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The pharmacophore of debromoaplysiatoxin responsible for protein kinase C activation

(3,4-dihydroxybutyrates/cyclic acylglycerols/diacylglycerols/aplysiatoxins/structural correlation)

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ABSTRACT **Protein kinase C is physiologically activated by 1,2-diacyl-*sn*-glycerol in the *S* configuration. The enzyme is also powerfully activated by structurally diverse tumor promotors. A model has been developed that demonstrates how the various tumor promotors and diacylglycerols can all be accommodated by the same binding site of the kinase. One prediction of this model concerns the structural nature of the pharmacophore in the tumor promotor debromoaplysiatoxin. This prediction is realized by synthesizing the analogs with the deduced pharmacophore and demonstrating that they are potent activators of protein kinase C. These findings provide strong experimental support for our structural model of protein kinase C activation.**

Protein kinase C (PKC) is an important regulatory enzyme involved in the control of diverse biological phenomena (1, 2). Under quiescent conditions, the enzyme is located in the cytoplasm, where it remains catalytically inactive (2). The enzyme is activated, in a transient fashion, when 1,2-diacyl-*sn*-glycerols in the *S* configuration are produced in the membrane by the action of a specific phospholipase C (2, 3). This latter enzyme cleaves inositol (poly)phospholipids to generate diacylglycerols and (poly)phosphoinositols (2, 4). The liberated diacylglycerol binds to the regulatory domain of PKC and activates PKC by increasing its affinity for calcium ions to the steady-state physiological range (2).

The specificity of the activation process is considerable, with only a narrow range of unmodified diacylglycerols in the *S* configuration being active (5–9). We recently proposed (10) a structural hypothesis concerning the nature of the diacylglycerol-binding region in PKC. Our major concern was to resolve how the same effector binding site in PKC can specifically recognize both diacylglycerols and the structurally diverse tumor promotors (10–13). We proposed the structural correlation among PKC activators summarized in Fig. 1 (10). We have demonstrated (14) that PKC activation is also stereospecific with respect to the second chiral center of 3-methylated 1,2-diacyl-*sn*-glycerols. Furthermore, the absolute configuration at C2 and C3 of the active 3-methylated diacylglycerols is the same as the absolute configuration at C29 and C30 of the naturally occurring tumor promotor debromoaplysiatoxin (DAT) (Fig. 1) (10). These observations support our structural hypothesis and forge a structural link between tumor promotors and diacylglycerols for PKC activation. In this report, this structural link is further explored by synthesizing and testing two classes of the hybrids of diacylglycerols and aplysiatoxins. It is demonstrated that the structural predictions made by our hypothesis are realized in both classes of hybrids and that the 3,4-dihydroxybutyrate moiety found in aplysiatoxins is the minimally essential pharmacophore of this class of tumor promotors.

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MATERIALS AND METHODS

Materials. Bovine phosphatidylserine was obtained from Avanti Polar Lipids. 1,2-Dioleoyl-*sn*-glycerol and histone type VS were from Sigma. Protease inhibitors were products of Boehringer Mannheim. [γ-^{32}P]ATP (≈3000 Ci/mmol; 1 Ci = 37 GBq) was from Amersham.

PKC Purification and Assays. PKC was partially purified from rat brain as described (7). PKC activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into lysine-rich histone type VS essentially as reported (7), with the following modifications: In the assay, mixtures of phosphatidylserine and diacylglycerol analogs in chloroform were dried down under nitrogen for 30 min and resuspended in 60 mM Tris·HCl, pH 7.5/3 mM EDTA, followed by sonication for 2 min with a Kontes microultrasonic cell disrupter. Incubations were carried out at 30°C for 5 min.

