Studies with Synthetic Peptide Substrates Derived from the Neuronal Protein Neurogranin Reveal Structural Determinants of Potency and Selectivity for Protein Kinase C†

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ABSTRACT: The neuronal protein neurogranin, also known as RC3, is a selective substrate for protein kinase C (PKC). We synthesized a peptide corresponding to the phosphorylation domain of neurogranin (amino acids 28–43) and characterized its properties as a PKC substrate. Neurogranin$_{(28-43)}$ was phosphorylated by purified PKC with a K_m of 150 nM. No significant phosphorylation of the peptide by either cAMP-dependent protein kinase or by calcium/calmodulin-dependent protein kinase II could be detected. Thus, neurogranin$_{(28-43)}$ is a potent and selective substrate for PKC. We tested several peptide analogues of neurogranin$_{(28-43)}$ for their substrate potency and specificity as kinase substrates, in order to help elucidate the structural determinants involved in the phosphorylation of substrates by PKC. Substituting Arg^{36} with Ile caused a significant reduction in the affinity for PKC. Replacing Lys^{30} with Arg enhanced the catalytic efficiency (V_{max}/K_m) for PKC but diminished the selectivity of the substrate for PKC. These results support the generally held model that basic amino acids on both sides of the phosphorylated Ser are important structural determinants in PKC substrates. However, the data also suggest that the presence of particular basic amino acids (Arg vs Lys) can contribute to the degree of selectivity of a substrate for PKC. Replacement with Ala of Phe^{35}, the amino acid adjacent to the Ser^{34} phosphorylation site, resulted in a peptide with greatly diminished potency as a PKC substrate. This finding indicates a critical role of Phe^{35} in modulating binding and phosphorylation of neurogranin-derived peptides by PKC. Substituting Ile in this same position caused only a slight decrease in potency as a PKC substrate, suggesting that the hydrophobic character of the amino acid in this position was an important potency determinant.

Protein kinase C (PKC)[1] is a calcium-activated, phospholipid-dependent kinase that plays an important role in signal transduction and the regulation of diverse cellular processes. PKC is present in a variety of tissues as multiple isoforms and is especially abundant in the brain (Nishizuka, 1988). Understanding the mechanisms by which PKC regulates cell activities requires the identification and characterization of substrates for PKC in vivo.

A few proteins, including the regulatory myosin light chain (Nishikawa et al., 1983), the S6 protein on the 40S ribosome (LePeuch et al., 1983), glycogen synthase (GS) (Ahmed et al., 1984), the EGF receptor (Hunter et al., 1984), the myristoylated alanine-rich C kinase substrate (MARCKS protein) (Graff et al., 1991), and histone H1 (Inoue et al., 1977), have been identified as potential substrates for PKC in vivo. The use of synthetic peptides derived from these protein substrates has allowed the definition of certain specificity determinants present in PKC substrates. It is generally agreed that basic amino acids on both sides of the phosphorylated Ser/Thr constitute a recognition determinant for PKC. However, the precise determinants for potency and selectivity of PKC substrates are not well understood.

Recently, two brain-specific proteins, neuromodulin (also known as GAP43/B50/F1) and neurogranin (also known as RC3), were identified as endogenous, selective substrate proteins for PKC (Alexander et al., 1987; Aloyo et al., 1983; Baudier et al., 1989; Watson et al., 1990). Both neuromodulin and neurogranin are present at high concentrations in the hippocampus (Represa et al., 1990), and phosphorylation of both proteins has been observed in intact cells in hippocampal slices (Baudier et al., 1991; Gianotti et al., 1992). These data suggest that the two proteins are physiologically relevant PKC substrates in this region of the central nervous system. Amino acid sequence analysis revealed a high degree of homology in their phosphorylation domains (Baudier et al., 1991). As both proteins are selective substrates for PKC, the sequence homology between neuromodulin and neurogranin is of particular interest because it may provide a basis for elucidating the amino acid sequence determinants underlying their selectivity as substrates for PKC. We hypothesized that a synthetic peptide corresponding to the sequence of the phosphorylation domain in these two proteins might be a selective PKC substrate in vitro. In this study, we synthesized a peptide with a sequence corresponding to amino acids 28–43 of neurogranin (NG$_{(28-43)}$) and characterized it as a PKC substrate. The high affinity and selectivity of NG$_{(28-43)}$ for PKC suggest that the specificity determinants of native neurogranin as a PKC substrate lie within amino acids 28–43 of its sequence. In addition, we investigated the potency and specificity determinants for PKC substrates by characterizing several substituted peptide analogues of NG$_{(28-43)}$ as substrates for PKC and other protein kinases. We found that NG$_{(28-43)}$ is a potent and highly selective substrate for PKC in vitro. In addition, we found that the presence of basic amino acids on

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[1] Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; CaMKII, calcium/calmodulin-dependent kinase II; DG, *sn*-1,2-dioctanoylglycerol; GS, glycogen synthase; MARCKS protein, myristoylated alanine-rich C kinase substrate; NG, neurogranin; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKM, protein kinase M; PMSF, phenylmethanesulfonyl fluoride; PS, L-α-phosphatidylserine.

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either side of the phosphorylated Ser/Thr contributed to the potency of the neurogranin-derived peptide, in agreement with previous work using other peptide substrates for PKC. Finally, we have found that a Phe immediately adjacent to the phosphorylation site is crucial in conferring potency as a PKC substrate and that the substitution of another hydrophobic amino acid (Ile) at this site can partially compensate for the removal of Phe. Overall, our studies suggest a recognition motif for potency and selectivity as a PKC substrate that includes not only positively charged amino acids near the phosphorylation site but also a hydrophobic amino acid carboxy-terminal adjacent to the phosphorylated Ser.

EXPERIMENTAL PROCEDURES

Materials. L-α-Phosphatidylserine (PS) and *sn*-1,2-dioctanoylglycerol (DG) were purchased from Calbiochem (La Jolla, CA). Calmodulin, PKC substrate peptide GS$_{(1-12)}$, Kemptide, and the catalytic subunit of PKA were purchased from Sigma (St. Louis, MO).

