Phosphorylation of GAP-43 (growth-associated protein of 43 kDa) by conventional, novel and atypical isotypes of the protein kinase C gene family: differences between oligopeptide and polypeptide phosphorylation

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GAP-43 (growth-associated protein of 43 kDa; also known as neuromodulin, P-57, B-50 and F-1) is a neuronal calmodulin binding protein and a major protein kinase C (PKC) substrate in mammalian brain. Here we describe the phosphorylation by and the site specificity of different PKC isotypes. The conventional PKC β_1 and the novel PKCs δ and ϵ effectively phosphorylated recombinant GAP-43 *in vitro*; atypical PKC ζ did not. The K_m values (between 0.6 and 2.3 μM) were very low, demonstrating a high-affinity interaction between kinase and substrate. All PKC isotypes were shown to phosphorylate serine-41 in GAP-43. When using a 19-amino-acid oligopeptide based on the GAP-43 phosphorylation site as substrate, there was a significant difference compared with polypeptide phosphorylation. The V_{max} values of PKC β_1 and PKC ϵ were much higher for this oligopeptide than for the complete protein (up to 10-fold); in contrast, their apparent affinities for the peptide were much lower (up to 100-fold) than for the intact GAP-43 polypeptide. Furthermore, phosphorylation of the GAP-43 oligopeptide by PKC β_1 was more sensitive to a catalytic-site inhibitor than was phosphorylation of intact GAP-43. These results suggest that there are multiple sites of interaction between GAP-43 and PKC.

INTRODUCTION

The hydrophilic, acidic, membrane protein GAP-43 (growth-associated protein of 43 kDa), which is also known as neuromodulin, B-50, P-57 and F-1, was originally identified as a protein the expression of which is greatly increased during development and regeneration of the vertebrate nervous system [1]. Phosphorylated GAP-43 has been linked directly to the persistence of long-term potentiation [2,3], synaptic plasticity [4] and neurotransmitter release [5]. Although the precise function of GAP-43 is unknown, there are several lines of evidence indicating that it is required for axonal growth [6–9].

A function of GAP-43 in signal tranduction can be deduced from mice lacking GAP-43. These mice die in the early postnatal period [10], and it was hypothesized that GAP-43 serves to amplify pathfinding signals from the growth cone of neurons. In fact, GAP-43 has been shown to interact with two important components of signal transduction pathways. Firstly, it has been reported to stimulate the binding of GTP to G_o [11], and increases the activity of G-protein-coupled receptors when injected into *Xenopus* oocytes [12]. Secondly, a domain of GAP-43 binds to Ca^{2+}-free calmodulin [13]; elevated Ca^{2+} levels or phosphorylation by protein kinase C (PKC) decrease the affinity of GAP-43 for calmodulin drastically [14,15].

GAP-43 is post-translationally modified by palmitoylation [16], which is required for targeting to the membrane [17]. It can be phosphorylated by casein kinase II *in vitro* (but this particular site probably is not phosphorylated *in vivo* [18]) and by phosphorylase kinase [19]. GAP-43 is not phosphorylated by cAMP-dependent protein kinase or calcium/calmodulin-dependent protein kinases [15]. Two novel phosphorylation sites were recently identified by Taniguchi and colleagues [20] by mass spectrometric studies of GAP-43 isolated from rat brain. These authors found that threonine-87 and serine-152 are the target sites of proline-directed protein kinases such as mitogen-activated protein (MAP) kinase. It is well established that GAP-43 is a major PKC substrate in the brain.

PKC encompasses a family of closely related serine/threonine kinases [21–23] which plays a crucial role in signal transduction for a variety of extracellular signals [24,25]. However, the molecular mechanisms of the processes leading to PKC activation, as well as those following activation, remain to be elucidated. The PKC family members can be divided into three distinct classes based on differences in their structures and biochemical properties [22]. The activity of conventional (cPKC) members (α, β_1, β_2 and γ) depends on the presence of phospholipids, Ca^{2+} and either diacylglycerol or tumour-promoting phorbol esters that mimic diacylglycerol. In contrast, the activity of novel (nPKC) members (δ, ϵ, η/L, θ and μ/PKD) is Ca^{2+}-independent, and that of atypical (aPKC) members (ζ and λ/ι) is not regulated by either Ca^{2+} or diacylglycerol. A similar complexity may exist with respect to the substrates of the PKC isotypes.