Synthesis of Analogs. Analysis of the structure–activity profiles of PKC activators led to the proposal that the 3,4-dihydroxybutyrate moiety of DAT is homologous to the backbone of diacylglycerols, which are endogenous PKC activators (Fig. 1). To test this hypothesis, the six chiral 3,4-dihydroxybutyrate derivatives **3** to **8** were synthesized (Fig. 2). The C3 stereochemistry of derivatives **3** to **5** corresponds to the C2 stereochemistry of active 1,2-diacyl-*sn*-glycerols in the *S* configuration, whereas that of derivatives **6** to **8** corresponds to that of inactive 1,2-diacyl-*sn*-glycerols in the *R* configuration. All the substrates synthesized gave satisfactory spectroscopic data, including the following: derivative **3**, $[\alpha]_D$ +9.2° (*c* 0.40, CH_2Cl_2); ^{1}H NMR (500 MHz, $C_6{}^2H_6$) δ 5.55 (2H, m), 5.45 (1H, m), 4.06 (1H, m), 3.51 (1H, t, J = 5.4 Hz), 2.61 (1H, dd, J = 7.7, 15.8 Hz), 2.52 (1H, dd, J = 5.3, 15.8 Hz), 0.96 (6H, t, J = 6.9 Hz); derivative **4**, $[\alpha]_D$ +3.0° (*c* 1.6, CH_2Cl_2); ^{1}H NMR (500 MHz, C^2HCl_3) δ 5.34 (4H, m), 5.16 (1H, ddd, J = 3.9, 5.6, 7.4 Hz), 4.06 (2H, t, J = 6.8 Hz), 3.93 (1H, m), 2.72 (1H, dd, J = 5.6, 15.8 Hz), 2.63 (1H, dd, J = 7.4, 15.8 Hz), 1.19 (3H, d, J = 6.4 Hz), 0.88 (6H, t, J = 7.1 Hz); derivative **5**, $[\alpha]_D$ +6.9° (*c* 1.4, CH_2Cl_2); ^{1}H NMR (500 MHz, C^2HCl_3) δ 5.34 (4H, m), 5.18 (1H, dt, J = 4.3, 6.3 Hz), 4.07 (2H, t, J = 6.8 Hz), 3.96 (1H, m), 2.65 (2H, m), 2.31 (2H, t, J = 7.6 Hz), 1.18 (3H, d, J = 6.5 Hz), 0.86 (6H, t, J = 6.1 Hz); derivative **6**, $[\alpha]_D$ −8.6° (*c* 0.88, CH_2Cl_2); ^{1}H NMR (500 MHz, C^2HCl_3) same as derivative **3**; derivative **7**, $[\alpha]_D$ −6.6° (*c* 0.50, CH_2Cl_2); ^{1}H NMR (500 MHz, C^2HCl_3) same as derivative **5**; derivative **8**, $[\alpha]_D$ −2.8° (*c* 0.28, CH_2Cl_2); ^{1}H NMR (500 MHz, C^2HCl_3) same as derivative **4**.

As the macrocyclic ring system found in DAT might be important for binding, the second class of hybrids of DAT and diacylglycerol was synthesized (Fig. 3). The absolute configuration of the C2 position of cyclic diacylglycerol derivatives **9** to **11** is the same as that of the C29 position of DAT and also the same as that of the C2 stereochemistry of active

Abbreviations: PKC, protein kinase C; DAT, debromoaplysiatoxin.
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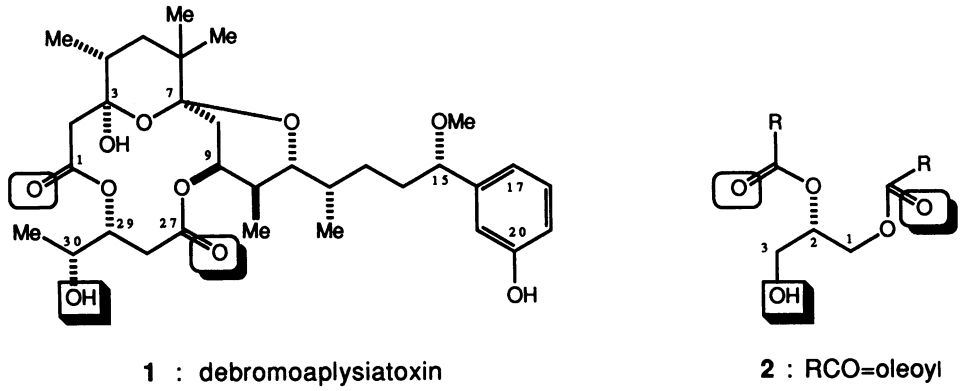

FIG. 1. Proposed PKC activation model. Identical symbols indicate equivalent atoms.

