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POTENCIES OF PROTEIN KINASE C INHIBITORS ARE DEPENDENT ON THE ACTIVATORS USED TO STIMULATE THE ENZYME

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Abstract—The aim was to examine systematically the potencies of protein kinase C inhibitors as a function of the kinase activator. Protein kinase C is activated by at least four stimulators: calcium plus phosphatidylserine (Ca/PS), phorbol 12-myristate 13-acetate plus PS (PS/PMA), arachidonic acid plus calcium (Ca/AA) and the synthetic peptide activator $PCK_{530-558}$. With histone or GS_{1-12} as substrates, protein kinase C was maximally activated by Ca/PS, or to maxima of 62%, 89% or 82% with PS/PMA, Ca/AA or $PKC_{530-558}$, respectively. One group of inhibitors, including H-7 and staurosporine, were equipotent, regardless of the activator. All other inhibitors showed variable selectivity, dependent upon the activator. A second group of inhibitors, including sphingosine and lipophosphoglycan, were eight or 200 times more potent for inhibition of PS/PMA-stimulated activity (relative to Ca/PS) and a third group, including retinal and palmitoylcarnitine, were 14 or 262 times more potent towards Ca/PS-stimulated activity. A final group (rhodamine 6G) was nine times more potent when Ca/AA was the activator. Similar results were obtained using the endogenous substrates dephosphin or MARCKS in synaptosol. Phosphorylation of MARCKS was stimulated by PS/PMA or Ca/PS, while phosphorylation of dephosphin was stimulated only by Ca/PS. The phosphorylation of either by Ca/PS-activated kinase was nine times more potently inhibited by palmitoylcarnitine, while phosphorylation of MARCKS by PS/PMA-activated kinase was 10 times more potently inhibited by sphingosine. H-7 inhibited both at similar concentrations. A model encompasses these differences in potency if the inhibitors are divided into four groups (A–D) according to their competitive inhibition with the appropriate activator or at the active site. The non-selective inhibitors interact at the active sites of protein kinase C (group A). The compounds which preferentially inhibit PS/PMA-activated kinase (sphingosine and lipophosphoglycan) are competitive inhibitors of PMA and 1,2-diacylglycerol (group B), those selective for Ca/PS-activated kinase (palmitoylcarnitine and retinal) are competitive with PS (group C) and those selective for Ca-AA activation (rhodamine 6G) are likely to be competitive with fatty acid (group D). Therefore, the effectiveness of protein kinase C inhibitors is dependent upon the activator employed.

Protein kinase C requires both Ca^{2+} and phospholipid for activity, and is further stimulated by 1,2-diacylglycerol (DAG†) in the presence of low micromolar concentrations of Ca^{2+} [1]. A transient production of DAG in the plasma membrane in response to various hormonal stimuli may be one of the main physiological mechanisms for its activation [1]. Protein kinase C can also be activated by tumor-promoting phorbol esters [2, 3] which compete with DAG for the same binding site [4, 5]. However, there are a number of significant differences between phorbol ester activation and DAG activation. As little as 50 nM phorbol 12-myristate 13-acetate (PMA) activates protein kinase C and causes an irreversible translocation of the kinase to the membranes, an effect not seen with DAG [5], and results in calcium-independent kinase activity [6]. PMA also stimulates autophosphorylation of the enzyme on distinct sites to that stimulated by (Ca plus L-phosphatidyl-L-serine (Ca/PS) [7]. Phorbol esters cause irreversible membrane insertion of protein kinase C in a calcium-independent manner [5] and the inserted enzyme is active, but independent of calcium [6, 8], while DAG activation of protein kinase C produces calcium-dependent membrane binding [5]. A third type of protein kinase C activator is *cis*-unsaturated fatty acids, such as arachidonic acid (AA) [9–12], or some of their oxygenation products [13–15]. This activation occurs in the presence or absence of calcium. Like the other activators, fatty acid activation results in translocation of protein kinase C from the cytosol to the membrane [16], but unlike other activators, Ca/AA does not induce autophosphorylation [12]. Although fatty acids do not interact with the PS/DAG binding site of protein kinase C, they may activate via either a distinct site or a site which overlaps with the PS site [9, 10, 12]. Other activators are also known [3, 17–19], but the synthetic peptide $PKC_{530-558}$ is one

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† Abbreviations: AA, arachidonic acid; Ca/AA, Ca plus AA; DAG, 1,2-diacylglycerol; PMA, phorbol 12-myristate 13-acetate; PS, L-phosphatidyl-L-serine; Ca/PS, calcium plus L-phosphatidyl-L-serine; PS/PMA, PMA plus PS; ACTH, adrenocorticotropic hormone.

activator that may act at a distinct site, i.e. by mimicking the substrate binding site it interacts with the pseudosubstrate inhibitory region of protein kinase C and also induces autophosphorylation [7]. Therefore, activation of protein kinase C has been studied widely using at least four activators: Ca/PS, PS/PMA, Ca/AA and $PKC_{530-558}$.

