Biochemical Pharmacology, Vol. 34, No. 20, pp. 3649–3653, 1985.
Printed in Great Britain.

0006–2952/85 $3.00 + 0.00

EFFECT OF TAMOXIFEN, A NONSTEROIDAL ANTIESTROGEN, ON PHOSPHOLIPID/CALCIUM-DEPENDENT PROTEIN KINASE AND PHOSPHORYLATION OF ITS ENDOGENOUS SUBSTRATE PROTEINS FROM THE RAT BRAIN AND OVARY*

HUAI-DE SU,† GONZALO J. MAZZEI, WILLIAM R. VOGLER and J. F. KUO‡
Departments of Pharmacology and Medicine (Division of Hematology and Oncology), Emory University School of Medicine, Atlanta, GA, 30322, U.S.A.

(*Received* 30 *November* 1984; *accepted* 20 *March* 1985)

Abstract—Antiestrogens (tamoxifen, clomiphene and nafoxidine) were found to inhibit phospholipid/Ca^{2+}-dependent protein kinase (PL/Ca-PK, or protein kinase C), whereas estrogens (estradiol and diethylstilbesterol) and the weakly estrogenic chlorotrianisene were inactive. Kinetic analysis indicated that the antiestrogens inhibited PL/Ca-PK competitively with respect to phosphatidylserine (K_i = 16–27 μM), but non-competitively with Ca^{2+} (K_i = 14–30 μM). Tamoxifen, but not diethylstilbesterol, also inhibited the phospholipid/Ca^{2+}-dependent phosphorylation of various endogenous proteins from the total, solubilized fraction of the rat brain and ovary. Myosin light chain kinase, a calmodulin/Ca^{2+}-dependent class of protein kinase, was similarly inhibited by tamoxifen; the drug, however, was without effect on cyclic AMP-dependent and cyclic GMP-dependent protein kinases. It is suggested that PL/Ca-PK, by virtue of the hydrophobic interactions required for the enzyme activation, may represent a potential site of action for the lipophilic antiestrogens, in addition to the commonly recognized intracellular estrogen receptors.

Nonsteroidal antiestrogen drugs are useful for the treatment of advanced breast cancer [1, 2]. Tamoxifen, a prototype of this class of drugs, appears to act by competitively binding to the intracellular estrogen receptors, thus blocking the entry of estrogens into the nucleus and inhibiting the subsequent macromolecule synthesis and cell proliferation [3]. This contention seems to be further supported by the findings that tamoxifen is effective against stage IV and II estrogen receptor-positive breast cancer [2] and inhibits the *in vitro* growth of human ovarian carcinomas containing high levels of estrogen receptors [4]. It has been reported that tamoxifen also causes a prolonged stabilization of advanced epithelial ovarian carcinomas with borderline or high levels of estrogen receptors [5]. Recent evidence indicates that antiestrogen receptor sites are present in normal and neoplastic tissues and that these sites are distinct from the estrogen receptors seen in the same tissues [6]. It is unclear whether the receptors for tamoxifen are functionally related to the antiproliferative action of the drug. Phospholipid/Ca^{2+}-dependent protein kinase (PL/Ca-PK, or protein kinase C) has been discovered recently [7]. This major enzyme, distributed widely in tissues [8], has been shown to be a site of action of a wide variety of agents [9] and to play a key role in membrane signal transductions [10]. In the present studies, we report that tamoxifen and other antiestrogens, but not estrogens, inhibited PL/Ca-PK or phosphorylation of its endogenous substrate proteins from the brain and ovary.

* This work was supported by USPHS Research Grants HL-15696, CA-36777, CA-29850 and NS-17608 and by a Postdoctoral Fellowship from the American Muscular Dystrophy Association of America (G. J. M.).

† On leave from the Department of Pharmacognosy, Faculty of Pharmacy, Beijing Medical College, Beijing, China.

‡ Author to whom all correspondence should be addressed.

