Biochemical Pharmacology, Vol. 32, No. 7, pp. 1259–1265, 1983.
Printed in Great Britain.

0006-2952/83/071259-07 $03.00/0

MODES OF INHIBITION BY ACYLCARNITINES, ADRIAMYCIN AND TRIFLUOPERAZINE OF CARDIAC PHOSPHOLIPID-SENSITIVE CALCIUM-DEPENDENT PROTEIN KINASE*

BRADLEY C. WISE† and J. F. KUO‡
Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322, U.S.A.

(*Received* 9 *June* 1982; *accepted* 30 *September* 1982)

Abstract—Palmitoylcarnitine, adriamycin, and trifluoperazine competively inhibited, with respect to phosphatidylserine (a phospholipid cofactor), purified cardiac phospholipid-sensitive Ca^{2+}-dependent protein kinase, with apparent K_i values of 3, 49 and 14 μM respectively. These compounds also inhibited the enzyme competitively with respect to Ca^{2+} (a metal activator), with corresponding apparent K_i values of 0.8, 140 and 9 μM. A synergistic inhibition was observed when palmitoylcarnitine and trifluoperazine were present in combination. A simple addition inhibition, on the other hand, was observed for the combination of either palmitoylcarnitine and adriamycin, or trifluoperazine and adriamycin. 1,3-Diolein decreased the inhibitory effect of trifluoperazine by increasing the affinity of the enzyme for phosphatidylserine. The results indicate that the recently identified phospholipid-sensitive species of Ca^{2+}-dependent protein kinase was inhibited by a variety of agents, probably via their abilities to interfere with a hydrophobic interaction between phospholipid and the enzyme, an interaction presumably required to confer upon the enzyme a Ca^{2+} sensitivity. Because other long-chain fatty acylcarnitines (stearoyl- and linoleoylcarnitine), short-chain fatty acylcarnitines (such as octanoylcarnitine) and palmitoyl CoA, compared to palmitoylcarnitine, were less active as inhibitors, it is further suggested that lipophilicity as well as other structural determinants are crucial for the ability of compounds to regulate the enzyme activity.

Regulation of cyclic nucleotide-dependent protein kinase has been reported. Protein inhibitor (inhibitory modulator) depresses cyclic AMP-dependent kinase activity by interacting with its catalytic subunit [1, 2], whereas stimulatory modulator [3–5] and oligonucleotides [6] enhance phosphorylation activity of cyclic GMP-dependent protein kinase by interacting with certain substrate proteins (i.e. histone subfractions). There are no other agents that have been shown to specifically regulate their catalytic activity. Unlike cyclic nucleotide-dependent enzymes, which are directly stimulated by cyclic AMP and cyclic GMP, phospholipid-sensitive Ca^{2+}-dependent protein kinase requires not only Ca^{2+} (analogous to cyclic nucleotides) as an activator but also phospholipid as a cofactor. In view of the apparent hydrophobic nature of the interactions between phospholipid and the enzyme protein that confers upon the enzyme a Ca^{2+} sensitivity, it seems that the activation process of the enzyme would be potentially regulated by various endogenous and exogenous substances. In fact, phenothiazine antipsychotic agents, such as chlorpromazine [7] and trifluoperazine [8], have been shown to inhibit phosphorylation of histone by phospholipid-sensitive Ca^{2+}-dependent protein kinase partially purified from the brain [7, 8], heart [8] and spleen [8], and of endogenous substrate proteins from the cerebral cortex [9]. Moreover, we reported that other lipophilic substances, notably palmitoylcarnitine [10] and adriamycin [11], also inhibit phosphorylation of endogenous substrate proteins in the heart. We report here the modes of inhibition by palmitoylcarnitine, trifluoperazine and adriamycin of the near homogenous (about 95%) cardiac enzyme.

MATERIALS AND METHODS

Materials. Carnitine, various fatty acylcarnitine and CoA esters, phosphatidylserine (bovine brain), adriamycin and lysine-rich histone (Type III-S, histone HI) were purchased from either the Sigma Chemical Co., St. Louis, MO, or P-L Biochemicals, Inc., Milwaukee, WI. Trifluoperazine·2HCl was provided by Dr. P. T. Ridley of Smith, Kline & French, Philadelphia, PA.

