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Photoaffinity Labeling of PKC Isozymes by Phorbol Ester Derivatives

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Abstract—Photoaffinity probes PPDA and PPTD, which have diazoacetyl and trifluorodiazopropionyl group at C-13 position in phorbol, respectively, were synthesized. Photoaffinity labeling of protein kinase C isozymes by both the probes resulted in specific cross-linking.

Introduction

Phorbol esters are tumor-promoting diterpene esters isolated from croton oil.[1–3] They are known to bind to and activate isozymes of the protein kinase C (PKC) family,[4–6] serine–threonine phosphorylating enzymes which play central roles in intracellular signal transductions. The structure of protein kinase C can be divided into two domains, regulatory and catalytic (Figure 1). The catalytic domain, which phosphorylates the substrate proteins, is homologous with that of other ATP dependent kinases such as protein kinase A (PKA). On the other hand, the structure of the regulatory domain, which consists of C1 and C2 regions, is characteristic of PKC. The C1 region of cPKC (conventional PKC; PKC α, βI/II, and γ) and nPKC (novel PKC; PKC δ, ε, η, and θ) has two repeats of a cysteine rich domain (CRD) whereas aPKC (atypical PKC; PKC ζ and λ/ι), which is not activated by phorbol esters, has only one CRD. The CRDs have been shown to be the binding site of protein kinase C activators such as diacylglycerol, phorbol esters, and teleocidin.[7–10] NMR studies[11–13] and X-ray analysis[14] have revealed the structure of the second CRD and the mode of interaction with phorbol esters. However, further studies are still necessary to understand the mechanism of PKC activation and to design new PKC modulators, since most of the previous studies such as the X-ray analysis[14] were carried out in the absence of phospholipids and/or the hydrophobic portion of phorbol esters, which are essential for PKC activation. Moreover, peptides containing only one repeat of CRDs were employed in those studies. Although it has been proposed that only one CRD repeat is responsible for phorbol ester binding,[15,16] Slater et al. recently reported that diacylglycerol and phorbol esters can bind simultaneously to PKCα, suggesting more complicated function of each CRD in native PKC.[17] We therefore focused on the photoaffinity labeling experiments as a method for analyzing interactions between native PKC and phorbol esters in the presence of phospholipids to clarify the role of individual CRDs in PKC. The development of an efficient photoaffinity probe of phorbol ester derivatives is very important for such an analysis.

Although there have been several reports in which the photoaffinity labeling of PKC was carried out using phorbol ester derivatives,[18,19] cross-linking to PKC was not, to our knowledge, achieved until we reported the synthesis of a novel photoaffinity probe PPDA(**5**) and cross-linking to crude PKC mixtures.[20] Wender and Irie et al. independently reported the photoaffinity labeling of PKC model peptides.[21] We suppose that the location of the photolabile group in the complexes is important: most previous ligands possessed an azidobenzoyl group at C12 in phorbol. Previous structure–activity relationship models[22–29] have proposed that a hydrophobic ester group is required at the C12 position in phorbol esters, suggesting that these groups are incorporated into phospholipid membranes. These models are consistent with the unsuccessful photoaffinity labeling

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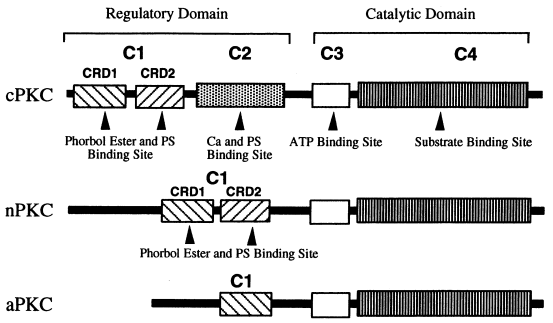
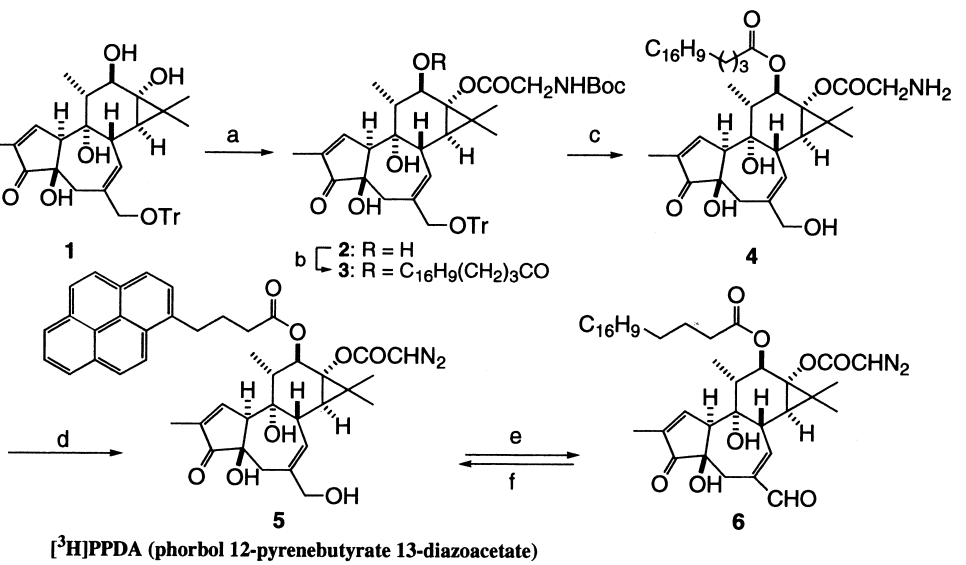


Figure 1. Structure of PKC family.

experiments of PKC by 12-azidobenzoyl phorbol derivatives, resulting in specific cross-linking not to proteins but only to phosphatidylserine. Our photoaffinity probe PPDA, and Wender and Irie's probe possessed a photolabile diazoacetyl group at the C13 position in phorbol. Concerning the C13 position in phorbol esters, we have pointed out the possible importance of the C13 ester group by synthesis and biological evaluation of 13-deoxyphorbol esters.[30] Some recent reports on molecular modeling supported the importance of the C13 ester group,[31,32] although models by other groups including X-ray data[14] have not suggested the importance of this group. Our results, the biological evaluation of 13-deoxyphorbol esters and photoaffinity labeling by 13-diazoacetyl phorbol ester derivative **5** (PPDA), suggest that the ester group at C-13 in phorbol esters is located in close proximity to PKC and interacts with PKC in phorbol ester–PKC–phospholipid complexes. In the course of our investigations towards the clarification of these interactions, we herein report the design and synthesis of the two photoaffinity probes and the photoaffinity labeling of pure native PKC isozymes in detail, which is essential for further analyses.

