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Cell Biology

Activation of early enzyme production in small lymphocytes in response to high, nonmitogenic concentrations of concanavalin A

(mitogenesis/*S*-adenosylmethionine decarboxylase/ornithine decarboxylase/thymidine kinase/DNA synthesis)

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ABSTRACT Lymphocyte mitogenesis is generally assessed by measuring the incorporation of [^{3}H]thymidine into DNA. By this criterion, small lymphocytes, which are activated by relatively low doses of concanavalin A, are either unresponsive to or inhibited by higher concentrations. Because lymphocytes begin to synthesize DNA about 24 hr after addition of mitogen, the response is far removed temporally from the initial stimulus. We have chosen to use the induction of *S*-adenosylmethionine decarboxylase (*S*-adenosyl-L-methionine carboxy-lyase, EC 4.1.1.50) to assess early activation events in bovine lymphocytes. Adenosylmethionine decarboxylase induction is bimodal, with an initial phase beginning 3 hr after addition of concanavalin A and a second wave coinciding with the onset of DNA synthesis. The initial accumulation of the decarboxylase (0–9 hr) in cultures treated with "nonmitogenic" levels of concanavalin A (108 μg/ml) was similar to that observed in cultures stimulated with optimally mitogenic doses (18 μg/ml). The early induction of ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) was also similar under these two culture conditions. However, the second phase of adenosylmethionine decarboxylase accumulation, the induction of thymidine kinase (ATP: thymidine 5′-phosphotransferase, EC 2.7.1.21), and DNA replication were blocked at the higher concentrations of concanavalin A. The inhibition of late events by high doses of concanavalin A was reversible. Cells treated with α-methyl-D-mannopyranoside 25 hr after addition of a high dose of lectin responded with a second period of adenosylmethionine decarboxylase accumulation, induction of thymidine kinase, and progression through S phase. These results suggest that initial lymphocyte activation occurs normally at high doses of concanavalin A, but that the cells are reversibly blocked prior to induction of "late" enzymes and progression through S phase.

Small lymphocytes are isolated in a quiescent state and can be stimulated to proliferate by a variety of mitogenic agents that interact at the cell surface (1). For example, the plant lectin concanavalin A (Con A), a protein whose structure (2) and binding specificity (3) have been well characterized, is a potent mitogen for T lymphocytes. The details of the relationship between the binding of mitogenic lectins to the surface of lymphocytes and the subsequent metabolic alterations have remained unclear. An interesting model for lymphocyte activation, involving association of receptors with cytoplasmic microtubular assemblies, has been proposed (4). A complicating aspect is that Con A stimulates cells to replicate DNA at relatively low doses, yet at higher concentrations it apparently either does not activate or inhibits (5, 6). Correlative evidence has suggested that the decreased mitogenicity of Con A at high doses may be related to a restriction of cell surface receptor mobility (4). Lack of mitogenesis caused by high levels of Con A might involve a specific cellular response mechanism or, alternatively, it could simply be a reflection of nonspecific cytotoxicity. In this paper we have asked whether any portion of the mitogenic program is activated under these conditions that are generally considered "nonmitogenic" by the criterion of DNA replication.

To study this problem, it seemed that the examination of an early response of lymphocytes to Con A might be informative. We chose to use the induction of *S*-adenosylmethionine decarboxylase (AdoMet decarboxylase) (*S*-adenosyl-L-methionine carboxy-lyase, EC 4.1.1.50), an enzyme in the pathway of polyamine biosynthesis (7), as a measure of cell activation. The accumulation of AdoMet decarboxylase activity is a relatively early response of lymphocytes to Con A (8, 9). The results reported here suggest that high, nonmitogenic concentrations of Con A activate some of the initial events that are observed in the normal mitogenic response. However, these high doses of Con A, although stimulatory, eventually interrupt the mitogenic program prior to the induction of "late" enzymes, such as thymidine kinase (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21), and the progression of cells through S phase. The inhibitory effects of high Con A were reversible. Addition of a competitive inhibitor of Con A binding, α-methyl-D-mannopyranoside, to cells blocked with high concentrations of the lectin, resulted in a second phase of AdoMet decarboxylase accumulation, elevation of thymidine kinase, and progression of cells through S phase.

MATERIALS AND METHODS

Cell Culture. Lymphocytes were prepared from bovine suprapharangeal lymph nodes by using a glass bead column and were cultured as described (10). Approximately 90% of the cells respond to Con A, as judged morphologically (10) and by [^{3}H]thymidine autoradiography (unpublished observations). Highly purified (grade IV) Con A was obtained from Sigma.