MATERIALS AND METHODS

Materials. Various antiestrogens and estrogens, histone HI (lysine-rich, type III-S), mixed histone (type II), phosphatidylserine (brain), cyclic AMP and cyclic GMP were purchased from the Sigma Chemical Co., St. Louis, MO; calmodulin was from the Sciogen Corp., Detroit, MI.

Methods. PL/Ca-PK was purified (80–95% homogenous) from pig brain extracts through the phosphatidylserine affinity step described previously for the heart enzyme [11]. Myosin light chain kinase (MLCK) [12] and unfractionated myosin light chains (MLC) [13] were purified to apparent homogeneity from pig heart. Cyclic AMP-dependent protein kinase (A-PK) [14] and cyclic GMP-dependent protein kinase (G-PK) [14] were partially purified from bovine heart extracts; there were no contaminating activities of other protein kinases in these preparations. [γ-^{32}P]ATP was prepared by the method of Post and Sen [15].

PL/Ca-PK using histone HI as substrate [11], MLCK using MLC as substrate [16], and A-PK and G-PK both using mixed histone as substrate [14] were assayed as described in the references cited.

Briefly, the purified PL/Ca-PK (0.05 to 0.10 μg) was assayed in the standard reaction mixture (0.2 ml) which contained 5 μmoles 1,4-piperazinediethanesulfonic acid (Pipes) buffer (pH 6.5), 2 μmoles $MgCl_2$, 40 μg histone HI, 0.04 μmoles ethyleneglycol bis (aminoethylether) tetraacetic acid (EGTA), with or without 0.1 μmoles $CaCl_2$, 5 μg phosphatidylserine, and 1 nmole [γ-^{32}P]ATP containing about 1×10^6 cpm. The reaction was carried out at 30° for 5 min. The incubation conditions for the phosphorylation of endogenous substrate proteins for PL/Ca-PK [17] were essentially the same as indicated above, except that the amounts of protein in the total, solubilized fraction of the tissues were 60–100 μg and there were additional modifications as indicated in Fig. 3. The enzyme activities were linear with respect to the amounts of the enzymes and the time of incubation. The rat tissues (brain and ovary) were homogenized in 5 vol. (1–5 ml) of ice-cold extraction solution containing 25 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM 2-mercaptoethanol, and 2 mM EGTA, and the entire homogenates were made to 0.2% Triton X-100. The homogenates were gently stirred for 1 hr at 4° and centrifuged at 30,000 *g* for 1 hr. The resultant supernatant fluids containing the cytosolic and solubilized membrane proteins (the total, solubilized fraction) were diluted 2- to 4-fold with the extraction solution and used directly for endogenous protein phosphorylation studies (Figs. 3 and 4). Phosphorylation of endogenous proteins and subsequent sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and autoradiography of the phosphorylated proteins were performed as described [17]. Antiestrogens were dissolved in dimethyl sulfoxide (DMSO). The final concentrations of DMSO in the incubation mixtures were less than 5% (v/v), which was without effect on the activities of the enzymes or phosphorylation of endogenous proteins. The same concentrations of DMSO served as controls for all experiments reported herein.

RESULTS

The nonsteroidal antiestrogens (tamoxifen, clomiphene and nafoxidine) were found to inhibit the PL/Ca-PK activity, with the IC_{50} values of 30–50 μM under the assay conditions (Fig. 1). In comparison, the estrogens (17α-estradiol, 17β-estradiol and diethylstilbesterol) had little effect; they inhibited the enzyme only up to 25% at a concentration as high as 500 μM. It was noted that chlorotrianisene, a weakly estrogenic compound having the basic structure of the antiestrogens, was also inactive (Fig. 1). All of the agents mentioned above were without effect on the basal enzyme activity seen in the absence of added phosphatidylserine and $CaCl_2$.

Analysis of inhibition kinetics revealed that antiestrogens inhibited the enzyme competitively with respect to phosphatidylserine, with K_i values of 16–27 μM (Fig. 2A). These agents, however, inhibited the enzyme non-competitively with respect to $CaCl_2$, with K_i values of 14–30 μM (Fig. 2B; double-reciprocal plots not shown).