Differences in the protein kinase C activating system result in apparently different substrate specificities of protein kinase C. Phosphorylation studies of endogenous protein kinase C substrates in subcellular fractions from various tissues show that the substrates can be divided into multiple groups according to the nature of the activator, Ca/PS, PS/PMA or Ca/AA [13, 19–23]. In intact cells, distinct activators of protein kinase C do not always induce the same cellular responses [19, 24–29]. Similarly, distinct inhibitors of protein kinase C do not always inhibit cellular responses stimulated by the different activators [28, 30–34]. For example, trifluoperazine and polymyxin B were less potent inhibitors of PMA-stimulated prolactin release than of calcium-stimulated release [30], and the potencies of protein kinase C inhibitors on NADPH oxidase activation in neutrophils appeared to vary with different activators used [31]. Furthermore, sphingosine, but not palmitoylcarnitine, prevented PMA-induced down-regulation of protein kinase C in lymphocytes [32]. Sphingosine inhibited PMA-induced, but 25-dihydroxyvitamin D3-induced, monocyte differentiation [33]. Sphingosine also inhibits PMA-activated Na^+/H^+ exchange in smooth muscle cells, but not thrombin or platelet-derived growth factor-activated exchange [34].

Activator dependence of protein kinase C inhibitors has also been described for *in vitro* assays of protein kinase C activity, but the underlying mechanisms were not understood [10, 12, 18, 28, 30, 31, 35]. For example, O'Brian and Weinstein [10] found rhodamine 6G to be 10 times more potent towards Ca/AA-activated kinase than towards Ca/PS or PS/PMA activation. Roghani *et al.* [18], found that chlorpromazine was less potent when PMA was used to activate protein kinase C rather than Ca/PS. El Touny *et al.* [12] found that sphingosine was more potent in inhibiting PS/DAG activation than fatty acid-activated protein kinase C. Such disparate effects of protein kinase C inhibitors might reflect the distinct mode of protein kinase C activation utilized by differing exogenous activators. The aim of this study was therefore to examine the effects of inhibitors of protein kinase C on the purified enzyme and on the phosphorylation of endogenous substrate proteins in rat brain in order to determine the underlying basis of the apparent disparate effects of protein kinase C inhibitors. Using a wide range of inhibitors with known, but distinct, mechanisms of action and using the different types of activators of protein kinase C, most inhibitors showed greatly increased potency when distinct activators were used. A model is proposed which encompasses these effects and which provides a framework for future studies which may employ protein kinase C inhibitors.

MATERIALS AND METHODS

Materials. The following reagents were from Sigma; palmitoylcarnitine; sphingosine, retinal, polymyxin B, acridine orange, chlorpromazine, calmodulin and histone III-S. Phorbol esters were from Avanti Polar Lipids. H-7, staurosporine and arachidonic acid were from Calbiochem. Lipophosphoglycan was isolated from *Leishmania donovani* and was a gift from M. McConville, the University of Melbourne, Australia. Synthetic peptides $PKC_{530-558}$ and GS_{1-12} were gifts from Dr. Bruce Kemp, St. Vincent's Institute of Medical Research, Melbourne, Australia. All other reagents were of Analytical Reagent grade or better.

Protein kinase C. Rat brain protein kinase C was purified as described [36] using calcium-dependent membrane binding, Q-sepharose high performance, a second Q-sepharose column in the presence of ATP and phenyl-sepharose chromatographies. The purified protein ran as a doublet on SDS–polyacrylamide gels, with the lower 80 kDa band representing isozymes β and γ and the larger 83 kDa band representing protein kinase C α [7]. Due to the initial calcium-dependent membrane binding step it is unlikely that significant protein kinase C δ, ε, ζ, or η are present in this preparation since these isozymes do not respond to calcium. Protein kinase assays were performed by the P81 phosphocellulose method of Roskoski [37] with the flat sheet modification of Sahal and Fujita-Yamaguchi [38]. The incubation contained 30 mM Tris–HCl pH 7.4, 10 mM $MgSO_4$, 1 mM EGTA, 200 μM [γ-^{32}P]ATP (New England Nuclear, 500 cpm/pmol), 1 mg/mL histone III-S and, where indicated in the text, either 1.2 mM $CaCl_2$ (200 μM free calcium, for Ca/PS conditions) or 2 mM $CaCl_2$ (1 mM free calcium, for Ca/AA conditions), 10 μg/mL PS plus 1 μg/mL 1,2-diolein, 1 μM PMA or 100 μM arachidonic acid. All solutions were kept on ice until the incubation. PS was prepared as follows: 400 μg PS in chloroform–methanol was dried under N_2 and sonicated on ice into 1 mL of 30 mM Tris–HCl pH 7.4 for 2 min with a fine probe sonicator to a stock concentration of 400 μg/mL. The PS vesicles were then frozen at −20°, and thawed and vortexed prior to each experiment (up to 10 freeze/thaw cycles). When present, 1,2-diolein was dried with the PS and they were co-sonicated. PMA was freshly prepared each time from a stock of 1 mg/mL in 100% ethanol (stored at −20°) by direct dilution in cold water to 10 μM and vigorously vortexed. PMA was added to the assay tubes separately from PS. AA was freshly prepared as 3 mM by dissolution in N_2-saturated 30 mM Tris–HCl pH 7.4. Frozen and thawed AA solutions proved occasionally unreliable as an activator of protein kinase C. When the activator $PKC_{530-558}$ was employed, the substrate was the synthetic peptide GS_{1-12} as described [7]. The reactions were terminated by addition of ice-cold 75 mM H_3PO_4 and aliquots were subsequently transferred to Whatman P81 paper. In all cases the assays were linear with respect to time and enzyme concentration. All inhibitors were freshly prepared in 20 mM Tris–HCl pH 7.4, except that H-7 and staurosporine were initially prepared as 1 mg/mL in dimethyl sulphoxide and were stored at −20°. Solvent controls were included where appropriate.