GAP-43 was previously shown to be phosphorylated by PKC at serine-41 [26] and was described to be more efficiently phosphorylated by PKC β_{II} (identical with PKCβ_1) than by PKCs β_I (i.e. β_2) and γ [27]. However, the specificity of nPKCs and aPKCs towards GAP-43 has not been studied so far. It is reported here that GAP-43 is differentially recognized as a substrate by the PKC isotypes investigated, both with the whole protein and with a synthetic oligopeptide the sequence of which resembles the PKC phosphorylation site of GAP-43. Further-

Abbreviations used: GAP-43, growth-associated protein of 43 kDa; GST, glutathione S-transferase; MARCKS, myristoylated alanine-rich C kinase substrate; PDB, phorbol 12,13-dibutyrate; PKC, protein kinase C; cPKC, conventional PKC; nPKC, novel PKC; aPKC, atypical PKC; PS, phosphatidylserine.

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more, we provide evidence suggesting that PKC interacts with GAP-43 not only via its catalytic domain.

MATERIALS AND METHODS

Purification of PKC isoenzymes, glutathione S-transferase (GST)–GAP-43 and His-GAP-43 fusion proteins

The full-length cDNAs coding for the murine PKC isotypes β_1 (also known as β_{II}, a representative of the cPKCs), δ and ϵ (nPKCs) and ζ (an aPKC) were expressed in baculovirus-infected insect cells and purified essentially as described [28]. The catalytic activities of the different preparations were balanced against that of the ϵ-peptide as a reference substrate in all PKC assays. The ϵ-peptide, which resembles the PKC ϵ pseudo-substrate site with a serine for alanine substitution [29], has been shown to be a mutual substrate for a variety of PKC isoenzymes. One unit of PKC activity is defined as that amount of enzyme which catalyses the incorporation of 1 nmol of phosphate into the ϵ-peptide per min under standard assay conditions as described below.

In order to obtain GAP-43 as a PKC substrate in sufficient amounts, we cloned the full-length coding sequence of GAP-43 downstream of the GST gene. Using PCR, a fragment containing 678 bp of the GAP-43 coding region starting with the second triplet (Leu) and ending with the stop codon of the rat cDNA was amplified. For PCR, a 5′ primer containing a *Bam*HI site, a 3′ primer containing an *Eco*RI site, the rat GAP-43 cDNA pF1 (kindly provided by A. Routtenberg) and Vent DNA polymerase (New England BioLabs Inc.) were used as described [30]. After digestion with the restriction enzymes *Bam*HI and *Eco*RI, the PCR fragment was ligated into the vector pGEX-2T (Pharmacia), which was digested with the same enzymes. The resulting plasmid was called pGEX-F1. In addition, we designed a His-tagged GAP-43 fusion protein in which the identical GAP-43 coding sequence as for the GST fusion was amplified, but with a 5′ primer containing a *Bam*HI restriction site and a 3′ primer with a *Kpn*I site. This PCR fragment was cloned downstream of the sequence coding for six histidine residues of the pQE30 vector (Qiagen). Correct amplification and in-frame ligation with the genes coding for GST and the His tag were confirmed by DNA sequencing of the respective constructs. The GST–GAP-43 fusion protein was purified by gluthatione–Sepharose 4B® (Pharmacia) chromatography as described previously [31]. *Escherichia coli* cells (XL-1 Blue; Stratagene) expressing the His-GAP-43 protein were lysed in the same manner and loaded on a Ni^{2+} nitrilotriacetate column as recommended by the manufacturer (Qiagen). Bound protein was eluted by a salt (300–1000 mM NaCl) and pH (pH 6.0–4.0) gradient in wash buffer. Typically, His-GAP-43 protein was specifically eluted at 750 mM NaCl and pH 5.0. Before using the isolated GST–GAP-43 and His-GAP-43 proteins as substrates in PKC phosphorylation assays, they were desalted by using a Sephadex G-25 column (Pharmacia). The concentrations of the full-size fusion proteins were determined by loading increasing amounts on SDS/polyacrylamide gels and comparison with BSA standards.

Cleavage of the GST fusion protein was carried out with thrombin (Sigma) essentially using the manufacturer's recommended protocol (Qiagen). Cleaved samples were heat-treated prior to use (recombinant GAP-43 is heat-stable; S. A. Oehrlein and T. Herget, unpublished work).