Results and Discussion

A photoaffinity probe [^{3}H]PPDA (phorbol 12-(1-pyrenebutyrate) 13-diazoacetate, **5**) was synthesized as shown in Scheme 1. After successful synthesis of [^{3}H]PPDA, we also designed the photoaffinity probe [^{3}H]PPTD (**11**) in a attempt to develop an even more efficient probe. [^{3}H]PPTD (**11**) has a trifluorodiazopropionyl group in place of a diazoacetyl group at the C-13 position in phorbol. It has been reported that the trifluorodiazopropionyl group is superior to the diazoacetyl group in that the trifluorodiazopropionyl group does not suffer from an undesirable Wolff rearrangement of the carbene intermediate generated by irradiation.[33] Although there have been several reports of successful photoaffinity labeling using trifluorodiazopropionyl derivatives,[34] direct comparisons of the diazoacetyl and trifluorodiazopropionyl groups in biological systems are scarce. Therefore we are interested in evaluating these two groups in our systems as well. The photoaffinity probe [^{3}H] PPTD (phorbol 12-(1-pyrenebutyrate) 13-(3,3,3-trifluoro-2-diazo-propionate), **11**) was synthesized as shown in Scheme 2.

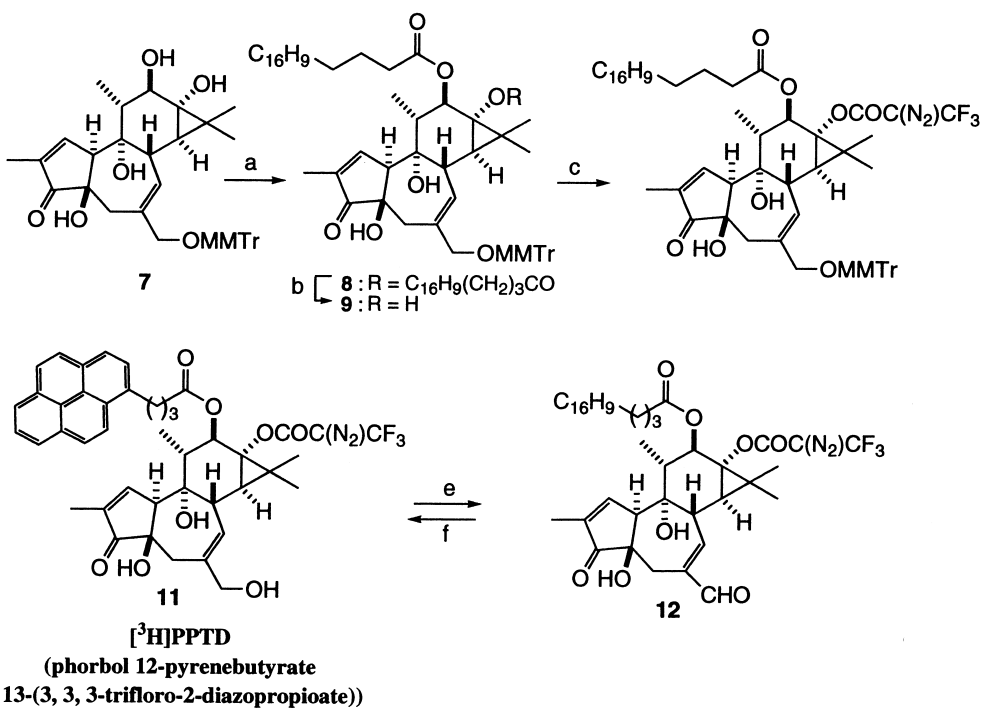


Scheme 1. (a) $BocNHCH_2CO_2H$, DCC, Et_3N, THF, r.t. (77%); (b) 1-pyrenebutyric acid, EDC·HCl, DMAP, CH_2Cl_2, r.t. (91%); (c) TFA, 0 °C (100%); (d) isoamyl nitrite, AcOH, $CHCl_3$, isoamyl alcohol, r.t. (58%); (e) MnO_2, CH_2Cl_2, r.t. (60%); (f) [^{3}H]$NaBH_4$, MeOH, 0 °C (65%).

First, the binding affinity of both ligands **5** (PPDA) and **11** (PPTD) to PKC βI was measured by evaluating their ability to inhibit [^{3}H]PDBu binding competitively using a reported procedure with minor modifications.[35] Both of the ligands showed a very strong affinity, which is comparable to PMA (phorbol 12-myristate 13-acetate, the most potent phorbol ester) (Figure 2(A)). The binding affinity to PKCα was also measured, and similar results were obtained. Thus confirming that these photoaffinity probes have a very strong binding affinity, we next investigated their ability to activate PKC catalytic activities (Figure 2(B)). It was also shown that these probes activate PKC βI with almost the same potency as PMA. It is very favorable that incorporation of photolabile groups was achieved without decreasing biological activities.

We next investigated conditions for the photolysis of the probes. The half-life of probe **5** and **11** irradiated at 254 nm was measured to be 5 and 20 s respectively, by following UV absorption. However, irradiation of PKC for longer than 30 s resulted in significant damage of PKC, judged by SDS–PAGE. To avoid this PKC damage, the reaction mixture was irradiated for only 10 s in the following experiments.

We then carried out photoaffinity labeling of PKC βI in the presence of phosphatidylserine vesicles (100 μg/ml), $CaCl_2$ (1 mM), [^{3}H]PPDA or [^{3}H]PPTD (1 μM), and PKC βI (280 nM) in a 50 mM Tris–HCl buffer (pH 6.8). After incubating for 3 min at 30 °C and for 2 min at 0 °C, the mixture was irradiated at 254 nm for 10 s at 0 °C. After separation of PKC βI by SDS–PAGE and silver staining, the gel was sliced every 3 mm and the radioactivity in each slice was measured after dissolving the gels in acidic H_2O_2. Radioactivities were detected only in the fraction containing PKC βI, indicating cross-linking of the probes to PKC βI (Figure 3). When the reaction was carried out in the presence of excess cold PMA (40 μM), the radioactivity in the fraction containing PKC βI was suppressed, suggesting that these cross-linking is specific. The yield for specific cross-linking by **5** and **11** was 1.2% and 1.3%, respectively. After investigations, it was observed that a small amount of cross-linking occurred also even in the absence of photolysis, and this cross-linking was not suppressed by the presence of excess cold PMA, suggesting that it was nonspecific (Figure 4, lane 3, 4, 7 and 8). The degree of the UV-independent cross-linking was larger in the case of [^{3}H]PPTD (**11**) than [^{3}H]PPDA (**5**). It has been reported that the diazoacetyl group can react with the SH group of a cysteine residue in cysteine protease and that the trifluorodiazopropionyl group can react with thiols such as mercaptoethanol and dithiothreitol to yield hydrazones in the dark.[36,37] Thus it is likely



Scheme 2. (a) 1-Pyrenebutyric acid, EDC·HCl, DMAP, CH_2Cl_2, r.t. (97%); (b) K_2CO_3, MeOH, THF, 0 °C (44%); (c) $CF_3C(N_2)COCl$, DMAP, CH_2Cl_2, r.t. (63%); (d) TFA, anisole, CH_2Cl_2, 0 °C (100%); (e) TPAP, NMO, CH_2Cl_2, r.t. (80%); (f) [^{3}H]$NaBH_4$, MeOH, 0 °C (63%).

that the UV-independent cross-linking was the result of a nonspecific reaction with the cysteine residues in PKC.