Sample preparation and protein phosphorylation.

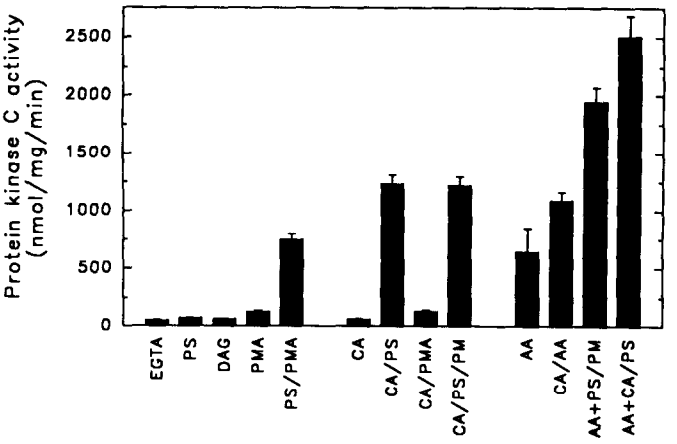

Fig. 1. Activation of protein kinase C by the three activators PS/PMA, Ca/PS and Ca/AA. Purified rat brain protein kinase C phosphorylation of histone was stimulated in the presence of the activators shown. Results are means and SEM (N = 3).

A purified synaptosomal fraction was freshly isolated from the cerebral cortex of a rat by differential centrifugation through Percoll, and the cytosolic fraction (synaptosol) isolated as described previously [39] and resuspended to a final protein concentration of 2.5–4.0 mg/mL. Phosphorylation of synaptosol in the presence of [γ-^{32}P]ATP was performed as described [23]. The reactions were terminated by the addition of 2% SDS (in 67 mM Tris, 25 mM β-mercaptoethanol, 2 mM EGTA, 10% glycerol pH 6.8) followed by rapid freezing, and the phosphoproteins were subsequently separated on polyacrylamide gels. Polyacrylamide slab gel electrophoresis and autoradiography were carried out as described [23] using 7.5–15% linear gradients and 20 cm gels (Bio-Rad, Protean II system). Regions of the dried polyacrylamide gel containing dephosphin or MARCKS were excised, using the autoradiograph as a template, and counted by liquid scintillation techniques [23]. Phosphorylation is expressed as cpm ^{32}P transferred to dephosphin or MARCKS per 30 μg of synaptosol protein per minute.

RESULTS

Studies with purified protein kinase C

Purified rat brain protein kinase C was activated in the presence of Ca/PS. Maximal activity was unaffected by further addition of DAG or PMA (Fig. 1). The combination of PS/PMA produced 62% of the maximum activation of the enzyme (varying with different preparations of protein kinase C, in the range 50–90%). Activation of protein kinase C by PS/PMA (in the absence of calcium) has been reported by others [3–6, 40, 41]. Protein kinase C was also activated by 100 μM AA in the absence of calcium or to 89% of maximum by AA in the presence of 1 mM calcium (Ca/AA), as described by others [10–12, 42]. Activation by AA (in the presence or absence of calcium) was approximately additive with both other activators. This shows that the site of interaction of AA with protein kinase C is distinct from sites used by the other activators. Stimulation of protein kinase C by multiple activators is not isozyme-specific since Ca/PS is known to activate isozymes α, βI, βII, and γ, PS/PMA is known to activate at least isozymes δ and ε [43–46] as well as α, βI, βII, and γ [47] and fatty acids activate at least proteins kinase C α, βI, βII, γ [11] and δ [45].