1,2-diacyl-*sn*-glycerols in the *S* configuration. On the other hand, the absolute configuration of the C2 stereochemistry of cyclic diacylglycerol derivative **12** is opposite to that of the C29 position of DAT and also to that of the C2 stereochemistry of active 1,2-diacyl-*sn*-glycerol in the *S* configuration. All the cyclic diacylglycerol derivatives synthesized gave satisfactory spectroscopic data, including the following: derivative **9**, $[\alpha]_D$ +3.5° (*c* 3.1, CH_2Cl_2); ^1H NMR (500 MHz, C^2HCl_3) δ 5.11 (1H, m), 4.37 (1H, dd, J = 2.2, 12.7 Hz), 4.30 (1H, dd, J = 5.9, 12.4 Hz), 3.78 (2H, m), 2.47 (1H, m), 2.40 (3H, m), and 2.04 (1H, t, J = 6.3 Hz); derivative **10**, $[\alpha]_D$ −0.68° (*c* 0.74, CH_2Cl_2); ^1H NMR (500 MHz, C^2HCl_3) δ 5.01 (1H, dt, J = 2.2, 6.5 Hz), 4.38 (1H, dd, J = 2.3, 12.6 Hz), 4.24 (1H, J = 6.6, 12.6 Hz), 4.04 (1H, m), 2.48 (1H, m), 2.40 (3H, m), 1.23 (3H, d, J = 6.5 Hz); derivative **11**, $[\alpha]_D$ −9.0° (*c* 0.59, CH_2Cl_2); ^1H NMR (500 MHz, C^2HCl_3) δ 4.94 (1H, m), 4.40 (1H, dd, J = 6.0, 12.4 Hz), 4.34 (1H, dt, J = 1.5, 12.4 Hz), 3.97 (1H, m), 2.47 (1H, m), 2.37 (3H, m), and 1.25 (3H, d, J = 6.4 Hz); derivative **12**, $[\alpha]_D$ −3.2° (*c* 3.2, CH_2Cl_2); ^1H NMR (500 MHz, C^2HCl_3) same as derivative **9**.

RESULTS

The model summarized in Fig. 1 focuses attention on the structural similarities between diacylglycerols in the *S* configuration and the 3,4-dihydroxybutyrate moiety of DAT. To study directly whether this moiety is the active pharmacophore of DAT, a series of 3,4-dihydroxybutyrate derivatives was synthesized and studied as potential PKC activators. The effects of 3,4-dihydroxybutyrate derivatives **3** to **8** on PKC were studied first (Table 1 and Fig. 4). Compounds for which no kinetic values are reported (Table 1) were basically inactive; they showed less than 2% of the activity of their active counterparts. This activity was not considered significant, since a minor contamination (less than 2%) from the active compounds would result in the observed activation. This amount of contamination would not have been detected by the analytical methods employed. This would explain also why saturation conditions could not be reached even at the high concentrations (up to 20 μM) of these compounds used (Fig. 4).

It is important to note that analog **3** was active but its enantiomer, analog **6**, was inactive, which was expected from our structural hypothesis. This is because the absolute configuration of the C3 position of analog **3** corresponds to that of the C2 position of active 1,2-diacyl-*sn*-glycerols in the *S* configuration. Furthermore, among the methylated analogs **4**, **5**, **7**, and **8**, only analog **4** activated PKC. The C4 absolute configuration of this analog is the same as the C3 absolute configuration of the active 3-methyldiacylglycerols (14), and the activity observed for the two compounds is similar. The corresponding diacylglycerol shows a K_d of 0.2 μM (14)