Photoaffinity labeling of other subtypes of PKC (α and ε, 140nM) also gave similar results (Figure 5). These results are consistent with the observation that both cPKC and nPKC have similar binding affinity to phorbol esters. Further analysis of the cross-linking site would reveal the role of individual CRD in either cPKC or nPKC. Photoaffinity labeling experiments were also carried out in other phospholipid systems (Figure 6). The phosphatidylserine(PS)-Triton X-100 mixed micelles (PS/Triton X-100 = 1/4) were prepared according to the method of Bell et al., followed by the addition of $CaCl_2$, [^{3}H]PPDA or PPTD (1 μM), and PKC βI (280 nM). The mixture was irradiated for 10 s, and after separation by SDS–PAGE, the radioactivities in the gel were measured as above. Under these conditions, interestingly, the results were somewhat different from those in the PS vesicle systems. Although [^{3}H]PPTD (**11**) resulted in specific cross-linking with PKC βI (1.1% yield), [^{3}H]PPDA (**5**) resulted only in a very low cross-linking yield (0.4%). The PS/Triton X-100 mixed micelles containing phorbol esters seem to have weaker interaction with PKC than PS vesicles containing phorbol esters because of lower surface concentration of PS. Assuming that a ketene intermediate was formed by a Wolff rearrangement of carbene spices generated from [^{3}H]PPDA (**5**), it is likely that less reactive, and long-living ketene

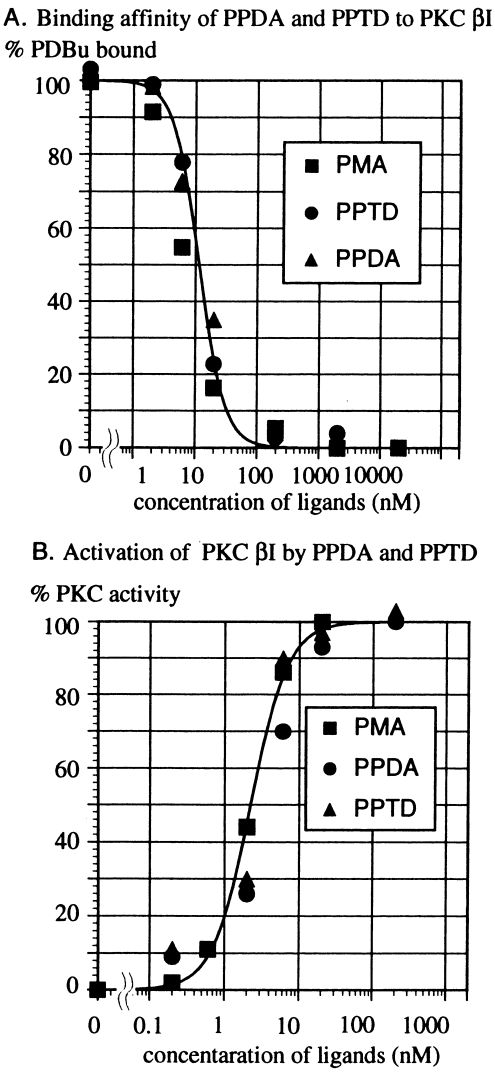


Figure 2. Biological activities of PPDA and PPTD. (A) %[^{3}H]PDBu bound to PKC βI was measured in the presence of various concentrations of ligands. (B) [^{32}P]Phosphate group incorporated into substrate peptides by PKC βI activated by various concentrations of ligands were measured.

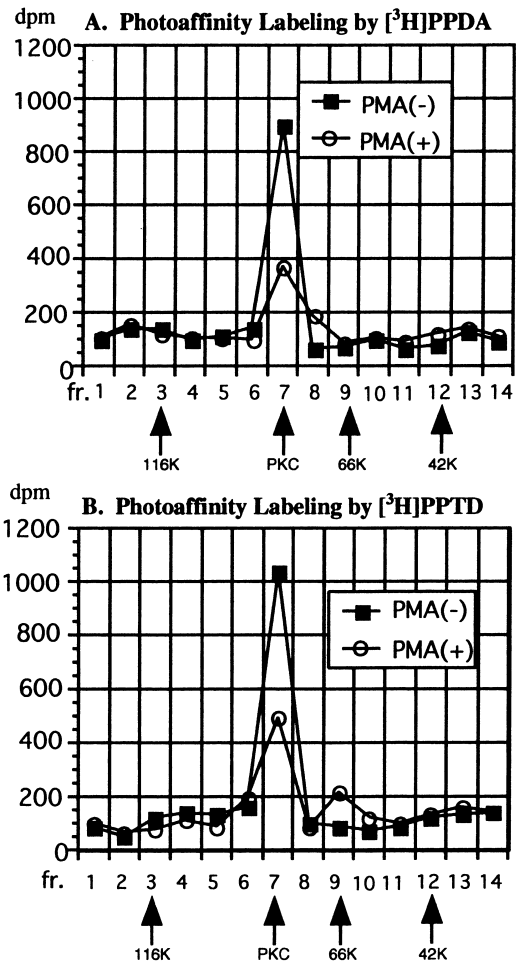


Figure 3. Photoaffinity labeling of PKC βI in PS vesicles by [^{3}H]PPDA (A) and [^{3}H]PPTD (B). Fourteen picomoles of PKC βI was used.

intermediates might be able to form cross-linking only in PS vesicle systems, where PKC has a stronger interaction with phospholipids containing phorbol esters. On the contrary, it is possible that the carbene adduct derived from [^{3}H]PPTD (**11**), which does not undergo a rearrangement to a ketene, can form cross-linking with PKC in both the PS vesicle and Triton X-100 mixed micelle systems. The lower degree of UV-independent nonspecific cross-linking in the PS-Triton X-100 mixed micelles system (Figure 6, lane 3, 4, 7 and 8) compared to the PS vesicle systems (Figure 4), in case of both probes, can also be interpreted as a result of a weaker interaction between PKC and PS-Triton X-100 mixed micelles containing phorbol esters. Thus, the results of photoaffinity labeling experiments were influenced by both the nature of the photolabile groups within the probes and the phospholipid systems employed. Although PS vesicle systems were essential for efficient cross-linking in the case of [^{3}H] PPDA (**5**) possibly because of formation of a less reactive ketene intermediate, it was shown that [^{3}H]PPTD (**11**), which had trifluorodiazopropionyl group in place of diazoaetyl group, resulted in cross-linking with similar efficiency in both PS vesicles and PS/Triton X-100 mixed micelle systems.