The effects of different protein kinase C inhibitors were examined on protein kinase C activity stimulated by the three activation systems, Ca/PS, PS/PMA and Ca/AA (the results are also summarized in Table 1). H-7 (Fig. 2) [48] equipotently inhibited kinase activity stimulated by all the activators. Other inhibitors, staurosporine [49–51], MDL 27,032 [52] and a potent peptide inhibitor of protein kinase C, PKC_{19-31} [53], also did not differentiate between protein kinase C activity stimulated by different activators (Table 1). However, all other inhibitors showed distinctly increased potencies when specific activators were used. For example, lipophosphoglycan (Fig. 2) showed much greater inhibitory potency when PS/PMA was the activator. Other inhibitors sphingosine and acridine orange showed similar, but less marked, selectivity (Table 1). Lipophosphoglycan, sphingosine and acridine orange blocked PS/PMA-activated kinase activity almost 200, eight and three times, respectively, more potently than either Ca/PS-activated kinase or Ca/AA-activated kinase activity (Table 1). Palmitoylcarnitine also showed highly selective inhibition, but towards the activators Ca/PS and Ca/AA (Fig. 2), with potencies that were 260 times greater than towards PS/PMA-activated kinase. Palmitoylcarnitine inhibited with an IC_{50} of approximately 15 μM when either Ca/PS or Ca/AA was the activator, but was virtually ineffective (IC_{50} of 3.8 mM) on PS/PMA-activated kinase (Fig. 2, Table 1). Other inhibitors, retinal, polymyxin B and chlorpromazine, also showed selective inhibition of Ca/PS-activated kinase, with potencies that were 14,

Table 1. Selectivity of protein kinase C inhibitors for inhibition of purified rat brain protein kinase C stimulated by either PS/PMA, Ca/PS or Ca/AA with histone III-S as substrate

Inhibitor class	Inhibitor	Protein kinase C activator			B:C*	C:D†
		PS/PMA B	Ca/PS C (IC_{50})	Ca/AA D		
(A) substrate:						
A1-Mg/ATP	H-7	34 μM	27 μM	57 μM	1.2	0.5
	Staurosporine	11 nM	15 nM	12 nM	0.7	1.3
	MDL 27,032	52 μM	45 μM	61 μM	1.2	0.7
A2-protein	PKC_{19-31}	0.5 μM	0.6 μM	0.6 μM	0.8	1.0
(B) PMA	Lipophosphoglycan	1.8 μM	>300 μM	ND	>170	—
	Sphingosine	21 μM	163 μM	141 μM	7.8	1.2
	Acridine orange	39 μM	113 μM	122 μM	2.8	0.9
(C) Phospholipid	Palmitoylcarnitine	3800 μM	14.5 μM	18 μM	262	0.8
	Retinal	131 μM	9.6 μM	25 μM	13.6	0.4
	Polymyxin B	22 μM	7.5 μM	38 μM	3.0	0.2
	Chlorpromazine	115 μM	35 μM	117 μM	3.2	0.3
(D) Fatty acid	Rhodamine 6G	1700 μM	900 μM	106 μM	1.9	8.5

IC_{50} values for inhibition of purified rat brain protein kinase C were derived from inhibitory concentration curves such as in Fig. 2.

ND, experiment not done.

* Ratio of PS/PMA:Ca/PS (IC_{50}) for inhibitor classes A and C; ratio of Ca/PS:PS/PMA (IC_{50}) for inhibitor class B.

† Ratio of Ca/PS:Ca/AA (IC_{50}) for all classes.

three and three times greater, respectively, than towards PS/PMA-activated kinase (Table 1). All compounds in this group (except palmitoylcarnitine) were also several-fold more selective towards Ca/PS-activated kinase than Ca/AA-activated kinase. Finally, rhodamine 6G was a 10-fold more potent inhibitor of Ca/AA-activated protein kinase C than of protein kinase C activated by either of the other activators (Table 1). This was reported previously by O'Brian and Weinstein [10]. Thus, the potency of most inhibitors is dependent on the kinase activator employed. On this basis, the inhibitors can be divided into four major groups or classes, termed A–D (Table 1) according to their activator selectivity (groups B–D) or non-selectivity (group A). This group division correlates precisely with the mode of competitive inhibition of each of these compounds with the appropriate activator or at the active site (see Discussion). Most of these compounds in groups B–D that have relatively weaker selectivities are also known to have multiple sites of interaction with protein kinase C.

When protein kinase C was activated by the synthetic peptide $PKC_{530-558}$, which is thought to represent the active site (substrate recognition site) and interacts with the pseudosubstrate site [7], similar differences in inhibitor potency were observed (Table 2). The active site inhibitor (group A1) equipotently blocked $PKC_{530-558}$- and Ca/PS-stimulated activities, while inhibitors directed to the regulatory domain, such as sphingosine, palmitoylcarnitine and polymyxin B (groups B and C), were 2–50 times less potent inhibitors of $PKC_{530-558}$-activated protein kinase C (Table 2). Note that sphingosine also has direct effects at the active site of protein kinase C, but at higher concentrations than at the PMA-binding site [54]. Polymyxin B may also act at both regulatory and catalytic subunit sites. Finally, allosteric interactions with the peptide activator cannot be ruled out.

