Proc. Natl. Acad. Sci. USA
Vol. 78, No. 10, pp. 6256–6260, October 1981
Cell Biology

Lectins activate lymphocyte pyruvate dehydrogenase by a mechanism sensitive to protease inhibitors

(mitogens/transmembrane signalling/receptor/lymphocyte activation)

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Communicated by Charles R. Park, June 8, 1981

ABSTRACT **The mitogenic lectins concanavalin A and phytohemagglutinin were found to stimulate pyruvate oxidation in rat mesenteric lymphocytes. Marked cell agglutination accompanied this response. Wheat germ agglutinin, a nonmitogenic lectin, also aggregated lymphocytes but did not cause alteration of pyruvate oxidation. Cell lysates from lectin-treated cells retained their ability to oxidize pyruvate at an elevated rate, indicating that the observed stimulation of pyruvate oxidation was not due to increased transport of labeled pyruvate into the cells. Pyruvate oxidation activity in such lysates was readily sedimented in a mitochondria-enriched cellular fraction, indicating that it reflectes mitochondrial pyruvate dehydrogenase. Stimulation of this activity by lectins in intact lymphocytes was inhibited when the cells were incubated under conditions expected to inhibit trypsin-like proteases. Thus, esters of arginine, but not of alanine or tyrosine, blocked stimulation of pyruvate dehydrogenase by the lectins. The data indicate that pyruvate dehydrogenase is activated in lymphocytes treated with mitogenic lectins by a mechanism involving one or more proteolytic reactions. The similarity between the results presented here and those recently reported for insulin action on its target cells [Seals, J. R. & Czech, M. P. (1980) *J. Biol. Chem.* 255, 6529–6531] suggests that these systems may have similar modes of transmembrane signalling.**

Lymphocytes can be stimulated *in vitro* by mitogens to undergo blastogenic alterations similar to those that follow antigenic stimulation of B and T lymphocytes *in vivo* (1–9, 12–14).†‡ These changes include an increase in anabolic reactions such as transport of nutrients, rate of glycolysis, and synthesis of protein, RNA, and DNA (1–4). The detailed molecular mechanisms that occur during lymphocyte activation have not been elucidated. The initial event in the mitogen-induced T-lymphocyte activation involves lectin binding to cell surface glycoproteins (3). This binding occurs rapidly in a manner consistent with a diffusion-controlled process (2) and is followed by patching, capping, and endocytosis of the glycoprotein–lectin complex (5). However, patching and capping alone are not sufficient to stimulate transformation of T lymphocytes, as wheat germ agglutinin causes capping of membrane glycoproteins but is nonmitogenic (6, 7). The intracellular mediation of lymphocyte activation presumably involves one or more metabolic regulators. Several possible regulators have been studied (4, 8), such as cyclic nucleotides or Ca^{2+}, but none has yet been documented by direct evidence as the primary initial mediator.

Recent work in this laboratory has suggested intriguing parallels between the insulin effector system in target tissues sensitive to this hormone and the immunogenic effector system in lymphocytes. For example, the insulin receptor structure has been shown to consist of two types of disulfide-linked subunits in the symmetrical arrangement (β-S-S-α)-S-S-(α-S-S-β), analogous to the general design of immunoglobulin molecules (9) that serve as receptors for the activation of B lymphocytes (2). Although the antigen receptor on T lymphocytes has not yet been identified, recent evidence suggests that it is composed of a disulfide-linked dimer that contains idiotypic (variable region) but not isotypic (constant region) determinants.†‡ Low levels of trypsin both mimic the action of insulin on adipocytes (10) and stimulate lipopolysaccharide-sensitive splenocytes to incorporate [^{3}H]thymidine into DNA (11) and enhance the antibody-forming cell response of hamster lymphocytes to sheep erythrocytes (12). Furthermore, the insulin–receptor interaction appears to trigger a plasma membrane proteolytic reaction that releases a putative peptide enzyme regulator that modulates the insulin-sensitive enzyme pyruvate dehydrogenase (13). Several studies have also implicated the activity of an esterase as a key feature of the activation of B lymphocytes by anti-immunoglobulin antiserum (14, 15). The present studies were designed to explore the hypothesis that the signaling processes for these two systems share common characteristics. It is demonstrated here that lectin action on lymphocytes also leads to rapid activation of pyruvate dehydrogenase and that this activation is inhibited by protease inhibitors.