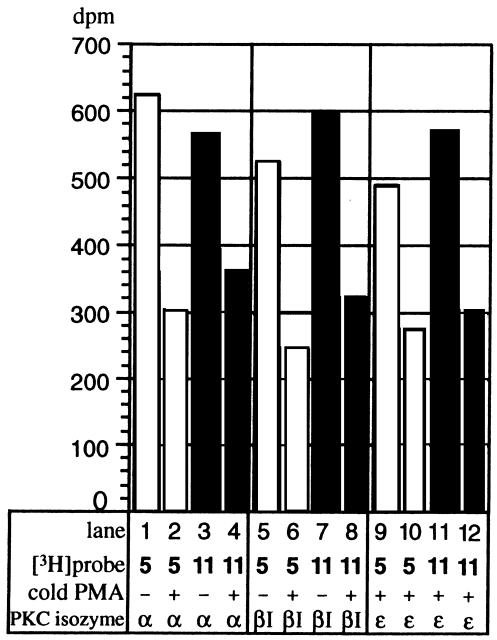


Figure 5. Photoaffinity labeling of PKC isozymes by [^{3}H] PPDA (lanes 1, 2, 5, 6, 9, and 10) or [^{3}H]PPTD (lanes 3, 4, 7, 8, 11, and 12) in PS vesicles. Reactions were carried out using 7 pmol of PKC α (lanes 1–4), PKC βI (lanes 5–8), and PKC ε (lanes 9–12), respectively.

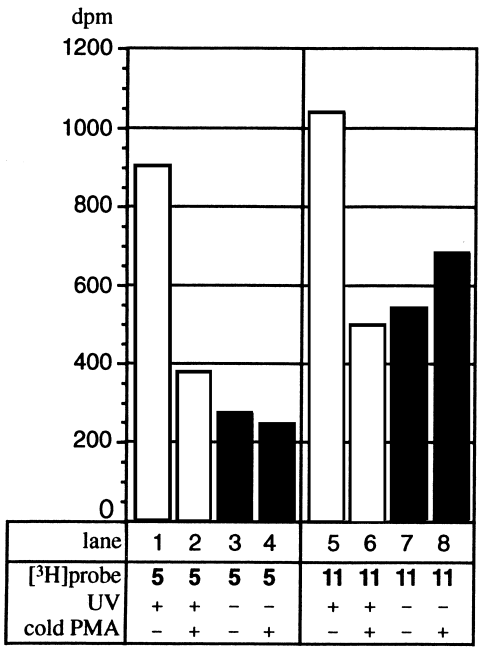


Figure 4. Photoaffinity labeling of PKC βI in PS vesicles. Reactions were carried out with (lanes 1, 2, 5, and 6) or without (lanes 3, 4, 7, and 8) UV irradiation (10 s) in the presence (lanes 2, 4, 6, and 8) or absence (lanes 1, 3, 5, and 7) of 40-fold excess of cold PMA. Fourteen picomoles of PKC βI was used in each experiment. Radioactivities found in the fractions containing PKC were shown.

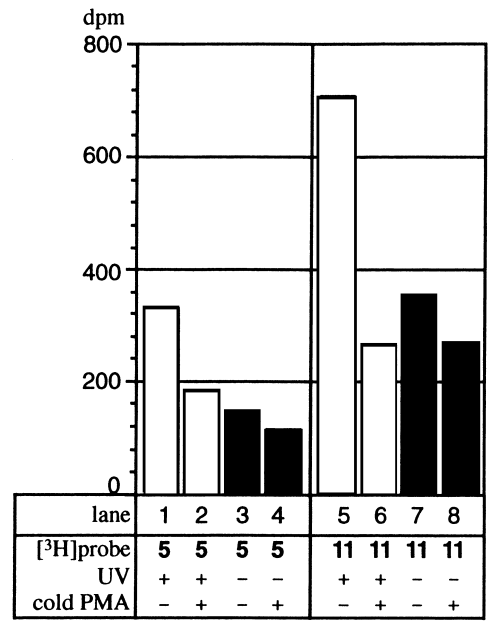


Figure 6. Photoaffinity labeling of PKC βI in PS-Triton X-100 mixed micelles. Reactions were carried out with (lanes 1, 2, 5, and 6) or without (lanes 3, 4, 7, and 8) UV irraditation (10 s) in the presence (lanes 2, 4, 6, and 8) or absence (lanes 1, 3, 5, and 7) of 40-fold excess of cold PMA. Fourteen picomoles of PKC βI was used in each experiment.

Conclusion

It was demonstrated that photoaffinity probes, which have photolabile groups at the C13 position in phorbol, can form cross-linking with native pure isozymes of PKC by irradiation. Although the cross-linking yield is not very high, we suppose that determination of the cross-linking site can be realized by taking advantage of subpicomole order analysis by mass spectroscopy and other methodologies to clarify the role of individual CRD in native PKC. Further studies along these lines are now in progress together with studies to develop more efficient photoaffinity probes.

Experimental

General procedures

Infrared (IR) spectra were recorded on a Perkin Elmer 1600 FT IR spectrometer. ^{1}H NMR spectra were measured with a JEOL JNM-EX 270 spectrometer. Chemical shifts are reported on the δ scale relative to $CHCl_3$ as an internal reference (7.26 ppm). Mass spectra (MS) were measured on a JEOL JMS-SX 102A instrument. In general, reactions were carried out in dry solvents under an argon atmosphere, unless otherwise mentioned. All phorbol esters should be handled with care, because they are potent tumor promoters and strong irritants. 3,3,3-Trifluoro-2-diazopropionyl chloride was purchased from Pierce. Phorbol was purchased from LC laboratories. [^{3}H]$NaBH_4$ (488.4 GBq/mmol) was purchased from NEN. Each isozyme of PKC (human, recombinant) was purchased from Calbiochem. Phosphatidylserine was purchased from SIGMA. The binding assay and PKC assay were carried out using reported procedure with minor modifications.[35,38] The results shown in the Figures are representative of three or four sets of experiments.

Preparation of compound 2

To a solution of phorbol 20-trityl ether[39] (11.8 mg, 19.4 μmol) in THF (0.3 mL) were added DCC (20.1 mg, 97.2 mmol), triethylamine (5.9 mg, 58.3 μmol), and *N*-*tert*-butoxycarbonylglycine (17.0 mg, 97.2 μmol) at 0 °C and the mixture was allowed to warm to r.t. After stirring for 2 h, the mixture was filtered, and the filtrate was concentrated. The residue was purified by silica gel column chromatography (AcOEt/Hex = 1/2) to give compound **2** (11.4 mg, 77%).

IR (neat) 3412, 2978, 1702, 1215, 1164, 1054 cm-1.

^{1}H NMR ($CDCl_3$, 270 MHz) δ 1.04 (1H, d, J = 5.4 Hz), 1.08 (3H, d, J = 6.5 Hz), 1.22 (3H, s), 1.27 (3H, s), 1.46 (9H, s), 1.78 (3H, dd, J = 1.5, 3.0 Hz), 1.99 (1H, m), 2.36 (1H, br-s), 2.37 (1H, d, J = 19.0 Hz), 2.48 (1H, d, J = 19.0 Hz), 3.00 (1H, br-s), 3.06 (1H, m), 3.11 (1H, dd, J = 5.0, 5.4 Hz), 3.30 (1H, br-s), 3.56 (2H, s), 3.98 (2H, m), 3.99 (1H, m), 4.98 (1H, m), 5.57 (1H, dd, J = 1.5, 5.0 Hz), 7.20–7.50 (15H, m), 7.56 (1H, dd, J = 1.4, 3.0 Hz).

