Journal of Cellular Biochemistry 29:37–44 (1985)
Biochemical and Molecular Epidemiology of Cancer 1–8

Stoichiometric Binding of Diacylglycerol to the Phorbol Ester Receptor

Bernhard König, Patricia A. DiNitto, and Peter M. Blumberg

Molecular Mechanisms of Tumor Promotion Section, Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, Bethesda, Maryland 20205

The major phorbol ester receptor is the Ca^{++}-activated, phospholipid-dependent protein kinase C. Diacylglycerol stimulates protein kinase C in a fashion similar to the phorbol esters. Likewise, it inhibits phorbol ester binding competitively. Both results suggest that diacylglycerol is the/an endogenous phorbol ester analogue. Alternatively, the diacylglycerol might simply be acting to modify the phospholipid environment of the protein. If diacylglycerol were indeed functioning as an analogue, it should interact with the receptor stoichiometrically. This interaction can be quantitated by measuring the perturbation in apparent diacylglycerol binding affinity as a function of the ratio of diacylglycerol to receptor. We report here that 1,2-dioleoylglycerol interacts with the receptor with the predicted stoichiometry.

Key words: problem kinase C, diacylglycerol, competitive inhibition of phorbol ester binding, stoichiometric binding, phorbol ester receptor, [^{3}H]phorbol 12,13-diacetate binding, tumor promotion

The biological effects of the phorbol esters appear to be mediated principally through a high-affinity receptor [1], which represents a complex between specific phospholipids, Ca^{++}, and the apoenzyme protein kinase C [2–7]. This complex is believed to play an important role in transducing the signals of extracellular messengers whose earliest effect is the enhanced breakdown of phosphatidylinositol -4,5-bisphosphate [8]. Diacylglycerol, one product of this breakdown, stimulates PKC under conditions of limiting Ca^{++} and phospholipid [9].

In analogy with the opiate receptor, the phorbol ester receptor displays high evolutionary conservation, strongly implying the existence of an endogenous analogue.

Abbreviations used: PKC, phospholipid and Ca^{++}-dependent protein kinase; [^{3}H]PDBu, [20-^{3}H]phorbol 12,13-dibutyrate; [^{3}H]PDA, [20-^{3}H]phorbol 12,13-diacetate; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid.

Bernhard König's present address is Boehringer Mannheim GmbH, Biochemica Werk Tutzing, Bahnhofstrasse 9-15, D-8132 Tutzing, Federal Republic of Germany.

Received March 15, 1985; revised and accepted June 7, 1985.

Diacylglycerol is a likely candidate for such an analogue because (1) both the phorbol esters and diacylglycerols stimulate protein kinase C activity in vitro [10], and (2) both induce similar biochemical and cellular responses, including phosphorylation of a 40K protein in platelets [11,12] and a 42K protein in chicken embryo fibroblasts [13].

Kikkawa et al [14] have demonstrated that [^{3}H]phorbol 12,13-dibutyrate binds to the complex of PKC and phosphatidylserine with a stoichiometry of approximately 1:1. Our laboratory has previously reported that 1,2-dioleoylglycerol inhibits phorbol ester binding competitively [15]; the apparent affinity was approximately 80-fold lower than that of the corresponding phorbol 12,13-dioleate [16]. As is expected for a lipophilic compound, the binding affinity of 1,2-dioleoylglycerol reflects its concentration in the lipid phase rather than its concentration averaged over the volume of the aqueous suspension. If diacylglycerol is indeed a phorbol ester analogue, it should also bind with 1:1 stoichiometry and interact at the same site on the enzyme. Neither result would be predicted if diacylglycerol simply functioned by perturbing the phospholipid bilayer. We present evidence here that diacylglycerol interacts stoichiometrically with the phorbol ester receptor/protein kinase C.

MATERIALS AND METHODS

Materials

L-α-phosphatidyl-L-serine (bovine brain), rac-1,2-dioleoylglycerol, leupeptin, phenylmethylsulfonyl fluoride, and bovine gamma globulin were from Sigma (St. Louis, MO). Diethylaminoethyl cellulose (DE-52) anion exchanger was from Whatman (Clifton, NJ). Polyethylene glycol (molecular weight—mol wt 6,000–7,500) was obtained from EM Science (Gibbstown, NJ). [^{3}H]PDA was synthesized from 20-oxo-20-deoxy-PDA (LC Services, Waltham, MA) according to published procedures [17] and purified by high-performance liquid chromatography (HPLC) on a RP-18 column in methanol/H_2O (55:45). The specific activity of [^{3}H]PDA was 1.49 Ci/mmol.

