Biochemistry

 Volume 26, Number 1 January 13, 1987

Accelerated Publications

Inhibition of Malic Enzyme by *S*-Oxalylglutathione, a Probable in Vivo Effector†

Roger K. Harris and Gordon A. Hamilton*

Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802

Received October 1, 1986; Revised Manuscript Received November 6, 1986

ABSTRACT: Various oxalyl thiol esters ($RSCOCOO^-$), especially *S*-oxalylglutathione (GS-Ox), were found to be very effective inhibitors of chicken liver malic enzyme. When the conditions are similar to those encountered physiologically [high reduced nicotinamide adenine dinucleotide phosphate (NADPH) concentrations], inhibition is detectable with less than 1 μM concentrations of GS-Ox. The amount of inhibition is not reversed by excess glutathione, thus indicating that it is not due to oxalyl transfer to some enzymic thiol group with release of glutathione. Detailed kinetic studies show that the inhibition by GS-Ox can be treated as a simple reversible binding to the enzyme; the double reciprocal plot patterns indicate that the inhibition is linear noncompetitive (mixed type), vs. both L-malate in the oxidative decarboxylation reaction and pyruvate in the reverse reaction. At pH 7.4 and 25 °C in the presence of 100–200 μM NADPH, the K_{is} and K_{ii} values for GS-Ox are 0.7 and 5 μM, respectively, and are the same for reactions run in either direction. The high specificity for GS-Ox is indicated by the observation that, under similar conditions, the K_{is} values for *S*-oxalyl coenzyme A and *S*-oxalyl-*N*-acetylcysteamine are 40 and 150 μM, respectively. Such high specificity indicates that the enzyme has evolved a specific binding site for the glutathione part of GS-Ox. The current results, when considered in conjunction with recent evidence that oxalyl thiol esters are present in animal tissues at concentrations up to 50 μM, imply that GS-Ox is an important in vivo regulator of malic enzyme. Probable mechanisms for the inhibition by GS-Ox, as well as the possibility that oxalyl thiol esters may be functioning as part of the intracellular messenger system for insulin, are also briefly discussed.

Several different lines of research in our laboratory have recently led to the hypothesis (Hamilton, 1985) that oxalyl thiol esters ($RSCOCOO^-$) may be functioning as metabolic effectors in animals and possibly as intracellular messengers for some hormones, especially insulin. Oxalyl thiol esters are formed as the direct product of the likely physiological reaction catalyzed by L-hydroxy acid oxidase (Brush & Hamilton, 1981, 1982; Hamilton & Brush, 1982; Gunshore et al., 1985) and could possibly result from further metabolism of the products formed in the suspected physiological reactions catalyzed by D-amino acid oxidase (Hamilton et al., 1979; Naber et al., 1982; Venkatesan & Hamilton, 1986) and D-aspartate oxidase (Burns et al., 1984). Regardless of the mechanism for their formation, it has very recently been demonstrated that oxalyl thiol esters are indeed present in animal tissues (Skorczynski & Hamilton, 1986) and in nontrivial amounts (up to 50 μM). Given their presence in animal cells, and many indications for their involvement in controlling animal metabolism (Hamilton, 1985; Hamilton et al., 1982; Hamilton & Buckthal, 1982), it seemed imperative to investigate the effects that such compounds have on the catalytic activities of various enzymes, especially those known to be modified by insulin. Their inhibitory effects on the activity of the catalytic subunit of phosphorylase phosphatase have recently been reported (Gunshore & Hamilton, 1986); summarized here is a study of their effects on malic enzyme.

Cytosolic malic enzyme (Frenkel, 1975; Hsu, 1982; Park et al., 1986) catalyzes the reaction shown in eq 1 and is thought

$$^-OOCCH_2\underset{OH}{\underset{|}{C}}HCOO^- + NADP^+ \rightleftharpoons CO_2 + CH_3\overset{O}{\overset{\|}{C}}COO^- + NADPH \quad (1)$$

to be responsible for generation of the NADPH[1] that supports

† This research was supported by Research Grants AM 13448 and AM 37451 from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, U.S. Public Health Service.