PKC phosphorylation assay *in vitro*

PKC activity was assayed in a modified mixed micellar assay [29]. The standard assay mixture contained 0.25 % (v/v) Triton X-100, 50 mM Hepes/NaOH, pH 7.5, 0.25 mM EDTA, 0.125 mM dithiothreitol, 12.5 mM $MgCl_2$, 0.75 mM $CaCl_2$, 0.5 mM EGTA and 100 μM [γ-^{32}P]ATP (specific radioactivity 170–350 c.p.m./pmol) in a final volume of 40 μl. The substrates used were as follows. (1) ϵ-Peptide is the synthetic peptide NH_2-ERMRPRKRQGSVRRRV-CO_2H, resembling the PKC ϵ pseudo-substrate site, with a serine for alanine substitution (underlined) [29]. (2) Histone III-S and protamine sulphate were purchased from Sigma. (3) GAP-43 peptide with the amino acid sequence NH_2-H^{32}KAATKIQASFRGHITRKK^{50}-CO_2H (according to the numbering of the rat sequence [32]) was synthesized in an Applied Biosystems synthesizer. (4) GST and GST–GAP-43 were purified by glutathione–Sepharose affinity chromatography. (5) The His-GAP-43 fusion protein was purified as described above. The concentrations of the substrates used are described in the text and respective figure legends. Autophosphorylation assays were performed under the same conditions but without adding substrate. After incubation at 30 °C for the indicated periods of time, the reactions were stopped by spotting 20 μl of the reaction mixture on to P81 phosphocellulose paper (Whatman; 1 cm^2) followed by immediate immersion in 5 % phosphoric acid. The papers were washed three times for 10 min each and counted for Cerenkov radiation. In some experiments the residual 20 μl of the reaction mixture was mixed with 2 × Laemmli sample buffer, loaded on to SDS/polyacrylamide gels and subsequently examined by autoradiography.

The inhibitor peptide used (Calbiochem) corresponded to the PKC pseudo-substrate regulatory sequence (NH_2-R^{19}FARKG-ALRQKNVHEVKN^{36}-CO_2H) of PKCs α and β, and is a potent and selective PKC inhibitor which binds to the active site of the kinase [33].

Two-dimensional phosphopeptide mapping

For tryptic peptide analyses, the phosphorylation reactions were performed as described above, terminated by adding Laemmli sample buffer and the phosphorylated His-GAP-43 protein and the GAP-43 peptide were loaded on to 7.5 % and 12.5 % polyacrylamide/SDS gels respectively. After transfer on to nitrocellulose membranes (BA85; Schleicher & Schüll) by semi-dry blotting, the bands were visualized by autoradiography and excised. The pieces of membrane were blocked with 0.5 % polyvinylpyrrolidine-40 in 100 mM acetic acid at 30 °C for 30 min, washed seven times with deionized water, and incubated for 18 h with 2 μg of trypsin (sequencing grade; Boehringer Mannheim) in 20 mM NH_4HCO_3, pH 7.8, as described [34]. More than 80 % of the incorporated radioactivity of the GAP-43 fusion protein and more than 60 % of the radioactivity of the GAP-43 peptide was released from the membranes by the trypsin digests. The resulting phosphopeptides in the supernatant were repeatedly lyophilized and then analysed on cellulose chromatogram plates (Merck, Darmstadt, Germany) by two-dimensional peptide mapping. Electrophoresis in the first dimension was at 1000 V for 30 min at pH 3.5 in pyridine/acetic acid/water (1:10:189, by vol.), and ascending chromatography in the second dimension was in n-butanol/pyridine/acetic acid/water (75:50:15:60, by vol.) at room temperature. The plates were dried, and phosphorylated peptides were detected by autoradiography.

RESULTS

Phosphorylation of GAP-43 by PKCs β_1, δ, ε and ζ *in vitro*

Bacteria-expressed purified GAP-43 proteins (GST- or His-tagged) were tested as substrates for baculovirus-expressed purified PKC isotypes. As shown in Figure 1(A), PKCs β_1, δ and