Peptides. Several synthetic peptides, NG$_{(28-43)}$, [R^{30}]-NG$_{(28-43)}$, [A^{34}]NG$_{(28-43)}$, [I^{35}]NG$_{(28-43)}$, [A^{35}]NG$_{(28-43)}$, [I^{36}]NG$_{(28-43)}$, [R^{3},A^{9,10}]GS$_{(1-10)}$, PKC$_{(19-36)}$, and S6$_{(229-249)}$, were used in the present study. Peptides were synthesized at the Baylor College of Medicine Protein Chemistry Facility. Peptides were made on an Applied Biosystems Model 430A peptide synthesizer and purified by reverse-phase HPLC using an Aquapore C8 column and acetonitrile gradient elution. Amounts of each peptide were determined and amino acid compositions were confirmed using a Waters amino acid analysis system.

Preparation of PKC. PKC was purified from adult rat forebrain by procedures previously described (Huang, 1986; Shearman et al., 1989). Purified kinase was stored in Tris-HCl buffer (pH 7.5, 20 mM) containing 50% glycerol, 0.5 mM EDTA, and 0.5 mM EGTA and kept at −20 °C; under these conditions, PKC activity was stable for several weeks. For prolonged storage, the kinase fraction was stored at −70 °C. The PKC preparation was essentially pure as determined by SDS–polyacrylamide gel electrophoresis and immunoblotting with a polyclonal antibody which recognized PKC (generously provided by Dr. Freesia Huang). A major band at 80 kDa and a minor band with molecular mass of 90 kDa were observed both on the Coomassie-blue stained gel and on the immunoblot. Separation of PKC isoforms was carried out using a hydroxylapatite column (100 × 7.8 mm, Bio-Rad) connected to an HPLC system, according to the methods of Shearman et al. (1989).

Preparation of PKM. To obtain the calcium/phospholipid-independent catalytic fragment of protein kinase C (protein kinase M, PKM), purified PKC was treated with trypsin (Boehringer Mannhein Biochemicals) at a PKC to trypsin ratio of 100:1 at 25 °C for 1 min. The digestion was stopped by adding 10 μg/mL leupeptin and the mixture was applied to a DEAE-cellulose column (1 × 1.2 cm). Intact PKC was eluted with Tris-HCl buffer (pH 7.5, 50 mM) containing 120 mM NaCl and PKM was eluted with same buffer containing 400 mM NaCl (Huang & Huang, 1986). The PKM fraction collected was dialyzed in buffer containing 50% glycerol and stored at −20 °C. The presence of PKM was confirmed by the appearance of an immunoreactive protein band of approximately 50 kDa apparent molecular mass on Western blots.

Phosphorylation of NG$_{(28-43)}$ and Substituted Peptides by PKC or PKM. The reaction mixture (50 μL) contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM sodium pyrophosphate, 0.1 mM PMSF, 25 μg/mL leupeptin, 1 mg/mL bovine serum albumin (BSA), 100 μM [γ-^{32}P]ATP (ICN Biomedicals Inc., Irvine, CA, or Amersham Corp., Arlington Heights, IL; approximately 1000 cpm/pmol for most studies and 4000 cpm/pmol when the K_m of peptide substrates was to be determined) and peptide substrate as indicated. Assays were carried out either in the presence of EGTA (2.5 mM final concentration) or in the presence of calcium (100 μM calcium in the presence of 500 μM EGTA) and lipid cofactors (final concentrations: PS 320 μg/mL; DG 30 μg/mL). Preliminary experiments carried out to determine the time course of NG$_{(28-43)}$ phosphorylation by purified PKC indicated that NG$_{(28-43)}$ could be phosphorylated by purified PKC even at 4 °C (data not shown). Therefore, all the kinase assays were started by adding substrate to the enzyme and incubated at 25 °C for the indicated time period. The reaction was stopped by adding 25 μL of ice-cold 450 mM H_3PO_4, and duplicate 25-μL aliquots of the reaction mixture were spotted onto Whatman P-81 phosphocellulose filter papers. The papers were then washed with four 5-min changes of 150 mM H_3PO_4, followed by a 2-min wash in 99% methanol. The papers were dried, immersed in 2.5 mL of Aquasol-2, and subjected to scintillation counting. For each experimental condition, values for control reactions lacking substrate peptide were subtracted as blanks. In all assays to determine K_ms and Hill coefficients, reaction rates were linear with respect to time for all concentrations of peptide, and less than 10% of the peptide substrate was converted to product.

Phosphorylation of Peptide Substrates by Other Protein Kinases. The catalytic subunit of PKA was assayed using methods similar to those described above using either Kemptide (25 μM) or S6$_{(229-249)}$ (50 μM) as substrates in the presence of 5 mg/mL dithiothreitol and 2.5 mM EGTA. Calcium/calmodulin-dependent protein kinase II (CaMKII) was prepared from rat forebrain using the method of Kuret and Schulman (1984). Unless indicated, CaMKII activity was determined by measuring [^{32}P]phosphate incorporation into a synthetic peptide analogue of a fragment of glycogen synthase, [R^{3},A^{9,10}]GS$_{(1-10)}$ (50 μM), or S6$_{(229-249)}$ (50 μM), in the presence of 100 μM calcium with 500 μM EGTA and 10 μg/mL of calmodulin (Pearson et al., 1985). In all cases, substrate phosphorylation was linear with respect to time and enzyme concentration.

Inhibition of Kinase Activity with Peptide Inhibitors. When an inhibitor peptide was included in the assay, the inhibitor peptide was mixed with the peptide substrate and both were added together to the enzyme for the time period indicated in the appropriate figure legend.

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                  24        *        49
Neurogranin:      A-N-A-A-A-A-K-I-Q-A-S-F-R-G-H-M-A-R-K-K-I-K-S-G-E-R

                  31        *        56
Neuromodulin:     A-H-K-A-A-T-K-I-Q-A-S-F-R-G-H-I-T-R-K-K-L-K-G-E-K-K
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FIGURE 1: Amino acid sequence homology between neurogranin and neuromodulin. These sequences correspond to the conserved phosphorylation site (*) and the putative calmodulin-binding domain on both neurogranin and neuromodulin. The sequence of the synthetic peptide substrate NG$_{(28-43)}$ is underlined. This figure is adapted from Baudier et al. (1991).