0006-2960/87/0426-0001$01.50/0

fatty acid synthesis. Insulin is known to stimulate fatty acid synthesis, and it increases the catalytic activity of malic enzyme by two distinct mechanisms (Drake et al., 1983). By one mechanism it increases the quantity of the enzyme, presumably by stimulating new synthesis, and by the other mechanism, it increases the specific activity of already existing enzyme. Since our correlations (Hamilton, 1985) suggest that oxalyl thiol esters may be negative messengers for insulin (i.e., that insulin causes a decrease in their concentration), it is predicted that such compounds should thus inhibit malic enzyme if they in fact participate in the intracellular messenger system for this hormone. As reported here, oxalyl thiol esters, especially *S*-oxalylglutathione, are indeed very potent inhibitors of malic enzyme.

Experimental Procedures

Unless otherwise noted, the materials and methods were the same as those previously described (Gunshore & Hamilton, 1986). All water used in this work was glass distilled and then passed through a Millipore (Milli Q) reverse osmosis water purification system. Chicken liver malic enzyme was obtained from Sigma Chemical Co. and was shown to be greater than 95% pure by SDS gel electrophoresis. Its catalytic activity varied from lot to lot, but unless otherwise noted, the experiments reported here were performed with material having a specific activity of approximately 21 units/mg (1 unit = 1 μmol/min) when measured at 25 °C and pH 7.4 (42 mM HEPES buffer) with 0.10 mM $NADP^+$, 0.10 mM NADPH, 1 mM $MgCl_2$, and 1.0 mM L-malate present. The commercial enzyme is supplied as a suspension in 2.9 M ammonium sulfate, 10 mM potassium phosphate buffer, pH 6.0, 0.5 mM 2-mercaptoethanol, 10 mM $MnCl_2$, and 3 mM EDTA. Stock solutions of this preparation were found to be more stable to storage at 0–5 °C (under N_2) when the solutions also contained 2 mM dithiothreitol, 10 mM maleate, and 5% (v/v) glycerol (these conditions were suggested by P. F. Cook). Thus, when diluted for the kinetic experiments, each reaction solution would contain, in addition to the components given in the tables, 1 mM ammonium sulfate, 3 μM maleate, 0.002% glycerol, 3 μM phosphate, 1 μM EDTA, 3 μM $MnCl_2$, 0.7 μM dithiothreitol, and 0.2 μM 2-mercaptoethanol.

Enzymic reactions were monitored by following the absorption due to the formation or disappearance of NADPH at 340 nm; a Gilford Model 240 spectrophotometer equipped with a thermostated cell compartment and a recorder was used. All assays were carried out at 25 °C and pH 7.4 with the buffer being 42 mM HEPES. The reactions were initiated by adding either L-malate or pyruvate to solutions of the other components that had been temperature equilibrated. The reactions were monitored for approximately 3 min, during which time less than 10% of the substrate is consumed with the amount of enzyme used (usually about 4 μg in 3 mL). Duplicate assays, which agreed within ±5%, were run in all cases. For several of the experiments with inhibitors, the rates are reported as percentages of a control rate; in the control reactions all other conditions were identical except that no oxalyl thiol ester was present.

The reported inhibition constants are those defined by Cleland (1963); K_{is} is the constant that is obtained from the slopes of Lineweaver–Burk plots at varying concentrations of inhibitor, and K_{ii} is the constant obtained from the intercepts of such plots. Data for each Lineweaver–Burk plot were collected at seven different L-malate or pyruvate concentrations (depending on the direction the reaction was run). Since L-malate is an inhibitor of the reaction at high concentrations (Schimerlik et al., 1977), the data with this substrate were obtained with concentrations of 1.0 mM or less, at which concentrations no evidence for substrate inhibition was noted. Data obtained from four different concentrations of inhibitor, in addition to that with no inhibitor, were used to calculate the K_{is} and K_{ii} values in each case. Only those replots of slope and intercept data that gave a correlation coefficient of 0.95 or better were used to calculate the reported constants. In all cases the data were subjected to least-squares linear regression analysis.