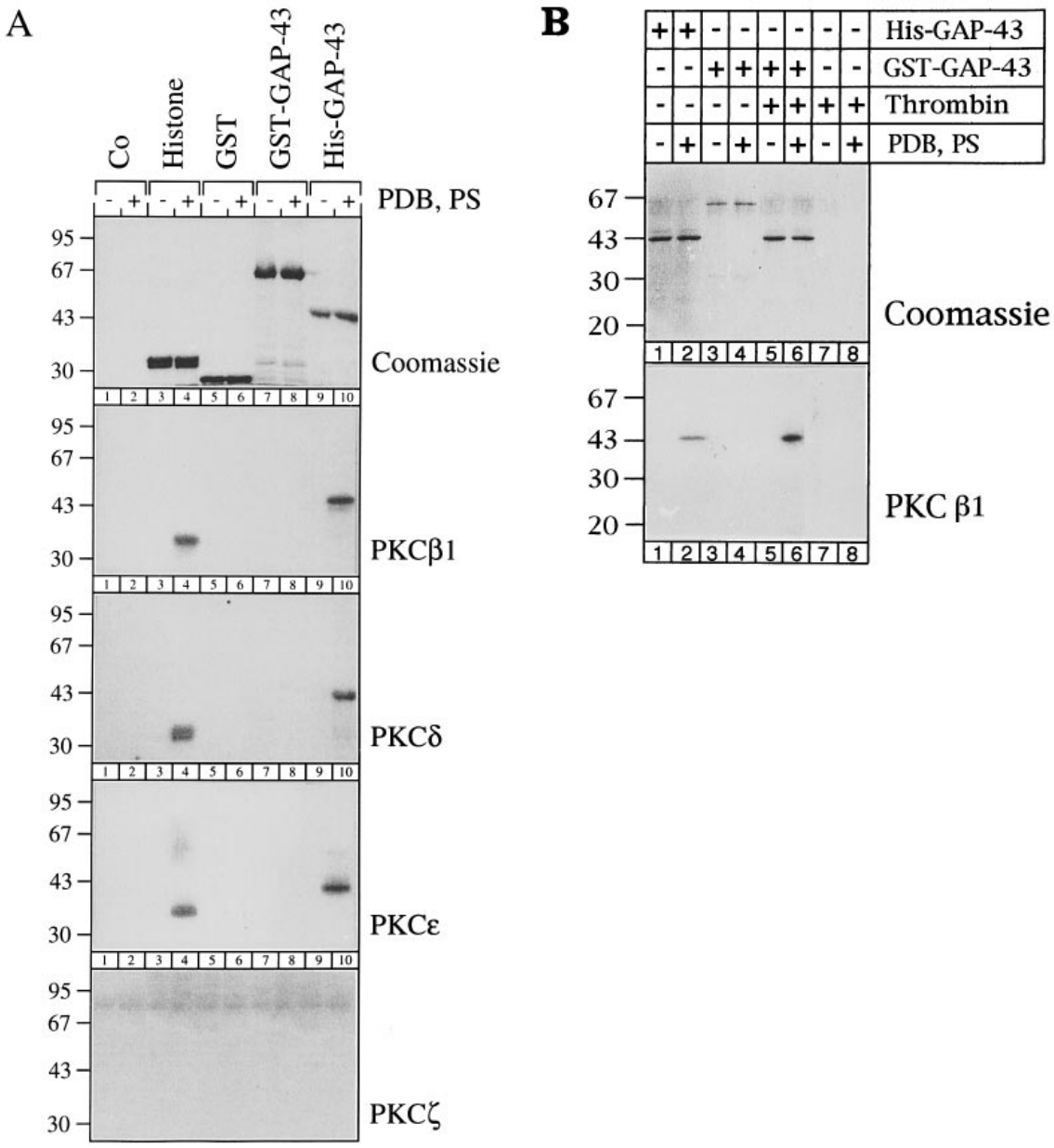

Figure 1 (A) Analysis of phosphorylation by PKC isotypes, and (B) phosphorylation of GAP-43, but not GST–GAP-43, by PKC

(**A**) The PKC isotypes β_1, δ, ϵ and ζ (0.05 unit/ml) were incubated in a mixed micellar assay without substrate (Co) or with histone IIIS (250 μg/ml), GST (50 μg/ml), GST–GAP-43 (50 μg/ml) or His-GAP-43 (50 μg/ml) as indicated. Incubation proceeded at 30 °C for 10 min in the absence (−) or presence (+) of 1.25 μg/ml phorbol 12,13-dibutyrate (PDB) and 1.25 mg/ml phosphatidylserine (PS), as described in the Materials and methods section. An aliquot of each reaction mixture was loaded on a 7.5% polyacrylamide/SDS gel, stained with Coomassie Blue (Coomassie) and analysed by autoradiography. Dried gels were exposured for 18 h (PKCs β_1, δ and ϵ) or for 3 days (PKC ζ) at −70 °C on Kodak XAR-5 films. Representative results of three to five experiments per PKC isotype are shown. The positions of protein markers (kDa) are indicated on the left. (**B**) The GST–GAP-43 fusion protein was digested with thrombin, and 0.5 μg of the purified GAP-43 was incubated with PKC β_1 in the presence of 1.25 mg/ml PS and 1.25 μg/ml PDB for 5 min (lane 6). As controls, His-GAP-43 (lane 2), GST–GAP-43 before thrombin digestion (lane 4) and thrombin alone (lane 8) were assayed as indicated. Reaction mixtures were separated by PAGE (10% gels); the upper panel shows the gel after staining with Coomassie blue. The lower panel depicts autoradiography of the same gel. Only His-GAP-43 (lane 2) and GAP-43 without GST tagging (lane 6) were phosphorylated in the presence of activators. Thrombin itself was not a substrate for PKC β_1 (lanes 7 and 8). The sizes of marker proteins (kDa) are delineated on the left.

