Biochemical Pharmacology, Vol. 33, No. 1, pp. 125–130, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

PHOSPHOLIPID-SENSITIVE Ca^{2+}-DEPENDENT PROTEIN KINASE INHIBITION BY R-24571, A CALMODULIN ANTAGONIST*

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(*Received* 7 *February* 1983; *accepted* 9 *June* 1983)

Abstract—R-24571 (calmidazolium), a derivative of the antimycotic agent miconazole, inhibited phospholipid-sensitive Ca^{2+}-dependent protein kinase (PL-Ca-PK), with an IC_{50} (the concentration causing 50% inhibition) of 5.3 μM. It also inhibited the calmodulin/Ca^{2+}-stimulated enzymes, with IC_{50} values of 1.6 and 0.1 μM for myosin light chain kinase (MLCK) and phosphodiesterase respectively. Analysis of inhibition by R-24571 of PL-Ca-PK and MLCK revealed complex kinetics, suggesting that the agent interacted with the cofactors, the enzyme, and/or the cofactor–enzyme complexes. At saturating concentrations of the cofactors, R-24571 inhibited PL-Ca-PK and MLCK noncompetitively with their respective cofactors. Inhibition of MLCK by R-24571 was completely overcome by phosphatidylserine, indicating a strong hydrophobic interaction between R-24571 and the phospholipid in the presence of calmodulin. R-24571 also inhibited phosphorylation of various endogenous proteins in brain stimulated specifically by phosphatidylserine/Ca^{2+} or calmodulin/Ca^{2+}. The present findings inducated that R-24571 has little specificity in inhibiting two types of Ca^{2+}-dependent protein kinases sensitive to phospholipid or calmodulin.

Trifluoperazine [1] and *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7) [2, 3] have been reported to inhibit a variety of enzyme reactions and biological processes that are stimulated by calmodulin in the presence of Ca^{2+}. Several lines of recent evidence indicate that trifluoperazine [4–6] and W-7 [6–8] also inhibit phospholipid-sensitive Ca^{2+}-dependent protein kinase (PL-Ca-PK), for which a phospholipid (notably phosphatidylserine), instead of calmodulin, serves as a cofactor to confer upon the enzyme a Ca^{2+} sensitivity. An antimycotic miconazole derivative, 1-[bis(*p*-chlorophenyl)methyl]-3-[2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl] imidazoliniumchloride (R-24571), has been reported recently to be a potent inhibitor of a number of calmodulin/Ca^{2+}-stimulated enzymes [9–11]. The IC_{50} values (concentrations causing 50% inhibition) for R-24571 were 0.5 μM [9], 0.15 to 0.35 μM [10], or 3 μM [11] for erythrocyte Ca^{2+}-ATPase, 2 μM for erythrocyte Ca^{2+} transport [9], 0.005 to 0.01 μM for brain phosphodiesterase (PDE) [10], and 5 μM for phosphorylase *b* kinase [10]. In this paper, we examined the effect of R-24571 on PL-Ca-PK as well as on myosin light chain kinase (MLCK), a calmodulin-sensitive Ca^{2+}-dependent protein kinase, and its mode of action on these enzymes. We found that R-24571, like other calmodulin antagonists (i.e. trifluoperazine and W-7) previously reported, inhibited both PL-Ca-PK and MLCK.

* This work was supported by USPHS Grant HL-15696, NS-17608, T32-GM-07594, ACS Grant CH-208, and Atlanta Carnival for Cure, Inc. Funds for Leukemic Research.

† Recipient of a Postdoctoral Fellowship from the Muscular Dystrophy Association of America.

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MATERIALS AND METHODS

Materials. R-24571 was purchased from Janssen Pharmaceutica, Beerse, Belgium; histone H1 (lysine-rich histone, type III-S), mixed histone (type II), phosphatidylserine, phenylmethylsulfonylfluoride (PMSF), cyclic AMP, and cyclic GMP were from the Sigma Chemical Co., St. Louis, MO; calmodulin was from the Sciogen Corp., Detroit, MI; and Hespan (hydroxyethyl starch) was from American Critical Care, McGaw Park, IL.