Results

In preliminary experiments [for details, see Harris (1986)], it was found that several different oxalyl thiol esters, including *S*-oxalylglutathione (GS-Ox), *S*-oxalyl coenzyme A (CoAS-Ox), and *S*-oxalyl-*N*-acetylcysteamine (NACS-Ox), inhibit malic enzyme. Since GS-Ox is considerably better as an inhibitor than either of the others, most of the subsequent experiments were performed with this compound. It was also found in preliminary experiments that the amount of inhibition does not depend on the length of time the oxalyl thiol ester is in contact with the enzyme; virtually the same amount of inhibition is observed when it is incubated with the enzyme for up to 35 min as is found when it incubates for only 2 min (shorter times were not examined).

Inhibition is observed when either Mg^{2+} or Mn^{2+} as the activator metal ion required for enzymic activity is used. However, all of the results reported here were obtained with Mg^{2+} because it is the probable activator in vivo. Under a given set of conditions, there is no detectable change in the percent inhibition caused by a particular concentration of GS-Ox when the Mg^{2+} concentration is varied from 0.1 to 1.0 mM. At higher Mg^{2+} concentrations there is an apparent decrease in the extent of inhibition (Harris, 1986), but this is probably due to a Mg^{2+}-catalyzed destruction of GS-Ox. This nonenzymic reaction occurs too slowly to affect results obtained with 1 mM or less Mg^{2+}, but at higher concentrations it becomes appreciable. For this reason, and because the physiological concentration of free Mg^{2+} is probably close to 1 mM (Veloso et al., 1973), most of the results reported here were obtained with 1 mM Mg^{2+}.

Summarized in Table I are some quantitative results illustrating the effects that the concentrations of $NADP^+$, NADPH, glutathione (GSH), and GS-Ox have on the catalytic activity of malic enzyme when measured in the direction of oxidative decarboxylation. One surprising observation, which contrasts with results obtained with the catalytic subunit of phosphorylase phosphatase (Gunshore & Hamilton, 1986), is that the amount of inhibition by GS-Ox is unaffected by the presence or absence of GSH. Consequently, most of the subsequent data were collected with 1.0 mM GSH present; the enzyme is more stable in the presence of thiols, and more reproducible kinetics could thus be obtained. Another finding illustrated by the results in Table I is that the amount of inhibition caused by a particular concentration of GS-Ox is greater at low $NADP^+$ concentrations than at high $NADP^+$ concentrations. Furthermore, when NADPH is also present in addition to $NADP^+$, there is a dramatic increase in the extent of inhibition caused by GS-Ox; as can be seen from the results in Table I, inhibition is then detectable with submicromolar concentrations.

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; GSH, glutathione; GS-Ox, *S*-oxalylglutathione; CoAS-Ox, *S*-oxalyl coenzyme A; NACS-Ox, *S*-oxalyl-*N*-acetylcysteamine; NADPH, reduced nicotinamide adenine dinucleotide phosphate; $NADP^+$, oxidized nicotinamide adenine dinucleotide phosphate.

Table I: Effects of Reagent Concentrations on Extent of Inhibition of Malic Enzyme by *S*-Oxalylglutathione (GS-Ox)[a]

