Calmodulin Antagonists Inhibit the Mitochondrial Pyruvate Dehydrogenase Complex*

(Received for publication, June 29, 1987)
Jan A. Miernyk†, Tung K. Fang, and Douglas D. Randall
From the Biochemistry Department, University of Missouri, Columbia, Missouri 65211

Calmodulin antagonists, including phenothiazine, sulfonamide, butyrophenone, and imidazolid derivatives, were in vitro inhibitors of pea mitochondrial pyruvate dehydrogenase complex activity. Inhibition was observed both during direct assay of the partially purified complex and during assay of pyruvate oxidation by isolated, intact mitochondria. When tested against the purified complex, the sulfonamide compound N-(6-aminohexyl)-3-chloro-1-naphthalene sulfonamide (W-7) was a competitive inhibitor with respect to coenzyme A and an uncompetitive inhibitor with respect to NAD and pyruvate. Inhibition of a process as crucial as mitochondrial respiration should serve to emphasize the care necessary in interpretation of whole-organism calmodulin antagonist studies.

The mitochondrial pyruvate dehydrogenase complex is regulated in part by reversible phosphorylation (1, 2). An intrinsic kinase (pyruvate dehydrogenase (lipoamide)) kinase (EC 2.7.1.99), inactivates the complex through Mg-ATP-dependent phosphorylation of the pyruvate dehydrogenase (EC 1.2.4.1) component. Reactivation of pyruvate dehydrogenase complex is catalyzed by a Mg-dependent [pyruvate dehydrogenase (lipoamide)]-phosphatase (EC 3.1.3.43). While calcium does not activate mammalian [pyruvate dehydrogenase (lipoamide)]-phosphatase, it does stimulate the magnesium-dependent activation (3). Stimulation is the result of tighter binding of the phosphatase to the dihydrolipoamide acetyltransferase (EC 2.3.1.12) core of the complex. This lowers the Km of the phosphatase for [pyruvate dehydrogenase (lipoamide)]-phosphatase by 20-fold (4). In contrast, micromolar concentrations of calcium antagonize the magnesium-dependent activation of plant mitochondrial [pyruvate dehydrogenase (lipoamide)]-phosphatase (5, 7). We recently proposed that inhibition of plant phosphatase activity could be mediated by calmodulin, and that CaMII might be a regulatory subunit of plant mitochondrial pyruvate dehydrogenase complex (7). Testing this proposal through the use of "specific" CaM antagonists was complicated by an apparent inhibition of overall complex activity. Herein we present a more thorough investigation of this inhibition.

MATERIALS AND METHODS

The CaM antagonists, calmidazolium, chlorpromazine, W-5, W-7, and W-13 were purchased from the Sigma. Squibb Canada provided the fluphenazine. Janssen Pharmaceutica, Inc. supplied pimozide, fluspirilene, and penfluridol. Stock solutions of all antagonists were made up in either 95% (v/v) ethanol or dimethyl sulfoxide. Controls verified that the amounts of solvent alone added to the assays were without effect.

The isolation of a mitochondria-enriched fraction from light-grown pea seedlings (Pisum sativum L. cv. Little Marvel) has been previously described (6). Mitochondria were then purified by rate-zonal sedimentation on two sequential discontinuous Percoll gradients. The final mitochondrial fractions were 97-98% intact and were completely free of cytosolic or chloroplast contamination. A detailed description of the isolation procedure will be presented elsewhere.

The activity of pyruvate dehydrogenase complex in isolated, intact mitochondria was measured using a Clark-type oxygen electrode system from Hansatech, Ltd. Reaction medium contained 300 mM mannitol, 3 mM MgCl2, 1 mM EDTA, 20 mM KCl, 5 mM KPi, 0.2 mM thiamine pyrophosphate, 0.12 mM CoA, 1 mM NAD, 5 mM pyruvate, 0.1 mM L-malate, 20 mM TES, pH 7.5, 0.1% (w/v) fatty acid-free bovine serum albumin, and 0.1 to 0.3 mg of mitochondrial protein in a 1-ml reaction vessel. It was assumed that air-saturated medium contained 250 μM O2.

All other materials and methods have been previously described (6-8).

RESULTS AND DISCUSSION

Having previously observed inhibition of pea mitochondrial pyruvate dehydrogenase complex activity by low concentrations of two different phenothiazine compounds (7), we chose to systematically examine representatives of three structurally diverse classes of CaM antagonists: phenothiazines, sulfonamides, butyrophenones, plus a single imidazolid derivative. Each of the antagonists inhibited the in vitro activity of partially purified pyruvate dehydrogenase complex (Fig. 1). Concentrations of antagonists giving 50% inhibition of enzyme activity (I50) ranged from 100 to 720 μM, at saturating concentrations of all substrates. A quantitative analysis of the inhibition by each of the nine antagonists studied is presented in Table I.

We have recently developed an assay for pyruvate dehydrogenase complex activity in isolated, intact pea mitochondria, based upon pyruvate-dependent oxygen uptake. Concentrations of W-7 which inhibited the partially purified pyruvate dehydrogenase complex also inhibited pyruvate oxidation by purified mitochondria (Fig. 2). These results indicate that the sulfonamide antagonist W-7 penetrates the mitochondrial membranes and inhibit the pyruvate dehydrogenase complex of whole-organism calmodulin antagonist studies.
CaM Antagonists Inhibit Pyruvate Dehydrogenase Complex Activity

**Fig. 1.** Inhibition of the partially purified pea mitochondrial pyruvate dehydrogenase complex by selected calmodulin antagonists. Original activity was 160 nmol min⁻¹ assay⁻¹.