Methods. Bovine heart phospholipid-sensitive Ca^{2+}-dependent protein kinase purified (80–95% homogeneous) through the step of phosphatidylserine-Affigel 102 chromatography [12] was employed in the present studies. The assay conditions were similar to those described earlier [12–14]. Briefly, the reaction mixture contained, in a final volume of 0.2 ml, 5 μmoles of 1,4-piperazine-diethanesulfonic acid (Pipes) (pH 6.5), 2 μmoles $MgCl_2$, various concentrations of phosphatidylserine and $CaCl_2$ as indicated in table and figure legends, 0.04 μmole EDTA, 40 μg histone HI, 1 nmole [γ-^{32}P]ATP containing 0.8 to 1.3 × 10^6 cpm, and

* This work was supported by USPHS Grants HL-15696, NS-17608 and T32-GM-07594.

† Present address: Laboratory of Prelinical Pharmacology, National Institute of Mental Health, St. Elizabeth's Hospital, Washington, DC 20032, U.S.A.

‡ To whom reprint requests should be sent.

appropriate amounts of enzyme protein as indicated. Drugs and other compounds were included in the assay at the concentrations indicated. The concentrations of $CaCl_2$, phosphatidylserine, drugs, and other added substances are given as total concentrations; no attempt was made to determine their actual free concentrations. The reaction was started by the addition of ATP and was carried out for 5–10 min at 30° and the phosphorylated histone was precipitated with trichloroacetic acid–tungstate and processed as previously described [12–14]. Cyclic AMP-dependent and cyclic GMP-dependent protein kinases were partially purified, and assays for their activities were the same as described earlier [5, 13]. Activity of the various enzymes was linear as a function of the incubation time and the enzyme amount in all experiments. Therefore, enzyme assays were conducted under initial rate conditions in all experiments. Where appropriate, data are expressed as means ± S.E.M.

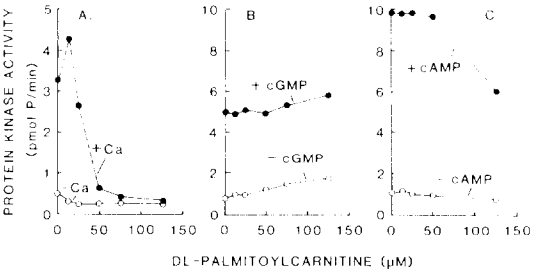

Fig. 1. Comparative effects of DL-palmitoylcarnitine on three classes of protein kinases from the bovine heart. (A) Phospholipid-sensitive Ca^{2+}-dependent protein kinase, 0.03 μg, (B) cyclic GMP-dependent protein kinase, 10 μg, and (C) cyclic AMP-dependent protein kinase, 10 μg, were incubated with phosphatidylserine (25 μg/ml) in the absence or presence of $CaCl_2$ (500 μM), cyclic GMP (0.5 μM) and cyclic AMP (0.5 μM), respectively, and in the presence of various concentrations of DL-palmitoylcarnitine, as indicated. Results similar to those shown in this figure were obtained in three other experiments.

RESULTS

DL-Palmitoylcarnitine, at 50 μM, caused a nearly complete inhibition of the Ca^{2+}-stimulated activity of cardiac phospholipid-sensitive Ca^{2+}-dependent protein kinase, with a 50% inhibition noted at 35 μM (Fig. 1A). The acylcarnitine at a lower concentration of 12.5 μM, on the other hand, slightly stimulated (about 30%) its activity (Fig. 1A); this observation was reproducible in three other experiments, but with a large variability (average stimulation was 15 ± 6%). It has been reported previously by others that the acylcarnitine, at low concentrations of 5–25 μM, also slightly stimulates cardiac sarcoplasmic reticulum Ca^{2+}-ATPase [15], while at concentrations higher than 70 μM inhibiting both Ca^{2+}-ATPase [15] and Na^+,K^+-ATPase [15–17]. In contrast to the Ca^{2+}-dependent protein kinase (Fig. 1A), the acylcarnitine, at a concentration as high as 125 μM, was virtually without effect on the cardiac cyclic GMP-dependent protein kinase (Fig. 1B) while inhibiting by only about 40% the cardiac cyclic AMP-dependent protein kinase (Fig. 1C). It has been reported that palmitoylcarnitine, a metabolic intermediate of long-chain fatty acids, accumulates greatly (to millimolar concentrations) in the ischemic heart [18–20].