RESULTS

Phosphorylation of NG$_{(28-43)}$ by PKC. The region of homologous amino acid sequence shared by neuromodulin (amino acids 31–58) and neurogranin (amino acids 24–51) is illustrated in Figure 1. This region contains a Ser surrounded

Table I: Phosphorylation of $NG_{(28-43)}$ by PKC[a]

cofactor	PKC activity (pmol/min)	
	EGTA	Ca^{2+}
	0.31 ± 0.04	0.36 ± 0.03
PS	0.98 ± 0.14	4.19 ± 0.27
DG	0.42 ± 0.03	0.46 ± 0.03
PS/DG	12.54 ± 0.14	15.30 ± 0.15

[a] $NG_{(28-43)}$ (10 μM) was phosphorylated by PKC in the presence of EGTA (2.5 mM) or calcium (100 μM calcium in the presence of 500 μM EGTA), with the addition of water, phosphatidylserine (PS), dioctanoylglycerol (DG), or phosphatidylserine/dioctanoylglycerol (PS/DG) to the reaction mixture, as described in Experimental Procedures. Values are the average ± SEM of four experiments. Similar cofactor dependency was observed when two other PKC preparations were used.

by several positively charged amino acids, which together comprise a putative consensus PKC phosphorylation site (Apel et al., 1990; House et al., 1987; Kemp & Pearson, 1990; Kennelly & Krebs, 1991). This Ser is known to be phosphorylated by PKC in both neurogranin and neuromodulin (Baudier et al., 1991; Apel et al., 1990) and appears to be of reasonably high affinity as the K_m of neuromodulin for PKC is around 1 μM (Apel et al., 1990). We synthesized a peptide (designated neurogranin$_{(28-43)}$, $NG_{(28-43)}$) corresponding to amino acids 28–43 of neurogranin and determined its potency and selectivity as a PKC substrate. Neurogranin and neuromodulin are highly homologous (81%) within this stretch of sequence. We extended the peptide only to amino acid 43 of neurogranin in order not to include the known calmodulin-binding domain of the protein (Baudier et al., 1991; Apel et al., 1990), so that the peptide would be unlikely to bind calmodulin.

Our initial experiments indicated that $NG_{(28-43)}$ could be phosphorylated by purified PKC in a kinase concentration-dependent and time-dependent manner (data not shown). A low level of phosphorylation of $NG_{(28-43)}$ was observed when PKC activity was assayed in the presence of excess EGTA, and the phosphorylation was significantly enhanced by adding lipid cofactors (PS plus DG) to the reaction mixture (Table I). The phosphorylation of $NG_{(28-43)}$ was further enhanced by adding a low concentration of calcium to the reaction mixture while addition of calcium alone had little effect on PKC phosphorylation of $NG_{(28-43)}$. The calcium and phospholipid dependency of $NG_{(28-43)}$ phosphorylation by PKC is similar to that of neurogranin and neuromodulin (Baudier et al., 1989). These properties suggest that $NG_{(28-43)}$ be classified as a type III PKC substrate (Bazzi & Nelsestuen, 1987).

Previous work by Sheu et al. (1990) has indicated that neuromodulin is a better substrate for PKC-β than other PKC isoforms. To determine if $NG_{(28-43)}$ could serve as a substrate for α, β, and γ isoforms of PKC, the PKC preparation was further fractionated through a hydroxylapatite column and fractions corresponding to type I (PKC-γ), type II (PKC-β plus PKC-ε), or type III (PKC-α) were tested for their abilities to phosphorylate $NG_{(28-43)}$. Phosphorylation of $NG_{(28-43)}$ by each isoform was compared to the phosphorylation of another well-characterized general PKC substrate, $S6_{(229-249)}$ peptide (House et al., 1987). For all three PKC isoforms, $NG_{(28-43)}$ appeared to be a better substrate than $S6_{(229-249)}$, as each peptide was used at a saturating concentration (10 μM) and the ratio of $NG_{(28-43)}$ phosphorylation to $S6_{(229-249)}$ phosphorylation was greater than 100% for each isoform. However, no significant difference in the phosphorylation ratio was observed among all three fractions (152.7 ± 26.1% for type I, 140.7 ± 14.0% for type II; 137.5 ± 21.2% for type III; n = 4), suggesting that $NG_{(28-43)}$ could serve as an effective

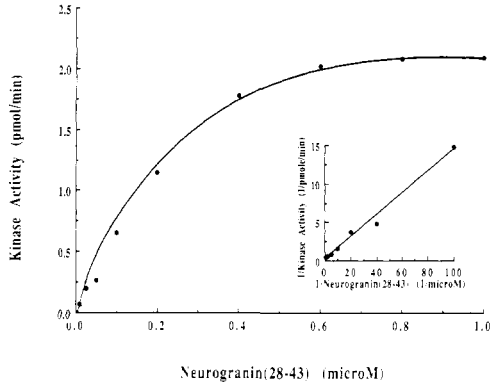

FIGURE 2: Concentration curve for phosphorylation of $NG_{(28-43)}$ by PKC. $NG_{(28-43)}$ at concentrations from 0.01 to 1 μM was phosphorylated by PKC in the presence of calcium, phosphatidylserine, and dioctanoylglycerol as described in Experimental Procedures. Each point represents the mean of four determinations assayed in duplicate; similar results were obtained in four different experiments. The inset shows a double-reciprocal plot of these data.

substrate for each PKC isoform. These data suggest that the differential phosphorylation of neuromodulin by various PKC isoforms that was observed by Sheu et al. (1990) is likely due to determinants on the protein outside of the primary sequence around the phosphorylation site.

In the presence of calcium and lipid cofactors, $NG_{(28-43)}$ was phosphorylated by PKC in a substrate concentration-dependent manner that was consistent with Michaelis–Menten kinetics (Figure 2, inset). Lineweaver–Burke analysis of the phosphorylation data resulted in a linear double-reciprocal plot, using $NG_{(28-43)}$ concentrations from 0.01 to 1 μM (Figure 2, inset). From these data, the apparent K_m for $NG_{(28-43)}$ was calculated to be 150 nM. This value is considerably lower than most of the known peptide substrates for PKC, with the exception of a peptide derived from the MARCKS protein (Graff et al., 1991). The calculated V_{max} for PKC phosphorylation of $NG_{(28-43)}$ was approximately 2.4 μmol/(min·mg of protein)$^{-1}$ at 25 °C. Hill plot analysis revealed a Hill coefficient of 0.97, suggesting noncooperative binding of the substrate peptide. This is in contrast to the behavior of the MARCKS protein-derived PKC substrate peptide, which exhibited positive cooperativity as a substrate for PKC (Graff et al., 1991). No significant inhibition of PKC activity was observed even at an $NG_{(28-43)}$ concentration as high as 100 μM (~700 times the K_m, data not shown), indicating little or no substrate inhibition effect. Maximal phosphorylation of $NG_{(28-43)}$ (10 μM) was achieved at 60 min under our assay conditions, as additional PKC did not increase phosphate incorporation into peptide at this time point. When the peptide was maximally phosphorylated, the amount of phosphate incorporation was calculated to be 0.94 mol of phosphate/mol of $NG_{(28-43)}$.