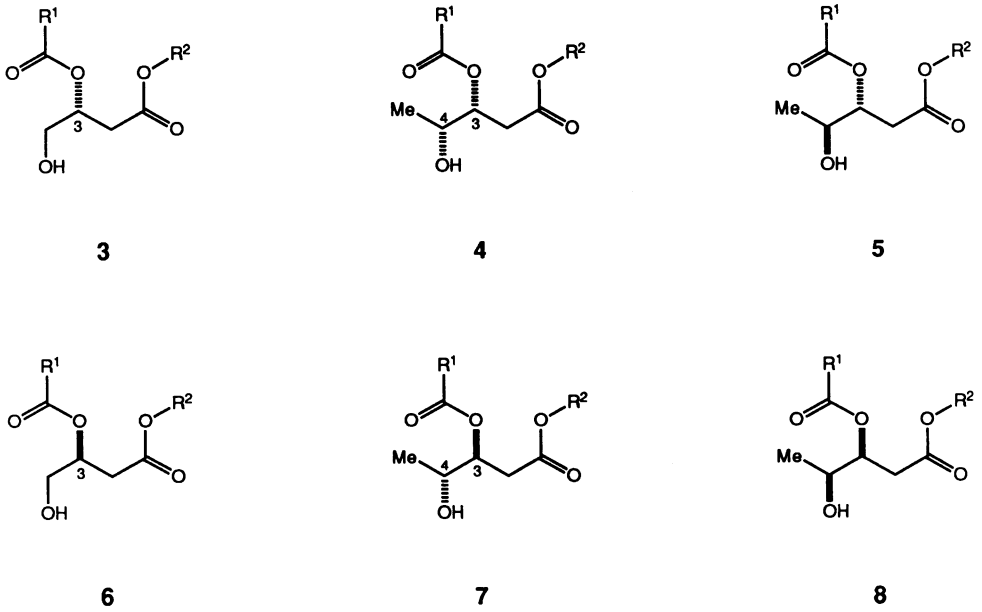

FIG. 2. Chemical structures of six chiral 3,4-dihydroxybutyrate analogs. R^1, (*Z*)-$Me(CH_2)_7CH{=}CH(CH_2)_7$-; R^2, (*Z*)-$Me(CH_2)_7CH{=}CH(CH_2)_8$-.