[NADP+] (μM)	[NADPH] (μM)	[GSH] (mM)	[GS-Ox] (μM)	catalytic activity: units/mg	catalytic activity: % of control[b]
220	0	0.0	25	8.3[c]	83
220	0	0.0	50	7.3[c]	73
220	0	0.0	100	5.7[c]	57
220	0	0.0	300	4.0[c]	40
220	0	0.0	1000	1.4[c]	14
220	0	1.0	25	17.7	86
220	0	1.0	50	15.1	74
220	0	1.0	100	11.2	52
220	0	1.0	300	6.7	35
220	0	1.0	1000	3.4	16
20	0	1.0	25	11.6	63
20	0	1.0	50	8.2	44
20	0	1.0	100	6.8	36
20[d]	10	1.0	25	6.3	41
20[d]	100	1.0	25	1.02	19
20[d]	200	1.0	25	0.34	11
20[d]	200	1.0	0.5	2.80	89
20[d]	200	1.0	2.0	2.20	70
20[d]	200	1.0	5.0	1.34	43
20[d]	200	1.0	10.0	0.62	21
10[d]	200	1.0	0.5	1.82	93
10[d]	200	1.0	5.0	0.88	45
10[d]	200	1.0	10.0	0.54	28
100[e]	100	0.0	2.0	9.3	50
100[e]	100	1.0	2.0	9.7	50
100[e]	100	2.0	2.0	8.3	39
100[e]	100	5.0	2.0	8.3	41
100[e]	100	10.0	2.0	7.1	38

[a] Reaction conditions: 25 °C, 42 mM HEPES buffer, pH 7.4, 2.0 mM L-malate (except those indicated), and 1.0 mM effective Mg^{2+} concentration (except where noted, the reaction solutions contained 1.5 mM total Mg^{2+} and 0.5 mM EDTA; in all cases where it was checked, the presence of EDTA did not affect the rate as long as the effective Mg^{2+} concentration remained the same). The reactions were initiated by adding L-malate after incubation of GS-Ox with the enzyme and all other components for 2–10 min. [b] Expressed as percent of the rate with all conditions the same except that no GS-Ox was present. [c] These experiments were done with an enzyme preparation that had a lower specific activity (10 units/mg) than usual. [d] No EDTA present so the total Mg^{2+} concentration was 1.0 mM. [e] L-Malate concentration was 1.0 mM.

The extent that the oxidative decarboxylation reaction is inhibited by a given concentration of GS-Ox is also dependent on the L-malate concentration. From Lineweaver–Burk plots of data obtained at varying L-malate and oxalyl thiol ester concentrations, it was found that GS-Ox and other oxalyl thiol esters behave as linear noncompetitive (mixed-type) inhibitors (Segel, 1975). Inhibition constants (K_{is} and K_{ii}, respectively) can be calculated from the slopes and intercepts of such plots; several of these, obtained at various $NADP^+$ and NADPH concentrations, and with various oxalyl thiol esters, are listed in Table II. Also given in Table II (third line) are constants obtained from one series of experiments in which the enzymic reaction was run in reverse, with pyruvate as the variable substrate. Of considerable interest is the finding that the inhibition pattern and inhibition constants are identical for reactions run in the two directions under comparable conditions.

The data in Table II illustrate that GS-Ox is a much better inhibitor than CoAS-Ox or NACS-Ox and that changing the Mg^{2+} concentration from 1.0 to 0.5 mM (second line) has no effect on the extent of inhibition caused by GS-Ox. Other points to note are that the inhibition constants have their lowest values at high NADPH concentrations and, under such conditions, are constant, regardless of the $NADP^+$ concentration. When there is no NADPH present, it is the value of K_{is} that especially increases.

Table II: Inhibition Constants for Inhibition of Malic Enzyme by Oxalyl Thiol Esters at Various Reagent Concentrations[a]

inhibitor (RS-Ox)	[RS-Ox] range (μM)	[$NADP^+$] (μM)	[NADPH] (μM)	K_{is} (μM)	K_{ii}[b] (μM)
GS-Ox	0–5	100	100	0.8	4
GS-Ox[c]	0–5	100	100	0.6	6
GS-Ox[d]	0–4	0	200	0.7	5
GS-Ox	0–5	20	200	0.7	5
GS-Ox	0–5	20	60	1.0	5
GS-Ox	0–5	20	20	1.2	
GS-Ox	0–20	20	0	6	3
GS-Ox	0–100	200	0	20	16
CoAS-Ox[e]	0–100	100	100	40	
NACS-Ox[e]	0–400	100	100	150	320