Methods. PL-Ca-PK was purified (80–95% homogeneous) from bovine heart extracts through the step of phosphatidylserine-Affi-Gel 102 [12] or purified to homogeneity from pig spleen extracts through the step of phosphatidylserine-Affi-Gel 10 [13] as reported recently. The enzyme was also purified (about 80% homogeneous) from pig brain extracts according to the procedures described for the cardiac enzyme (R. S. Turner and J. F. Kuo, unpublished data). Acute monocytic leukemia (AML) cells were obtained from human leukemic patients undergoing therapeutic leukapheresis. The cells were further enriched by being allowed to sediment for 1 hr at room temperature in the presence of 10% Hespan. The cells were then suspended and sonicated in 5 vol. (2.5 ml) of ice-cold 50 mM Tris/HCl (pH 7.5) containing 50 mM 2-mercaptoethanol, 1 mM PMSF, 2 mM ethyleneglycolbis(amino-ethylether)tetraacetate (EGTA) and 0.1% Triton X-100. The sonicate was gently stirred for 1 hr at 4° and finally centrifuged at 100,000 *g* for 1 hr. The resultant supernatant fluid (total, solubilized fraction) of AML

cells was used directly as the source of PL-Ca-PK. The activity level of PL-Ca-PK in AML cells was found to be very high, comparable to the levels seen in rat brain and spleen [14] and human normal neutrophils [15]. MLCK [16] and unfractionated myosin light chains [17] were purified from bovine heart by the methods previously reported. Calmodulin/Ca^{2+}-stimulated phosphodiesterase [18], cyclic AMP-dependent protein kinase (A-PK) [19], cyclic GMP-dependent protein kinase (G-PK) [19] and its stimulatory modulator [20], all from bovine heart, also were prepared as described previously. [γ-P^{32}]ATP was prepared by the method of Post and Sen [21], and protein was determined according to Bradford [22].

PL-Ca-PK using histone H1 as substrate [12, 14] MLCK using myosin light chains as substrate [15], A-PK and G-PK both using mixed histone as substrate [19, 20] and PDE using cyclic AMP as substrate [18, 23] were assayed as described in the references cited. The enzyme activities were linear with respect to the amount of the enzyme and the time of incubation under the assay conditions. Phosphorylation of endogenous proteins in the particulate fraction of rat brain and subsequent sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography of the phosphorylated proteins were performed as described [24]. R-24571 was dissolved in dimethyl sulfoxide (DMSO); the agent was stable for about 4 days at 4°. The final concentration of DMSO in the incubation mixtures (including the controls) was 0.5% (v/v), which was without effect on the enzyme activities. All assays were carried out in plastic tubes, because R-24571 sticks to the glass surface in aqueous solutions due to its lipophilicity [9].

RESULTS

The antimycotic miconazole derivative R-24571, at 7 μM, was found to inhibit 68–81% of the Ca^{2+}-dependent activity of PL-Ca-PK (a phospholipid/Ca^{2+}-stimulated protein kinase) from various tissues and to inhibit 97% of that of the cardiac MLCK (a calmodulin/Ca^{2+}-stimulated protein kinase) (Table 1). At the same concentration, it also markedly inhibited (93%) a cardiac calmodulin/Ca^{2+}-stimulated PDE, an effect similar to that reported previously by others [10]. The compound, in comparison, was essentially without effect on the cyclic nucleotide-dependent class of protein kinases, i.e. A-PK and G-PK (Table 1).

The Ca^{2+}-dependent enzymes were inhibited by R-24571 in a concentration-related manner (Fig. 1). The IC_{50} values for the effect of R-24571 on the enzymes, under the present assay conditions, were: PL-Ca-PK, 5.3 μM; MLCK, 1.6 μM; and PDE, 0.1 μM. R-24571 appeared to inhibit specifically the Ca^{2+}-dependent activities (in the presence of either phosphatidylserine or calmodulin) of the enzymes without appreciably affecting their basal activities.