ϵ all phosphorylated His-GAP-43 in an effector-dependent fashion and at a rate comparable with their histone kinase activities. PKC ζ showed autophosphorylation, but only very low activity towards His-GAP-43 or histone.

GST–GAP-43 was found not to be a substrate. However, on cleavage of GAP-43 from the GST fusion, the protein was readily phosphorylated (Figure 1B). Again, this phosphorylation was effector-dependent. It would appear that the dimerization of the GST [35] is responsible for preventing phosphorylation.

The comparative activities of these PKC isotypes with His-GAP-43 and other common substrates [23,29,36] are summarized in Figure 2. The lipid-dependent phosphorylation of His-GAP-

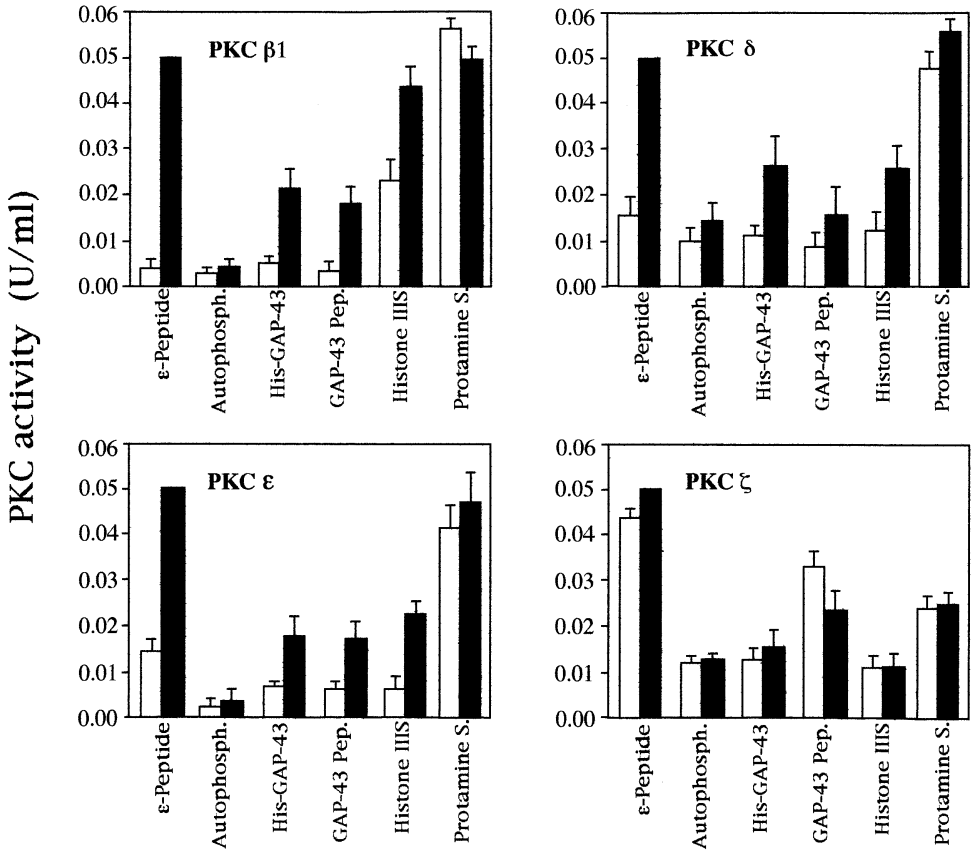

Figure 2 Substrate specificity of PKCs β_1, δ, ε and ζ

PKC activity was determined in the mixed micellar assay under standard conditions in the absence (open bars) or presence (closed bars) of 1.25 mg/ml PS and 1.25 μg/ml PDB as activators. The following substrates were used: ϵ-peptide (25 μg/ml), His-GAP-43 (50 μg/ml), GAP-43 peptide (GAP-43 Pep.; 25 μg/ml), histone IIIS (250 μg/ml) and protamine sulphate (protamine S.; 250 μg/ml). To estimate autophosphorylation of PKC isotypes, buffer was added instead of substrate (Autophosph.). Details of the filter-binding assay are described in the Materials and methods section. All PKC preparations were diluted so that their enzymic activities were identical (0.05 unit/ml) when balanced against the incorporation of ^{32}P into the ϵ-peptide in the presence of activators. The values depicted here represent means $\pm$ S.E.M. of three to five experiments, each performed in duplicate.