MATERIALS AND METHODS

Cell Isolation. Mesenteric lymph nodes from male Sprague–Dawley rats, 150–200 g, were collected in RPMI 1640 tissue culture medium/2% horse serum at 37°C and carefully trimmed to remove connective tissue. The nodes were gently teased between ground glass slides to yield a single-cell suspension. The cells were then suspended in Krebs–Ringer phosphate buffer, pH 7.4/3% bovine serum albumin at 6×10^7/ml. Cell viability was >90% by trypan blue exclusion. All these manipulations were carried out at 37°C.

Pyruvate Oxidation in Lymphocytes. Production of $^{14}CO_2$ from [1-^{14}C]pyruvate was determined as follows. Lymphocytes (3×10^3 cells) were incubated at 37°C with 0.25 mM [1-^{14}C]pyruvate (1 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels) in the presence of various lectins or proteolytic inhibitors (or both) in a total volume of 150 μl in sealed test tubes. The incubation was ended after 20 min by the addition of 0.2 ml of 0.5 M sulfuric acid to the lymphocyte mixture. Phenethylamine (0.2 ml; New England Nuclear) was added immediately to pieces of Whatman filter paper suspended in plastic wells above the lymphocytes.

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‡ Marchalonis, J. J. (1980) *4th International Congress of Immunology*, Paris, France, July 1980, Abstract No. 2.6.08.

The liberated $^{14}CO_2$ precipitated on the filter paper was measured in a Packard liquid scintillation counter using Triton X-100 in toluene, 33% (wt/vol) containing Omnifluor at 4 g/liter (New England Nuclear). All values were corrected for oxidation of [1-^{14}C]pyruvate to $^{14}CO_2$ in the absence of lymphocytes (0.1 mmol of $^{14}CO_2$/20 min), which always accounted for <10% of the basal oxidation level. Basal oxidation levels varied from 0.68 to 2.10 nmol of $^{14}CO_2$ evolved per 3×10^6 cells per 20 min, so the values from each experiment were normalized to the basal level for that day.

The effect of previous incubation with concanavalin A on lymphocyte pyruvate oxidation was assayed by incubation of lymphocytes (3×10^6 cells) with the indicated concentration of lectin in a total volume of 148 ul for various times at 37°C. [1-^{14}C]Pyruvate (1 mCi/mol) was added at a final concentration of 0.25 mM, and the reaction was carried out at 37°C for 7 min. The calculated value, Δ nmol of [1-^{14}C]pyruvate converted to $^{14}CO_2$, was obtained by subtracting the basal level of oxidation of pyruvate from the level of $^{14}CO_2$ produced from [1-^{14}C]pyruvate by lymphocytes previously incubated with concanavalin A. Lymphocytes used to determine basal levels of oxidation were incubated with buffer and then assayed under conditions identical to those used with lymphocytes previously incubated with concanavalin A.

The oxidation of [1-^{14}C]pyruvate to $^{14}CO_2$ was also measured in cell lysates. Lymphocytes (1.6×10^7 cells) were incubated with various concentrations of concanavalin A in the absence or presence of *p*-tosyl-L-arginine methyl ester (2.7 mM) in a total volume of 600 μl for 20 min at 37°C. The incubation was stopped by the addition of 3 ml of ice-cold Krebs–Ringer phosphate buffer, pH 7.4/0.1% bovine serum albumin, and the mixture was centrifuged. Cell pellets were placed on ice and lysed with 100 μl of 50 mM potassium phosphate buffer, pH 7.4, at 4°C. The assay was begun by the addition of assay buffer to give final concentrations of 50 mM potassium phosphate, pH 8.0, 0.12 mM coenzyme A, 0.12 mM cocarboxylase, 1.2 mM dithiothreitol, 0.3 mM [1-^{14}C]pyruvate (1 mCi/mmol), and 0.6 mM β-NAD, as described by Seals and Jarett (16). The tube was immediately sealed, and the release of $^{14}CO_2$ was determined after a 7-min incubation.

Background values were determined from samples that were incubated exactly as described above except that lymphocytes were omitted and were ≈10% of the values for samples with lymphocytes. The background values also showed a slight drift during the assay, which was minimized by keeping the assay buffer on ice. Corrections for this drift were made by determining background values at the beginning and end of the assay, interpolating values throughout the experiment, and subtracting the appropriate background value from each experimental result. All experimental determinations were done in triplicate, and each experiment was repeated at least three times except where noted.