Tamoxifen also inhibited the phospholipid/Ca^{2+}-stimulated phosphorylation of several endogenous

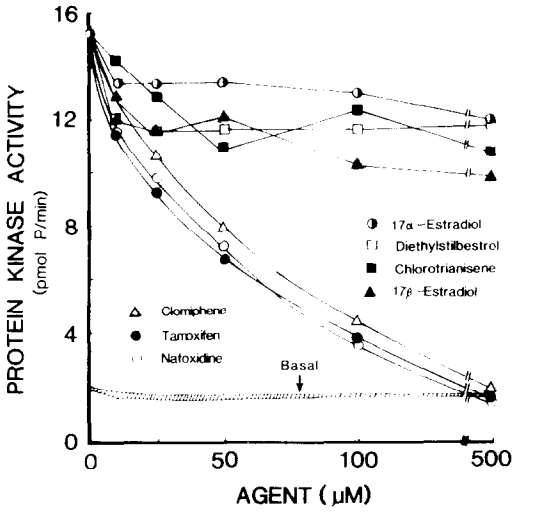

Fig. 1. Comparative effects of estrogens and antiestrogens on PL/Ca-PK. The enzyme was assayed (in 0.2 ml) in the presence of phosphatidylserine (5 μg), with either EGTA (200 μM, basal) or $CaCl_2$ (200 μM), and various concentrations (up to 500 μM) of the agents, as indicated. Detailed incubation conditions are given in Materials and Methods.

proteins (notably the species having M_r of 81,000, 67,000, 54,000, 50,000, 18,000 and 14,000) in the total, solubilized fraction of rat brains (Fig. 3). The 18,000 and 14,000 dalton proteins have been identified previously as large and small myelin basic protein respectively [18]. Because the antiestrogen inhibits growth of ovarian carcinomas [4, 5], we examined in the present studies the effect of the drug on phosphorylation of endogenous proteins in rat ovaries. The results indicated that there were several substrate proteins (notably the species having M_r of 50,000, 44,000, 39,000 and 14,000) for PL/Ca-PK in the total, solubilized fraction of the ovary, and that phosphorylation of these proteins by the enzyme was inhibited markedly by tamoxifen (Fig. 4A). The synthetic estrogen diethylstilbesterol, in comparison, had no effect (Fig. 4B). As shown above for the brain (Fig. 3), tamoxifen was without effect on other proteins whose phosphorylation was not stimulated by phospholipid and Ca^{2+}. For both brain (Fig. 3) and ovary (Fig. 4A), 50 μM tamoxifen totally or almost totally abolished the stimulation. These findings made with endogenous protein phosphorylation were in agreement with those made with histone phosphorylation by the purified PL/Ca-PK shown earlier (Fig. 1), i.e. antiestrogen, but not estrogen, inhibited specifically the phospholipid/Ca^{2+}-dependent protein phosphorylation reaction.

The effects of tamoxifen on other protein kinases were investigated (Table 1). It is of interest that MLCK, a calmodulin/Ca^{2+}-dependent protein kinase, was also found to be inhibited by the drug, with an IC_{50} value estimated to be about 35 μM, a value similar to that seen for PL/Ca-PK. A-PK and G-PK, the cyclic nucleotide-dependent protein kinases, on the other hand, were not inhibited by the drug at a concentration as high as 100 μM.