Protein kinase C in synaptosol

These disparate effects of different protein kinase C inhibitors could also be demonstrated with a preparation of cytosolic fraction from rat cortical synaptosomes, which was used as the source of both protein kinase C and endogenous substrates. Ca/PS-stimulated the phosphorylation of a 96 kDa synaptosomal phosphoprotein called dephosphin [23, 39] and an 83 kDa protein called MARCKS [55, 56] (Fig. 3, lane 8). Ca/AA mimicked this response very closely (not shown). The synaptosol also had a high level of endogenous calmodulin-dependent kinase activity that was activated upon addition of calcium alone (Fig. 3, lane 7); however, MARCKS and dephosphin are not substrates of this kinase. PS/PMA stimulated the phosphorylation of MARCKS, but not dephosphin (Fig. 3), but in contrast to the situation with purified protein kinase C (Fig. 1) and dephosphin (Fig. 4), MARCKS phosphorylation was also stimulated by PMA alone, and PS or calcium enhanced this effect (Fig. 4). The stimulatory effect of PMA alone is most likely due to the presence of phospholipids or other endogenous activator in the synaptosol. Using the selective activators and substrates (i) PS/PMA stimulation of MARCKS or (ii) Ca/PS stimulation of dephosphin, protein kinase C inhibitors showed differential

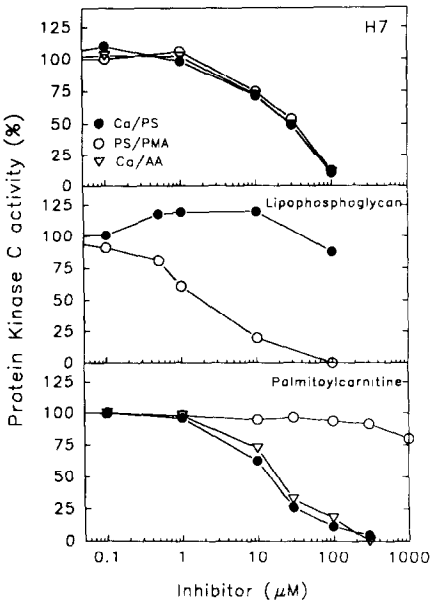

Fig. 2. Inhibition of purified rat brain protein kinase C. Protein kinase C phosphorylation of histone was activated by PS/PMA (open circles), Ca/PS (closed circles) or Ca/AA (open triangles). Kinase activity is expressed as a per cent of maximal activity with each activator (see Fig. 1) and was determined in the presence of various concentrations of H-7, lipophosphoglycan or palmitoylcarnitine. Results are representative from two or three experiments, each performed in duplicate or triplicate.

Table 2. Selectivity of protein kinase C inhibitors for inhibition of purified rat brain protein kinase C stimulated by either $PKC_{530-558}$ or Ca/PS with the synthetic peptide GS_{1-12} as substrate

Inhibitor	PKC activator: $PKC_{530-558}$ A	PKC activator: Ca/PS C	A:C
	IC_{50}		
(A1) H-7	11 μM	9 μM	1.2
(B) Sphingosine	28 μM	6 μM	4.7
(C) Palmitoylcarnitine	>1000 μM	20 μM	>50
Polymyxin B	8.7 μM	3.9 μM	2.2

IC_{50} values for inhibition of purified rat brain protein kinase C were derived from inhibitory concentration curves.

sensitivities. Some inhibitors, PKC_{19-31}, H-7 and staurosporine, equally inhibited phosphorylation of dephosphin and MARCKS. The IC_{50} for PKC_{19-31} inhibition of MARCKS was 2.2 μM and for dephosphin was 1.4 μM. Similarly, H-7 and staurosporine inhibited the phosphorylation of both phosphoproteins at the same concentrations (39 μM and 95 nM, respectively). Dephosphin phosphorylation was inhibited by palmitoylcarnitine with an IC_{50} of 62 μM, but was weakly inhibited by sphingosine with an IC_{50} of 270 μM (Fig. 5). In contrast, MARCKS phosphorylation (activated by PS/PMA) was nine times less potently inhibited by palmitoylcarnitine, with an IC_{50} of 530 μM, and 10 times more potently inhibited by sphingosine, with an IC_{50} of 26 μM.