**Table I**

Quantitative analyses of the inhibition of pyruvate dehydrogenase complex activity by calmodulin antagonists

| Antagonist  | I₅₀ | Kᵣ |
|-------------|-----|-----|
| Fluphenazine| 175 | 20  |
| Chlorpromazine| 230 | ND* |
| W-5         | 720  | 154 |
| W-7         | 190  | 29  |
| W-13        | 140  | ND* |
| Calmidazolium| 100 | ND* |
| Fluspirilene| 450  | 48  |
| Pimozide    | 300  | ND* |
| Penfluridol | 340  | ND* |

* ND, not determined.

**Fig. 2.** Inhibition of pyruvate-dependent oxygen uptake by isolated, intact pea mitochondria incubated with the sulfonamide antagonist W-7.

Having demonstrated that each of the four classes of antagonists was inhibitory, kinetic analyses were performed with representative compounds. By holding two substrates at fixed concentrations and the antagonist at fixed variable concentrations and by varying the concentration of the third substrate, it was established that inhibition by W-7 was uncompetitive with respect to NAD and pyruvate, but competitive with respect to CoA (Fig. 3). Similar results were obtained with pimozide or fluphenazine as inhibitors (data not presented). The nature of inhibition by calmidazolium was not further analyzed due to its low solubility in aqueous systems (9). The observed patterns of inhibition are consistent with the hexa-uni ping-pong mechanism proposed for the pyruvate dehydrogenase complex (10). Replots of the inhibition data allowed calculation of Kᵣ values for W-5, W-7, pimozide, and fluphenazine (Table I).

There is a wide structural diversity among calmodulin antagonists. It is thought that the antagonists bind directly to CaM by both hydrophobic and ionic interactions (11). It has, however, been clearly demonstrated that neither hydrophobicity nor charge alone conveys inhibitory activity. All of the antagonists tested as inhibitors contain a planar aromatic nucleus. It seems likely that this structure interferes with the interaction of coenzyme A and a hydrophobic binding site in the pyruvate dehydrogenase complex.

Among the sulfonamide compounds tested, W-5 was a much weaker inhibitor of pyruvate dehydrogenase complex activity than W-7 or W-13. The only structural difference between W-5 and W-7 is substitution with chlorine at position 5 of the naphthalene ring system (12). It is interesting to note that the order of inhibition of pyruvate dehydrogenase complex activity, W-13 > W-7 > W-5, is identical to the pattern of CaM antagonist activity (12).

The role of CaM as a mediator of several plant processes is understood at the molecular level (13). Other regulatory roles have been inferred from experiments using ostensibly specific CaM antagonists (e.g. see Refs. 14-16). Gilroy et al. (17) recently proposed one alternate explanation for some of the effects of CaM antagonists upon diverse plant processes. Our results also suggest that some apparent effects could be secondary responses and emphasize that care must be taken in
interacting the results of whole-plant studies using these antagonists.

REFERENCES
1. Hucho, F., Randall, D. D., Roche, T. E., Burgett, M. W., Pelley, J. W., and Reed, L. J. (1972) Arch. Biochem. Biophys. 151, 329-340
2. Miernyk, J. A., Camp, P. J., and Randall, D. D. (1985) Curr. Top. Plant Biochem. Physiol. 4, 175-190
3. Denton, R. M., Randle, P. J., and Martin, B. R. (1972) Biochem. J. 128, 161-163
4. Pettit, F. H., Roche, T. E., and Reed, L. J. (1972) Biochem. Biophys. Res. Commun. 49, 563-571
5. Randall, D. D., Williams, M., and Rapp, B. J. (1981) Arch. Biochem. Biophys. 207, 437-444
6. Miernyk, J. A., and Randall, D. D. (1987) Plant Physiol. (Bethesda) 83, 306-310
7. Miernyk, J. A., and Randall, D. D. (1987) Plant Physiol. (Bethesda) 83, 311-315
8. Camp, P. J., and Randall, D. D. (1985) Plant Physiol. (Bethesda) 77, 571-577
9. Gietzen, K., Wuthrich, A., and Bader, H. (1981) Biochem. Biophys. Res. Commun. 101, 418-425
10. Cleland, W. W. (1973) J. Biol. Chem. 248, 8353-8355
11. Weiss, B., Sellinger-Barnette, M., Winkler, J. D., Schechter, L. E., and Prozialeck, W. C. (1986) in Calmodulin Antagonists and Cellular Physiology (Hidaka, H., and Hartshorne, D. J., eds) pp. 45-62, Academic Press, Orlando, FL
12. Hidaka, H., Sasaki, Y., Tanaka, T., Endo, T., Ohno, S., Fujii, Y., and Nagata, T. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 4354-4357
13. Marmé, D. (1985) Curr. Top. Plant Biochem. Physiol. 4, 166-174
14. Roux, S. J., McEntire, K., Slocum, R. D., Cedel, T. E., and Hale, C. C., II (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 283-287
15. Elliott, D. C., Batchelor, S. M., Cassas, R. A., and Maners, G. M. (1983) Plant Physiol. (Bethesda) 72, 219-224
16. Veluthambi, K., and Pooviah, B. W. (1986) Plant Physiol. (Bethesda) 81, 836-841
17. Gilroy, S., Hughes, W. A., and Trewavas, A. J. (1987) FEBS Lett. 212, 133-137