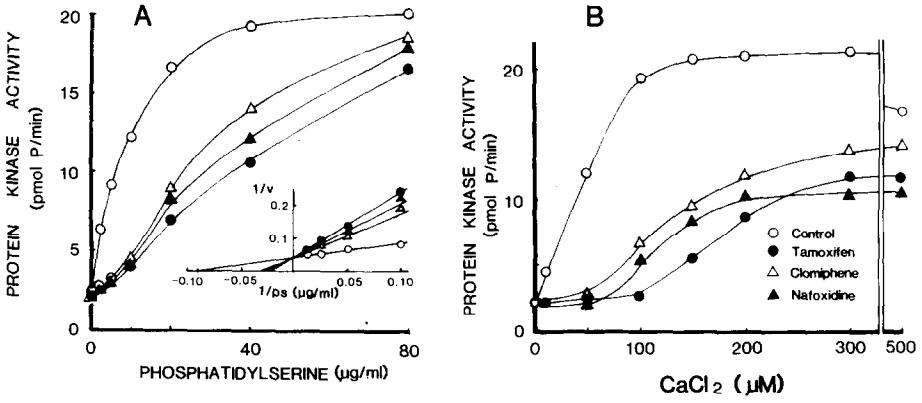

Fig. 2. Inhibition of PL/Ca-PK by antiestrogens. (A) Inhibition by antiestrogens (50 μM each) of the enzyme incubated with $CaCl_2$ (200 μM) and various concentrations of phosphatidylserine, as indicated. (B) Inhibition by antiestrogens (50 μM each) of the enzyme incubated with phosphatidylserine (25 μg/ml) and various concentrations of $CaCl_2$ as indicated.

DISCUSSION

One commonly accepted mechanism by which antiestrogens exert their therapeutic and antiproliferative effects on certain tumors is that they bind to the intracellular estrogen receptors and, therefore, competitively inhibit estrogen action [2]. Recent lines of evidence, however, suggest that tamoxifen does not block all endogenous estrogen action even in the postmenopausal women having low circulating estrogen levels [2], and that antiestrogen receptors distinct from estrogen receptors have been found in normal and neoplastic tissues [6]. It appears that tamoxifen may have some sites of action besides estrogen receptors, and that PL/Ca-PK might be one of these. Because tamoxifen also similarly inhibits MLCK, it is further suggested that this enzyme may be a potential site of the drug

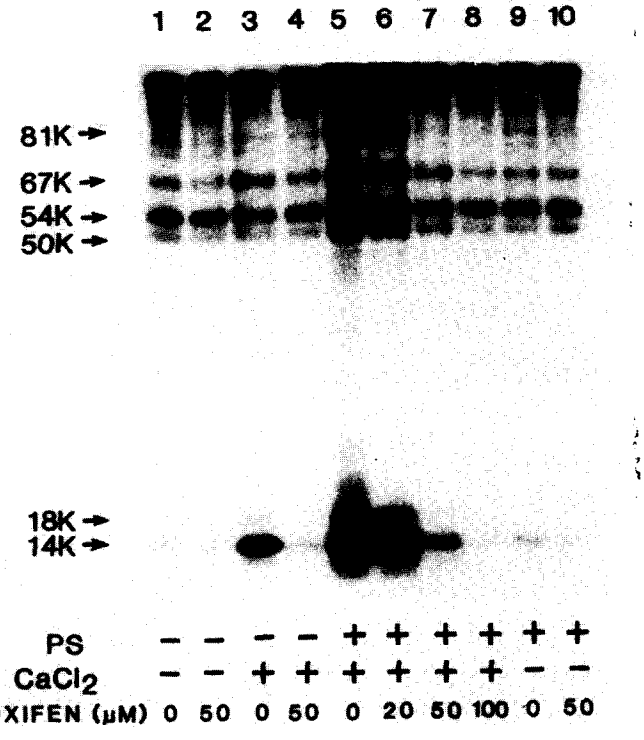

Fig. 3. Autoradiograph showing inhibition by tamoxifen of the phospholipid/Ca^{2+}-dependent phosphorylation of endogenous proteins in the total, solubilized fraction of the rat brain. The fraction was incubated in 0.2 ml of 25 mM Pipes, pH 6.5, for 10 min at 30°. When present, the additions were phosphatidylserine (PS, 5 μg), $CaCl_2$ (300 μM), and various concentrations of tamoxifen, as indicated. EGTA (200 μM) was present when no exogenous $CaCl_2$ was added. The separating gel contained 10% acrylamide and 0.1% sodium dodecyl sulfate.