Purification of the Phorbol Ester Receptor

The phorbol ester aporeceptor from mouse brain cytosol was partially purified by chromatography on DE-52 as described previously [18].

[^{3}H]PDA Binding Assay

[^{3}H]PDA binding was assayed basically as described for [^{3}H]PDBu [16]. The incubation mixtures contained 0.5 μg/ml of phosphatidylserine, 0.05 M Tris-Cl, pH 7.4, 100 μg/ml of DE-52 purified protein (unless indicated otherwise), 7–340 nM [^{3}H]PDA, 3 mg/ml of bovine gamma globulin, 2 mM Ca^{++}, and 0.5 mM EGTA. (Additional chelators in the enzyme preparation [edetic acid—EDTA, EGTA] increase the total concentration of chelators to approximately 1.8 mM.) To determine the dissociation constant (K_D) for [^{3}H]PDA, the concentration of radiolabeled phorbol ester was varied between 5 and 200 nM. To measure inhibition of phorbol ester binding, aliquots of phosphatidylserine and rac-1,2-dioleoylglycerol were mixed in chloroform, the solvent was removed under nitrogen, and the lipids were dispersed in 50 mM Tris-Cl, pH 7.4 (saturated with nitrogen) by gentle sonication. The lipids were then added to the assay mixture to give the final concentrations indicated. Nonspecific binding was measured in control samples containing at least a 150-fold

molar excess of nonradioactive phorbol ester. Specific binding represents the difference between total and nonspecific binding. To determine the actual dissociation constant (K_i) for rac-1,2-dioleoylglycerol, inhibition of [^{3}H]PDA binding was measured as described above, except that the incubation mixture contained 100 μg/ml of phosphatidylserine and 0.1 mM $CaCl_2$ with a final concentration of ~1.3 mM chelators present in the enzyme preparation.

RESULTS AND DISCUSSION

Because of the insolubility of the long-chain diacylglycerols, the measurement of their direct binding to protein kinase C is difficult. Since the diacylglycerol remains in the lipid phase used to reconstitute the receptor, whether it is specifically bound or not, physical separation of the bound and free ligand is not feasible. Therefore, the approach we adopted relies on the decrease of free ligand in the lipid phase as ligand binds to the receptor. This decrease in free ligand can be detected as a shift in the apparent dissociation constant (K_{app}), expressed in terms of total ligand concentration, as a function of the ratio of ligand to receptor in the system.

The K_{app} for the diacylglycerol was determined from inhibition of radioactive phorbol ester binding according to the relationship:

$$K_{app} = \frac{I_{50}}{1 + L/K_D} \quad (1)$$

where I_{50} = concentration of *total* diacylglycerol yielding 50% inhibition of phorbol ester binding, L = concentration of radioactive phorbol ester, and K_D = dissociation constant for phorbol ester binding. Because $I_{50} = I_{50}(\text{free}) + I_{50}(\text{bound})$ and $I_{50}(\text{bound}) = n\,(R/2)$, equation 1 can be rewritten as:

$$K_{app} = K_i + \frac{n\,(R/2)}{1 + L/K_D} \quad (2)$$

where K_i = actual dissociation constant for the diacylglycerol, expressed in terms of free ligand [19], R = concentration of reconstituted receptor, and n = number of diacylglycerol molecules bound per receptor molecule. The value of n can be determined by varying either R or L and measuring K_{app}.

The relatively low affinity of the diacylglycerol made it essential to optimize the assay conditions in order to get significant variation in K_{app}.

First, we maximized the concentration of receptors in the assay and minimized the concentration of phosphatidylserine. The motivation was that we had previously shown that the K_i for 1,2-dioleoylglycerol, expressed in molar concentrations, was proportional to the lipid concentration, ie, the receptor actually recognizes the concentration of diacylglycerol in the lipid phase [15]. Previous work in this laboratory had also shown that the potency of phosphatidylserine and other phospholipids to reconstitute phorbol ester binding activity was dramatically dependent on the concentration of free Ca^{++} [7]; ie, Ca^{++} reduced the requirements for phospholipids substantially. A factor limiting how much the phosphatidylserine concentration could

be decreased is that 50–100 molecules of phosphatidylserine per receptor are required for reconstitution under our assay conditions.