Phosphorylation of $NG_{(28-43)}$ by PKM. Proteolytic degradation of PKC generates the cofactor-independent, catalytically active fragment of PKC, referred to as PKM. In some cases, PKM has been shown to exhibit a different substrate specificity relative to native PKC (Nakabayashi et al., 1991). We tested the phosphorylation of $NG_{(28-43)}$ by PKM. Like PKC, PKM phosphorylated $NG_{(28-43)}$ in a substrate concentration-dependent manner and the phosphorylation reaction appeared to follow Michaelis–Menten kinetics (Figure 3). No inhibition of phosphorylation was detected at a high substrate concentration (100 μM, data not shown). The apparent K_m for this reaction was estimated to be 147 nM by Lineweaver–Burke analysis (Figure 3, inset).

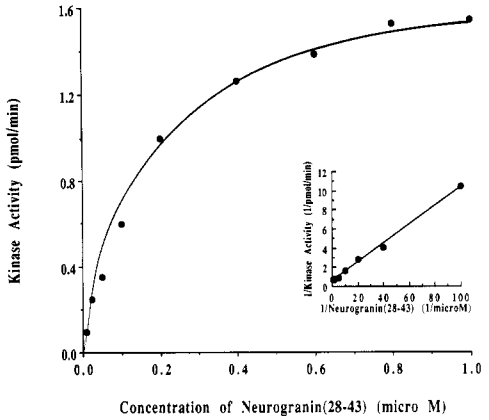

FIGURE 3: Phosphorylation of $NG_{(28-43)}$ by PKM. $NG_{(28-43)}$ at concentrations from 0.01 to 1 μM was phosphorylated by PKM in the presence of 2 mM EGTA as described in Experimental Procedures. Each point represents the average of six determinations; similar results were obtained in a different experiment. The inset shows the double-reciprocal plot of these data.

This value approximates the K_m for the phosphorylation of $NG_{(28-43)}$ by native PKC. Hill plot analysis showed no cooperativity for $NG_{(28-43)}$ as a substrate for PKM (Hill coefficient 1.07). These results together with those described above indicate that $NG_{(28-43)}$ is a potent substrate for both cofactor-dependent PKC and cofactor-independent PKM.

It is known that phosphorylation of neuromodulin and neurogranin by PKC can be inhibited by calmodulin (Chan et al., 1986; Alexander et al., 1987; Baudier et al., 1991), an effect most likely due to binding of calmodulin by neuromodulin and neurogranin. We found no inhibition of $NG_{(28-43)}$ phosphorylation at calmodulin concentrations lower than 10 μM. A higher concentration of calmodulin only partially inhibited PKC phosphorylation of $NG_{(28-43)}$ (36% inhibition with 12 μM calmodulin added). A similar inhibition by calmodulin was observed when $S6_{(229-249)}$ was used as a substrate (28% inhibition with 12 μM calmodulin added). Therefore, the inhibitory effects of calmodulin are probably due to an interaction between calmodulin and PKC rather than a specific interaction between calmodulin and $NG_{(28-43)}$. It is known that the binding of calmodulin to neurogranin and neuromodulin is calcium-independent (Apel et al., 1990; Baudier et al., 1991). In order to determine if calmodulin could attenuate the phosphorylation of $NG_{(28-43)}$ in the absence of calcium, we determined if calmodulin addition affected the phosphorylation of $NG_{(28-43)}$ by PKM, in the presence of excess EGTA. Calmodulin addition had no significant effect on the ability of PKM to phosphorylate $NG_{(28-43)}$, suggesting that calmodulin is unable to bind significantly to $NG_{(28-43)}$ even in the absence of calcium.

Phosphorylation of $NG_{(28-43)}$ by Other Protein Kinases. To determine the specificity of $NG_{(28-43)}$ as a protein kinase substrate, we compared the ability of $NG_{(28-43)}$ to serve as a substrate for PKC, CaMKII, and the catalytic subunit of PKA. After equalizing the phosphorylating activity of each of the three protein kinases using $S6_{(229-249)}$ peptide as substrate, all three protein kinases were tested for their ability to utilize $NG_{(28-43)}$ as substrate (Table II). At all concentrations tested (from 10 to 500 μM) $NG_{(28-43)}$ was readily phosphorylated by PKC while very little phosphate incorporation could be detected when either PKA or CaMKII was used. These results demonstrate that $NG_{(28-43)}$ is not an effective substrate for PKA and CaMKII. In a separate experiment, phosphorylation of $NG_{(28-43)}$ by PKA was compared to Kemptide, a well-characterized PKA substrate.

Table II: Phosphorylation of $NG_{(28-43)}$ by PKC, PKA, and CaMKII[a]

kinase	PKC	CaMKII (pmol/min)	PKA
substrate			
$S6_{(229-249)}$			
50 μM	46.70 ± 0.74	47.03 ± 5.85	57.11 ± 4.47
$NG_{(28-43)}$			
10 μM	67.96 ± 4.10	0.99 ± 0.85	0.96 ± 0.81
50 μM	77.78 ± 0.42	1.13 ± 0.97	1.24 ± 1.02
100 μM	74.90 ± 2.63	1.07 ± 1.06	1.18 ± 1.54
500 μM	71.31 ± 2.22	<0.1	1.71 ± 1.07

[a] $NG_{(28-43)}$ at the concentrations indicated was phosphorylated by PKC, catalytic subunit of PKA, or CaMKII under standard assay conditions described in Experimental Procedures. Each value represents the mean ± SEM of four determinations.

Even with PKA catalytic activity as high as 14.7 μmol/min (100 μM Kemptide as substrate), no phosphorylation of $NG_{(28-43)}$ could be detected using a concentration range from 1 to 500 μM (data not shown).