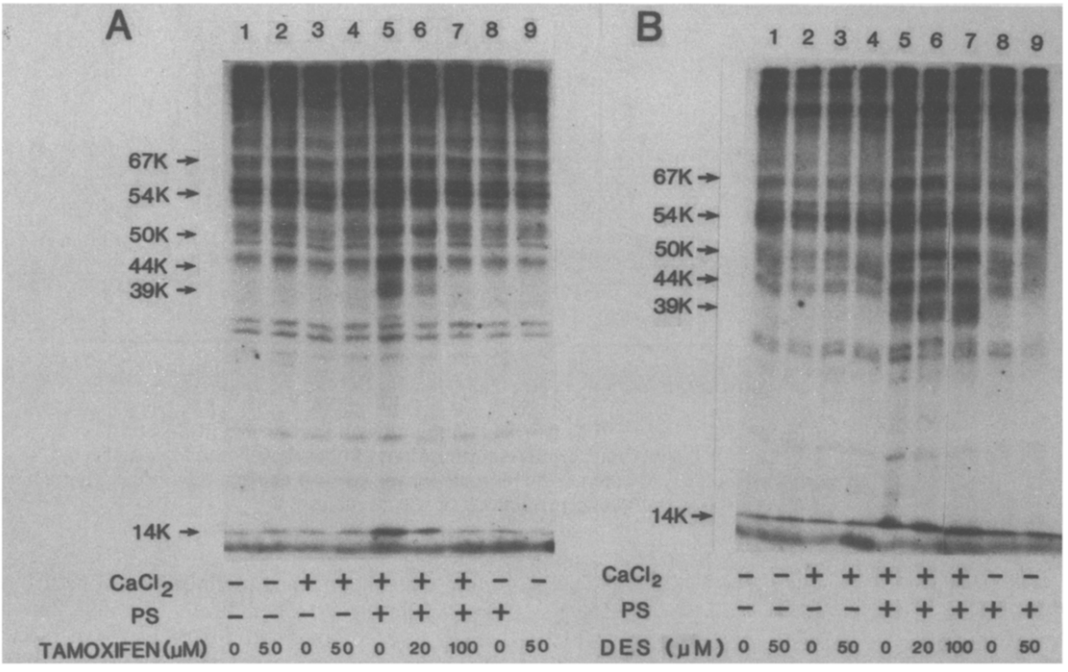

Fig. 4. Autoradiographs showing comparative effects of tamoxifen and diethylstilbesterol on the phospholipid/Ca^{2+}-dependent phosphorylation of endogenous proteins in the total, solubilized fraction of the rat ovary. The incubation conditions were similar to those shown in Fig. 3 for the rat brain except that the effects of (A) tamoxifen and (B) diethylstilbesterol (DES) were directly compared.

Table 1. Comparative effects of tamoxifen on various protein kinases*

	Enzyme activity (pmoles P transferred/min)			
	Tamoxifen (μM)			
Protein kinase	0	20	50	100
PL/Ca-PK	16.9	10.6	7.6	3.2
MLCK	10.6	8.2	2.5	0.5
A-PK	24.3	24.0	22.8	22.8
G-PK	6.0	6.6	7.3	7.1

* PL/Ca-PK was assayed in the presence of phosphatidylserine (5 μg/0.2 ml) and $CaCl_2$ (200 μM), MLCK in the presence of calmodulin (0.5 μg/0.2 ml) and $CaCl_2$ (100 μM), A-PK in the presence of cyclic AMP (0.5 μM), and G-PK in the presence of cyclic GMP (0.5 μM). Other assay conditions were as described in Materials and Methods.

action as well. It is conceivable that many other Ca^{2+} effector systems regulated by hydrophobic interactions with phospholipids or calmodulin could be potentially and generally influenced by tamoxifen and other lipopholic antiestrogens.