43 is evident for all except PKC ζ which, as indicated above, showed very low activity towards the intact protein.

To validate the kinetic analysis of GAP-43 phosphorylation by these PKC isotypes, site mapping was carried out. Prolonged incubation of GAP-43 with PKCs β_1, δ, ϵ and ζ resulted in the phosphorylation of the same single site in each case (Figure 3, left-hand panels). This phosphopeptide migrated with a net charge of $+0.5$ and an R_F of 0.46 relative to ϵ-dinitro-pyridyllysine. This is consistent with phosphorylation on the tryptic fragment NH_2-I^{38}QAS*FR^{43}-CO_2H, previously identified as a PKC site [15,20]. Confirmation was obtained by phosphorylation of the synthetic GAP-43 peptide (residues 32–50), tryptic digestion and mapping (Figure 3, right-hand panels). The same tryptic phosphopeptide was identified. Additional confirmation was obtained by N-terminal Edman degradation [37] (results not shown).

Kinetics of GAP-43 phosphorylation by PKCs β_1, δ, ε and ζ

For kinetic studies, the activities of all PKC isotypes were determined in the presence of PDB and PS with the common oligopeptide substrate ϵ-peptide, and activities were diluted to 0.5 unit/ml. Apparent K_m and V_{max} values for the substrates His-GAP-43, GAP-43 peptide and, for comparison, ϵ-peptide are summarized in Table 1. For PKCs β_1, δ and ϵ there was little difference in K_m and V_{max} values with respect to His-GAP-43; PKC ζ activity against His-GAP-43 was too low for adequate kinetic analysis. By contrast with His-GAP-43, phosphorylation of the GAP-43 peptide that encompasses the PKC site (see above) yielded quite varied kinetic values. The V_{max} for PKC β_1 with this substrate was nearly 40-fold higher than that determined for PKC δ.

In comparing the phosphorylation of His-GAP-43 with that of the GAP-43 peptide, it is notable that PKCs β_1 and ϵ displayed V_{max} values that were 9-fold and 2-fold respectively higher for the peptide. By contrast, PKC δ showed an 8-fold decreased V_{max} for the GAP-43 peptide. Additionally, the apparent K_m values for PKCs β_1 and ϵ with His-GAP-43 as substrate were 16–94-fold lower than with the GAP-43 peptide; this was not the case for PKC δ, where no difference was observed.

An explanation for the discrepant kinetic behaviour of the GAP-43 protein and the GAP-43 peptide as substrates for PKC is that there is more than one site of contact for the protein, i.e. interaction not just through the immediate catalytic site. In order to address this, use was made of a catalytic-site competitive inhibitor by comparing its potency as an inhibitor with the two GAP-43 substrates. As illustrated in Figure 4, the inhibitor peptide blocked both His-GAP-43 and GAP-43 peptide