Table 1. Comparative effects of R-24571 on various Ca^{2+}-dependent and cyclic nucleotide-dependent enzymes*

Enzyme	Activator	Enzyme activity (pmoles P transferred or cyclic AMP hydrolyzed/min)	
		Control	R-24571
PL-Ca-PK			
Bovine heart	None	0.37	0.41
	$CaCl_2$	5.20	1.32 (81)
Pig brain	None	0.43	0.30
	$CaCl_2$	13.30	3.40 (76)
Pig spleen	None	0.08	0.13
	$CaCl_2$	2.50	0.90 (68)
Human AML cells	None	14.60	14.50
	$CaCl_2$	41.22	21.01 (76)
MLCK	None	2.50	2.57
	$CaCl_2$	43.37	3.80 (97)
PDE	None	0.34	0.30
	$CaCl_2$	2.34	0.44 (93)
A-PK	None	2.70	3.50
	Cyclic AMP	32.34	31.55 (5)
G-PK	None	0.81	0.90
	Cyclic GMP	5.51	5.21 (8)

* PL-Ca-PK from bovine heart (0.1 μg), pig brain (0.9 μg), pig spleen (0.1 μg) or human AML cells (8.5 μg) was assayed (in 0.2 ml) in the presence of phosphatidylserine (2 μg) and EGTA (0.2 mM), with or without $CaCl_2$ (0.5 mM); MLCK from bovine heart (2.5 μg) was assayed (in 0.2 ml) in the presence of calmodulin (0.5 μg) and EGTA (0.05 mM), with or without $CaCl_2$ (0.1 mM); A-PK from bovine heart (5 μg) or G-PK from bovine lung (3 μg) was assayed (in 0.2 ml) with or without cyclic AMP (0.5 μM) or cyclic GMP (0.5 μM); PDE from bovine heart (3 μg) was assayed (in 0.1 ml) in the presence of calmodulin (1 μg) and EGTA (0.05 mM), with or without $CaCl_2$ (0.1 mM). When R-24571 was present, the concentration was 7 μM. The percent inhibitions by R-24571 (indicated in parentheses) were defined as follows: (the net activities stimulated by the activators seen in the presence of R-24571/the net activities stimulated by the activators seen in the absence of R-24571) × 100. The values presented are the average of duplicate incubations, with assay errors being less than 3%.