Second, analysis was simplified provided we could restrict the increase in phorbol ester concentration (L) over the competition curve as the phorbol ester was displaced from the receptor by the diacylglycerol. This condition necessitated maintaining high concentrations of L. Because we had to measure binding activity at concentrations of L below its K_D, a phorbol ester of low binding affinity was required. We therefore synthesized and used [^{3}H]PDA. Under our assay conditions [^{3}H]PDA bound with a K_D of 45 ± 0.2 nM (mean ± SE, three experiments) as determined by Scatchard analysis [20] of binding data (Fig. 1). In contrast [^{3}H]PDBu, the more usual derivative for phorbol ester binding studies, bound under similar conditions with a K_D of 0.8 ± 0.09 nM (mean ± SE, seven experiments) [7].

To determine the actual dissociation constant (K_i) for 1,2-dioleoylglycerol, inhibition of [^{3}H]PDA binding by the diacylglycerol was measured under conditions of an excess of inhibitor over receptor. As shown in Figure 2, the measured values for inhibition of [^{3}H]PDA binding by 1,2-dioleoylglycerol fit the theoretical curve for a competitive inhibitor. The results confirm our previously published findings

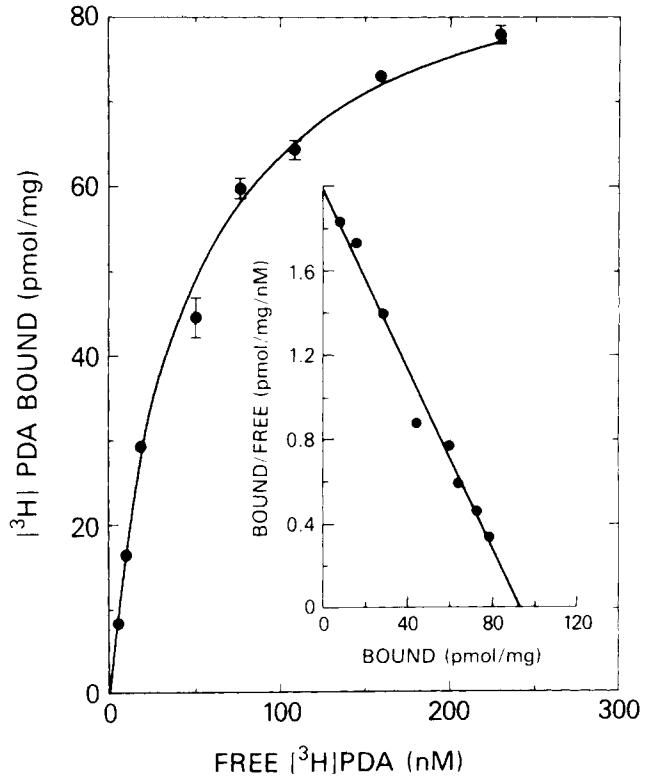

Fig. 1. Specific binding of [^{3}H]PDA to the phorbol ester aporeceptor. [^{3}H]PDA binding was assayed in the presence of 0.5 μg/ml of phosphatidylserine as described in Materials and Methods. Each point represents the mean value for triplicate samples. Results are representative of three independent experiments. Inset: Scatchard analysis of binding data.

using [^{3}H]PDBu and somewhat different assay conditions [15]. The measured value for the I_{50} of 1,2-dioleoylglycerol was 0.75 μg/ml (see Fig. 2), and the concentration of free [^{3}H]PDA at the I_{50} was 235 nM. Using these values and the K_D value of 45 nM (see Fig. 1), a K_i for 1,2-dioleoylglycerol of 0.12% (w/w, relative to phosphatidylserine) was determined according to equation 1.

Having determined the K_D for [^{3}H]PDA binding and the K_i for inhibition of phorbol ester binding by 1,2-dioleoylglycerol, we used two approaches for affecting K_{app} (equation 2).