Table 1. Comparative effects of various acylcarnitine and CoA esters on the cardiac phospholipid-sensitive Ca^{2+}-dependent protein kinase*

Addition	Protein kinase activity (pmoles P/min)
None (control)	2.9 (100)
L-Carnitine (30 μM)	2.7 (94)
L-Carnitine (60 μM)	2.4 (82)
Palmitate, Na-salt (30 μM)	3.3 (114)
Palmitate, Na-salt (60 μM)	2.8 (95)
DL-Palmitoylcarnitine (30 μM)	1.2 (41)
DL-Palmitoylcarnitine (60 μM)	0.2 (7)
L-Palmitoylcarnitine (30 μM)	1.5 (52)
L-Palmitoylcarnitine (60 μM)	0.2 (7)
DL-Stearoylcarnitine (60 μM)	1.4 (48)
L-Linoleoylcarnitine (60 μM)	2.1 (72)
DL-Octanoylcarnitine (60 μM)	2.8 (97)
DL-Hexanoylcarnitine (60 μM)	2.1 (72)
DL-Acetylcarnitine (60 μM)	2.4 (83)
Palmitoyl CoA (60 μM)	2.4 (83)

* The enzyme (0.05 μg) was incubated with cardiolipin (50 μg/ml), instead of phosphatidylserine (25 μg/ml), in the absence or presence of $CaCl_2$ (500 μM) and indicated compounds (30 or 60 μM). The data presented have been corrected for the basal activity values seen in the absence of added $CaCl_2$, which amounted to 8–10% of those seen in its presence. The numbers in parentheses are the percent of the control value obtained in the absence of added compounds, which was taken as 100%. All compounds tested had no effect on the basal enzyme activity. Results similar to those shown in this table were obtained in two separate experiments.

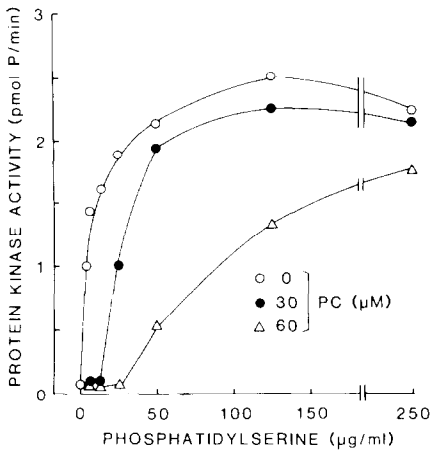

Fig. 2. Reversal by phosphatidylserine of DL-palmitoylcarnitine inhibition of phospholipid-sensitive Ca^{2+}-dependent protein kinase. The enzyme (0.02 μg) was incubated in the absence or presence of DL-palmitoylcarnitine (PC, 30 or 60 μM), $CaCl_2$ (500 μM) and various concentrations of phosphatidylserine, as indicated. The data presented have been corrected for the respective basal values obtained in the absence of $CaCl_2$, which amounted to about 5–7% of those seen in the presence of $CaCl_2$.

In order to study the specificity of acylcarnitines in inhibiting the enzyme, a number of structurally related compounds were examined for their effects (Table 1). While L-carnitine and palmitate had only little effect, DL- and L-palmitoylcarnitine were the most inhibitory and, moreover, they were nearly equipotent. Other acylcarnitines of long-chain fatty acids (i.e. stearoyl- and linoleoylcarnitine) and of short-chain fatty acids (i.e. octanoyl-, hexanoyl- and acetylcarnitine) were less effective. Palmitoyl CoA, which has also been shown to accumulate in ischemic heart [18–20], was relatively ineffective.

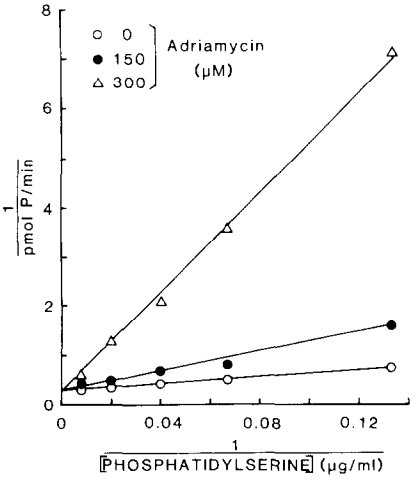

Fig. 3. Kinetics showing competitive inhibition with respect to phosphatidylserine by adriamycin of phospholipid-sensitive Ca^{2+}-dependent protein kinase. The enzyme (0.02 μg) was incubated with $CaCl_2$ (500 μM) in the absence or presence of adriamycin (150 or 30 μM), and in the presence of various concentrations (7–250 μg/ml) of phosphatidylserine, as indicated. The data have been corrected for the respective basal values obtained in the absence of $CaCl_2$, which amounted to about 5–7% of those seen in the presence of $CaCl_2$.

