and DG for all three isoenzymes. As with histone III-S, PS-dependent activity of PKCα was insensitive to the addition of Ca^{2+} or DG alone, while PS-dependent activity of PKCβ was stimulated by DG in the absence of Ca^{2+} (40.2% of maximum). PKCγ displayed high levels of PS-independent activity (~36% of maximum) which was not stimulated by the addition of PS alone or in combination with either Ca^{2+} or DG. With peptide ε sub-

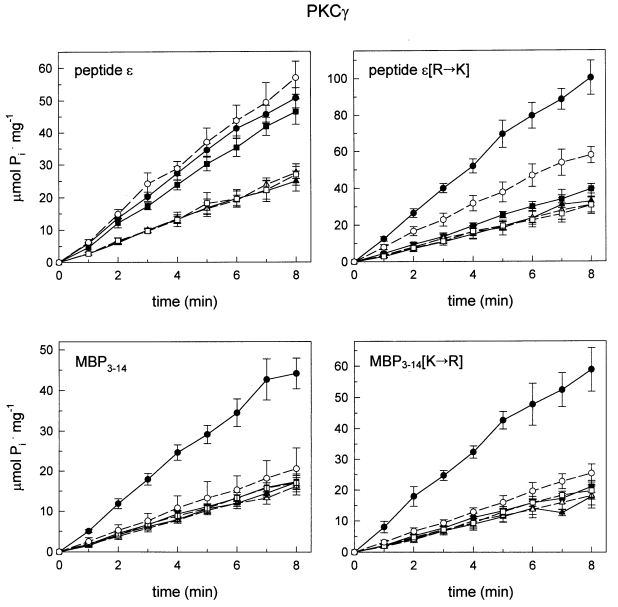

Fig. 4. Effector dependence of PKCγ with various peptide substrates. Reactions contained substrate concentrations of 31.4 μM (peptide ε), 52.9 μM (peptide ε[R→K]), 36.5 μM (MBP_{3-14}), or 30.1 μM (MBP_{3-14}[K→R]). Symbols as in Fig. 1.

strate, the effector requirements of PKCs α, β and γ were significantly reduced as compared to histone III-S and MBP_{3-14}. For all three isoenzymes, approximately the same activity was observed in the presence of PS and either Ca^{2+} or DG as when all three effectors were present. In addition, both PKCs β and γ displayed high levels of PS-independent basal activity (~50% of maximum).

With MBP_{3-14}[K→R], no changes in effector requirements were observed for any of the isoenzymes, similar to that found with another Arg for Lys substituted peptide (FRRSFRL) and PKCα [19]. These results agree with the finding that a critical number of Arg residues must flank the target sequence in order to elicit effector-independent activity [8]. As predicted, when peptide ε[R→K] was used as substrate the overall effector dependence for each isoenzyme was increased, approaching those observed with the MBP peptides. The most dramatic change occurred with PKCγ whose PS-dependent activity in the presence of Ca^{2+} or DG alone was near basal levels. Activity of PKCα in the absence of Ca^{2+} or DG was reduced significantly (32.4% and 67.4% of maximum, respectively) as was the activity of PKCβ in the absence of DG (26.9% of maximum). To eliminate the possibility that the ability of enzyme to bind peptide ε[R→K], but not peptide ε, was impaired by the absence of Ca^{2+} or DG, their kinetic constants with PKCα were determined in the absence of these effectors (Table 2). As compared with the Ca^{2+}/PS/DG condition, DG had no influence on K_m, while Ca^{2+} decreased the K_m for both peptide ε and peptide ε[R→K] to a similar degree (2.2 and 2.9 times, respectively). These results demonstrate that effector conditions do not differentially influence the binding of enzyme to Arg- or Lys-rich substrates. Instead, the initial rates of phosphorylation correlate directly with the V_{max}. These results also indicate that the K_m values we have reported are valid for comparing the ability of enzyme to bind various substrates.

Although the exact mechanism of PKC activation by Arg-rich substrates is unknown, as with conventional activators there is a conformational change in which the pseudosubstrate is displaced from the catalytic site [20]. It has been proposed that Arg-rich polypeptides may bind distal to the active site and activate PKC by an allosteric mechanism, since protamine and poly-L-Arg, but not histone or poly-L-Lys, stimulate effector-independent PKC autophosphorylation with positive cooperativity [7]. On the other hand, protamine and poly-L-Arg do not influence the phosphorylation of histone by PKC [8] and therefore are not general effectors of PKC. Alternatively, it has been suggested that Arg-rich proteins activate PKC by neutralizing the acidic patch that maintains the pseudosubstrate domain in the active site, thereby releasing the pseudosubstrate by effectively competing for contacts [20]. In that case, it may be predicted that a greater degree of effector independence is directly correlated with higher substrate affinity, as indicated by the K_m. While our data provide many examples of such a correlation, this is not a consistent observation. For instance: (i) the K_m values of PKCγ for peptide ε, MBP_{3-14} and MBP_{3-14}[K→R] are very similar but the effector requirements with peptide ε as substrate are quite different from the MBP peptides; (ii) the K_m values of PKCβ for MBP_{3-14} and MBP_{3-14}[K→R] differ by ~2-fold, but the effector requirements with these two substrates are essentially identical; (iii) the difference in K_m between peptide ε and peptide ε[R→K] is smallest for PKCα and largest for PKCγ, while the opposite trend is observed with respect to changes in effector requirements. Although the mode of PKC activation by Arg-rich substrates remains unresolved, both Arg and Lys are fully protonated at pH values at which assays are performed; therefore, the distinct effector requirements of PKC with Arg- and Lys-rich substrates is not due simply to a charge effect, indicating that side chain structure is important.

Overall, the complex substrate-dependent enzymatic activities observed with PKC isoenzymes have significant implications regarding methods used to monitor PKC activity in vitro and the interpretation of how PKC is regulated in a physiological context. It may be considered that the use of certain artificial substrates is not appropriate for evaluating PKC activity. Alternatively, it is possible that different substrates dictate the conditions under which they will be phosphorylated by various PKC isoenzymes, thereby adding another level of complexity to the physiological regulation of PKC.

Table 2
Kinetic parameters of PKCα with peptide ε and peptide ε[R→K] as substrate under different conditions

		Peptide ε	Peptide ε[R→K]
EGTA/PS/DG	K_m	10.2 ± 0.6	40.8 ± 7.4
	V_{max}	6.7 ± 0.1	3.9 ± 0.3
	V_{max}/K_m	0.7	0.1
Ca^{2+}/PS	K_m	4.5 ± 0.7	15.0 ± 4.9
	V_{max}	5.2 ± 0.2	11.7 ± 1.5
	V_{max}/K_m	1.2	0.8
Ca^{2+}/PS/DG	K_m	4.7 ± 0.8	14.3 ± 2.2
	V_{max}	5.2 ± 0.3	16.1 ± 0.9
	V_{max}/K_m	1.1	1.1

Substrate concentrations were 1, 2, 4, 6, 8, 10, 25, 50 and 100 μM. K_m values are in μM and V_{max} values are in μmol P_i mg^{-1} min^{-1}.

Acknowledgements: This work was supported by a grant from the Medical Research Council of Canada (M.T.-13101). C.K.W. was a recipient of a Summer Studentship from the Alberta Heritage Foun-

dation for Medical Research (AHFMR) and M.P.W. is an AHFMR Medical Scientist.

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