In the primary set of experiments, L was varied while R was kept constant (Fig. 3). The K_{app} was shifted over a fivefold range as the [^{3}H]PDA concentration was varied from 7 nM (L/K_D = 0.2) to 340 nM (L/K_D = 7.6). Higher concentrations of L could not be used because of excessive nonspecific binding. The variation of K_{app} with L was consistent with a direct interaction of 1,2-dioleoylglycerol with the receptor/kinase C. The data closely fit the theoretical curve calculated for a value of n = 2 and the K_i of 0.12% (w/w, relative to phosphatidylserine), which was determined under conditions of a vast excess of 1,2-dioleoylglycerol over receptor. The actually determined value of n was 1.72 ± 0.11 (mean ± SE, 14 determinations). Studies on the conformation of potent and nonpotent PKC activators revealed that specific structural features are required for stimulation of the enzyme [21]. Among the diacylglycerols, 1,2-dioleoylglycerol is known to be one of the most potent PKC activators whereas the 1,3-stereoisomer is basically inactive [22]. Furthermore, Rando

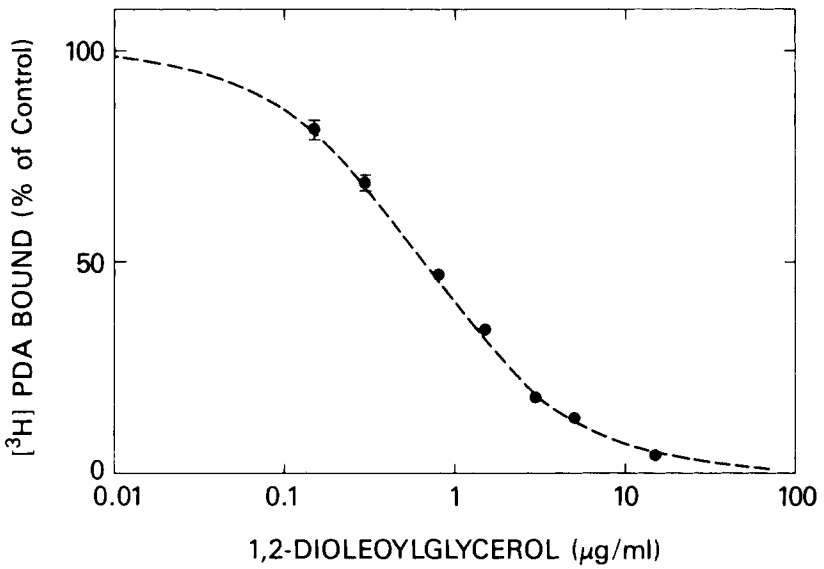

Fig. 2. Inhibition of specific [^{3}H]PDA binding by rac-1,2-dioleoylglycerol. Specific binding of [^{3}H]PDA was measured in the presence of 100 μg/ml of phosphatidylserine and 0.1 mM Ca^{++} as described in Materials and Methods. Indicated are the mean values ± SE of triplicate determinations with duplicate determinations of nonspecific binding at each inhibitor concentration. The concentration of total [^{3}H]PDA was 255 nM; that of free [^{3}H]PDA was 235 nM at the I_{50} and varied from 223–251 nM over the competition curve. The concentration of reconstituted receptor was 30.3 nM (100% = 25,000 dpm). Results are representative of three independent experiments.