We also performed an additional experiment to determine if $NG_{(28-43)}$ could serve as a substrate for CaMKII. Under the standard assay conditions (100 μM calcium and 10 μg/mL calmodulin), CaMKII phosphorylated $[R^3,A^{9,10}]GS_{(1-10)}$ efficiently (108 pmol/min at 50 μM substrate concentration) while only a low level (2.8 pmol/min) of phosphorylation of $NG_{(28-43)}$ (50 μM) was detected. Although this low level of phosphorylation of $NG_{(28-43)}$ by CaMKII exhibited substrate concentration dependence, no saturation of phosphorylation was observed at $NG_{(28-43)}$ concentrations up to 500 μM. These results indicate that the K_m of $NG_{(28-43)}$ for CaMKII is higher than 250 μM and are consistent with the results presented above suggesting that $NG_{(28-43)}$ is a poor substrate for CaMKII.

There is one caveat to the interpretation that $NG_{(28-43)}$ is a poor CaMKII substrate: because $NG_{(28-43)}$ contains part of the putative calmodulin-binding sequence of the native protein, $NG_{(28-43)}$ may interact with calmodulin and thus interfere with CaMKII activity by depleting calmodulin from the reaction mixture. To explore this possibility, we measured the catalytic activity of CaMKII in the presence of $NG_{(28-43)}$ using $[R^3,A^{9,10}]GS_{(1-10)}$ (30 μM) as a CaMKII substrate. If $NG_{(28-43)}$ binds calmodulin and thereby inhibits CaMKII activity, the presence of $NG_{(28-43)}$ should attenuate phosphorylation of $[R^3,A^{9,10}]GS_{(1-10)}$. The presence of up to 100 μM $NG_{(28-43)}$ in the reaction mixture showed no significant inhibition of the phosphorylation of $[R^3,A^{9,10}]GS_{(1-10)}$ by CaMKII (134.2 ± 18.3 pmol/min in the absence of $NG_{(28-43)}$; 110.0 ± 5.5 pmol/min in the presence of 100 μM $NG_{(28-43)}$). These results rule out the possibility that $NG_{(28-43)}$ inhibits CaMKII activity and favor the interpretation that $NG_{(28-43)}$ is a poor substrate for purified CaMKII.

Phosphorylation of Peptide Analogues of $NG_{(28-43)}$ by PKC and Other Protein Kinases. Previous studies by House et al. (1987) indicated that basic amino acids, particularly Arg, on both sides of the Ser/Thr phosphorylation site were important determinants in the consensus sequence for phosphorylation of substrates by PKC. An analogue of $NG_{(28-43)}$ with Arg^{36} replaced by an Ile was synthesized and characterized in order to assess the importance of this carboxy-terminal proximal basic amino acid in conferring substrate potency for PKC. $[I^{36}]NG_{(28-43)}$ exhibited a 1000-fold decrease in potency as a PKC substrate (Table III, Figure 4). This result confirms the positive effect of a proximal carboxy-terminal basic amino acid for $NG_{(28-43)}$ as a PKC substrate.

An interesting feature of the sequence of $NG_{(28-43)}$ is the lack of an Arg on the amino-terminal side of the phospho-

Table III: Phosphorylation of $NG_{(28-43)}$ and Peptide Analogues by PKC

	V_{max} (μmole/(min·mg)$^{-1}$)	K_m (μM)	V_{max}/K_m
$NG_{(28-43)}$ AAKIQASFRGHMARKK	2.69	0.15	17.90
$[R^{30}]NG_{(28-43)}$ AARIQASFRGHMARKK	3.00	<0.10	>30.00
$[A^{35}]NG_{(28-43)}$ AAKIQASARGHMARKK	0.52	150	0.003
$[I^{35}]NG_{(28-43)}$ AAKIQASIRGHMARKK	2.60	5.0	0.52
$[I^{36}]NG_{(28-43)}$ AAKIQASFIGHMARKK	0.79	46.0	0.017

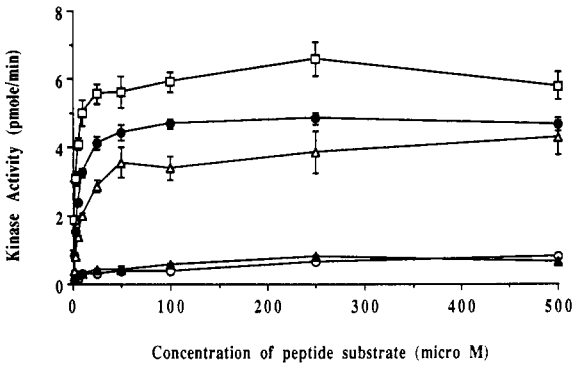

FIGURE 4: Phosphorylation of $NG_{(28-43)}$ and peptide analogues by PKC. $NG_{(28-43)}$ and peptide analogues at concentrations from 1 to 500 μM was phosphorylated with purified PKC in the presence of calcium and lipid cofactor for 2 min as described in Experimental Procedures. Each point represents the mean of three determinations assayed in duplicate; similar results were obtained in three different experiments. Symbols: open square, $[R^{30}]NG_{(28-43)}$; closed circle, $NG_{(28-43)}$; open circle, $[A^{35}]NG_{(28-43)}$; open triangle, $[I^{35}]NG_{(28-43)}$; closed triangle, $[I^{36}]NG_{(28-43)}$.

rylation site. The only basic amino acid amino-terminal to the phosphorylated Ser is Lys^{30}, which is located four amino acids away. Often, replacement of an Arg with a Lys results in a poor substrate for those protein kinases such as PKA and CaMKII which utilize an amino-terminal Arg as a primary specificity determinant (Colbran et al., 1992; Pearson et al., 1985). Thus, we hypothesized that Lys^{30} may serve as a specificity determinant for phosphorylation by PKC versus other kinases. To test this hypothesis, a peptide analogue was synthesized in which the Lys^{30} was replaced by an Arg ($[R^{30}]NG_{(28-43)}$) and the peptide was tested for its specificity as a kinase substrate. $[R^{30}]NG_{(28-43)}$ appeared to be a better substrate for PKC than $NG_{(28-43)}$ (Table III, Figure 4). However, $[R^{30}]NG_{(28-43)}$ was also significantly phosphorylated by CaMKII (Table IV). Phosphorylation of $[R^{30}]NG_{(28-43)}$ by CaMKII was dependent on substrate concentration with the apparent K_m calculated to be 24 μM and a low V_{max} (0.14 μmol/(min·mg of protein)$^{-1}$). $[R^{30}]NG_{(28-43)}$ also displayed an inhibitory effect on CaMKII activity (100 μM of $[R^{30}]$-$NG_{(28-43)}$ inhibited CaMKII phosphorylation of $[R^3,A^{9,10}]$-$GS_{(1-10)}$ (50 μM) by 42%). Therefore, replacing Lys^{30} with Arg greatly enhances the affinity of the peptide for CaMKII and thus abolishes the specificity of $[R^{30}]NG_{(28-43)}$ as a PKC substrate.