DL-Palmitoylcarnitine inhibited the enzyme in a dose-dependent manner, and this inhibition appeared to be overcome by increasing concentrations of phosphatidylserine (Fig. 2). Complete inhibition of the enzyme by 30 or 60 μM acylcarnitine was still evident in the presence of phospholipid at concentrations of 16 and 25 μg/ml, respectively, suggesting that a stoichiometric interaction might exist between the two lipid substances. It is conceivable that such an interaction would lead to alterations of certain phosphatidylserine vesicular or micellar structures that are required for enzyme activation. It should be noted that the inhibition by the acylcarnitine did not obey classical Michaelis–Menten kinetics. However, double-reciprocal plots and kinetic analysis of certain experimental data obtained with higher phosphatidylserine concentrations from Fig. 2 revealed that inhibition by the acylcarnitine appeared to be competitive with respect to the phospholipid cofactor, with an apparent K_i value of 2.6 ± 0.3 μM (three determinations; figure not shown).

Adriamycin, a phospholipid-interacting and DNA-intercalating anti-neoplastic drug with a unique cardiotoxicity [21], was found to inhibit the enzyme competitively with respect to phosphatidylserine, with an apparent K_i of 49 ± 3 μM (three determinations) (Fig. 3). Similarly, trifluoperazine, a phenothiazine antipsychotic drug, inhibited the enzyme competitively with respect to the phospholipid cofactor, with an apparent K_i of 14 ± 3 μM (five determinations) (Fig. 4). We have reported pre-

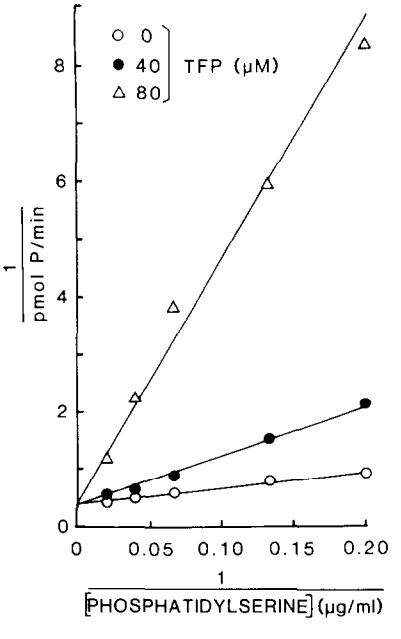

Fig. 4. Kinetics showing competitive inhibition with respect to phosphatidylserine by trifluoperazine (TFP) of phospholipid-sensitive Ca^{2+}-dependent protein kinase. The enzyme (0.02 μg) was incubated with $CaCl_2$ (500 μM) in the absence or presence of trifluoperazine (40 or 80 μM), and in the presence of various concentrations (5–100 μg/ml) of phosphatidylserine, as indicated. The data have been corrected for the respective basal values obtained in the absence of $CaCl_2$, which amounted to about 5–7% of those seen in the presence of $CaCl_2$.

Table 2. Effects of DL-palmitoylcarnitine, trifluoperazine and adriamycin, present singly or in combinations, on the cardiac phospholipid-sensitive Ca^{2+}-dependent protein kinase*

Addition	Protein kinase activity (pmoles P/min)
None (control)	2.79 (100) [100]
DL-Palmitoylcarnitine (10 μM)	2.94 (105)
DL-Palmitoylcarnitine (30 μM)	0.98 (35)
Trifluoperazine (40 μM)	2.53 (91)
Trifluoperazine (60 μM)	2.08 (75)
Adriamycin (100 μM)	2.03 (73)
Adriamycin (200 μM)	1.55 (56)
DL-Palmitoylcarnitine (10 μM) + trifluoperazine (40 μM)	1.82 (65) [96]
DL-Palmitoylcarnitine (10 μM) + trifluoperazine (60 μM)	0.61 (22) [79]
DL-Palmitoylcarnitine (30 μM) + trifluoperazine (40 μM)	0.30 (11) [32]
DL-Palmitoylcarnitine (30 μM) + trifluoperazine (60 μM)	0.04 (1) [26]
DL-Palmitoylcarnitine (10 μM) + adriamycin (100 μM)	2.31 (83) [77]
DL-Palmitoylcarnitine (10 μM) + adriamycin (200 μM)	1.93 (69) [59]
DL-Palmitoylcarnitine (30 μM) + adriamycin (100 μM)	0.77 (28) [26]
DL-Palmitoylcarnitine (30 μM) + adriamycin (200 μM)	0.53 (19) [20]
Trifluoperazine (40 μM) + adriamycin (100 μM)	2.17 (78) [66]
Trifluoperazine (40 μM) + adriamycin (200 μM)	1.86 (67) [51]
Trifluoperazine (60 μM) + adriamycin (100 μM)	1.90 (68) [55]
Trifluoperazine (60 μM) + adriamycin (200 μM)	1.07 (38) [42]
DL-Palmitoylcarnitine (10 μM) + trifluoperazine (40 μM) + adriamycin (100 μM)	1.80 (65) [70]