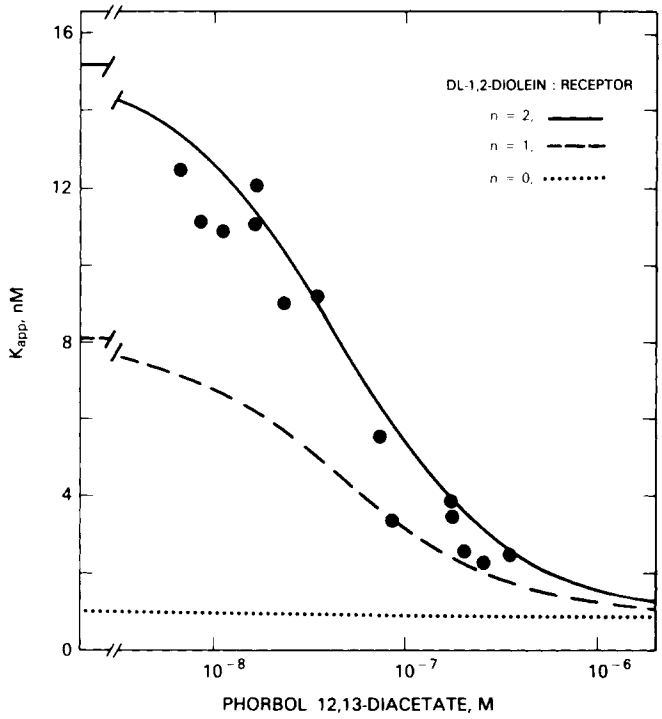

Fig. 3. Apparent binding affinity of rac-1,2-dioleoylglycerol as a function of [^{3}H]PDA concentration. Binding of [^{3}H]PDA to the partially purified phorbol ester aporeceptor from mouse brain was assayed as described in Materials and Methods. K_{app} values were determined at fixed concentrations of [^{3}H]PDA (7–340 nM) by competition with varying concentrations of rac-1,2-dioleoylglycerol incorporated into phosphatidylserine (0.5 μg/ml). The concentration of reconstituted aporeceptor, determined by specific phorbol ester binding, was kept approximately constant at 13–17 nM. Each competition curve spanned at least six inhibitor concentrations, with triplicate determinations of specific [^{3}H]PDA binding and duplicate determinations of nonspecific binding at each concentration. The K_{app} values were derived from the 50% inhibitory concentrations of rac-1,2-dioleolyglycerol in each competition curve according to equation 1 (see text). The theoretical curves for K_{app} as a function of L, indicated for n = 0 (. . . .), n = 1 (----), and n = 2 (——), were calculated according to equation 2 (see text). The R value was 14.25 ± 0.39 nM (mean ± SE, 14 experiments); the K_i value of 0.12% (w/w, relative to phosphatidylserine) measured under conditions of excess diacyglycerol over receptor (see Fig. 2) corresponds to 0.95 ± 0.04 nM (mean ± SE, three determinations) under the conditions of the present experiment.

and Young [23] reported that the stimulation of PKC by 1,2-dioleoylglycerol is stereospecific and that only the 1,2-dioleoyl-sn-glycerol is active, whereas the enantiomer, 2,3-dioleoyl-sn glycerol, is inactive. In our studies, we used the racemic mixture of 1,2-dioleoylglycerol. The active enantiomer could not be used because of considerable racemization during the sonication procedure that is required to generate uniform liposomes. Based on the concentration of the active enantiomer, the value of n is thus 0.86 ± 0.05 (mean ± SE, 14 determinations).

We were able to obtain basically the same results if the concentration of ligand (L) was kept fixed and the receptor concentration (R) was varied (Fig. 4). Increasing the receptor concentration from 2.2 to 16.4 nM led to a 3.3-fold increase in K_{app}. A plot of K_{app} versus $R/2(1 + L/K_D)$ resulted in a linear function with a slope of

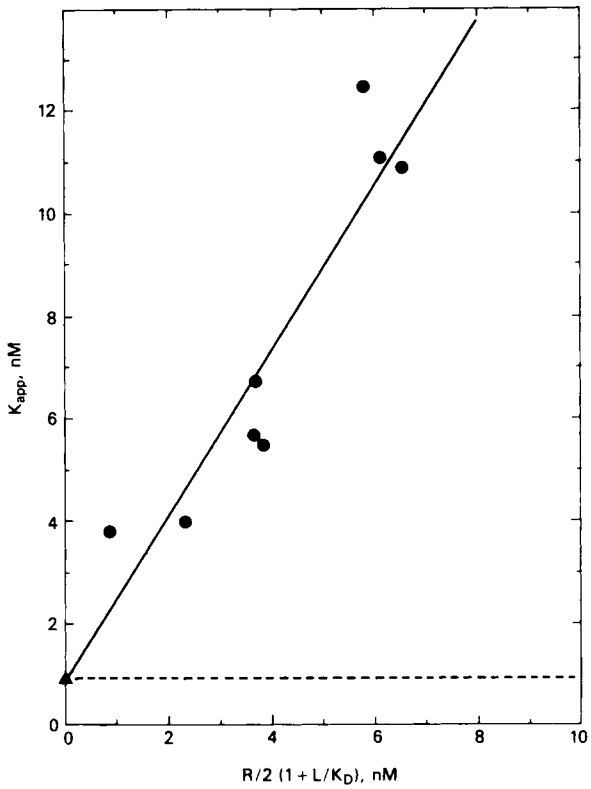

Fig. 4. Apparent binding affinity of rac-1,2-dioleoylglycerol as a function of the concentration of reconstituted receptor. The assay conditions were similar to those described in Figure 1, except that the receptor concentration was varied between 2.2 and 16.4 nM (20–100 μg/ml of DE-52 purified protein) and L was kept approximately constant between 7 and 11 nM ($1 + L/K_D = 1.15$–1.26). The Ca^{++} concentration in individual experiments was adjusted to give an approximately 0.2 mM excess over chelators (EGTA, EDTA) present in the receptor preparation. The value of n was calculated by least-squares analysis assuming the K_i value determined from Figure 2 as indicated (▲). The solid line corresponds to the least squares value of $n = 1.60$, the dashed line corresponds to $n = 0$. The three data points at high receptor concentrations are derived from Figure 1.