It has been demonstrated that hydrophobic interactions exist between calmodulin and its target enzymes in the presence of Ca^{2+}; such interactions are thought to be essential for the Ca^{2+}-dependent activation of the enzymes and disruptions of such interactions may modify the enzyme activities [19]. Similar hydrophobic interactions may also exist between phospholipid and PL/Ca-PK in the presence of Ca^{2+}, because many lipophilic agents shown to inhibit calmodulin/Ca^{2+}-dependent enzymes (including MLCK and phosphodiesterases) are similarly inhibitory to PL/Ca-PK (for reviews, see Refs. 9, 20, 21). In this regard, the tamoxifen inhibition seen in the present studies may represent yet another example underscoring the similarity between the two Ca^{2+}-effector systems utilizing either phospholipid or calmodulin as a cofactor. Although no agents specifically inhibiting the calmodulin/Ca^{2+}-dependent enzymes have been found, certain amphipathic polypeptides, notably antibiotic polymyxin B [22] and cobra venom cytotoxin I [23], are specific and potent inhibitors of PL/Ca-PK. These observations suggest that, despite the apparent similarity in activation mechanisms for these two Ca^{2+}-effector systems, there are certain additional structural determinants required for the PL/Ca-PK activation. This contention is supported, based upon the analysis of inhibition kinetics, in part, by the fact that the active ternary complex (i.e. consisting of the enzyme, the

cofactor and Ca^{2+}) for PL/Ca-PK assumes a "cyclic" configuration, whereas that for the calmodulin/Ca^{2+}-dependent enzymes assumes a "linear" configuration [24].

PL/Ca-PK is distributed in both cytosolic and particulate fractions [8, 9]. Our initial immunocytochemical studies indicate that, in the particulate fraction, the enzyme is localized in the membranes and nuclei of the brains [25] and leukemic cells (M. Shoji, P. R. Girard, G. J. Mazzei, W. R. Vogler and J. F. Kuo, unpublished observations). These findings also suggest that tamoxifen could interact with the enzyme at various cellular locations as the free form of the drug or possibly as complexes with receptors for estrogens or antiestrogens.

The K_i values for antiestrogens of PL/Ca-PK were determined to be about 14–30 μM. The values appear to be in line with the therapeutic dose of tamoxifen, which is about 40 mg/day for the duration of up to 6 years [2]. It has been shown that a continued presence of 2 μM tamoxifen is required for the demonstration of its antiproliferative effect on human ovarian carcinomas *in vitro* [4]. It is likely that, under these conditions, high concentrations of the drug may be reached at some cellular locations to inhibit PL/Ca-PK and other Ca^{2+}-effector systems.

PL/Ca-PK has been shown to be a receptor protein for phorbol esters [26, 27] and directly stimulated by the tumor promoters [28]. In this respect, it is of some interest that antitumor agents, such as alkyllysophospholipid [29], adriamycin [30] and antiestrogens (the present studies), are inhibitory to the enzyme. Because the enzyme, a major protein phosphorylation system [9], has been shown to play a key role in transductions of membrane signals, cellular regulation and tumor promotion [10], direct modulation of the enzyme system by antiestrogens may be of some biological significance.