DISCUSSION

Protein kinase C was shown to be activated by four distinct activators, $PKC_{530-558}$, PS/PMA, Ca/PS and Ca/AA. The potency of protein kinase C inhibitors was dependent upon which activator was used. Thus, the kinase was potently inhibited by some compounds, such as palmitoylcarnitine, only when activated by Ca/PS or Ca/AA, potently inhibited by other compounds, such as sphingosine, only when stimulated with PS/PMA, but was equipotently inhibited by compounds such as H-7, staurosporine and PKC_{19-31}, regardless of the activator. These protein kinase C inhibitors can also be divided broadly into at least four classes according to their site of interaction with the enzyme (operationally defined here as competitive inhibition) as follows: (A) the active sites: A1, the ATP binding site e.g. H-7, staurosporine, quercetin, MDL 27,032 and erbstatin [48, 50–52, 57–59], or A2, the substrate binding site e.g. the pseudosubstrate synthetic peptide PKC_{19-31} [53] and chelerythrine [60]; (B) the PMA binding site e.g. sphingosine, lipophosphoglycan, acridine orange, gossypol, Adriamycin-iron(III) and calphostin [54, 57, 61–64]; (C) the phospholipid binding site e.g. palmitoylcarnitine, polymyxin B, retinal, Adriamycin®, tamoxifen, and phenothiazines such as chlorpromazine and trifluoperazine [57, 65–68]; and (D) the activation site for fatty acids such as AA and oleic acid [12, 35]. Although no competitive inhibitors of site D have yet been demonstrated, rhodamine 6G [10] is a likely candidate for a competitive inhibitor at this site. Multiple interactions of inhibitors between two or more sites can also occur, but usually with different affinities [54, 57]. Some inhibitors are also competitive inhibitors at more than one site [69, 70]. It can be seen that this division of inhibitors into groups according to their site of interaction with the enzyme also matches the division of inhibitors described in this report into their selectivity or lack of selectivity for the type of activator used. Therefore the apparent disparity of inhibitor effects is greatly reduced if their apparent site of action is taken into consideration.

The protein kinase C sequence can be divided into four constant (C1–C4) and five variable domains (V1–V5), based on sequence homology between the isozymes [71]. Isozymes α, βI, βII and γ can be cleaved into two major fragments, comprising the regulatory and catalytic domains, by trypsin or calpain in the V3 domain. Several sites of interaction of substrates, cofactors and activators are possible: A1: the ATP binding sequence, Gly-X-Gly-X-X-Gly . . . . Lys, lies within the C3 domain. A2: the precise location of the protein substrate binding site is unknown, but may lie in an acidic region of C4 [7]. B: phorbol esters, such as PMA and DAG

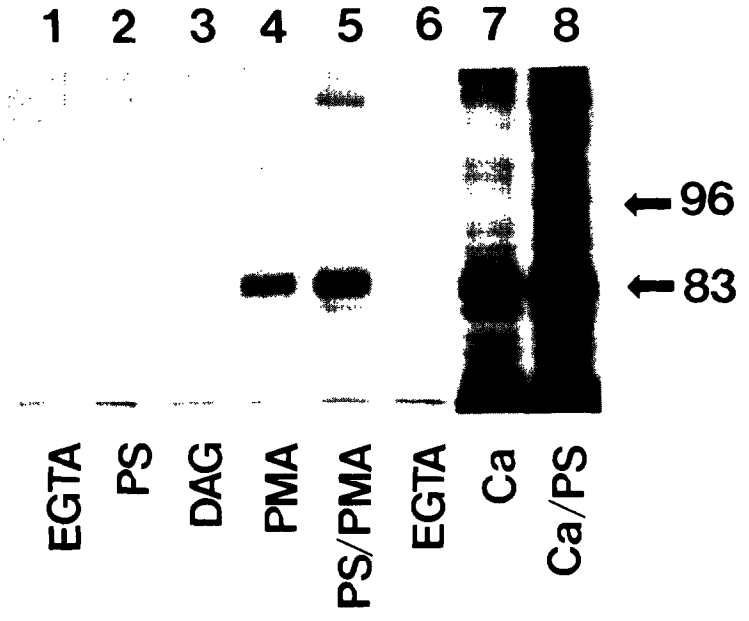

Fig. 3. Autoradiograph of phosphoproteins in synaptosol phosphorylated by protein kinase C. Phosphorylation by endogenous kinase proceeded for 1 min in the presence of the activators shown. Phosphoproteins were then separated on a polyacrylamide gel and detected by autoradiography. Dephosphin is indicated by an arrow at 96 kDa and the MARCKS protein by an arrow at 83 kDa. Results are representative of at least three experiments.

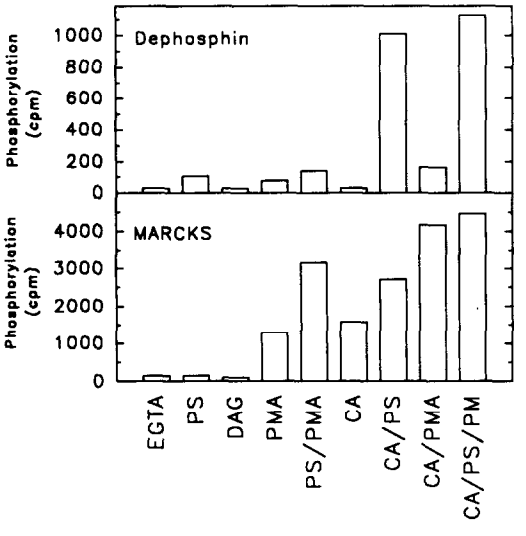

Fig. 4. Effect of different protein kinase C activators on phosphorylation of dephosphin or MARCKS. Phosphorylation of dephosphin (96 kDa) in synaptosol is shown in the upper panel while phosphorylation of the MARCKS protein (83 kDa) in synaptosol is shown in the lower panel. Phosphorylation of dephosphin or MARCKS was determined by excision of the proteins from dried polyacrylamide gels and liquid scintillation counting. Phosphorylation is expressed as cpm ^{32}P incorporated per min for 30 μg of synaptosol and results are means of two experiments.