* The enzyme (0.04 μg) was incubated with phosphatidylserine (25 μg/ml) in the absence or presence of $CaCl_2$ (500 μM), DL-palmitoylcarnitine (10 or 30 μM), trifluoperazine (40 or 60 μM) and adriamycin (100 or 200 μM), as indicated. The data presented have been corrected for the basal activity values seen in the absence of added $CaCl_2$, which amounted to 6–7% of those seen in its presence. The numbers in parentheses are the percent of the control value obtained in the absence of added compounds, which was taken as 100%. The numbers in brackets are the theoretical values expressed as percent of the control activity to be observed in the combined presence of the inhibitors, assuming their individual effects are additive. All compounds tested under the incubation conditions had no effect on the basal enzyme activity. Results similar to those shown in this table were obtained in two separate experiments.

viously that DL-palmitoylcarnitine [10], adriamycin [11] and trifluoperazine [9] inhibit phospholipid/Ca^{2+}-stimulated phosphorylation of endogenous substrate proteins from the cytosolic or particulate fractions of several rat or guinea pig tissues.

A synergism of inhibition of the enzyme by DL-palmitoylcarnitine (10 or 30 μM) and by trifluoperazine (40 or 60 μM) was observed when they were present in combination (Table 2). This observation suggested that interaction of both the acylcarnitine and trifluoperazine with phosphatidylserine would greatly diminish the ability of the phospholipid to serve as a cofactor or, alternatively, the two agents could form a possible complex and act as a highly effective inhibitor. In comparison, the degree of inhibition seen in the presence of DL-palmitoylcarnitine (10 or 30 μM) plus adriamycin (100 or 200 μM), or trifluoperazine (40 or 60 μM) plus adriamycin (100 or 200 μM), was similar to, or slightly lower than, the sums of inhibitions seen with the individual compounds (Table 2). The overall inhibition seen in the combined presence of all three compounds was nearly additive (Table 2).

Diolein decreased the apparent K_a of the enzyme for phosphatidylserine (Fig. 5), in line with previous findings of Kishimoto *et al.* [22] and us [12, 14]: it, however, was without a clear effect on the V_{max} of the enzymes under the assay conditions, at variance with some previous reports using partially purified enzyme [14, 22]. This discrepancy is most likely due to the purity of the enzyme used in this and other studies [12, 13] compared to early studies [14, 22] in which only partially purified enzyme was employed. Although the highly purified enzyme (80–95% homogeneous) was stimulated by low concentrations of phosphatidylserine and Ca^{2+} [12], changes in the kinetic parameters (i.e. lowering of K_a values for phosphatidylserine and Ca^{2+} and increasing V_{max}) induced by diolein were not as pronounced as those obtained for the less purified enzyme preparations [14, 22]. Another factor that may be responsible for the above discrepancy is the assay conditions for the

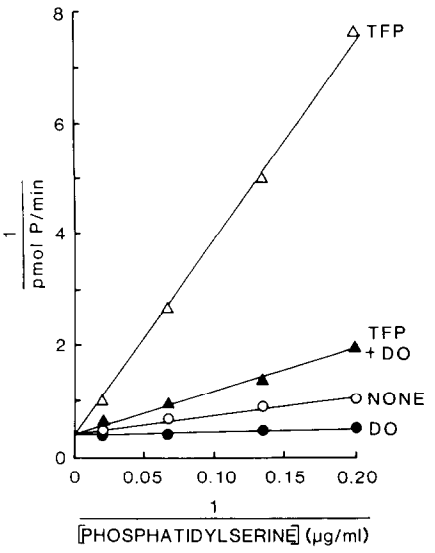

Fig. 5. Kinetics showing effects of 1,3-diolein (DO) and trifluoperazine (TFP) present singly or in combination, on phospholipid-sensitive Ca^{2+}-dependent protein kinase. The enzyme (0.02 μg) was incubated with $CaCl_2$ (300 μM) in the absence or presence of 1,3-diolein (2.5 μg/ml), or trifluoperazine (50 μM), or both, and in the presence of various concentrations (5–50 μg/ml) of phosphatidylserine, as indicated. The data have been corrected for the respective basal values obtained in the absence of $CaCl_2$, which amounted to about 5–7% of those seen in the presence of $CaCl_2$.

highly purified enzyme compared to that for less purified enzyme preparations. In this and other studies [12, 13], the enzyme was assayed at pH 6.5, the optimal pH for the highly purified enzyme [12]. However, in other studies [14, 22] where diolein increased the V_{max}, a higher pH of 7.5 was used for the assay. We noted that diolein reversed the inhibitory effect of trifluoperazine, without affecting the competitive nature of the inhibition by the antipsychotic drug, by decreasing the apparent K_a for phosphatidylserine from 60 μg/ml (in the presence of trifluoperazine) to 15 μg/ml (in the presence of trifluoperazine and diolein), which was accompanied by a concomitant increase in the apparent K_i for trifluoperazine from 4 to 19 μM (Fig. 5).