FAB HRMS (NBA) m/z 786.3565, ($M + Na^+$) calculated for $C_{46}H_{53}O_9NNa$: 786.3618.

Preparation of compound 3

To a solution of compound **2** (54.8 mg, 71.7 μmol) in methylene chloride (1.5 mL) were added EDCI·HCl (27.5 mg, 144 mmol), DMAP (8.8 mg, 71.7 μmol), and 1-pyrenebutyric acid (41.4 mg, 144 μmol) at 0 °C and the mixture was allowed to warm to r.t. The mixture was stirred for 3 h and filtered through SiO_2 (AcOEt/Hex = 1/1), and the filtrate was concentrated. The residue was purified by silica gel column chromatography (AcOEt/benzene = 1/12) to give compound **3** (67.2 mg, 91%).

IR (neat) 3422, 2974, 1708, 1448, 1367, 1161, 845 cm^{-1}.

^{1}H NMR ($CDCl_3$, 270 MHz) δ 0.95 (3H, d, J = 6.1 Hz), 1.15 (1H, d, J = 5.0 Hz), 1.27 (3H, s), 1.29 (3H, s), 1.47 (9H, s), 1.82 (3H, dd, J = 1.1, 2.6 Hz), 2.10 (1H, s), 2.16 (1H, m), 2.21 (2H, m), 2.46 (1H, d, J = 18.0 Hz), 2.53 (2H, t, J = 7.1 Hz), 2.57 (1H, d, J = 18.0 Hz), 3.21 (1H, dd, J = 5.0, 5.0 Hz), 3.30 (1H, m), 3.46 (2H, t, J = 7.6 Hz), 3.57 (2H, s), 3.93 (1H, dd, J = 18.0, 5.1 Hz), 4.02 (1H, dd, J = 18.0, 6.5 Hz), 5.03 (1H, m), 5.19 (1H, s), 5.43 (1H, d, J = 10.5 Hz), 5.63 (1H, d, J = 5.0 Hz), 7.20–7.50 (15H, m), 7.56 (1H, m), 7.80–9.30 (9H, m).

FAB HRMS (NBA) m/z 1033.4677, (M^+) calculated for $C_{66}H_{67}O_{10}N$: 1033.4765.

Preparation of compound 4

To compound **3** (31.9 mg, 30.8 μmol) was added TFA (0.5 mL) at 0 °C, and the mixture was stirred for 0.5 h. The mixture was concentrated, and the residue was purified by silica gel column chromatography (MeOH/$CHCl_3$ = 1/10) to give compound **4** (21.3 mg, 100%).

IR (neat) 3393, 2923, 1704, 1376, 1207, 1140, 844 cm^{-1}.

^{1}H NMR ($CDCl_3$, 270 MHz) δ 0.95 (3H, d, J = 6.3 Hz), 1.22 (1H, d, J = 5.2 Hz), 1.23 (3H, s), 1.28 (3H, s), 1.69 (3H, dd, J = 1.2, 1.9 Hz), 2.19 (2H, m), 2.29 (1H, m), 2.47 (2H, m), 2.56 (2H, t, J = 6.9 Hz), 3.17 (1H, m), 3.31 (1H, m), 3.46 (2H, dd, J = 6.1, 8.5 Hz), 3.92 (1H, d, J = 13.0 Hz), 3.98 (1H, d, J = 13.0 Hz), 4.12 (1H, d, J = 16.0 Hz), 4.20 (1H, d, J = 16.0 Hz), 5.59 (1H, d, J = 10.5 Hz), 5.63 (1H, d, J = 5.5 Hz), 7.52 (1H, m), 7.90–8.60 (9H, m).

FAB HRMS (NBA) m/z 692.3250, ($M + H^+$) calculated for $C_{42}H_{46}O_8N$: 692.3223.

Preparation of compound 5 (PPDA)

To a solution of compound **4** (5.3 mg, 7.7 μmol) in $CHCl_3$ (0.19 mL) and isoamyl alcohol (0.02 mL) were added isoamyl nitrite (27.1 mg, 232 μmol), 40 mM acetic acid/$CHCl_3$ (0.19 mL, 7.7 μmol) at 0 °C and the mixture was allowed to warm to r.t. The mixture was stirred for 1 h and K_2CO_3 (3.0 mg) was added. After filtration, the filtrate was concentrated. The residue was purified by silica gel column chromatography (AcOEt/hexane = 1/1) to give compound **5** (3.2 mg, 58%).

IR (neat) 3397, 2118, 1670, 1370, 845 cm^{-1}.

^1H NMR ($CDCl_3$, 270 MHz) δ 0.93 (3H, d, J = 6.6 Hz), 1.18 (1H, m), 1.19 (3H, s), 1.22 (3H, s), 1.78 (3H, dd, J = 1.1, 2.6 Hz), 2.18 (1H, m), 2.19 (1H, s), 2.20 (2H, m), 2.48 (2H, m), 2.51 (2H, t, J = 7.5 Hz), 3.25 (2H, m), 3.40 (2H, t, J = 7.9 Hz), 4.02 (2H, s), 4.84 (1H, br-s), 5.57 (1H, d, J = 10.2 Hz), 5.69 (1H, br-s), 5.70 (1H, d, J = 5.0 Hz), 7.59 (1H, m), 7.80–8.40 (9H, m).

FAB HRMS (NBA) m/z 702.2906, (M^+) calculated for $C_{42}H_{42}O_8N_2$: 702.2941.

Preparation of compound 6

To a solution of compound **5** (5.6 mg, 7.9 μmol) in methylene chloride (1.0 mL) was added MnO_2 (6.9 mg, 79 μmol) at r.t., and the mixture was stirred for 3 h. After filtration through SiO_2 (acetone) and concentration, the residue was purified by silica gel column chromatography (AcOEt/Hex = 1/1) to give 6 (3.3 mg, 60%).

IR (neat) 3383, 2924, 2118, 1676, 1545, 1373, 1193, 909, 845.

^1H NMR ($CDCl_3$, 270 MHz) δ 0.93 (3H, d, J = 6.2 Hz), 1.23 (6H, s), 1.34 (1H, d, J = 5.1 Hz), 1.78 (3H, m), 2.20 (1H, m), 2.22 (2H, m), 2.37 (1H, s), 2.47 (1H, d, J = 19.6 Hz), 2.49 (2H, t, J = 7.0 Hz), 2.94 (1H, d, J = 19.6 Hz), 3.06 (1H, m), 3.41 (2H, t, J = 7.6 Hz), 3.62 (1H, dd, J = 5.1, 5.1 Hz), 4.88 (1H, br-s), 5.59 (1H, d, J = 10.3 Hz), 5.91 (1H, br-s), 6.73 (1H, dd, J = 2.0, 5.1 Hz), 7.54 (1H, m), 7.80–8.40 (9H, m), 9.43 (1H, s).

FAB HRMS (NBA) m/z 700.2891, (M^+) calculated for $C_{42}H_{40}O_8N_2$: 700.2785.