We noted the presence of a Phe adjacent to the phosphorylated Ser (SFK/R) among several substrates known to be selective for PKC, including a potent PKC substrate peptide derived from the MARCKS protein which contains three Ser phosphorylation sites with a similar recognition motif (SFK) (Turner et al., 1985; Romhanyi et al., 1985; Chakravarthy et al., 1991; Graff et al., 1991). To assess the importance of

Table IV: Phosphorylation of Substituted $NG_{(28-43)}$ by PKC, PKA, and CaMKII[a]

substrate concn (μM)	PKC	CaMKII (pmole/min)	PKA
$S6_{(229-249)}$			
50	30.74 ± 3.77	53.59 ± 8.07	39.33 ± 1.96
$[R^{30}]NG_{(28-43)}$			
50	74.04 ± 11.74	3.09 ± 0.39	<0.1
500	67.08 ± 19.06	0.17 ± 0.11	<0.1
$[A^{35}]NG_{(28-43)}$			
50	3.76 ± 0.17	0.22 ± 0.01	<0.1
500	11.07 ± 3.33	<0.1	<0.1
$[I^{35}]NG_{(28-43)}$			
50	42.52 ± 11.98	0.14 ± 0.06	<0.1
500	63.43 ± 12.27	0.22 ± 0.03	<0.1
$[I^{36}]NG_{(28-43)}$			
50	10.88 ± 2.05	0.21 ± 0.10	<0.1
500	7.28 ± 2.65	<0.1	<0.1

[a] Peptides at the concentrations indicated were phosphorylated by PKC, catalytic subunit of PKA, or CaMKII under standard assay conditions described in Experimental Procedures. Each value represents the mean ± SEM of three determinations.

Phe^{35} in determining affinity of the neurogranin-derived peptide for PKC, we synthesized a peptide in which the bulky, aromatic side chain of Phe^{35} was replaced by a small, aliphatic side chain (Ala) and tested its phosphorylation by PKC. As Phe is among one of the most hydrophobic amino acids, we also tested if another hydrophobic amino acid, Ile, could substitute for Phe as an affinity determinant. Substitution of the Phe^{35} with an Ala resulted in a 1000-fold increase in K_m and a 6000-fold decrease in catalytic efficiency (V_{max}/K_m) of the peptide, indicating a crucial role for Phe^{35} in conferring potency and efficacy for $NG_{(28-43)}$ as a PKC substrate (Table III). The loss of reactivity was much smaller when Ile was substituted for Phe^{35} (roughly a 30-fold increase in K_m and essentially no effect on V_{max}) (Table III). These data provide strong support for the hypothesis that the local hydrophobicity near the phosphorylation site serves as an important potency determinant for PKC substrates. However, other factors such as the size of the side chain or the presence of aromatic versus aliphatic groups may also play an important role in optimizing substrate–kinase interactions.

Inhibition of PKC Activity by $[A^{34}]NG_{(28-43)}$. We also tested the possibility that substituting the single Ser in $NG_{(28-43)}$ with an Ala might result in a peptide that served as a PKC inhibitor, as has been described previously for other PKC substrates (Graff et al., 1991). As a positive control, we used the PKC inhibitor peptide, $PKC_{(19-36)}$. Phosphorylation by PKC and PKM of $NG_{(28-43)}$ and three other PKC peptide substrates, $S6_{(229-249)}$, $GS_{(1-12)}$, and $[A^{35}]NG_{(28-43)}$, was inhibited by the addition of $PKC_{(19-36)}$. The IC_{50} of $PKC_{(19-36)}$ was approximately 0.5 μM when 10 μM $NG_{(28-43)}$ was used as substrate and nearly complete inhibition of the phosphorylation of $NG_{(28-43)}$ by PKC was achieved by 10 μM $PKC_{(19-36)}$ (data not shown). $[A^{34}]NG_{(28-43)}$ at low concentrations did not inhibit $NG_{(28-43)}$ and $S6_{(229-249)}$ phosphorylation, and at a high concentration only partial inhibition was observed (~20% inhibition at 500 μM $[A^{34}]NG_{(28-43)}$). $[A^{34}]NG_{(28-43)}$ exhibited similarly poor inhibitory effects when $NG_{(28-43)}$ and $S6_{(229-249)}$ were phosphorylated by PKM (data not shown). $[A^{34}]NG_{(28-43)}$ showed a dose-dependent inhibition of the phosphorylation of two other peptide substrates of fairly high K_m for PKC. PKC phosphorylatino of both $GS_{(1-12)}$ and $[A^{35}]NG_{(28-43)}$ phosphorylation was inhibited by $[A^{34}]NG_{(28-43)}$. The K_i of $[A^{34}]NG_{(28-43)}$ was calculated to be 22 μM when $GS_{(1-12)}$ was used as a substrate (Figure 5C), indicating that $[A^{34}]NG_{(28-43)}$ is a relatively poor inhibitor

compared to PKC$_{(19-36)}$ (K_i = 0.15 μM; House & Kemp, 1987). The inhibition appeared to be of a mixed type mechanism (Figure 5B). We also tested the effect of [A^{34}]NG$_{(28-43)}$ on PKA and CaMKII activity and observed no inhibition of either protein kinase (data not shown). Therefore, the [A^{34}]NG$_{(28-43)}$ peptide does not appear to be a potent inhibitor of PKC or the other kinases tested.

The low potency of the inhibitor peptide, which presumably binds to the active site of PKC in a manner similar to that of substrate peptides, may indicate a crucial role for Ser^{34} in determining the affinity of binding of peptides to PKC. However, at low concentrations the inhibitor peptide caused some stimulation of PKC activity (Figure 5A). A similar stimulatory effect has been reported for inhibitor peptides derived from the MARCKS protein sequence (Graff et al., 1991). This stimulatory effect of the inhibitor peptide may elevate its apparent K_i for PKC and thus cause an underestimate of its true affinity for PKC.