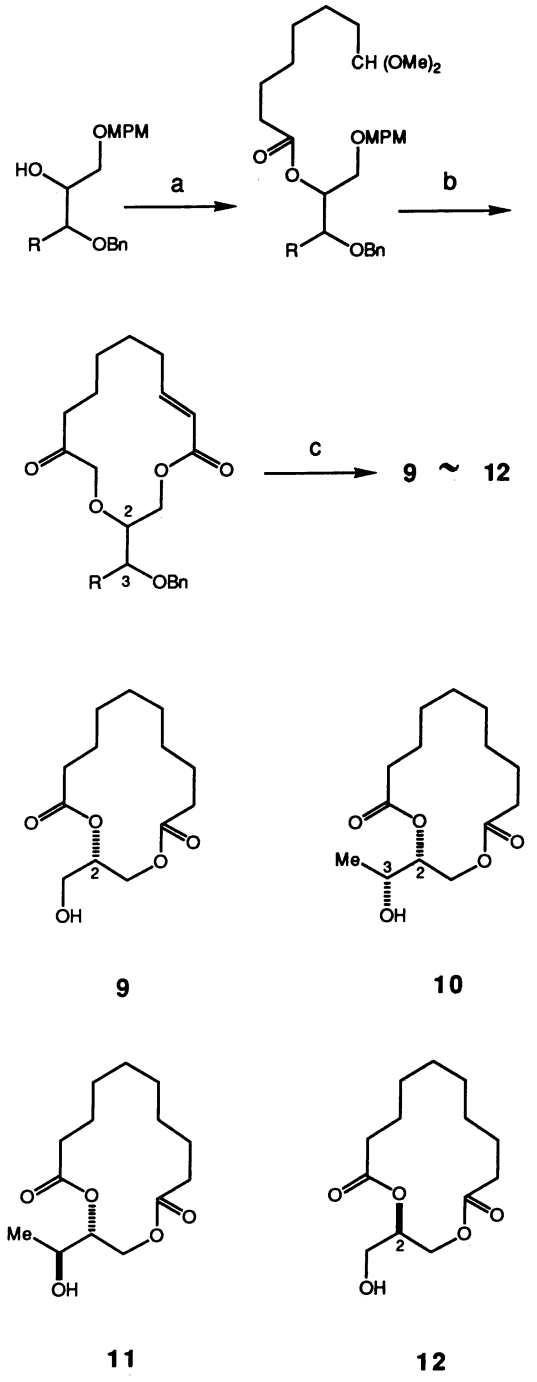

FIG. 3. Chemical syntheses and structures of four chiral cyclic diacylglycerol analogs. Reagents and reaction conditions are as follows. Reaction **a.** $(MeO)_2CH(CH_2)_6CO_2H$/1,3-dicyclohexylcarbodiimide/C_6H_6. Reaction **b.** Steps: 1, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone/H_2O/CH_2Cl_2; 2, $Ph_3P{=}C{=}CO$/80°C/C_6H_5Me, followed by treatment with 0.1 M HCl then pH = 8.4 buffer at room temperature. Reaction **c.** H_2/$Pd(OH)_2$ on C/MeOH. The starting materials for the synthesis of derivatives **9** and **12** (i.e., R=H) were prepared in four steps. Steps: 1, $C_6H_5CH_2Br$/NaH; 2, aqueous AcOH; 3, *p*-$MeOC_6H_4CH(OMe)_2$/pyridinium *p*-toluenesulfonate, 4, $NaBH_3CN$/trifluoroacetic acid from D- and L-1,2-isopropylideneglycerols, respectively, whereas those for derivatives **10** and **11** (i.e., R=Me) were prepared in five steps. Steps: 1, MeMgBr; 2, $C_6H_5CH_2Br$/NaH, followed by chromatographic separation [the major product (2,3-erythro) was used for the preparation of derivative **11** and the minor (2,3-threo) was for derivative **10**; 3, aqueous AcOH; 4, *p*-$MeOC_6H_4CH(OMe)_2$/pyridinium *p*-toluenesulfonate; 5, $NaBH_3CN$/trifluoroacetic acid] from D-isopropylideneglyceroaldehyde.

Table 1. Activation of PKC by 3,4-dihydroxybutyrates and cyclic diacylglycerols

Addition	Relative activation	V_{max}, nmol·min^{-1}·mg^{-1}	K_d, μM	V_{max}/K_d
Diolein	3.9	9.36 ± 0.29	0.09 ± 0.02	104
None	1.0	2.41 ± 0.11	—	—
Analog				
3	3.5	8.42 ± 0.38	0.78 ± 0.15	11
6		Inactive	Inactive	
4	3.2	7.74 ± 0.32	0.66 ± 0.11	12
5		Inactive	Inactive	
7		Inactive	Inactive	
8		Inactive	Inactive	
9	3.2	7.79 ± 0.31	1.30 ± 0.25	6
10	2.1	5.01 ± 0.39	2.72 ± 0.88	2
11	1.1	2.53 ± 0.02	—	—
12	1.4	3.42 ± 0.15	6.82 ± 3.94	0.5

Phosphatidylserine and various compounds, as indicated, were added to the reaction mixture. The results shown are average values ± standard deviation from the mean of triplicate experiments.

compared to a K_d of 0.66 μM for analog **4**. Of great importance is the observation that the stereochemical preference found for the chiral 3-methyldiacylglycerol is mirrored in the stereochemical preference found in both the aplysiatoxin and the 3,4-dihydroxybutyrate series. These observations further establish the stereochemical preference at the binding site of PKC activators and, moreover, forge a strong structural link between the pharmacophore of diacylglycerol and the pharmacophore of DAT.

The binding of cyclic diacylglycerols **9** to **12** to PKC was also studied. The behavior of these analogs towards PKC was found to be consistent with that observed for aplysiatoxins, diacylglycerols, and 3,4-dihydroxybutyrates, although they proved to be quantitatively slightly less active than their dioleoyl counterparts (Table 1). These studies demonstrate that the macrocyclic ring system of DAT is not in and of itself an important contributor to the potency of these compounds.