Materials. Bovine serum albumin was obtained from Armour (Phoenix, AZ). RPMI 1640 tissue culture medium and horse serum were obtained from Microbiological Associates (Bethesda, MD). Concanavalin A, phytohemagglutinin, and wheat germ agglutinin were purchased from Miles. Sodium pyruvate, dithiothreitol, coenzyme A, cocarboxylase, and β-NAD were obtained from Sigma. All proteolytic inhibitors and substrates were obtained from Sigma. Phenethylamine and [1-^{14}C]pyruvate were obtained from New England Nuclear.

RESULTS

As shown in Fig. 1, the lectins concanavalin A and phytohemagglutinin stimulated the ability of rat mesenteric lymph node cells to oxidize exogeneous pyruvate. Phytohemmagglutinin

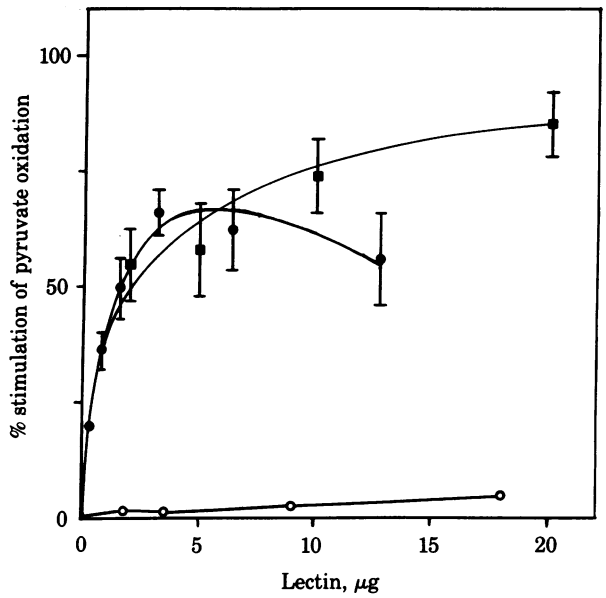

FIG. 1. Stimulation of pyruvate oxidation by lectins. Lymphocytes (3×10^6 cells) were incubated for 20 min at 37°C in Krebs–Ringer phosphate buffer, pH 7.4/3% bovine serum albumin containing 0.25 mM [1-^{14}C]pyruvate and various concentrations of concanavalin A (■), phytohemagglutinin (●), or wheat germ agglutinin (○). $^{14}CO_2$ production was determined at the end of a 20-min incubation. Background values, determined from samples incubated without lymphocytes, were 10% of values for samples with lymphocytes, and the relative increase in pyruvate oxidation was calculated. All determinations were done in triplicate. Values for phytohemagglutinin and concanavalin A stimulation are averages of four and eight experiments, respectively, while the effect of wheat germ agglutinin is a representative experiment. Results are mean ± SEM.

maximally stimulated pyruvate oxidation in lymphocytes by ≈66% at 3.2 μg per 3×10^6 cells in 150 μl. The level of oxidation of pyruvate in the absence and presence of phytohemagglutinin (3.2 μg per 3×10^6 cells) was 0.96 ± 0.10 and 1.57 ± 0.10 nmol of $^{14}CO_2$ produced per 3×10^6 per 20 min, respectively (n = 4). Concanavalin A also increased pyruvate oxidation in lymphocytes in a dose-dependent manner. The level of oxidation of pyruvate in the absence and presence of concanavalin A (20 μg per 3×10^6 cells) was 1.39 ± 0.15 and 2.49 ± 0.18 nmol of $^{14}CO_2$ produced per 3×10^6 cells per 20 min, respectively (n = 8). However, wheat germ agglutinin did not significantly increase the oxidation of pyruvate by lymphocytes under the assay conditions used. Addition of 3.6 or 36 μg of wheat germ agglutinin to 3×10^6 cells in the presence of 20 μg of concanavalin A decreased only slightly the stimulation of pyruvate oxidation in lymphocytes by concanavalin A alone (data not shown), indicating that the wheat germ agglutinin preparation did not contain a metabolic inhibitor. All three mitogens caused aggregation of lymphocytes but did not affect cell viability during the 20-min assay.