DISCUSSION

Many protein kinases utilize basic amino acids in the region of the phosphorylated Ser/Thr as the primary specificity determinants in their substrates. This generalization holds for phosphorylase *b* kinase (Tessmer et al., 1977), PKA (Kemp et al., 1977), cGMP-dependent protein kinase (Glass & Krebs, 1982), PKC (Turner et al., 1985), smooth muscle myosin light chain kinase (Kemp & Pearson, 1983), histone H4 kinase (Eckols et al., 1983), and CaMKII (Pearson et al., 1985). However, there are subtle differences in consensus sequences for individual kinases. For example, an amino-terminal Arg–Arg doublet allows optimal efficiency for PKA (Kemp et al., 1983). Similar to PKA, the cGMP-dependent protein kinase also possesses a stringent requirement for multiple basic residues at the amino-terminal side (Kennelly & Krebs, 1991). A Lys to Arg substitution greatly diminished the ability of a peptide derived from cGMP-binding cGMP-specific phosphodiesterase to serve as substrate for cGMP-dependent kinase and eliminated the ability of PKA to phosphorylate the peptide substrate (Colbran et al., 1992). Pearson et al. (1985) suggest that Arg-X-Y-Ser (Thr) may represent the minimum specificity determinant of skeletal muscle CaMKII and in these studies a Lys-substituted peptide analogue was a poor CaMKII substrate. Our data indicate that the presence of a Lys four amino acids amino-terminal to the phosphorylation site is not sufficient for efficient phosphorylation by PKA or CaMKII.

It has been established that phosphorylation efficiency by PKC can be improved by increasing the number of basic residues close to the phosphorylated serine, on either their carboxy- or amino-terminal side, or both (Ferrari et al., 1985). However, the use of various synthetic peptides to study the structural requirements for PKC phosphorylation has yielded somewhat conflicting conclusions. In studies using peptides based on a site from myelin basic protein, the enzyme appeared to exhibit a requirement for a basic residue amino-terminal to the Ser/Thr phosphorylation site (Turner et al., 1985). While PKC could phosphorylate proteins and peptides at sites with only amino-terminal basic amino acids in vitro, these sites have not yet been shown to be phosphorylated by PKC in the cell (Nishizuka et al., 1978). In contrast, another report suggested that basic amino acids carboxy-terminal to the target phosphorylation site were of primary importance for recognition by PKC (Ferrari et al., 1985).

Our analyses of the synthetic peptide derived from the phosphorylation site of a selective PKC substrate, neurogranin, provide some insight into the selective recognition motif for

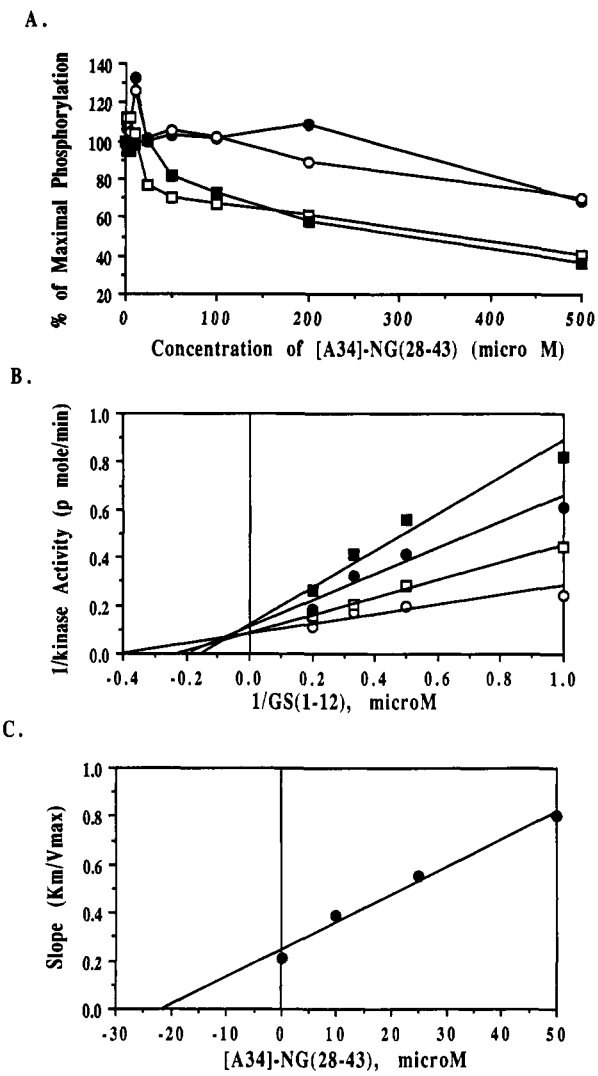

FIGURE 5: Effects of [A^{34}]NG$_{(28-43)}$ on PKC activity. (A) Various concentrations of [A^{34}]NG$_{(28-43)}$ were used to inhibit PKC-dependent phosphorylation of NG$_{(28-43)}$ (10 μM) (open circle), S6$_{(229-249)}$ (10 μM) (closed circle), GS$_{(1-12)}$ (20 μM) (open squre), and [A^{35}]NG$_{(28-43)}$ (200 μM) (closed square) as described in Experimental Procedures. The inhibitor and substrate peptide were added together to the reaction mixture containing Ca/PS/DAG, and the reaction was carried out for 2 min. Results are plotted as a percentage of kinase activity measured in the absence of inhibitor peptide. Each point represents the average of four experiments. (B) Kinetics of the [A^{34}]NG$_{(28-43)}$ inhibition of PKC. Various concentrations of [A^{34}]NG$_{(28-43)}$ were used to inhibit PKC-dependent phosphorylation of GS$_{(1-12)}$ peptide. Double-reciprocal plot of the data demonstrate a noncompetitive inhibition. Concentration of [A^{34}]NG$_{(28-43)}$ used: open circle, 0; open square, 10 μM; closed circle, 25 μM; closed square, 50 μM. (C) K_i determination of [A^{34}]NG$_{(28-43)}$ using GS$_{(1-12)}$ as substrate. The plot of slope (K_m/V_{max}) versus inhibitor indicates that the K_i is about 22 μM.

PKC and support the latter model for recognition by PKC of a phosphorylation site. Kinetic data from [I^{36}]NG$_{(28-43)}$ show that the presence of a basic amino acid on the carboxy-terminal side is required to achieve optimal phosphorylation efficiency. However, as [I^{36}]NG$_{(28-43)}$ was still a minimally effective PKC substrate, the presence of a single Lys amino-terminal to the phosphorylated Ser on NG$_{(28-43)}$ apparently satisfied the minimum positive charge requirement necessary in PKC substrates. Although the presence of an amino-terminal Arg (vs Lys) in our NG$_{(28-43)}$ analogues decreased the K_m for PKC, our results point out that it could exert a negative effect on the specificity of the substrate because the peptide is rendered more effective as a CaMKII substrate. Taken together, our

results indicate that the location as well as the identity of the basic amino acid can have a great impact on PKC substrate specificity.