[a] General reaction conditions: 25 °C, 42 mM HEPES buffer, pH 7.4. Except where noted, the reactions were run in the oxidative decarboxylation direction and the solutions also contained 1.0 mM GSH, 1.0 mM $MgCl_2$, and L-malate, which was varied from 0.14 to 1.0 mM. Under these conditions the malate K_m was 0.32 ± 0.09 mM. [b] Where no number is given the data were not considered accurate enough to give a reliable value. [c] The reaction solutions contained only 0.5 mM $MgCl_2$. [d] These reactions were run in the reductive carboxylation direction; the reaction solutions contained no L-malate but instead had 5.0 mM $NaHCO_3$ and pyruvate, which was varied from 0.5 to 3 mM. Under these conditions the apparent K_m for pyruvate was 0.3 mM. [e] The 1.0 mM GSH in the usual reaction solutions was replaced with either 1.0 mM coenzyme A (in the experiments involving CoAS-Ox) or 1.0 mM *N*-acetylcysteamine (in the experiments involving NACS-Ox). This was necessary because oxalyl exchange among thiols occurs rapidly (Law & Hamilton, 1986) under the reaction conditions employed. Thus, if 1 mM GSH were present, most of the oxalyl thiol ester in solution after a few minutes would be GS-Ox rather than the desired CoAS-Ox or NACS-Ox. In the absence of the oxalyl thiol ester, 1 mM *N*-acetylcysteamine, like 1 mM GSH, had no effect on the rate of the enzymic reaction, but 1 mM coenzyme A decreased the enzymic rate approximately 35% under the conditions of the CoAS-Ox experiments.

Discussion

Perhaps the most important result of the current investigation is the general finding that GS-Ox is a very potent inhibitor of malic enzyme. Such a finding has considerable implications for the control of this enzyme in vivo, but before discussing these, some mechanistic aspects will be considered. The main focus of this study was not to determine its detailed mechanism but rather to generally characterize the inhibition, so that one could evaluate how physiologically significant it might be. Nevertheless, the data allow one to arrive at some conclusions concerning mechanism.

The general mechanistic possibilities that need to be considered for the inhibition are illustrated in eq 2. Malic enzyme

$$\mathrm{ESH + GS\text{-}Ox} \underset{a}{\rightleftharpoons} \mathrm{ESH\cdot GS\text{-}Ox} \underset{b}{\rightleftharpoons} \mathrm{ES\text{-}Ox\cdot GSH} \underset{c}{\rightleftharpoons} \mathrm{ES\text{-}Ox + GSH} \quad (2)$$

is known to have many reactive thiol groups, at least some of which, when modified, cause the enzyme to be inhibited (Tang & Hsu, 1974; Hsu, 1982). Consequently, the enzyme is symbolized as ESH in eq 2. Theoretical possibilities for the inhibition by GS-Ox are (a) simple binding to the enzyme (ESH·GS-Ox), not necessarily at a thiol site, (b) oxalyl transfer to an enzymic thiol with the GSH remaining tightly bound (ES-Ox·GSH), or (c) oxalyl transfer to an enzymic thiol with the GSH being released into solution (ES-Ox + GSH). When the present research was initiated, possibility c seemed very likely because oxalyl group transfer from one thiol to another occurs in seconds to minutes in nonenzymic systems under

physiological conditions (Law & Hamilton, 1986; Hamilton, 1985), and the inhibition of the catalytic subunit of phosphorylase phosphatase by oxalyl thiol esters (Gunshore & Hamilton, 1986) appears to occur, at least partially, by this mechanism. The present data, however, eliminate (c) as a possibility for the inhibition of malic enzyme. The most persuasive evidence is that the inhibition by GS-Ox is not reversed by excess GSH (Table I). This result, coupled with the fact that the amount of inhibition by GS-Ox in the absence of GSH is concentration dependent (i.e., that the equilibrium of eq 2 is not completely to the right), indicates that (c) is an untenable possibility.