Incubation of lymphocytes with concanavalin A prior to the addition of labeled pyruvate enhanced the stimulation of pyruvate oxidation (Fig. 2). At concentrations of concanavalin A <20 μg per 3×10^6 cells in 150 μl, there is, in general, a time-dependent increase in effect on pyruvate oxidation. At 20 μg of concanavalin A per 3×10^6 cells, maximum activation occurs at 5 min, as the same activity was noted for cells treated 5, 10, or 20 min prior to addition of [1-^{14}C]pyruvate. Incubation of lymphocytes with concanavalin A for 20 min prior to the addition of labeled pyruvate caused maximal stimulation of pyruvate oxidation at all concentrations of concanavalin A tested.

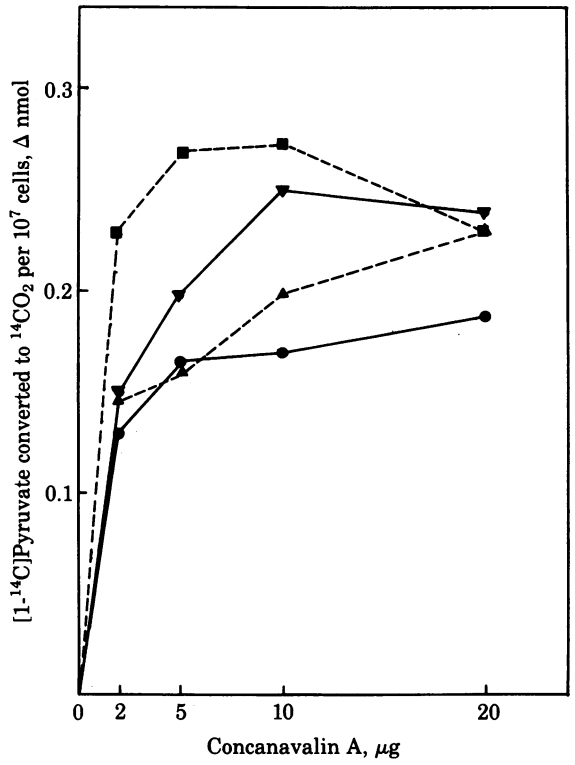

FIG. 2. Effect of previous incubation with concanavalin A on pyruvate oxidation in lymphocytes. Lymphocytes (3×10^6 cells in 148 μl) were incubated at 37°C with various concentrations of concanavalin A for 0 (●), 5 (▲), 10 (▼), or 20 (■) min. [1-^{14}C]Pyruvate (1 mCi/nmol) was added to a final concentration of 0.25 mM and the tube was immediately capped. After 7 min, the reaction was stopped. Determinations were done in duplicate, and results represent the average of three experiments.