Preparation of compound [^{3}H]5 ([^{3}H]PPDA)

To a solution of [^{3}H]$NaBH_4$ (0.76 μmol, 370 MBq) in 50 mM aqueous NaOH (30 μL) was added a solution of **5** (2.6 mg, 3.6 μmol) in 5 mM AcOH/MeOH (300 μL) at 0 °C. After stirring for 20 min, 40 mM AcOH/$CHCl_3$ (160 μL), and phosphate buffer (pH 6.8) were added to the reaction mixture. The organic layer was dried over Na_2SO_4 and concentrated after filtration. The residue was purified by silica gel column chromatography (AcOEt/Hex = 1/1) to give [^{3}H] **5** (1.33 mg, 50 GBq/mmol, 65%). The radiochemical purity of the product was confirmed by radioscanning of the TLC plate.

Preparation of compound 8

To a solution of phorbol 20-monomethoxytrityl ether[18] (31.3 mg, 49.2 μmol) in methylene chloride (1 mL) were added EDCI·HCl (28.3 mg, 147 mmol), DMAP (18.0 mg, 147 μmol), and 1-pyrenebutyric acid (34.0 mg, 118 μmol) at 0 °C and the mixture was allowed to warm to r.t. The mixture was stirred for 12 h and filtered through SiO_2 (AcOEt/Hex = 1/1), and the filtrate was concentrated. The residue was purified by silica gel column chromatography (AcOEt/Hex = 1/3) to give compound **8** (57.9 mg, 100%).

IR (neat) 3404, 1711, 1604, 1080 cm^{-1}.

^1H NMR (270 MHz, $CDCl_3$) δ 0.93 (3H, d, J = 6.1 Hz), 1.05 (1H, d, J = 5.2 Hz), 1.07 (3H, s), 1.24 (3H, s), 1.78 (3H, m), 2.10–2.30 (5H, m), 2.35–2.65 (6H, m), 3.17 (1H, m), 3.28 (1H, m), 3.30–3.50 (4H, m), 3.52 (2H, s), 3.76 (3H, s), 5.55 (1H, s), 5.57 (1H, d, 10.5 Hz), 5.66 (1H, m), 6.80–7.40 (14H, m), 7.6 (1H, m), 7.78–8.40 (18H, m).

FAB HRMS (NBA) m/z 1176.5143, (M^+) calculated for $C_{80}H_{72}O_9$: 1176.5176.

Preparation of compound 9

To a solution of compound **8** (5.8 mg, 4.9 μmol) in THF (0.25 mL) and MeOH (0.25 mL) was added K_2CO_3 at −10 °C, and the mixture was stirred for 4.5 h. The reaction mixture was poured into cold water and extracted with AcOEt, washed with brine, and dried over Na_2SO_4. After filtration and concentration, the residue was purified by silica gel column chromatography (AcOEt/Hex = 1/3) to give compound **9** (2.5 mg, 56%).

IR (neat) 3426, 2922, 1702, 1250, 1217, 1180, 1076, 755 cm^{-1}.

^1H NMR (270 MHz, $CDCl_3$) δ 0.90 (1H, d, J = 6.0 Hz), 1.02 (3H, d, J = 6.3 Hz), 1.06 (3H, s), 1.22 (3H, s), 1.78 (3H, m), 2.10 (1H, s), 2.10 (1H, m), 2.21 (2H, m), 2.36 (1H, d, J = 19.0 Hz), 2.46 (1H, m), 2.51 (2H, t, J = 7.0 Hz), 3.04 (2H, m), 3.41 (2H, t, J = 5.0 Hz), 3.53 (1H, d, J = 12.0 Hz), 3.58 (1H, d, J = 12.0 Hz), 3.8 (3H, s), 4.71 (1H, s), 4.89 (1H, d, J = 9.5 Hz), 5.63 (1H, m), 6.80–7.40 (14H, m), 7.58 (1H, m), 7.82–8.36 (9H, m).

FAB HRMS (NBA) *m/z* 906.4180, (M^+) calculated for $C_{60}H_{58}O_8$: 906.4132.

Preparation of compound 10

To a solution of compound **9** (24.2 mg, 26.7 μmol) in methylene chloride (0.5 mL) was added DMAP (3.9 mg, 32.0 mmol) and 2-diazo-3,3,3-trifluoropropionyl chloride (4.6 mg, 26.7 μmol) at 0 °C. The mixture was allowed to warm to r.t. and stirred for 2.5 h. The mixture was concentrated, and the residue was purified by silica gel column chromatography (AcOEt/Hex = 1/4) to give compound **10** (17.5 mg, 63%).

IR (neat) 3434, 2928, 2141, 1701, 1348, 1143, 1063 cm^{-1}.

^1H NMR (270 MHz, $CDCl_3$) δ 0.92 (3H, d, J = 6.3 Hz), 1.23 (3H, s), 1.24 (3H, s), 1.25 (1H, m), 1.77 (3H, m), 2.10 (1H, s), 2.10–2.30 (3H, m), 2.40 (1H, d), 2.48 (2H, t, J = 7.5 Hz), 2.52 (1H, d, J = 18.9 Hz), 3.15–3.28 (2H, m), 3.40 (2H, t, J = 7.1 Hz), 3.46 (2H, s), 3.80 (3H, s), 5.08 (1H, s), 5.56 (1H, d, J = 10.5 Hz), 5.67 (1H, m), 6.80–7.22 (14H, m), 8.58 (1H, m), 7.85–8.35 (9H, m).

FAB HRMS (NBA) *m/z* 1042.3922, (M^+) calculated for $C_{63}H_{57}F_3N_2O_9$: 1042.4016.

Preparation of compound 11 (PPTD)

To a solution of compound **10** (10.1 mg, 9.7 μmol) in methylene chloride (0.5 mL) was added anisole (10.0 mg, 92.0 μmol) and TFA (10 mL) at 0 °C, and the mixture was stirred for 1 h. To the mixture was added saturated aqueous $NaHCO_3$, and the resulting mixture was extracted with AcOEt, washed with brine, and dried over Na_2SO_4. After filtration and concentration, the residue was purified by silica gel column chromatography to give compound **11** (7.7 mg, 100%).

IR (neat) 3420, 2141, 1700, 1684, 1559, 1540, 1457, 1339, 1140, 1064 cm^{-1}.

^1H NMR (270 MHz, $CDCl_3$) δ 0.92 (3H, d, J = 6.1 Hz), 1.22 (3H, s), 1.24 (3H, s), 1.25 (1H, m), 1.78 (3H, m), 2.13 (1H, s), 2.13–2.30 (3H, m), 2.40–2.55 (4H, m), 3.23–3.30 (2H, m), 3.40 (2H, t, J = 7.0 Hz), 4.03 (2H, m), 5.15 (1H, s), 5.56 (1H, d, J = 10.2 Hz), 5.69 (1H, m), 7.59 (1H, m), 7.80–8.31 (9H, m).

FAB HRMS (NBA) *m/z* 770.2805, (M^+) calculated for $C_{43}H_{41}F_3N_2O_8$: 770.2815.