REFERENCES

1. M. P. Cole, C. T. A. Jones and I. D. H. Todd, *Br. J. Cancer* **25**, 270 (1971).
2. O. H. Pearson, A. Manni and B. M. Arafah, *Cancer Res.* **42**, 3424s (1982).
3. R. I. Nicholson, N. M. Borthwich, C. P. Daniel, J. C. Syne and P. Davies, in *Nonsteroidal Antiestrogens* (Eds. R. L. Sutherland and V. C. Jordan), pp. 281–301. Academic Press, New York (1981).
4. J. C. Lazo, P. E. Schwartz, N. J. MacLusky, D. C. Labaree and A. J. Eisenfield, *Cancer Res.* **44**, 2266 (1984).
5. P. E. Schwartz, G. Keating, N. MacLusky, F. Naftolin and A. J. Eisenfield, *Obstet. Gynec., N.Y.* **59**, 583 (1982).
6. L. C. Murphy, M. S. Foo, M. D. Green, K. B. Milthorpe, A. M. Whybourne, Z. S. Krozowski and R. L. Sutherland, in *Nonsteroidal Antoestrogens* (Eds. R. L. Sutherland and V. C. Jordan), pp. 317–37. Academic Press, New York (1981).
7. Y. Takai, A. Kishimoto, Y. Iwasa, Y. Kawahar, T. Mori and Y. Nishizuka, *J. biol. Chem.* **254**, 3692 (1979).
8. J. F. Kuo, R. G. G. Andersson, B. C. Wise, L. Mackerlova, I. Salomonsson, I. Brackett, N. Katoh, M. Shoji and R. W. Wrenn, *Proc. natn. Acad. Sci. U.S.A.* **77**, 7039 (1980).
9. J. F. Kuo, R. C. Schatzman, R. S. Turner and G. J. Mazzei, *Molec. cell. Endocr.* **35**, 65 (1984).
10. Y. Nishizuka, *Nature, Lond.* **308**, 693 (1984).
11. B. C. Wise, R. L. Raynor and J. F. Kuo, *J. biol. Chem.* **257**, 8481 (1982).
12. M. P. Walsh, B. Vallet, F. Autric and J. G. Demaille, *J. biol. Chem.* **254**, 12136 (1979).
13. W. T. Perrie and S. V. Perry, *Biochem. J.* **119**, 31 (1970).
14. M. Shoji, J. G. Patrick, C. W. Davis and J. F. Kuo, *Biochem. J.* **161**, 213 (1977).
15. R. L. Post and A. K. Sen, *Meth. Enzym.* **10**, 773 (1967).
16. N. Katoh, R. L. Raynor, B. C. Wise, R. C. Schatzman, R. S. Turner, D. M. Helfman, J. N. Fain and J. F. Kuo, *Biochem. J.* **202**, 217 (1982).
17. R. W. Wrenn, N. Katoh, B. C. Wise and J. F. Kuo, *J. biol. Chem.* **225**, 12042 (1980).
18. R. S. Turner, C-H. J. Chou, R. F. Kibler and J. F. Kuo, *J. Neurochem.* **39**, 1397 (1982).
19. T. Tanaka and H. Hidaka, *J. biol. Chem.* **255**, 11078 (1980).
20. R. C. Schatzman, R. S. Turner and J. F. Kuo, in *Calcium and Cell Function* (Ed. W. Y. Cheung), Vol. 5, pp. 33–66. Academic Press, New York (1984).
21. R. S. Turner and J. F. Kuo, in *Phospholipids and Cellular Regulation* (Ed. J. F. Kuo), Vol. 2, pp. 75–110. CRC Press, Boca Raton (1985).
22. G. J. Mazzei, N. Katoh and J. F. Kuo, *Biochem. biophys. Res. Commun.* **109**, 1129 (1982).
23. J. F. Kuo, R. L. Raynor, G. J. Mazzei, R. C. Schatzman, R. S. Turner and W. R. Kem, *Fedn Eur. Biochem. Soc. Lett.* **153**, 183 (1983).
24. D-F. Qi, R. S. Schatzman, G. J. Mazzei, R. S. Turner, R. L. Raynor, S. Liao and J. F. Kuo, *Biochem. J.* **213**, 281 (1983).
25. P. R. Girard, G. J. Mazzei, J. G. Wood and J. F. Kuo, *Proc. natn. Acad. Sci. U.S.A.* **82**, 3030 (1985).
26. J. E. Niedel, L. J. Kuhn and G. R. Vandenbark, *Proc. natn. Acad. Sci. U.S.A.* **80**, 36 (1983).
27. U. Kikkawa, Y. Takai, Y. Tanaka, Y. Miyake and Y. Nishizuka, *J. biol. Chem.* **258**, 11442 (1983).
28. M. Castagna, Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa and Y. Nishizuka, *J. biol. Chem.* **257**, 7847 (1982).
29. D. M. Helfman, K. C. Barnes, J. M. Kinkade, Jr., W. R. Vogler, M. Shoji and J. F. Kuo, *Cancer Res.* **43**, 2955 (1983).
30. N. Katoh, B. C. Wise, R. W. Wrenn and J. F. Kuo, *Biochem. J.* **198**, 199 (1981).