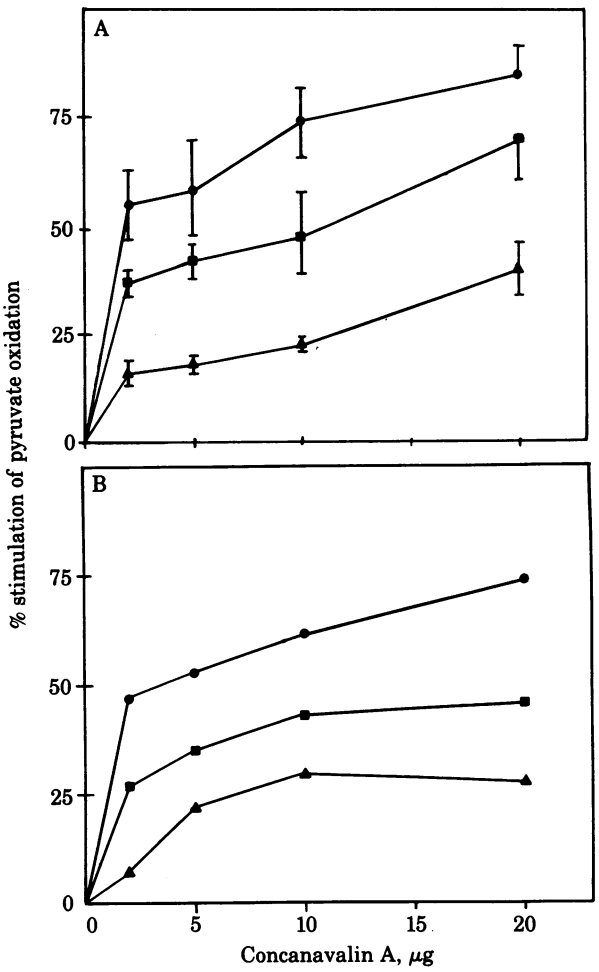

FIG. 3. Effect of proteolytic substrates on concanavalin A stimulation of pyruvate oxidation in whole cells. Lymphocytes (3×10^6 cells) were incubated with 0.25 mM pyruvate at 37°C for 20 min with various concentrations of concanavalin A in the presence and absence of *p*-tosyl-L-arginine methyl ester (*A*) or α-*N*-benzoylarginine methyl ester (*B*). The reaction was terminated after 7 min. Determinations were done in duplicate. Results for the tosyl ester represent mean ± SEM for five experiments; results for the benzoyl ester represent a single experiment. (*A*) ●, Control; ■, 0.27 mM; ▲, 2.7 mM. (*B*) ●, control; ■, 2.7 mM; ▲, 13.5 mM.

Previous incubation of lymphocytes with concanavalin A for 40 min did not further significantly increase the level of pyruvate oxidation (data not shown).

To begin to test whether the activation of pyruvate oxidation by lectins might involve proteolytic release of an intracellular activator, the ability of protease inhibitors to block the effect of concanavalin A was tested. Benzamidine (10 mM) and ε-aminocaproic acid (10 mM) inhibited the stimulation of pyruvate oxidation due to lectin when added to lymphocytes together with concanavalin A (data not shown). However, these inhibitors and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (0.2 mM) greatly stimulated the level of pyruvate oxidation of lymphocytes in the absence of concanavalin A. The alcohol-soluble proteolytic inhibitors phenylmethylsulfonyl fluoride and *p*-nitrophenyl-*p*-guanidino benzoate were also tested. However, alcohol alone greatly decreased basal and concanavalin A-stimulated pyruvate oxidation. Only inhibitors that did not affect basal levels of oxidation were further studied.

As shown in Fig. 3*A*, treatment of cells with 0.27 mM *p*-tosyl-L-arginine methyl ester decreased the stimulation of pyruvate oxidation at all concentrations of concanavalin A tested and treatment with 2.7 mM ester decreased the stimulation of oxidation due to low concentrations of the lectin to 25% of the basal level. Basal levels of pyruvate oxidation were not altered by inclusion of the protease substrate in the incubation mixture as lymphocytes incubated with 0, 0.27, and 2.7 mM *p*-tosyl-L-arginine methyl ester yielded 1.32 ± 0.16, 1.35 ± 0.22, and 1.33 ± 0.20, respectively, nmol of $^{14}CO_2$ evolved per 3×10^6 cells per 20 min. *p*-Tosyl-L-arginine methyl ester at a final concentration of 10 mM further diminished the response to concanavalin A but did not totally abolish the ability of the lectin to stimulate pyruvate oxidation in whole cells (data not shown). Cellular viability as determined by trypan blue exclusion was not affected by incubation of lymphocytes with 10 mM *p*-tosyl-L-arginine methyl ester. As shown in Fig. 3*B*, α-*N*-benzoyl-L-arginine methyl ester at 2.7 and 13.5 mM also decreased the concanavalin A stimulation of pyruvate oxidation but had no effect on the basal levels of pyruvate oxidation. Two other proteolytic substrates, *N*-acetyltyrosine ethyl ester and *N*-acetyl-L-alanyl-L-alanyl-L-alanyl methyl ester, at 2.7 mM did not significantly alter either basal levels or concanavalin A-stimulated levels of pyruvate oxidation in intact lymphocytes (data not shown).

To investigate whether the activation of pyruvate oxidation by lectins survives cell lysis, lymphocytes were treated with concanavalin A and then disrupted. The cell lysates were assayed for their ability to convert [1-^{14}C]pyruvate to $^{14}CO_2$. As shown in Fig. 4, previous incubation of lymphocytes with concanavalin A stimulated the subsequent ability of the cellular lysate to oxidize pyruvate in a dose-dependent manner. This stimulation was significantly decreased by the addition of 2.7