The finding that the GS-Ox inhibition can be subjected to kinetic analysis, assuming simple equilibrium binding to the enzyme, suggests possibility a as the mechanism of inhibition, but it would be consistent with possibility b if the interconversion of ESH·GS-Ox and ES-Ox·GSH occurs rapidly. The data would, of course, also be consistent with the inhibited form of the enzyme being the tetrahedral intermediate involved in the conversion of (a) to (b). In any event, the much greater inhibition in the presence of high concentrations of NADPH indicates that GS-Ox preferentially binds to an NADPH–enzyme complex. It has been suggested (Schimerlik et al., 1977) that the rate-controlling step for the overall reaction involves a conformation change of an NADPH–enzyme complex. Possibly the binding of GS-Ox prevents the change from occurring.

Because of the structural similarity of oxalyl thiol esters and pyruvate, the possibility that GS-Ox inhibits by binding at the pyruvate site needs to be considered. The current results do not favor such a mechanism, but one could imagine a set of unlikely circumstances where they would be consistent with it. However, the observations, that GS-Ox is a noncompetitive inhibitor vs. both malate and pyruvate, that K_{is} and K_{ii} are the same for reactions run in either direction, and that they do not vary as the $NADP^+$ concentration is altered when NADPH is in excess, are more consistent with a mechanism involving the binding of GS-Ox at an allosteric site on the NADPH–enzyme complex rather than at the active site. An intriguing possibility is that GS-Ox binds at the bromopyruvate site that Hsu and his co-workers (Chang & Hsu, 1977; Pry & Hsu, 1978; Hsu, 1982) have identified. They have found that treatment of malic enzyme with bromopyruvate under appropriate conditions leads to alkylation of a specific sulfhydryl group on only two of the four subunits of this tetrameric enzyme. The finding that such alkylation completely prevents the enzyme from catalyzing the overall oxidative decarboxylation of malate has been cited by Hsu and his group as strong evidence that malic enzyme catalyzes its reaction by a "half of the sites" mechanism. The observations, that pyruvate does not protect the enzyme from alkylation by bromopyruvate and that the alkylated enzyme still catalyzes the reduction of pyruvate, indicate that the bromopyruvate reacting site is distinct from the pyruvate binding site. It seems possible that the reason this specific α keto acid binding site is present on malic enzyme is to act as a GS-Ox receptor. Binding of GS-Ox to such a site would be an efficient mechanism for controlling the enzyme's activity, because only two GS-Ox molecules would need to bind to eliminate the catalytic activity of all four subunits. Since there is a thiol at this bromopyruvate site, the possibility becomes greater that GS-Ox may in fact be functioning by causing some covalent modification, as considered earlier.

It seems very likely that the inhibition of malic enzyme by GS-Ox is an important mechanism for controlling this enzyme's activity in vivo. The high specificity for GS-Ox suggests that the enzyme has evolved a specific binding site for this compound, which in turn implies, therefore, that it is a normal physiological effector of the enzyme. As indicated earlier, oxalyl thiol esters have now been identified in animal tissues (Skorczynski & Hamilton, 1986), and in rat liver their concentration can be as high as 25 μM. Since glutathione is by far the most abundant thiol in the cell (Cooper, 1983; Meister & Anderson, 1983), at equilibrium, most of the cellular oxalyl thiol ester molecules would be GS-Ox. Consequently, its concentration is in the range that causes effective inhibition of malic enzyme when NADPH concentrations are high. That is exactly the situation the enzyme encounters in vivo; the ratio of NADPH to $NADP^+$ concentrations in liver cytosol, where malic enzyme resides, is about 80 (Krebs & Veech, 1969).

Malic enzyme is now the second enzyme shown to be inhibited by oxalyl thiol esters at known physiological concentrations. The other one is the catalytic subunit of phosphorylase phosphatase (Gunshore & Hamilton, 1986). Since insulin is known to increase the intracellular activities of both these enzymes, the current results add further credence to the hypothesis (Hamilton, 1985) that oxalyl thiol esters may be important insulin messengers, with insulin causing a decrease in their intracellular concentrations. Because messengers for one hormone are frequently messengers for others as well, it thus seems very probable that oxalyl thiol esters may form the basis for a whole new system of metabolic control in animals.