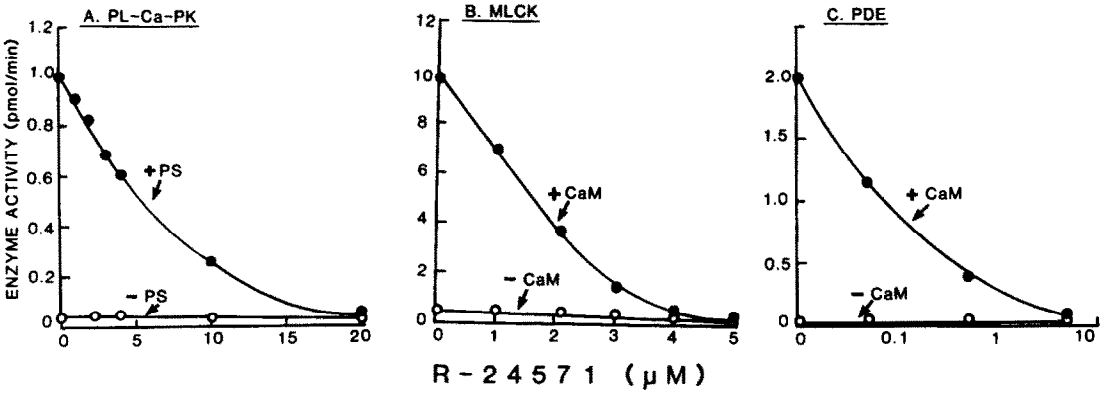

Fig. 1. Inhibition of various Ca^{2+}-dependent enzymes by R-24571. (A) PL-Ca-PK (0.05 μg) was incubated (0.2 ml) in the presence of $CaCl_2$ (0.5 mM) and EGTA (0.2 mM), with or without phosphatidylserine (10 μg/ml); (B) MLCK (2.5 μg) was incubated in the presence of $CaCl_2$ (0.1 mM) and EGTA (0.05 mM), with or without calmodulin (2.5 μg/ml); (C) PDE (2.5 μg) was incubated in the presence of $CaCl_2$ (0.1 mM) and EGTA (0.05 mM), withor without calmodulin (10 μg/ml). The enzymes were purified from bovine heart extracts and assayed by the methods mentioned. The enzyme activities were expressed as pmoles (phosphate transferred or cyclic AMP hydrolyzed)/min.

Inhibition of PL-Ca-PK by R-24571 was progressively reversed by increasing concentrations of phosphatidylserine, with an apparently complete recovery seen at the highest concentration (50 μg/ml) of the phospholipid cofactor tested (Fig. 2A). Similarly, inhibition of MLCK (Fig. 2B) and PDE (Fig. 2C) by R-24571 was also overcome by increasing concentrations of calmodulin, the protein cofactor for the enzymes. These results clearly suggested that the lipophilic agent R-24571 interacted with phosphatidylserine or a hydrophibic region on calmodulin of the calmodulin–Ca^{2+} complex, leading to a diminished ability of the cofactors to activate the enzymes in the presence of Ca^{2+}. Furthermore, the findings of a complete inhibition by 4 μM R-24571 of PL-Ca-PK, stimulated by a low concentration (1.5 μg/ml) of phosphatidylserine (Fig. 2A), and of MLCK, stimulated by low concentrations (up to 1.5 μg/ml) of calmodulin (Fig. 2B), indicated the existence of certain stoichiometric interactions between the agent and the cofactors.

Detailed kinetic analysis of the R-24571 inhibition of PL-Ca-PK and MLCK was performed using saturating concentrations of the cofactors and concentrations of the agent lower than those used in Fig. 2. Dixon plots of the inhibition of PL-Ca-PK (Fig. 3A) and MLCK (Fig. 3B) by R-24571 revealed a noncompetitive type of inhibition, with K_i values of 14.5 ± 0.2 μM (mean ± S.E., N = 3) and 2.1 ± 0.2 μM (N = 3) respectively. The data from Figs. 2 and 3 supported possible complex interactions of R-24571 with the enzymes, cofactors, and/or the enzyme–cofactor complexes for the two Ca^{2+}-dependent protein kinases.

The above possibility was further supported by the observation that phosphatidylserine was able to overcome completely the R-24571 inhibition of MLCK stimulated by calmodulin and Ca^{2+} (Fig. 4). Because phosphatidylserine, at all concentrations tested, was without effect on the MLCK activity (Fig. 4), it was concluded that the phospholipid interacted with R-24571 even in the presence of calmodulin, thus preventing the agent from inhibiting MLCK activation.