In addition, we have examined the three inhibitors mentioned above for their effects on the enzyme as a function of $CaCl_2$ concentrations (Fig. 6). It was found that, analogous to phosphatidylserine shown earlier in Figs. 2–4, they inhibited the enzyme competitively with respect to $CaCl_2$, with average apparent K_i values (means ± range of two determinations) of 0.8 ± 0.1, 140 ± 56 and 9 ± 2 μM for DL-palmitoylcarnitine, adriamycin and trifluoperazine respectively.

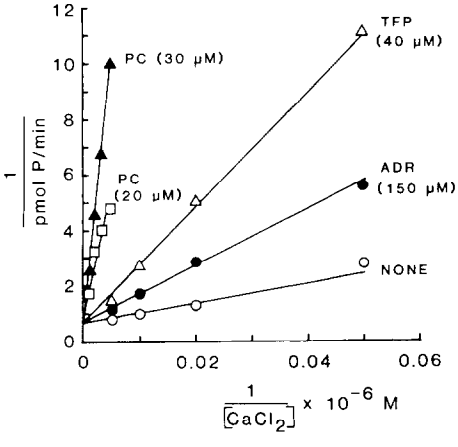

Fig. 6. Kinetics showing competitive inhibition with respect to $CaCl_2$ by DL-palmitoylcarnitine (PC), adriamycin (ADR) and trifluoperazine (TFP) of phospholipid-sensitive Ca^{2+}-dependent protein kinase. The enzyme (0.02 μg) was incubated with phosphatidylserine (25 μg/ml) in the absence or presence of DL-palmitoylcarnitine (20 or 30 μM), adriamycin (150 μM) or trifluoperazine (40 μM), and in the presence of various concentrations (20–500 μM) of $CaCl_2$, as indicated.

DISCUSSION

We have observed that neither inhibitory modulator (protein inhibitor) of cyclic AMP-dependent protein kinase nor stimulatory modulator (also a heat-stable, acidic protein) of cyclic GMP-dependent protein kinase has any effect on the present phospholipid-sensitive Ca^{2+}-dependent protein kinase [12]. On the other hand, there are certain agents that inhibit the phospholipid/Ca^{2+}-stimulated phosphorylation of histone or endogenous substrate proteins, without appreciably affecting the cyclic nucleotide-dependent reactions. These agents include palmitoylcarnitine ([10, 13]; Fig. 1), adriamycin [11, 13] and trifluoperazine [8, 9, 13]. These findings clearly indicate that the three species of protein kinases are also distinguishable based upon regulation of their activities.

Phenothiazines, such as trifluoperazine and chlorpromazine, have been shown to inhibit a number of calmodulin-sensitive Ca^{2+}-dependent enzymes by interacting with calmodulin [23]. These findings have led to the notion that these drugs are selective calmodulin antagonists [23]. Recent and present evidence, however, indicates that phenothiazines also inhibit the phospholipid-sensitive Ca^{2+}-dependent species of protein kinase in phosphorylating histone ([7, 8]; Figs. 4–6) as well as various endogenous proteins from the brain [9], with an inhibitory potency comparable to that seen for phosphodiesterase [23]. We noted that W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, previously shown by others to inhibit the calmodulin/Ca^{2+}-stimulated enzymes [24, 25] such as phosphodiesterase, myosin light chain kinase and ATPase, also inhibits phosphorylation of histone by phospholipid-sensitive Ca^{2+}-dependent protein kinase purified from heart [13] and that of myelin basic protein in rat brain myelin catalyzed by the enzyme [26]. It appears, therefore, that phenothiazines and W-7 cannot be regarded as selective inhibitors of the calmodulin-sensitive species of Ca^{2+}-dependent enzymes. Hydrophobic interactions between the cofactors (i.e. calmodulin and phosphatidylserine) with their respective enzymes seem to be a common mechanism leading to the enzyme activation. This contention is further supported by our findings that palmitoylcarnitine [10], adriamycin [11], and trifluoperazine

[9] similarly inhibit the phospholipid/Ca^{2+}- and calmodulin/Ca^{2+}-stimulated phosphorylation of specific endogenous proteins in heart and brain. Furthermore, we found that melittin [27] (an amphipathic polypeptide toxin), as well as polymyxin B (a surface-active polypeptide antibiotic) and R-24571 (an imidazolinium chloride derivative previously shown to be a potent inhibitor of calmodulin/Ca^{2+}-activated enzymes) [28, 29], all inhibit both phospholipid-sensitive Ca^{2+}-dependent protein kinase and myosin light chain kinase (G. J. Mazzei and J. F. Kuo, unpublished observations).