ACKNOWLEDGMENTS

We thank Lisa Ney for obtaining some of the early results indicating that malic enzyme is inhibited by oxalyl thiol esters.

REFERENCES

Brush, E. J., & Hamilton, G. A. (1981) *Biochem. Biophys. Res. Commun. 103*, 1194–1200.

Brush, E. J., & Hamilton, G. A. (1982) *Ann. N.Y. Acad. Sci. 386*, 422–425.

Burns, C. L., Main, D. E., Buckthal, D. J., & Hamilton, G. A. (1984) *Biochem. Biophys. Res. Commun. 125*, 1039–1045.

Chang, G. G., & Hsu, R. Y. (1977) *Biochemistry 16*, 311–320.

Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 173–187.

Cooper, A. J. L. (1983) *Annu. Rev. Biochem. 52*, 187–222.

Drake, R. L., Parks, W. C., & Thompson, E. W. (1983) *J. Biol. Chem. 258*, 6008–6010.

Frenkel, R. (1975) *Curr. Top. Cell. Regul. 9*, 157–181.

Gunshore, S., & Hamilton, G. A. (1986) *Biochem. Biophys. Res. Commun. 134*, 93–99.

Gunshore, S., Brush, E. J., & Hamilton, G. A. (1985) *Bioorg. Chem. 13*, 1–13.

Hamilton, G. A. (1985) *Adv. Enzymol. Relat. Areas Mol. Biol. 57*, 85–178.

Hamilton, G. A., & Brush, E. J. (1982) *Dev. Biochem. 21*, 244–249.

Hamilton, G. A., & Buckthal, D. J. (1982) *Bioorg. Chem. 11*, 350–370.

Hamilton, G. A., Buckthal, D. J., Mortensen, R. M., & Zerby, K. W. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2625–2629.

Hamilton, G. A., Buckthal, D. J., & Kalinyak, J. (1982) in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S., & Morrison, M., Eds.) pp 447–459, Pergamon, New York.

Harris, R. K. (1986) Ph.D. Thesis, The Pennsylvania State University, University Park, PA.

Hsu, R. Y. (1982) *Mol. Cell. Biochem. 43*, 3-26.
Krebs, H. A., & Veech, R. L. (1969) *Adv. Enzyme Regul. 7*, 397-413.
Law, W. A., & Hamilton, G. A. (1986) *Bioorg. Chem. 14*, 378-391.
Meister, A., & Anderson, M. E. (1983) *Annu. Rev. Biochem. 52*, 711-760.
Naber, N., Venkatesan, P. P., & Hamilton, G. A. (1982) *Biochem. Biophys. Res. Commun. 107*, 374-380.
Park, S.-H., Harris, B. G., & Cook, P. F. (1986) *Biochemistry 25*, 3752-3759.
Pry, T. A., & Hsu, R. Y. (1978) *Biochemistry 17*, 4024-4029.
Schimerlik, M. I., Grimshaw, C. E., & Cleland, W. W. (1977) *Biochemistry 16*, 571-576.
Segel, I. H. (1975) *Enzyme Kinetics*, pp 170-177, Wiley, New York.
Skorczynski, S. S., & Hamilton, G. A. (1986) *Biochem. Biophys. Res. Commun.* (in press).
Tang, C. L., & Hsu, R. Y. (1974) *J. Biol. Chem. 249*, 3916-3922.
Veloso, D., Guynn, R. W., Oskarsson, M., & Veech, R. L. (1973) *J. Biol. Chem. 248*, 4811-4819.
Venkatesan, P. P., & Hamilton, G. A. (1986) *Bioorg. Chem. 14*, 392-402.