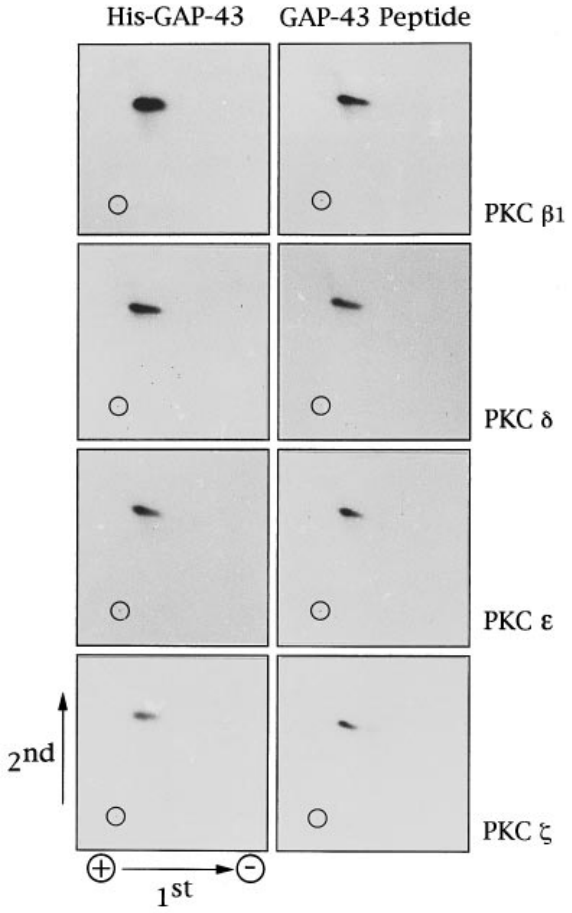

Figure 3 Two-dimensional phosphopeptide mapping

Recombinant His-GAP-43 and the synthetic GAP-43 oligopeptide were maximally phosphorylated by incubating with cPKC β_1, nPKC δ, nPKC ϵ or aPKC ζ for 4 h under standard conditions (see the Materials and methods section), resolved by PAGE and transferred to a nitrocellulose membrane. His-GAP-43 protein and the GAP-43 peptide bound to the membrane were digested by trypsin at 37 °C overnight. The tryptic phosphopeptides were resolved in the first dimension by thin-layer electrophoresis at pH 3.5 and in the second dimension by TLC, as indicated. The circle marks the start position. The chromatography plates were exposed at −70 °C for 1 day (3 days for PKC ζ and His-GAP-43) on Fuji X-ray films.

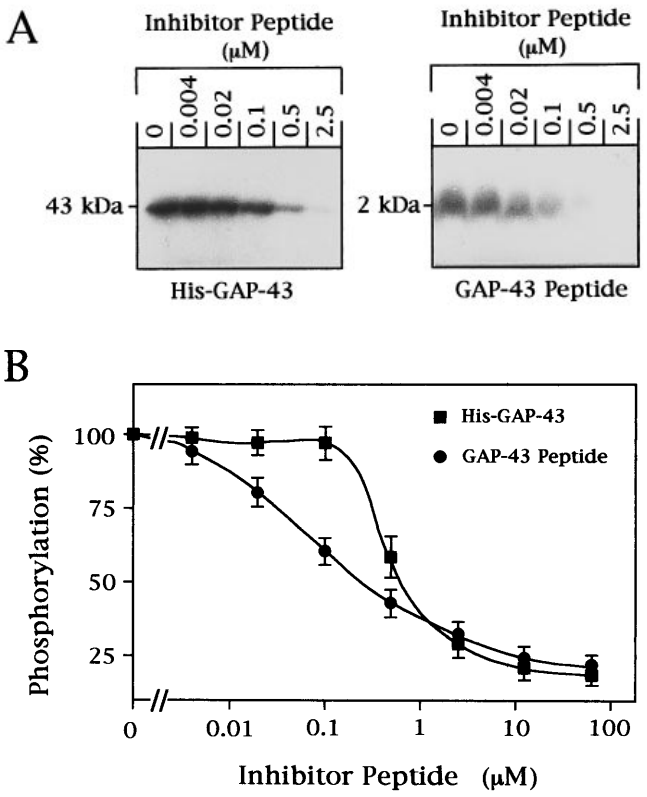

Figure 4 Phosphorylation in the presence of inhibitor peptide

Samples of 5 μmol of His-GAP-43 and of the GAP-43 peptide were incubated for 3 min with PKC β_1 (0.25 unit/ml) under standard conditions with activators in the presence of increasing amounts of PKC inhibitor peptide (4 nM–62.5 μM). (**A**) To visualize phosphorylation of the substrates, 10 μl of the phosphorylation mixture was separated by SDS/7.5%-PAGE for the His-GAP-43 protein (left panel) and by SDS/15%-PAGE for the GAP-43 peptide (right panel). Gels were dried and exposed at −70 °C for 24 h. Prestained protein markers (Bio-Rad) and reference peptides were used as standards for calibrating the size of His-GAP-43 and GAP-43 peptide respectively. (**B**) In parallel, incorporation of radioactivity was determined by binding the phosphorylated substrates to P81 phosphocellulose paper followed by Cerenkov counting, and expressed as a percentage of radioactivity in the absence of inhibitor peptide (set as 100%). The points on the dose–response curves for His-GAP-43 (■) and GAP-43 peptide (●) are means ± S.E.M. of seven independent experiments.