$n = 1.60 \pm 0.08$ (least squares ± SE, eight determinations). Once again, based on the concentration of the active enantiomer of 1,2-dioleoylglycerol, a value of n close to one was obtained ($n = 0.8$).

The stoichiometric interaction of 1,2-dioleoylglycerol with the phorbol ester receptor corroborates the indirect evidence that diacylglycerols are endogenous phorbol ester analogues. The experiments obviously cannot address the issue of whether other classes of endogenous analogues of phorbol esters exist. Other classes of structurally dissimilar exogenous analogs of phorbol esters are known, including the indole alkaloids dihydroteleocidin B and lyngbyatoxin, and the polyacetate aplysiatoxin [24]. As we have discussed elsewhere, competitive binding activity does not mean that compounds will function identically [15]. Marked differences in the pharmacokinetic behavior of phorbol esters and diacylglycerols would be predicted.

The stoichiometric binding of diacylglycerol to the phorbol ester receptor/protein kinase C enhances the likelihood that pharmacological antagonists can be designed. Given the important role postulated for protein kinase C in signal transduction, such compounds could be of considerable interest.

ACKNOWLEDGMENTS

We thank Dr. S.H. Yuspa for careful reading of the manuscript.

REFERENCES

1. Blumberg PM, Dunn JA, Jaken S, Jeng AY, Leach KL, Sharkey NA, Yeh, E: In Slaga TJ (ed): "Mechanisms of Tumor Promotion," Vol 3. Boca Raton, Florida: CRC press, 1984, pp 143–184.
2. Ashendel CL, Staller JM, Boutwell RK: Biochem Biophys Res Commun 111:340, 1983.
3. Sando JJ, Young MC: Proc Natl Acad Sci USA 80:2642, 1983.
4. Niedel JE, Kuhn LJ, Vandenbark GR: Proc Natl Acad Sci USA 80:36, 1983.
5. Leach KL, James ML, Blumberg PM: Proc Natl Acad Sci USA 80:4208, 1983.
6. Parker PD, Stabel S, Waterfield MD: EMBO J 5:953, 1984.
7. König B, DiNitto PA, Blumberg PM: J Cell Biochem 27:255, 1985.
8. Berridge MJ: Biochem J 220:345, 1984.
9. Nishizuka Y: Nature 308:693, 1984.
10. Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U, Nishizuka Y: J Biol Chem 257:7847, 1982.
11. Sano K, Takai Y, Yamanishi J, Nishizuka Y: J Biol Chem 258:2010, 1983.
12. Kaibuchi K, Takai Y, Sawamura M, Hoshijima M, Fujikura T, Nishizuka Y: J Biol Chem 258:6701, 1983.
13. Gilmore T, Martin GS: Nature 306:487, 1983.
14. Kikkawa U, Takai Y, Tanaka Y, Miyake R, Nishizuka Y: J Biol Chem 258:11442, 1983.
15. Sharkey NA, Leach KL, Blumberg PM: Proc Natl Acad Sci USA 81:607, 1984.
16. Sharkey NA, Blumberg PM: Cancer Res 45:19, 1985.
17. Kreibich G, Hecker E: Z Krebsforsch 74:448, 1970.
18. Kikkawa U, Takai Y, Minakuchi R, Inohara S, Nishizuka Y: J Biol Chem 257:13341, 1982.
19. Cheng YC, Prusoff WH: Biochem Pharmacol 22:3099, 1973.
20. Scatchard G: Ann NY Acad Sci 51:660, 1949.
21. Brasseur R, Cabiaux V, Huart P, Castagna M, Baztar S, Ruysschaert JM: Biochem Biophys Res Commun 127:969, 1985.
22. Couturier A, Bazgar S, Castagna M: Biochem Biophys Res Commun 121:448, 1984.
23. Rando RR, Young N: Biochem Biophys Res Commun 122:818, 1984.
24. Sugimura T: Gann 73:499, 1982.