Monoclonal Antibodies That Coimmunoprecipitate the 1,4-Dihydropyridine and Phenylalkylamine Receptors and Reveal the Ca^{2+} Channel Structure†

Sylvie Vandaele, Michel Fosset,* Jean-Pierre Galizzi, and Michel Lazdunski
Centre de Biochimie du CNRS, Parc Valrose, 06034 Nice Cedex, France
Received October 1, 1986; Revised Manuscript Received November 10, 1986

ABSTRACT: Monoclonal hybridoma cell lines secreting antibodies against the (+)-PN 200-110 and the (−)-demethoxyverapamil binding components of the voltage-dependent calcium channel from rabbit transverse-tubule membranes have been isolated. The specificity of these monoclonal antibodies was established by their ability to coimmunoprecipitate (+)-[^{3}H]PN 200-110 and (−)-[^{3}H]demethoxyverapamil receptors. Monoclonal antibodies described in this work cross-reacted with rat, mouse, chicken, and frog skeletal muscle Ca^{2+} channels but not with crayfish muscle Ca^{2+} channels. Cross-reactivity was also detected with membranes prepared from rabbit heart, brain, and intestinal smooth muscle. These antibodies were used in immunoprecipitation experiments with ^{125}I-labeled detergent [3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and digitonin] solubilized membranes. They revealed a single immunoprecipitating component of molecular weight (M_r) 170 000 in nonreducing conditions. After disulfide bridge reduction the CHAPS-solubilized (+)-PN 200-110–(−)-demethoxyverapamil binding component gave rise to a large peptide of M_r 140 000 and to smaller polypeptides of M_r 30 000 and 26 000 whereas the digitonin-solubilized receptor appeared with subunits at M_r 170 000, 140 000, 30 000, and 26 000. All these results taken together are interpreted as showing that both the 1,4-dihydropyridine and the phenylalkylamine receptors are part of a single polypeptide chain of M_r 170 000.

Organic Ca^{2+} channel inhibitors have proved to be of great importance (Janis & Triggle, 1984; Miller & Freedman, 1984) in studies of the mechanism and of the molecular structure of the slow type of Ca^{2+} channel. The best known Ca^{2+} channel inhibitors include (i) 1,4-dihydropyridines such as nitrendipine and (+)-PN 200-110, (ii) verapamil-like compounds, and (iii) other types of molecules such as diltiazem and bepridil.

Skeletal muscle transverse tubule (T-tubule) membranes are the best source to study the properties of the 1,4-dihydropyridine-sensitive Ca^{2+} channel (Fosset et al., 1983; Ferry et al., 1984). The (+)-[^{3}H]PN 200-110 binding component of the channel protein has been detergent-solubilized and purified. However, different purification procedures in different laboratories have provided different evaluations of the subunit structure of the putative Ca^{2+} channel. It has been found in this laboratory that the 1,4-dihydropyridine receptor is a protein of M_r 170 000 assembled from a large subunit of M_r 140 000 and a smaller subunit of M_r 33 000–29 000, the two subunits being covalently linked by disulfide bridge(s) (Borsotto et al., 1984a, 1985; Schmid et al., 1986). It has also been found that the large subunit is the target of cAMP-dependent phosphorylation (Hosey et al., 1986). Conversely, it has been reported by another laboratory that the 1,4-dihydropyridine receptor is made by the assembly of three noncovalently linked polypeptides of M_r 160 000/130 000–50 000–33 000 (Curtis & Catterall, 1984), the peptide of M_r 50 000 being the one that is phosphorylated by a cAMP-dependent kinase (Curtis & Catterall, 1985). The first purpose of this work is to approach the problem of the subunit structure of the Ca^{2+} channel by a different route, which does not require purification procedures and uses monoclonal antibodies. The second purpose is to show that the two distinct receptors (Janis & Triggle, 1984) for 1,4-dihydropyridines and verapamil-like compounds are present in the same protein.

Experimental Procedures

Chemicals. (+)-[^{3}H]PN 200-110 and (−)-[^{3}H]demethoxyverapamil were from Amersham. *Staphylococcus aureus* V8 protease was from Sigma. Digitonin was from Serva. Methylcellulose was from Fluka and Pansorbin from Cal-

† This work was supported by the Association des Myopathes de France, the Centre National de la Recherche Scientifique, the Fondation sur les Maladies Vasculaires, and the Ministère de la Recherche et de la Technologie (Grant 85 C 1137).
* Address correspondence to this author.

0006-2960/87/0426-0005$01.50/0