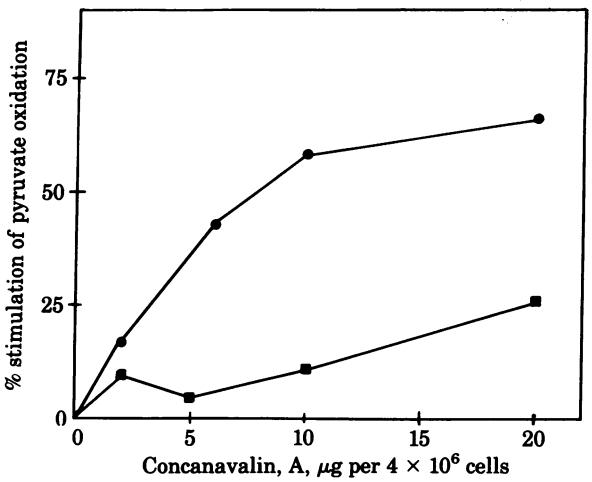

FIG. 4. Pyruvate oxidation in cell lysates after incubation of lymphocytes with concanavalin in A in the presence or absence of *p*-tosyl-L-arginine methyl ester. Lymphocytes (1.6×10^7 cells) were incubated at 37°C for 20 min with concanavalin A in the absence (●) or presence (■) of 2.7 mM *p*-tosyl-L-arginine methyl ester in a total volume of 600 μl of Krebs–Ringer phosphate buffer/3% bovine serium albumin. The incubation was stopped by the addition of 3 ml of ice-cold Krebs–Ringer phosphate buffer, pH 7.4/0.1% bovine serum albumin, and the mixture was centrifuged. Cell pellets were lysed with 100 μl of 10 mM potassium phosphate buffer, pH 7.4, at 4°C. Oxidation of pyruvate was measured for 7 min at 37°C after the addition of [1-^{14}C]pyruvate and cofactors. The data depict a representative experiment.

mM *p*-tosyl-L-arginine methyl ester to the incubation mixture of cells and lectin at all concentrations of concanavalin A. Again, addition of *p*-tosyl-L-arginine methyl ester did not alter the basal levels of oxidation in this assay system.

The pyruvate oxidation activity in the cell lysate could be readily sedimented with a cellular fraction rich in mitochondria. In contrast, the specific activity of pyruvate oxidation in lymphocyte supernatant after centrifugation of the cellular lysate at 16,000 × *g* for 30 min was low in the presence and absence of 2.5 mM magnesium and calcium (0.1–0.3 nmol of $^{14}CO_2$ evolved per mg per 5 min). However, the particulate fraction demonstrated a higher level of pyruvate oxidation (1.2 nmol of $^{14}CO_2$ produced per mg per 5 min), which was stimulated to 7.1 nmol of $^{14}CO_2$ produced per mg per 5 min by the inclusion of 2.5 mM magnesium and calcium in the assay mixture. It is known that mitochondrial pyruvate dehydrogenase is readily stimulated by calcium and magnesium and remains with the mitochondria during isolation (13). Thus, the pyruvate-oxidizing activity in the cellular lysates probably reflects the activity of pyruvate dehydrogenase, a mitochondrial enzyme.

DISCUSSION

The studies described in this paper show that binding of the mitogenic lectins concanavalin A and phytohemagglutinin to rat mesenteric lymph node cells as demonstrated by cellular agglutination stimulates the oxidation of [1-^{14}C]pyruvate to $^{14}CO_2$ in a dose-dependent manner (Fig. 1). The concentrations of lectin that gave maximal mitogenic response were 2–4 μg of concanavalin A and 2.6 μg of phytohemagglutinin per 3×10^6 cells (Miles Laboratory Analysis Report). These are comparable with the doses of lectins that produced stimulation of pyruvate oxidation as reported here. Wheat germ agglutinin, a nonmitogenic lectin (6, 7) aggregated rat mesenteric lymph node cells but did not stimulate pyruvate oxidation. Thus, increases in pyruvate oxidation due to agents such as mitogens may be an indicator of or play a role in lymphocyte activation. Furthermore, the effect of concanavalin A or phytohemagglutinin on pyruvate oxidation in lymphocytes could not be due to increased transport of pyruvate as cell lysates from lectin-treated cells retained their ability to oxidize pyruvate at an elevated level (Fig. 4). The pyruvate oxidation activity in the cell lysate was readily sedimented, as would be expected of a mitochondrial enzyme. Thus, the data suggest that lectin action on lymphocytes leads to rapid activation of pyruvate dehydrogenase.