All agents shown to inhibit the Ca^{2+}-dependent enzymes are lipophilic (such as trifluoperazine) or contain non-polar regions (such as melittin). It is likely that interactions of these agents with calmodulin or phosphatidylserine would interfere with the normal interactions of these cofactors with their respective enzymes, leading to inhibition of the Ca^{2+}-dependent activities. The observations that a synergistic effect was seen for the combination of palmitoylcarnitine and trifluoperazine but not for that of palmitoylcarnitine and adriamycin, or trifluoperazine and adriamycin, in inhibiting phospholipid-sensitive Ca^{2+}-dependent protein kinase (Table 2) suggest that the agents form complexes with phosphatidylserine in different manners, yielding phospholipid vesicles or micelles having variable ability to serve as a cofactor. This notion seems to be also supported by the observations of Kaibuchi *et al.* [30] that various phospholipids either potentiate, attenuate or have no effect on the ability of phosphatidylserine to activate this enzyme. Finally, it should be emphasized that lipophilicity *per se* is not the sole determinant for the agents to be inhibitors, or for that matter, activators, of this enzyme. This conclusion is supported by the following observations: (1) other long-chain fatty acylcarnitines (such as stearoyl- and linoleoylcarnitine) and palmitoyl CoA, compared with palmitoylcarnitine, were less effective (Table 1); (2) W-5, a structural analog of W-7 without chloride substitution at position 5 and having a similar lipophilicity as W-7, was much less effective than W-7 as an inhibitor of the enzyme (R. C. Schatzman and J. F. Kuo, unpublished observations); and (3) of many phospholipids examined, phosphatidylserine is the most effective as a cofactor for the enzyme [12, 31]. W-5 has been shown previously by Kanamori *et al.* [32] to be also less effective than W-7 as an inhibitor of the calmodulin/Ca^{2+}-stimulated enzymes.

Earlier reports using less purified enzyme preparations [7, 8] indicated that Ca^{2+} could not totally overcome the phenothiazine inhibition of enzyme activity, whereas in the present study a competitive interaction between Ca^{2+} and trifluoperazine was found. This discrepancy may be due to the inclusion in the earlier [7, 8], but not in the present, studies of diolein, which not only decreases the K_a for Ca^{2+} and phospholipid but also increases the V_{max} of the partially purified enzyme. In the presence of trifluoperazine (or for that matter adriamycin or palmitoylcarnitine), Ca^{2+} may not be able to completely restore enzyme activity to its maximal level seen in the presence of diolein and, therefore, the inhibition of the partially purified enzyme is apparently non-competitive. However, with the highly purified enzyme where it has been shown that diolein has no effect on maximal enzyme activity under optimal enzyme assay conditions [12], Ca^{2+} can competitively interact with these inhibitory agents in the absence of diolein (Fig. 6). Alternatively, diolein, by altering the interaction of Ca^{2+} and phospholipid with the enzyme, may also change the nature of the inhibition of enzyme activity by trifluoperazine and other substances.

In addition to the possibility that the enzyme inhibition by the drugs used in this study is due to an interaction with the phospholipid cofactor, it should also be considered that the agents interact with the enzyme itself, particularly in light of the competitive inhibition of the compounds with respect to Ca^{2+}. It is possible that Ca^{2+} and the phospholipid cofactor bind to the same domain, but to different sites within the domain, of the enzyme and thereby cause enzyme activation. To be competitive with respect to phosphatidylserine and Ca^{2+}, the drugs could bind to this regulatory domain and, by doing so, inhibit activation. It is also possible that the inhibition of palmitoylcarnitine, adriamycin and trifluoperazine may be due in part to the potential ability of these agents to chelate Ca^{2+}.