interact with the zinc fingers, which comprise much of the C1 domain [72]. C: the location of the binding site for PS and other phospholipids has not been determined precisely, but may lie within C1. D: the binding site for unsaturated fatty acids is also unknown but may overlap with the PS binding site in the C1 domain [12]. Fatty acid activation of all isozymes is not equivalent at low concentrations [71], but all isozymes appear to be activated [45]. A binding site for calcium is not known and it has yet to be shown to interact directly with the kinase, rather than or as well as with the acidic phospholipid head group, and no specific competitive inhibitors of calcium have yet been described. Parker originally proposed it to be in the V3 or C2 domain [73, 74], but since the δ, ε, ζ, and η isozymes (collectively termed nPKC) lack the C2 domain and are calcium-independent it is also possible that calcium interacts with the C2 domain. Protein kinase C inhibitors can be divided into the same A–D categories (other categories are possible) according to their mode of kinetic inhibition of the enzyme (see Table 1). It is proposed that each inhibitor that interacts in the regulatory domain of the enzyme will more potently inhibit activity when the activator used is also targeted to the same site (however, multiple sites of drug interaction can occur for some inhibitors). This model encompasses the known sites of interactions of inhibitors with protein kinase C and correlates with the presumed mechanisms of actions of these compounds of competitive inhibition with respect to phospholipids [65, 67], phorbol ester [61] and the active site of protein kinase C [48, 50, 51, 53]. The model provides a new framework for understanding

the apparent disparate effects of these inhibitors. Previous models have considered protein kinase C only as regulatory and catalytic domains. While this has proved of some value it does not allow for different biochemical interactions at other sites on protein kinase C within the regulatory domain. The new model substantially expands on this original concept.

The results obtained with purified protein kinase C were reproduced and extended with studies on the phosphorylation of synaptosol phosphoproteins. These fractions are an excellent source of brain protein kinase C substrates. Dephosphin is a phosphoprotein in intact synaptosomes that is rapidly dephosphorylated on depolarization [75] and is also a protein kinase C substrate *in vitro* [23, 39] and in intact synaptosomes [47]. MARCKS was identified as the "80 kDa" protein substrate described in other laboratories [55, 56] due to its acidic pI and since it yielded characteristic 13 kDa and 9 kDa phosphopeptides after V8 protease digestion [55]. Both dephosphin and MARCKS were phosphorylated by synaptosomal protein kinase C in this study when Ca/PS or Ca/AA was the activator. However, phosphorylation of both differed from that of histone. While MARCKS was phosphorylated in the presence of PS/PMA, it was also phosphorylated in the presence of PMA or calcium alone. In contrast, dephosphin was never phosphorylated in the presence of PS/PMA, calcium or PMA alone. Dephosphin is therefore another example of a protein kinase C substrate whose phosphorylation is activator dependent [13, 19–23, 27]. However, note that other endogenous protein kinase C activators were probably present in the cytosolic extracts. The phosphorylation of MARCKS and dephosphin by the distinct types of protein kinase C activators described in this report showed markedly different sensitivities to inhibition by sphingosine [61] and palmitoylcarnitine [65, 67]. The PS/PMA-stimulated phosphorylation of MARCKS was 10 times more potently inhibited by sphingosine and the Ca/PS-dependent phosphorylation of dephosphin was nine times more potently inhibited by palmitoylcarnitine. However, phosphorylation of both proteins was equipotently inhibited by H-7, staurosporine and $PKC_{19\text{-}31}$, indicating that both activities are indeed due to the action of protein kinase C. This shows that the activity of endogenous protein kinase C in synaptosol also demonstrates activator-dependent inhibitor specificity, as seen for the purified enzyme. The phosphorylation in synaptosomal cytosol is a slightly more physiological system than histone phosphorylation by purified kinase and, although it is a less informative system, it points to a greater physiological relevance of the inhibitor effect.

Two recent studies demonstrate that the inhibitor potency dependence upon activator reported here has important implications in the use of inhibitors in studies with intact cells. Recent work with MARCKS and dephosphin showed that the same inhibitor selectivity observed here in synaptosomal cytosol also occurs in intact synaptosomes [47], suggesting that the effect is of physiological relevance. Secondly, in cultured ovine anterior pituitary cells, we demonstrated recently that adrenocorticotropic hormone (ACTH) release stimulated by arginine-vasopressin is dependent upon protein kinase C-mediated pathways [76]. Using a representative inhibitor from groups A–C we demonstrated that each inhibited ACTH release stimulated by the hormone arginine-vasopressin, but only group A and B inhibitors were effective against PMA-induced ACTH release. Therefore, group C inhibitors are likely to have no or highly variable effectiveness when employed against PMA-activated cells, as described in numerous other studies [28, 30–34]. These apparent discrepancies in these more physiologically relevant situations can now be understood within the framework of the model proposed here, and the model itself can be used effectively to predict the potency of each type of protein kinase C inhibitor [76].