The possibility that a proteolytic event occurs in response to concanavalin A interaction with plasma membranes and leads to increased pyruvate dehydrogenase activity was investigated by using proteolytic inhibitors. The ability of concanavalin A to stimulate pyruvate oxidation in intact lymphocytes was markedly inhibited by incubation of lymphocytes with the proteolytic substrates *p*-tosyl-L-arginine methyl ester and α-*N*-benzoyl-L-arginine methyl ester (Fig. 3). These two inhibitors displayed no significant effect on the basal rate of pyruvate oxidation. Other proteolytic substrates, *N*-acetyltyrosine ethyl ether and *N*-acetyl-L-alanyl-L-alanyl-L-alanyl methyl ester, had no effect on either basal or concanavalin A-stimulated levels of pyruvate oxidation. These data are consistent with the concept that the mediation of mitogen-induced stimulation of pyruvate oxidation involves activation of an arginine-specific protease. The results presented here are indirect, however, and this concept requires rigorous evaluation by further investigation.

Anti-immunoglobulin stimulation of B lymphocytes also appears to be mediated by a mechanism that involves one or more proteolytic events. Hirschorn *et al.* (17) demonstrated that proteolytic inhibitors decreased phytohemagglutinin stimulation of DNA, RNA, and protein synthesis in human peripheral blood lymphocytes. Treatment of splenic lymphocytes with anti-immunoglobulin antiserum induced movement of B lymphocytes and required the activation of a serine esterase with arginine specificity (14). Also, anti-immunoglobulin antiserum treatment of B lymphocytes activated a membrane-bound serine protease, also with arginine specificity, that generated a cytoplasmic factor (15). This factor was capable of stimulating phosphorylation of nonhistone chromatin proteins in isolated nuclei of untreated cells. It should be noted that, in several other cell types, activation of a cellular function by specific stimuli can also be depressed by potent inhibitors of serine esterases. Serine protease inhibitors having chymotrypsin specificity depressed the uptake of a chemotactic peptide (18) and inhibited chemotaxis (19) in human peripheral neutrophils. Chymostatin but not other proteolytic inhibitors inhibited phytohemagglutinin-induced aggregation of human peripheral blood lymphocytes (20).

Our results indicating that mitogenic lectins activate lymphocyte pyruvate dehydrogenase and that this activation is sensitive to antiproteases represent further intriguing parallels between cellular responses accompanying lymphocyte activation and insulin action on its target cells. In both of these models of transmembrane signalling, a number of common cellular responses are elicited synchronously, including activation of membrane transport systems (21–24), increases in certain enzyme activities (25–27), and changes in the phosphorylation state of specific proteins (28, 29). It is also striking that lectins mimic the actions of insulin on isolated fat cells (30), as well as trigger a mitogenic response in lymphocytes (1–4). The activation of membrane transport systems in adipocytes or muscle by insulin and in lymphocytes by lectins exhibit similar rapid time courses, indicating that they are not secondary to *de novo* protein synthesis (21, 30). These similarities between the lymphocyte immunogenic response and the insulin effector system led us to the hypothesis that they share a common mechanism.

Recent experiments by Seals and Jarett (16) have shown that a factor capable of regulating pyruvate dehydrogenase *in vitro*

can be released from isolated adipocyte plasma membrane in response to insulin or concanavalin A. Seals and Czech (13), further showed that generation of this factor by membranes in the presence of insulin was blocked by antiproteases and protease substrate analogues of arginine. The bioactivity of the factor could also be destroyed by treatment with proteases, suggesting that it is a peptide-like substance released from the membrane in response to insulin–receptor interaction. Preliminary experiments in our laboratory have shown that lymphocyte plasma membranes treated with concanavalin A or phytohemagglutinin release a soluble factor that stimulates pyruvate dehydrogenase activity in either lymphocyte or adipocyte mitochondria (data not shown).

The data presented here are consistent with a model for lymphocyte activation and insulin action whereby the stimulation of an endogenous membrane protease by ligand binding results in the release of an intracellular mediator from a precursor protein.

We thank Dr. Jonathan R. Seals for helpful discussions and critical reading of the manuscript. We thank Sandra Brenner for skillful secretarial assistance. This work was supported by Grants AM 17893 and AM 27027 from the National Institutes of Health and a grant from the Kroc Foundation. M.P.C. is the recipient of Research Career Development Award AM 00249 from the U. S. Public Health Service.

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