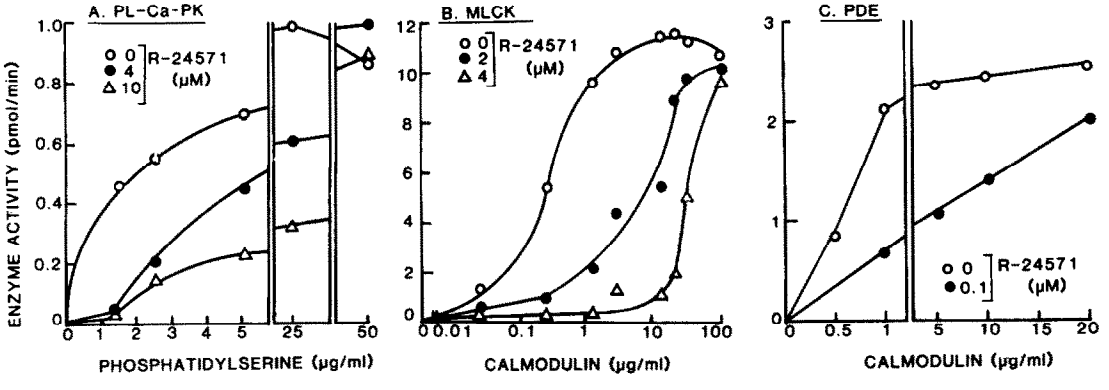

Fig. 2. Restoration of R-24571 inhibition of Ca^{2+}-dependent enzymes by phosphatidylserine or calmodulin. (A) PL-Ca-PK, (B) MCLK, and (C) PDE were incubated with $CaCl_2$ (in the presence of EGTA) as mentioned in Fig. 1, and in the presence or absence of various concentrations of R-24571 and one of the cofactors (phosphatidylserine or calmodulin), as indicated.

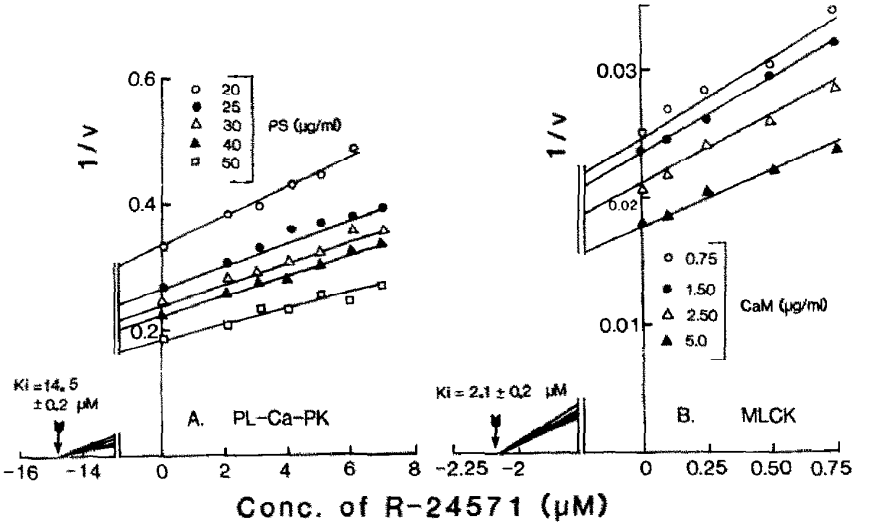

Fig. 3. Dixon plots showing inhibition of cardiac PL-Ca-PK (A) and cardiac MLCK (B) by R-24571 as a function of phosphatidylserine and calmodulin respectively. The protein kinases were assayed at saturating concentrations of cofactors, ATP and substrates. The enzyme activities were corrected for the basal values seen in the absence of the cofactors. Data represented are the means of three independent experiments.

We reported previously that the particulate fraction of rat brain contains two substrate proteins (mol. wt 18,000 and 14,000) for PL-Ca-PK and two other substrates (mol. wt 66,000 and 58,000) for a calmodulin/Ca^{2+}-stimulated protein kinase [5, 24]. The 18,000 and 14,000 mol. wt proteins were identified subsequently to be large and small myelin basic proteins, respectively [25], and myelin basic protein has been shown to be an exceptionally effective substrate for PL-Ca-PK [6]. In the present studies, we found that R-24571 inhibited phosphorylation of all these brain proteins (Fig. 5). It appeared that phosphorylation of the 66,000 and 58,000 mol. wt proteins, compared to that of the 18,000 and 14,000 mol. wt proteins, was more sensitive to inhibition by R-24571 (Fig. 5). These results with endogenous substrate phosphorylation were in line with the data shown earlier in Figs. 1–3, in which the agent inhibited the calmodulin-sensitive phosphorylation of myosin light chain by the purified MLCK slightly more effectively than it inhibited the phospholipid-sensitive phosphorylation of an exogenous protein (histone H1) by the purified PL-Ca-PK.