Preparation of compound 12

To a solution of compound **11** (8.0 mg, 10 μmol) in methylene chloride (0.5 mL) were added TPAP (0.5 mg, 1.4 μmol) and NMO (1.2 mg, 10 μmol) at r.t., and the mixture was stirred for 30 min. After filtration through SiO_2 (acetone) and concentration, the residue was purified by silica gel column chromatography (AcOEt/Hex = 1/3) to give compound **12** (5.6 mg, 70%).

IR (neat) 3422, 2925, 2142, 1702, 1459, 1351, 1142, 846 cm^{-1}.

^1H NMR (270 MHz, $CDCl_3$) δ 0.95 (3H, d, J = 6.1 Hz), 1.24 (3H, s), 1.26 (3H, s), 1.40 (1H, d, J = 6.9 Hz), 1.78 (3H, m), 2.15–2.30 (3H, m), 2.40 (1H, d, J = 1.2 Hz), 2.47 (1H, d, J = 25.0 Hz), 2.48 (2H, t, J = 9.0 Hz), 2.94 (1H, d, J = 25.0 Hz), 3.07 (1H, m), 3.41 (2H, t, J = 9.5 Hz), 3.63 (1H, m), 5.40 (1H, s), 5.58 (1H, d, J = 13.0 Hz), 6.72 (1H, dd, J = 2.5, 7.0 Hz), 7.52 (1H, m), 7.80–8.32 (9H, m), 9.42 (1H, s).

FAB HRMS (NBA) *m/z* 768.2722, (M^+) calculated for $C_{43}H_{39}F_3N_2O_8$: 768.2659.

Preparation of compound [^{3}H]11 ([^{3}H]PPTD)

To a solution of [^{3}H]$NaBH_4$ (0.76 μmol, 370 MBq) in 50 mM aqueous NaOH (30 μL) was added a solution of **12** (3.1 mg, 4.1 μmol) in 5 mM AcOH/MeOH (300 μL) at 0 °C. After stirring for 20 min, 40 mM AcOH/$CHCl_3$ (160 μL), and phosphate buffer (pH 6.8) were added to the reaction mixture. The organic layer was dried over Na_2SO_4 and concentrated after filtration. The residue was purified by silica gel column chromatography (AcOEt/Hex = 1/1) to give [^{3}H] **11** (PPTD)(1.48 mg, 50 GBq/mmol, 63%). The radiochemical purity of the product was confirmed by radioscanning of the TLC plate.

Evaluation of binding affinity of compound 5 and 11 to PKC

Plastic tubes of each binding assay mixture (300 μL) contained 50 mM Tris–HCl (pH 7.5), 1 mM $CaCl_2$, 100 μg/mL phosphatidyl-L-serine (L-PS), 10 nM [^{3}H]PDBu (20.7 Ci/mmol, from NEN), 0.5% DMSO, 4 mg/mL bovine serum albumin, 0.8 μg/mL PKC βI and 2nM–20 μM cold PMA, PPDA(**5**), or PPTD(**11**). L-PS was sonicated in 50 mM Tris–HCl (pH 7.5) at 0 °C prior to use. After incubation at 30 °C for 30 min, the mixture was filtered through a glass-fiber filter which was pretreated with freshly prepared 0.3% polyethyleneimine for 1 h. The filter was washed three times with 3 mL of ice cold 0.5% DMSO. The radioactivity of each filter was counted in a scintillation vial with 10 mL of scintillator (Aquasol-2, NEN) using a liquid scintillation counter. The count in the presence of 10 μM of PMA which accounted for nonspecific binding was subtracted from each of the counts to account for the specific bindings.

Evaluation of an ability of compound 5 and 11 to activate PKC

PKC activity was determined by measuring the incorporation of ^{32}P from [γ–^{32}P]ATP into EGF-R fragment peptide (651–658, BIOMOL) using the method reported by Bell et al. with minor modifications. The amorphous powder of L-PS (SIGMA) in 0.3% Triton X-100 solution in 50 mM Tris–HCl buffer (pH 7.5) was vortexed for 1 min, and incubated for 10 min at 25 °C, followed by the addition of $CaCl_2$, DTT, and peptide. To this mixture was added 0.5 μL of various concentrations of PMA, PPDA(**5**), or PPTD(**11**) in DMSO, after which it was vigorously vortexed. After incubation for 6 min at 25 °C, 25 μL of the PKC solution in Tris buffer was added, and incubated for 7 min at 25 °C. The enzyme reaction was started by adding 25 μL of solution containing ATP, [γ–^{32}P]ATP, and $MgCl_2$. The final reaction mixture (75 μL) contained $CaCl_2$ (1 mM), $MgCl_2$ (15 mM), ATP (50 μM), [γ–^{32}P]ATP (6 μCi), peptide (75 μM), 20 mol % of L-PS dispersed in 0.025% (w/v) Triton X-100 in 50 mM Tris–HCl buffer (pH 7.5), and various concentration of activators. The reaction was conducted for 15 min at 25 °C, and stopped by the addition of 100 μL ice-cold 25% trichloroacetic acid. The resulting precipitate was collected on a binding paper (P-81, Whatman Ltd., England) and washed twice with 250 mL of 75 mM phosphoric acid. The filter was placed in a scintillation vial containing 10 mL of Aquasol-2 solution, and ^{32}P radioactivities were quantitated by a scintillation counter. The radiocount for the control assay without PMA was determined and was subtracted from each of the radiocounts to account for nonspecific kinase activities. The result shown is representative of three sets of experiments.

Photoaffinity labeling of PKC in PS vesicles

The reaction mixture (50 μL) in a 2.0 mL plastic tube contained PKC (280 nM), [^3H]PPDA or PPTD (1 μM), phosphatidylserine (100 μg/mL), $CaCl_2$ (1 mM) in 50 mM Tris–HCl (pH 6.8) in the presence or absence of cold PMA (40 μM). After incubation for 3 min at 30 °C and for 2 min at 0 °C, the mixture was irradiated at 254 nm for 10 s from 0.5 cm above (Pen-Ray Lamp 11 SC-1, UVP). To the mixture was added 12.5 μL of Tris–HCl buffer (pH 6.8) containing 10% SDS, 25% sucrose, and 10 mM EGTA, and the mixture was applied to SDS–PAGE (7.5%T, 2.6%C) using standard procedures. After silver staining (Silver Stain Plus, Bio Rad), the gel was sliced every 3 mm. Each slice was placed in a scintillation vial containing 30% H_2O_2 (0.4 mL) and 70% $HClO_4$ (0.1 mL) and incubated for 2 h at 80 °C. A scintillator (Aquasol-2, 7 mL) and saturated $Na_2S_2O_3$ solution (0.1 mL) were added, and the radioactivities were then measured by a liquid scintillation counter.