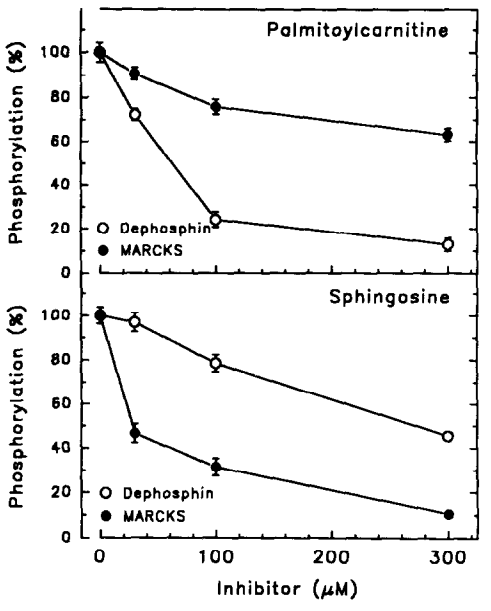

Fig. 5. Inhibition of dephosphin or MARCKS phosphorylation by palmitoylcarnitine (upper panel) or sphingosine (lower panel). Protein kinase C activity was stimulated by PS/PMA (MARCKS) or Ca/PS (dephosphin) for 1 min, as described in the legend to Fig. 3. The phosphorylation of dephosphin (open circles) or MARCKS (filled circles) was determined by excision of the bands from a dried polyacrylamide gel and liquid scintillation counting. Results are means and SEM from six experiments and are expressed as a per cent of the maximum cpm for that protein. In this series of experiments maximum phosphorylation for dephosphin was 2219 ± 97.6 cpm and for MARCKS was 3598 ± 173 cpm.

There are several limitations to this model of inhibitor specificity towards protein kinase C. Firstly, many inhibitors such as polymyxin B, acridine orange and gossypol have multiple sites of interaction with protein kinase C [56, 63]. Therefore, selectivity between inhibition of the two protein kinase C activation systems may be less for such inhibitors. Other inhibitors have poorly defined sites of interaction, for example, palmitoylcarnitine is competitive with respect to phospholipid [65] and

does not interact at the PMA- or DAG-binding sites [67], but it is not clear whether it interacts directly with protein kinase C or is a lipophilic modulator [67]. However, its selectivity for inhibition of both Ca/PS- and Ca/AA-activated kinase supports this role as a lipophilic modulator. The second limitation is the role of kinase substrates, which could dictate the effectiveness of specific inhibitors [77]. Substrates may be grouped into many categories according to their requirement for (A) no activator, (B) PS alone, (C) Ca/PS [78] (see also [23]), (D) PS/PMA (this report) or (E) unsaturated fatty acids [21, 28]. However, all substrates used in this study (except dephosphin) were phosphorylated in the presence of all activators. Finally, another important consideration related to the use of inhibitors in cell lysates or intact cells is the possible lack of inhibitor specificity towards protein kinase C [48, 51, 76]. For example, H-7, staurosporine and retinal also inhibit cAMP-dependent protein kinase [48, 76, 79]. Sphingosine [80], staurosporine [80], chlorpromazine and polymyxin B (not shown) are also calmodulin-dependent protein kinase inhibitors with potencies similar to their effects on protein kinase C. In contrast, palmitoylcarnitine did not inhibit calmodulin-dependent protein kinase (not shown), nor cAMP- and cGMP-dependent protein kinases [81], suggesting that it is the most specific group C protein kinase C inhibitor.

The division of protein kinase C inhibitors into specific categories according to their possible sites of interaction with protein kinase C provides a novel basis for understanding the mechanism of action of such inhibitors and provides a framework for the selection of inhibitors in studies of intact cells. Group A inhibitors blocked all protein kinase C activity regardless of the activator; however, group B, C and D inhibitors were significantly more potent when employed with kinase activated by differing types of activators. The basis of the distinct inhibition is likely to be competitive interaction at distinct (possibly overlapping) activation sites on protein kinase C. One solution to understanding the roles of activators in the inhibition of protein kinase C has been to utilize assays on native protein kinase C in concert with assay of the inhibitor effect on the catalytic fragment of the enzyme, PKM [57, 77]. Although such studies support the findings in this report, they have the limitation that they cannot take into account differences between group B, C and D inhibitors. The group B and C inhibitors employed in this study are also effective protein kinase C inhibitors in intact cells [47, 76, 82], but have shown inconsistent selectivities in inhibiting PMA-promoted processes [29, 32–34, 76, 82]. The results of the present study, however, would indicate that the apparent disparate effects of different drugs could partly reside in their differential selectivities to the type of activator used, particularly if the drugs employed were chosen from group B, C or D. The judicious use of selective inhibitors from these three groups, as well as the non-discriminating, active site inhibitors from group A, will therefore provide a better approach to elucidating the biological roles of protein kinase C.

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