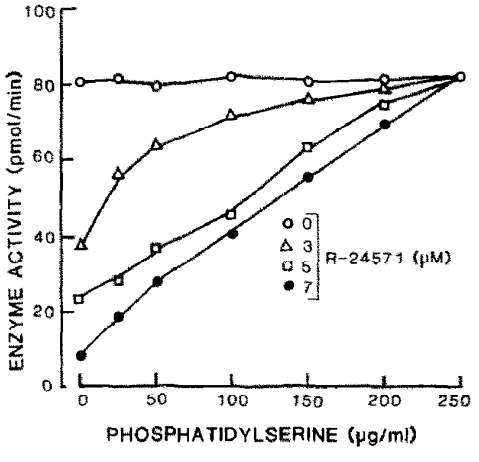

Fig. 4. Restoration of R-24571 inhibition of MLCK by phosphatidylserine. The enzyme (1.2 μg) was incubated (in 0.2 ml) in the presence of calmodulin (0.5 μg), $CaCl_2$ (0.1 mM) and EGTA (0.5 mM), with various concentrations of R-24571 and phosphatidylserine, as indicated. The stimulation of the enzyme by calmodulin and Ca^{2+} was 35-fold in the absence of R-24571, which had no effects on the basal activity of the enzyme.

DISCUSSION

The present studies indicate that R-24571 inhibited MLCK, as it has been shown previously to inhibit other species of the calmodulin/Ca^{2+}-stimulated enzymes [9–11]. The present studies also showed that PL-Ca-PK, an enzyme stimulated by phosphatidylserine/Ca^{2+}, was also inhibited by the agent with a comparable potency. It is of some interest that the Ca^{2+}-dependent protein kinases appeared to be more resistant than PDE to R-24571 inhibition. The IC_{50} values for PL-Ca-PK and MLCK were 5.3 and 1.6 μM respectively (Fig. 1); for phosphorylase *b* kinase it was reported as 5 μM [10], compared to much lower values of 0.1 μM (Fig. 1) and 0.005 to 0.01 μM [10] shown for PDE.

R-24571 appeared to inhibit PL-Ca-PK, MLCK and PDE competitively with respect to phosphatidylserine and calmodulin because the cofactors seemed to be able to overcome the inhibition (Fig. 2). The analysis of the data shown in Fig. 2 (A andB) revealed that the inhibition was complex and did not obey classical Michaelis–Menten kinetics. This was particularly true at lower concentrations of the cofactors where the PL-Ca-PK and MLCK were totally or nearly totally inhibited by R-24571, prob-