REFERENCES

1. D. A. Walsh, C. D. Ashby, C. Gonsalez, D. Calkins, E. H. Fischer and E. G. Krebs, *J. biol. Chem.* **246**, 1977 (1971).
2. C. D. Ashby and D. A. Walsh, *J. biol. Chem.* **247**, 6637 (1972).
3. T. E. Donnelly, J. F. Kuo, P. L. Reyes, Y-P. Liu and P. Greengard, *J. biol. Chem.* **248**, 190 (1973).
4. W-N. Kuo and J. F. Kuo, *J. biol. Chem.* **251**, 4283 (1976).
5. M. Shoji, N. L. Brackett, J. Tse, R. Shapira and J. F. Kuo, *J. biol. Chem.* **253**, 3427 (1978).
6. M. Yamamoto, Y. Takai, E. Hashimoto and Y. Nishizuka, *J. Biochem., Tokyo* **81**, 1857 (1977).
7. T. Mori, Y. Takai, R. Minakuchi, B. Yu and Y. Nishizuka, *J. biol. Chem.* **255**, 8378 (1980).
8. R. C. Schatzman, B. C. Wise and J. F. Kuo, *Biochem. biophys. Res. Commun.* **98**, 669 (1981).
9. R. W. Wrenn, N. Katoh, R. C. Schatzman and J. F. Kuo, *Life Sci.* **29**, 725 (1981).
10. N. Katoh, R. W. Wrenn, B. C. Wise, M. Shoji and J. F. Kuo, *Proc. natn. Acad. Sci. U.S.A.* **78**, 4813 (1981).
11. N. Katoh, B. C. Wise, R. W. Wrenn and J. F. Kuo, *Biochem. J.* **198**, 199 (1981).
12. B. C. Wise, R. L. Raynor and J. F. Kuo, *J. biol. Chem.* **257**, 8481 (1982).
13. B. C. Wise, D. B. Glass, C-H. J. Chou, R. L. Raynor, N. Katoh, R. C. Schatzman, R. S. Turner, R. J. Kibler and J. F. Kuo, *J. biol. Chem.* **257**, 8489 (1982).
14. J. F. Kuo, R. G. G. Andersson, B. C. Wise, L. Makerlova, I. Salomonsson, N. L. Brackett, N. Katoh, M. Shoji and R. W. Wrenn, *Proc. natn. Acad. Sci. U.S.A.* **77**, 7039 (1980).
15. R. J. Adams, D. W. Cohen, S. Gupte, J. D. Johnson, E. T. Wallick, T. Wang and A. Schwartz, *J. biol. Chem.* **254**, 12404 (1979).
16. K. Ahmed and B. S. Thomas, *J. biol. Chem.* **246**, 103 (1971).
17. J. M. J. Lamers and W. C. Hulsmann, *J. molec. cell. Cardiol.* **9**, 343 (1977).

18. J. T. Whitmer, J. A. Idell-Wenger, M. J. Rovetto and J. R. Neely, *J. biol. Chem.* **253**, 4305 (1978).
19. J. A. Idell-Wenger, L. W. Grotyohann and J. R. Neely, *J. biol. Chem.* **253**, 4310 (1978).
20. A. L. Shug, J. H. Thomasen, J. D. Folts, N. Bittar, M. I. Klein, J. R. Koke and P. J. Huth, *Archs Biochem. Biophys.* **187**, 25 (1978).
21. E. Goormaghtigh, P. Chatalain, J. Caspers and J. M. Ruysschaert, *Biochim. biophys. Acta* **597**, 1 (1980).
22. A. Kishimoto, Y. Takai, T. Mori, U. Kikkawa and Y. Nishizuka, *J. biol. Chem.* **255**, 2273 (1980).
23. B. Weiss and T. L. Wallace, in *Calcium and Cell Function* (Ed. W. Y. Cheung), Vol. 1, pp. 329–79. Academic Press, New York (1980).
24. H. Hidaka, T. Yamaki, M. Naka, T. Tanaka, H. Hayashi and R. Kobayashi, *Molec. Pharmac.* **17**, 66 (1980).
25. T. Tanaka and H. Hidaka, *J. biol. Chem.* **255**, 11078 (1980).
26. R. S. Turner, C-H. J. Chou, R. F. Kibler and J. F. Kuo, *J. Neurochem.* **39**, 1397 (1982).
27. 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).
28. H. Van Belle, *Cell Calcium* **2**, 483 (1981).
29. K. Gietzen, A. Wuthrich and H. Bader, *Biochem. biophys. Res. Commun.* **101**, 418 (1981).
30. K. Kaibuchi, Y. Takai and Y. Nishizuka, *J. biol. Chem.* **256**, 7146 (1981).
31. Y. Takai, A. Kishimoto, Y. Isawa, Y. Kawahara, T. Mori and Y. Nishizuka, *J. biol. Chem.* **254**, 3692 (1979).
32. M. Kanamori, M. Naka, M. Asano and H. Hidaka, *J. Pharmac. exp. Ther.* **217**, 494 (1981).