Photoaffinity labeling of PKC in PS-Triton X-100 mixed micelles

The amorphous powder of L-PS (SIGMA) in 0.3% Triton X-100 solution in 50 mM Tris–HCl buffer (pH 7.5) was vortexed for 1 min, and incubated for 10 min at 25 °C. The reaction mixture (50 μL) in a 2.0 mL plastic tube contained PKC (280 nM), [^3H]PPDA or PPTD (1 μM), 20 mol% of L-PS dispersed in 0.025% (w/v) Triton X-100, and $CaCl_2$ (1 mM) in 50 mM Tris–HCl (pH 6.8) in the presence or absence of cold PMA (40 μM). After incubation for 3 min at 30 °C and for 2 min at 0 °C, photoaffinity labeling experiments were carried out as described above.

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References

1. Hecker, E.; Adolf, W.; Hergenhahn, M.; Schmidt, R.; Sorg, B. *Cellular Interactions by Environmental Tumor Promoters*; Hecker, E.; Adolf, W.; Hergenhahn, M.; Schmidt, R.; Sorg, B., Eds; Japan Sci. Soc., Tokyo; VNU Science, Utrecht, The Netherlands, 1984, pp 3–36.

2. Blumberg, P. M. *Cancer Res.* **1988**, *48*, 1–8.

3. Castagna, M.; Takai, Y.; Kaibuchi, K.; Sano, K.; Kikkawa, U.; Nishizuka, Y. *J. Biol. Chem.* **1982**, *257*, 7847–7851.

4. Nishizuka, Y. *Nature* **1988**, *334*, 661–665.

5. Nishizuka, Y. *Science* **1992**, *258*, 607–614.

6. Lester, D. S.; Epand, R. M. *Protein Kinase C: Current Concepts and Future Perspectives*; Ellis Horwood Ltd.: West Sussex, 1992.

7. Ono, Y.; Fujii, T.; Ogita, K.; Kikkawa, U.; Igarashi, K.; Nishizuka, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 3099–3103.

8. Burns, D. J.; Bell, R. M. *J. Biol. Chem.* **1991**, *266*, 18330–18339.

9. Wender, P. A.; Cribbs, C. M. *Advances in Medicinal Chemistry*; Wender, P. A.; Cribbs, C. M., Eds; JAI PRESS INC.: London, 1992; Vol. 1, pp 1–54.

10. Quest, A. F. G.; Bardes, E. S. G.; Bell, R. M. *J. Biol. Chem.* **1994**, *269*, 2961–2970.

11. Hommel, U.; Zurini, M.; Luyten *Nat. Struct. Biol.* **1994**, *1*, 383–387.

12. Ichikawa, S.; Hatanaka, H.; Takeuchi, Y.; Ohno, S.; Inagaki *J. Biochem.* **1995**, *117*(3), 566–574.

13. Xu, R. X.; Pawelczyk, T.; Xia, T.-H.; Brown, S. C. *Biochemistry* **1997**, *36*, 10709–10717.

14. Zhang, G.; Kazanietz, M. G.; Blumberg, P. M.; Hurley, J. H. *Cell* **1995**, *81(6)*, 917–924.

15. Szallasi, Z.; Bogi, K.; Gohari, S.; Biro, T.; Acs, P.; Blumberg, P. M. *J. Biol. Chem.* **1996**, *271*, 18299–18301.

16. Irie, K.; Yanai, Y.; Oie, K.; Ishizawa, J.; Nakagawa, Y.; Ohigashi, H.; Wender, P. A.; Kikkawa, U. *Bioorg. Med. Chem.* **1997**, *5*, 1725–1737.

17. Slater, S. J.; Ho, C.; Kelly, M. B.; Larkin, J. D.; Taddeo, F. J.; Yeager, M. D.; Stubbs, C. D. *J. Biol. Chem.* **1996**, *271*, 4627–4631.

18. Delclos, K. B.; Yeh, E.; Blumberg, P. M. *Proc. Natl. Acad. Sci. USA* **1983**, *80*, 3054–3058.

19. Schmidt, R.; Heck, K.; Sorg, B.; Hecker *Cancer Lett.* **1985**, *26*(1), 97–111.

20. Sodeoka, M.; Uotsu, K.; Shibasaki, M. *Tetrahedron Lett.* **1995**, *36*, 8795–8798.

21. Irie, K.; Ishii, T.; Ohigashi, H.; Wender, P. A.; Miller, B. L.; Takeda, N. *J. Org. Chem.* **1996**, *61*, 2164–2173.

22. Jeffrey, A. M.; Liskamp, R. M. *J. Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 241–245.

23. Wender, P. A.; Koehler, K. F.; Sharkey, N. A.; Dell'Aquila, M. L.; Blumberg, P. M. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 4214–4218.

24. Wender, P. A.; Cribbs, C. M.; Koehler, K. F.; Sharkey, N. A.; Herald, C. L.; Kamano, Y.; Pettit, G. R.; Blumberg, P. M. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 7197–7201.

25. Itai, A.; Kato, Y.; Tomioka, N.; Iitaka, Y.; Endo, Y.; Hasegawa, M.; Shudo *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 3688–3692.

26. Thomson, C.; Wilkie, J. *Carcinogenesis* **1989**, *10*(3), 531–540.

27. Nakamura, H.; Kishi, Y.; Pajares, M. A.; Rando, R. R. *Proc. Natl. Acad. Sci. USA.* **1989**, *86*, 9672–9676.

28. Leli, U.; Hauser, G.; Froimowitz *Mol. Pharmacol.* **1990**, *37*, 286–295.

29. Rando, R. R.; Kishi, Y. *Biochemistry* **1992**, *31*, 2211–2218.

30. Sugita, K.; Sawada, D.; Sodeoka, M.; Sasai, H.; Shibasaki, M. *Chem. Pharm. Bull.* **1996**, *44*, 463–465.

31. Wang, S.; Kazanietz, M. G.; Blumberg, P. M.; Marquez, V. E.; Milne, G. W. A. *J. Med. Chem.* **1996**, *39*, 2541–2553.

32. Krauter, G.; von der Lieth, C.-W.; Schmidt, R.; Hecker, E. *Eur. J. Biochem.* **1996**, *242*, 417–427.

33. Chowdhry, V.; Vaughan, R.; Westheimer, F. H. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 1406–1408.

34. Casanova, J.; Horowitz, Z. D.; Copp, R. P.; McIntyre, W. R.; Pascual, A.; Samuels, H. *J. Biol. Chem.* **1984**, *259*, 12084–12094.

35. Tanaka, Y.; Miyake, R.; Kikkawa *J. Biochem.* **1986**, *99(1)*, 257–261.

36. Leary, R.; Larsen, D.; Watanabe, H.; Shaw, E. *Biochemistry* **1977**, *16*, 5857–5861.

37. Takagaki, Y.; Gupta, C. M.; Gobind, K. H. *Biochem. Biophys. Res. Comm.* **1980**, *95*, 589–595.

38. Hannun, Y. A.; Loomis, C. R.; Bell, R. M. *J. Biol. Chem.* **1985**, *260*, 10039–10043.

39. Sorg, B.; Fürstenberger, G.; Berry, D. L.; Hecker, E.; Marks, F. *J. Lipid Res.* **1982**, *23*, 443–437.