We found that a Phe to Ala substitution adjacent to the phosphorylated Ser caused a dramatic (~1000-fold) increase in K_m for PKC, suggesting that the presence of a Phe near the phosphorylated residue can greatly affect affinity of substrates for PKC. This hypothesis is supported by the observation that a similar sequence exists in another potent PKC substrate peptide, a peptide derived from the MARCKS protein. In the MARCKS peptide, three of the four Ser phosphorylation sites are adjacent to a Phe. Our hypothesis would predict that Ser^{16} in the MARCKS protein, which is not adjacent to a Phe, should have lower affinity for PKC than the other three Ser phosphorylation sites in the peptide. Indeed, several reports from other laboratories have shown that Ser^{16} is the least preferred phosphorylation site in the MARCKS peptide and the native MARCKS protein (Graff et al., 1989; McIlroy et al., 1991). This hypothesis can also explain the observation that the maximally phosphorylated MARCKS protein or peptide only incorporate 3 mol of phosphate/mol of protein (Graff et al., 1989, 1991).

The results using $[I^{35}]NG_{(28-43)}$ and $[A^{35}]NG_{(28-43)}$ suggest that a hydrophobic interaction is important in determining the binding between substrate and PKC. This interpretation is supported by the observation that 23 of 37 PKC phosphorylation site sequences contain an adjacent hydrophobic amino acid on the carboxy-terminal side of the phosphorylated Ser/Thr (Pearson & Kemp, 1991). The amino acid with the shortest side chain found is Val or Cys while most PKC substrates have either Leu or Phe at this position, suggesting that the length of the nonpolar side chain, rather than the aromatic property, is the main determinant for this interaction. The presence of a hydrophobic amino acid is also found in the proposed pseudosubstrate sequence of several PKC isoforms (Kemp et al., 1991).

Overall, the results of our studies suggest several possible modifications to the currently-accepted model for a PKC consensus phosphorylation sequence (see Kennelly and Krebs (1991) for a review). We have found that a positively-charged amino acid four residues amino-terminal to the phosphorylated Ser can have an effect on both PKC affinity and selectivity. In addition, we have observed a dramatic effect on substrate binding of hydrophobic amino acids immediately adjacent to the phosphorylation site. This suggests that the model of a high-affinity PKC phosphorylation site might include not only positively-charged amino acids but also hydrophobic amino acids near the phosphorylated Ser.

We found that $NG_{(28-43)}$ can serve as a substrate for various PKC isoforms (types I, II, and III; γ, β, and α, respectively). In addition, we have found no difference in the affinity between PKC and PKM for $NG_{(28-43)}$. A recent study indicates that both PKC-α and PKC-β display a requirement for a basic amino acid amino-terminal to the phosphorylation site while PKC-γ showed an additional requirement for a basic amino acid carboxy-terminal to the phosphorylated Ser (Marais et al., 1990). The sequence of $NG_{(28-43)}$ meets the sequence requirements for all three PKC isoforms, which is consistent with our observation that $NG_{(28-43)}$ can serve as a substrate for all three types of PKC.

The MARCKS protein, neuromodulin, and neurogranin are potent PKC substrates, and they are all calmodulin-binding proteins. However, certain structural features distinguish $NG_{(28-43)}$, a selective and potent PKC substrate, from the peptide derived from the MARCKS protein, a potent but less selective substrate. For example, the MARCKS protein and peptides derived from the MARCKS protein bind to calmodulin in a calcium-dependent manner with high affinity (K_d 2.8 nM) (McIlroy et al., 1991) whereas neurogranin, neuromodulin, and related peptides bind to calmodulin in the absence of calcium with a much lower affinity (K_d 4.2 μM) (Chapman et al., 1991). This difference in calmodulin-binding ability appears to be reflected in their effects on CaMKII. In spite of being a poor CaMKII substrate, the MARCKS protein and related peptides are potent inhibitors of CaMKII and other calmodulin-dependent protein kinases (Graff et al., 1991). In contrast, $NG_{(28-43)}$ has little effect on CaMKII activity and does not interact with calmodulin with high affinity. A second difference between $NG_{(28-43)}$ and the MARCKS peptide is that the MARCKS peptide can be phosphorylated by PKA with low affinity (K_m = 37 μM) while no phosphorylation of $NG_{(28-43)}$ by PKA could be detected even with extremely high concentrations of peptide substrate and protein kinase. In the sequence of $NG_{(28-43)}$, both the lack of multiple basic residues at an appropriate spacing amino-terminal to the phosphorylation site and the presence of multiple basic amino acids at close proximity carboxy-terminal to the phosphorylation site present negative determinants as a PKA substrate (Kemp et al., 1977). In addition, the presence of a Phe carboxy-terminal to the phosphorylation site could also serve as a negative determinant for PKA phosphorylation (Colbran et al., 1992).

$NG_{(28-43)}$ should be useful for measuring PKC activity even in crude cell extracts. The ability to selectively measure a low level of PKC activity in a crude cellular homogenate requires a substrate with high affinity and high selectivity. $NG_{(28-43)}$ appears to meet these requirements. $NG_{(28-43)}$ can be phosphorylated by both cofactor-dependent PKC and cofactor-independent PKM with high efficacy and is among the most potent PKC substrates reported to date. However, unlike other potent PKC substrates such as the MARCKS protein-derived peptide, $NG_{(28-43)}$ can be used at a high substrate concentration without causing inhibition. This attribute should make $NG_{(28-43)}$ useful as a PKC substrate for tissue containing low PKC activity. In common with its parent protein, $NG_{(28-43)}$ cannot be significantly phosphorylated by either PKA or CaMKII (Baudier et al., 1989; Chan, 1986; Baudier et al., 1989). This attribute of $NG_{(28-43)}$ should allows its use in selectively measuring PKC activity in the presence of these other protein kinases. The favorable kinetic parameters of $NG_{(28-43)}$, coupled with its selectivity for phosphorylation by PKC, should facilitate measurement of PKC activity in crude tissue and cell extracts.

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