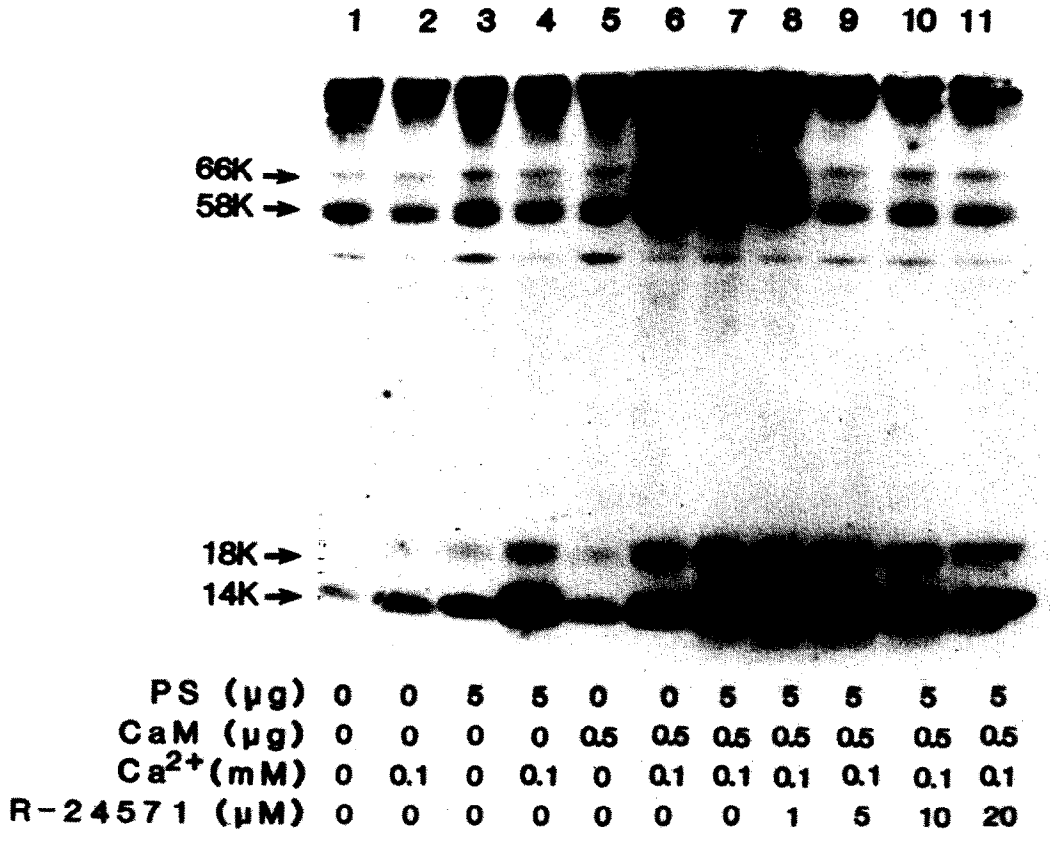

Fig. 5. Inhibition by R-24571 of Ca^{2+}-dependent phosphorylation of endogenous proteins in the particulate fraction of rat brain. The phosphorylation (in 0.2 ml) was carried out in the presence of EGTA (0.05 mM), with or without additions, as indicated. Abbreviations: PS, phosphatidylserine; and CaM, calmodulin.

ably due to certain stoichiometric interactions of R-24571 with the cofactors. Detailed kinetic studies in separate experiments, using saturating concentrations of the cofactors, however, indicated that R-24571 inhibited both PL-Ca-PK and MLCK noncompetitively with respect to their respective cofactors (Fig. 3). These results suggested that, under these reaction conditions, R-24571 does not interact with the enzyme in the same manner as, or at the same sites where, the cofactors would interact; R-24571, therefore, appeared to interact with the cofactors and/or the cofactor–enzyme complexes. It was reported that R-24571 directly interacts with the proteolysis-activated Ca^{2+}-ATPase (another calmodulin/Ca^{2+}-dependent enzyme) in the absence of calmodulin [11]. It is conceivable, therefore, that the inhibitory effect of R-24571 on PL-Ca-PK and MLCK may result from complex interactions of the agent with the enzymes, the cofactors, and the enzyme–cofactor complexes.

It is obvious that there was little difference in the inhibitory potency of R-24571 toward various Ca^{2+}-stimulated protein kinases utilizing either phospholipid (PL-Ca-PK) or calmodulin (MLCK and phosphorylase *b* kinase) as cofactor. R-24571, however, was far more effective in inhibiting calmodulin/Ca^{2+}-stimulated PDE. The inhibition by other calmodulin antagonists or anticalmodulins, notably trifluoperazine [4–6, 25] and W-7 [6–8, 25], of the PL-Ca-PK system has been reported previously. In addition, we have reported that many other agents, including palmitoylcarnitine [26], alkyllysophospholipid [27], adriamycin [26], polyamines [28], and a number of amphipathic polypeptides such as melittin [15], polymyxin B [29] and a number of cobra and marine worm toxins [30], in most cases inhibit PL-Ca-PK and MLCK with comparable potencies. From the foregoing, it is obvious that the mechanisms underlying the pharmacological effect of these agents, including those commonly known as calmodulin antogonists, need to be interpreted with care.

REFERENCES

1. B. Weiss and T. L. Wallace, *Calcium and Cell Function* (Ed. W. Y. Cheung), Vol. 1, pp. 329–79. Academic Press, New York (1980).