Table 1 Summary of kinetic analysis of His-GAP-43 and GAP-43 peptide phosphorylation by PKCs β_1, δ, ε and ζ

The K_m and V_{max} values of the PKC isoenzymes β_1, δ, ϵ and ζ for His-GAP-43, GAP-43 peptide and ϵ-peptide were determined. Accordingly, various amounts of His-GAP-43 (0.1, 0.25, 0.5, 1.0, 2.5 and 5 μM), GAP-43 peptide (0.05, 0.1, 0.5, 1.0, 10 and 50 μM) and ϵ-peptide (0.1, 0.5, 1.0, 2.5, 10 and 50 μM) were incubated with the respective PKC isotype at 30 °C for 3 min. Kinetic analyses were performed in the presence of 1.25 mg/ml PS and 1.25 μg/ml PDB as activators. Incorporation of radioactivity was measured by filter binding assay and Cerenkov counting. For PKC ζ only the K_m and V_{max} values for GAP-43 peptide and ϵ-peptide could be estimated. Results were generated using the Enzfitter program and are means ± S.E.M. of duplicate determinations from three independent experiments. The V_{max}/K_m ratio expresses catalytic efficiency [40]. n.d., not determined

	His-GAP-43			GAP-43 peptide			ϵ-Peptide		
Isoform	K_m (μM)	V_{max} (units/ml)	V_{max}/K_m (units/ml · μM)	K_m (μM)	V_{max} (units/ml)	V_{max}/K_m (units/ml · μM)	K_m (μM)	V_{max} (units/ml)	V_{max}/K_m (units/ml · μM)
PKC β_1	0.6 ± 0.2	0.12	0.19	56 ± 26	1.11	0.020	6.3 ± 2.4	0.5	0.08
PKC δ	1.7 ± 0.8	0.23	0.13	1.1 ± 0.5	0.03	0.025	1.7 ± 0.8	0.5	0.29
PKC ϵ	2.3 ± 1.4	0.38	0.17	38 ± 19	0.72	0.019	3.5 ± 1.7	0.5	0.14
PKC ζ	n.d.	n.d.	n.d.	1.2 ± 0.6	0.43	0.36	0.5 ± 0.2	0.5	1.1

phosphorylation by PKC β_1 at submicromolar concentrations. However, consistent with the notion of more extensive GAP-43–PKC interactions, the inhibitor was 6-fold less potent in inhibiting His-GAP-43 phosphorylation ($IC_{50} = 0.87 \pm 0.1\ \mu M$) than in inhibiting phosphorylation of the peptide ($IC_{50} = 0.15 \pm 0.03\ \mu M$) (Figure 4B). Similar results were obtained for PKC ϵ, with IC_{50} values of 0.4 μM for His-GAP-43 and 0.09 μM for GAP-43 peptide.

DISCUSSION

We define here the relative abilities of recombinant PKCs β_1, δ, ϵ and ζ to phosphorylate the established PKC neuronal substrate GAP-43. These PKC isotypes are representative of cPKC (β_1), nPKC (δ,ϵ) and aPKC (ζ) isoforms. Each is shown to be able to phosphorylate full-length GAP-43, although PKC ζ is clearly very poor in this regard. The site phosphorylated by each PKC isotype is mapped to serine-41, consistent with this assignment for (mixed) tissue purified PKC [26].

The kinetic analysis of the phosphorylation of GAP-43 by PKC isotypes provides an interesting insight into the kinase–substrate interaction, particularly when compared with phosphorylation of an oligopeptide encompassing the serine-41 site. Perhaps the key distinction is the very much higher apparent K_m values for PKCs β_1 and ϵ when the peptide is employed as a substrate compared with the intact protein. This type of selectivity is in contrast with that reported for PKC η. In this latter instance substrate selectivity has been shown to be governed in part through competition with the intrinsic pseudo-substrate site of the kinase; specific peptide substrates have been shown to compete better than the protein [38]. The opposite is true here, implying that GAP-43 competes better than the (exclusively) substrate-site-binding GAP-43 peptide. This property of GAP-43 suggests that it interacts with phospholipids or with PKC itself beyond the immediate catalytic site. Consistent with this view, a catalytic site competitive inhibitor is shown to be more effective in inhibiting GAP-43 peptide phosphorylation than that of the GAP-43 protein. This interpretation is in line with the finding that, alongside an increase in apparent K_m, the V_{max} for GAP-43 peptide phosphorylation by PKCs β_1 and ϵ also increases; product dissociation is presumably faster. Similar observations were made for the ubiquitiously expressed myristoylated alanine-rich C kinase substrate (MARCKS) and an oligopeptide based on the phosphorylation site of MARCKS [37]. Furthermore, Fujise and co-workers [39] recently showed, using overlay assays, that MARCKS binds to both the catalytic and regulatory domains of PKC. Therefore it seems that the contact between a PKC isotype and an intact substrate may not be restricted to the catalytic domain and the phosphorylation site. The present studies indicate that this is also the case for PKC and GAP-43.

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