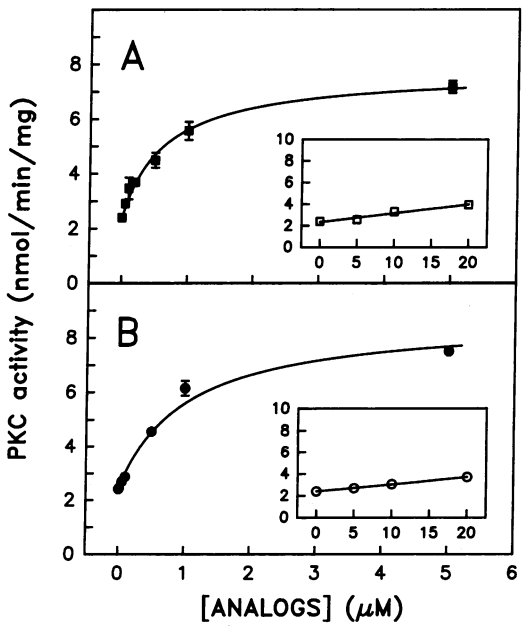

FIG. 4. Activation of PKC by 3,4-dihydroxybutyrate derivatives. The following analogs were assayed as PKC activators. (*A*) Analogs **4** (■) and **5** (*Inset*). (*B*) Analogs **3** (●) and **6** (*Inset*). The results are average values ± standard deviation from the mean of triplicate experiments.

DISCUSSION

The studies described here establish the 3,4-dihydroxybutyrate moiety found in aplysiatoxins to be the minimum pharmacophore required for PKC activation, and lend strong independent support to our structural model for PKC activation (10).

It is instructive to compare the potency of DAT and its active pharmacophore, 3,4-dihydroxybutyrate derivative **4**. The K_d for DAT binding to PKC is approximately 2 nM, whereas it is 660 nM for derivative **4**. Therefore, DAT is greater than 300-fold more active than derivative **4**. The substantially increased potency of DAT over that of derivative **4** may suggest that additional favorable contacts occur between DAT and PKC, beyond those possible between the simple 3,4-dihydroxybutyrate and PKC.

One possibility for the increased potency of DAT over that of diacylglycerols and 3,4-dihydroxybutyrates is the macrocyclic ring system found in DAT. However, the macrocyclic diacylglycerol analogs **9** and **10** proved to be actually somewhat less potent than their dioleoyl counterparts. It is likely that this result can be attributed to the lessened hydrophobicity of the macrocycles **9** and **10**, compared to the dioleoyl analogs. It is clear from structure–activity studies (7, 11, 15–17) that all the active diacylglycerols contain relatively hydrophobic acyl chains (7, 18). The fact that the macrocyclic diacylglycerols are not more potent as PKC activators than their acyclic counterparts may suggest that the high potency of DAT is probably not due to energetically favorable conformers found only in the macrocycle. Of course, the macrocycle found in DAT is conformationally much more rigid than that found in analogs **9** and **10**, and a simple comparison may be meaningless. However, the finding that the macrocyclic analogs are active suggests that the fatty acid chains in the acyclic series may be folded in such a way as to approximate the conformation found in the macrocycle. This might be especially pertinent to the unsymmetrical diacylglycerols, such as 1-oleoyl-2-acetylglycerol (15), in which the two ends of the acyl chains might be held in close proximity.

In addition to the macrocyclic ring of DAT discussed above, other moieties of DAT need also to be considered as being of quantitative importance in binding to PKC. Additional hydrophilic and hydrophobic contacts are possible with DAT that are not found in the simplified pharmacophores. Since 3-deoxy-DAT is as active as DAT itself, the hydroxyl group at the C3 position can be removed from consideration as a possible hydrophilic contact. However, the macrocyclic hemiketal oxygens at the C7 and C11 positions cannot be eliminated as hydrogen bond acceptors of secondary importance. Furthermore, the side chain of DAT could provide favorable hydrophobic interactions with the enzyme, which may enhance binding.

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