2. M. Kanamori, M. Naka, M. Asano and H. Hidaka, *J. Pharmac. exp. Ther.* **217**, 494 (1981).
3. H. Hidaka, M. Naka and T. Yamaki, *Biochem. biophys. Res. Commun.* **90**, 649 (1979).
4. R. C. Schatzman, B. C. Wise and J. F. Kuo, *Biochem. biophys. Res. Commun.* **98**, 669 (1981).
5. R. W. Wrenn, N. Katoh. R. C. Schatzman and J. F. Kuo, *Life Sci.* **29**, 725 (1981).
6. B. C. Wise, D. B. Glass, C-H. J. Chou, R. L. Raynor, N. Katoh, R. C. Schatzman, R. S. Turner, R. F. Kibler and J. F. Kuo, *J. biol. Chem.* **257**, 8489 (1982).
7. T. Tanaka, T. Ohmura, T. Yamakado and H. Hidaka, *Molec. Pharmac.* **22**, 408 (1982).
8. R. C. Schatzman, R. L. Raynor and J. F. Kuo, *Biochim. biophys. Acta* **755**, 144 (1983).
9. K. Gietzen, A. Wuthrich and H. Bader, *Biochem. biophys. Res. Commun.* **101**, 418 (1981).
10. H. Van Belle, *Cell Calcium* **2**, 483 (1981).
11. E. S. Adunyah, B. Niggli and E. Carafoli, *Fedn Eur. Biochem. Soc. Lett.* **143**, 65 (1982).
12. B. C. Wise, R. L. Raynor and J. F. Kuo, *J. biol. Chem.* **257**, 8481 (1982).
13. R. C. Schatzman, R. L. Raynor, R. B. Fritz and J. F. Kuo, *Biochem. J.* **209**, 435 (1983).
14. J. F. Kuo, R. G. G. Andersson, B. C. Wise, L. Mackerlova, I. Salomonsson, N. L. Brackett, N. Katoh, M. Shoji and R. W. Wrenn, *Proc. natn. Acad. Sci. U.S.A.* **77**, 7039 (1980).
15. 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).
16. M. P. Walsh, B. Vallet, F. Autric, and J. G. Demaille, *J. biol. Chem.* **254**, 12136 (1979).
17. W. T. Perrie and S. V. Perry, *Biochem. J.* **119**, 31 (1970).
18. H. C. Ho, E. Wirch, F. C. Stevens and J. H. Wang, *J. biol. Chem.* **252**, 43 (1977).
19. M. Shoji, J. G. Patrick, C. W. Davis and J. F. Kuo, *Biochem. J.* **161**, 213 (1977).
20. M. Shoji, N. L. Brackett, J. Tse, R. Shapira and J. F. Kuo, *J. biol. Chem.* **253**, 3427 (1978).
21. R. L. Post and A. K. Sen, *Meth. Enzym.* **10**, 773 (1967).
22. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
23. N. Katoh, D. M. Helfman, R. W. Wrenn and J. F. Kuo, *Biochim. biophys. Acta* **714**, 129 (1982).
24. R. W. Wrenn, N. Katoh, B. C. Wise and J. F. Kuo, *J. biol. Chem.* **255**, 12042 (1980).
25. R. S. Scott, C-H. J. Chou, R. F. Kibler and J. F. Kuo, *J. Neurochem.* **39**, 1397 (1982).
26. B. C. Wise and J. F. Kuo, *Biochem. Pharmac.* **32**, 1259 (1983).
27. D. M. Helfman, K. C. Barnes, J. M. Kinkade, Jr., W. R. Vogler, M. Shoji and J. F. Kuo, *Cancer Res.* **43**, 2955 (1983).
28. D-F. Qi, R. C. Schatzman, G. J. Mazzei, R. S. Turner, R. L. Raynor, S. Liao and J. F. Kuo, *Biochem. J.* **213**, 281 (1983).
29. G. J. Mazzei, N. Katoh and J. F. Kuo, *Biochem. biophys. Res. Commun.* **109**, 1129 (1982).
30. 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).