Harvest of Cell Culture and Enzyme Assays. At the time of collection, lymphocyte cultures were cooled on ice and a 10% volume of 1.0 M α-methyl-D-mannopyranoside (grade III from Sigma) in serum-containing medium was added. The cultures remained on ice for 1 hr before the cells were collected by centrifugation at 300 $\times$ *g* for 10 min. This procedure eliminated Con A-precipitated serum glycoproteins from the cell pellets, which interfered with the determination of cellular protein. The cells were washed in Puck's saline G (11), again collected by centrifugation, and stored frozen overnight at -20°C. When the above procedure was performed on control cultures stimulated with optimal concentrations of Con A (18 μg/ml), there was no effect on the yield of harvested cells (as judged by protein content of cell extracts) or the specific activity of AdoMet decarboxylase.

AdoMet decarboxylase activity in cell-free extracts was measured by the release of $^{14}CO_2$ from *S*-adenosyl-L-[*carboxyl*-^{14}C]methionine (9). The activity is expressed as units/mg of protein in the cell extract, where a unit has been defined as

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Abbreviations: Con A, concanavalin A; AdoMet decarboxylase, *S*-adenosylmethionine decarboxylase.

the amount of enzyme that will catalyze the release of 1 nmol of CO_2 per hr. Ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) activity in lymphocyte extracts was determined as described (12). Frozen cell pellets (1.5×10^8 cells) were thawed and homogenized in a glass homogenizer in 0.5 ml of 25 mM Tris·HCl buffer (pH 7.4) containing 0.1 mM EDTA, 5 mM dithiothreitol, and 0.2 mM pyridoxal phosphate. The homogenate was centrifuged at $100,000 \times g$ for 100 min at 4°C and dialyzed against 200 vol of homogenization buffer. Ornithine decarboxylase was measured by the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine at 37°C. The standard incubation mixture, total volume 0.3 ml, was buffered with 0.1 M Tris·HCl (pH 7.2) and contained 0.2 mM pyridoxal phosphate, 5 mM dithiothreitol, 0.2 mM L-[1-^{14}C]ornithine (specific activity, 3.3 Ci/mol; New England Nuclear; 1 Ci = 3.7×10^{10} becquerels), and cell extract containing approximately 100 μg of protein. The CO_2 evolved was trapped and its radioactivity was measured as described (13) after the addition of 20 μl of 100% (wt/vol) trichloroacetic acid. Assays were routinely run for 30 min. The reaction is linear with time for at least 1 hr, and the rate was a linear function of protein concentration to at least 500 μg per 0.3-ml assay (12). Protein content of cell extracts was determined by the method of Lowry *et al.* (14) with a bovine serum albumin standard (Calbiochem, grade A). Ornithine decarboxylase activity is expressed as described for AdoMet decarboxylase.

The thymidine kinase assay was based on the method described by Ives *et al.* (15). Cell extracts were prepared from frozen cell pellets (3×10^8 cells) which were thawed and homogenized in a glass homogenizer in 0.5 ml of 10 mM Tris·HCl buffer (pH 7.4) containing 1.0 mM dithiothreitol and 20% (wt/vol) glycerol. The homogenate was centrifuged at 100,000 $\times g$ for 60 min at 4°C. Thymidine kinase activity was measured by the conversion of [*methyl*-^{3}H]thymidine to [*methyl*-^{3}H]-thymidylate at 37°C. The incubation mixture, total volume 0.2 ml, was buffered with 0.1 M Tris·HCl (pH 8.0 at 37°C) and contained 10 mM $MgCl_2$, 5 mM ATP, 0.4 mM [*methyl*-^{3}H]-thymidine (specific activity, 100 Ci/mol; Amersham), and cell extract containing approximately 300 μg of protein. After a 20-min incubation at 37°C the reactions were terminated by transfer to a boiling water bath for 2.5 min. All remaining steps were essentially as described (15). Enzyme assays were linear with respect to protein concentration and time at all times after lymphocyte activation. Thymidine kinase activity is expressed as units/mg of protein in the cell extract, where a unit has been defined as the amount of enzyme that will catalyze the conversion of 1 nmol of thymidine to thymidylate per min.

[^{3}H]Thymidine Incorporation and Autoradiography. The rate of DNA synthesis in lymphocyte cultures was determined by measuring the incorporation of [*methyl*-^{3}H]thymidine (5 μCi/ml, 6.7 Ci/mmol; New England Nuclear) into DNA after exposure to label for 2 hr (10). For autoradiography, cells labeled as above were collected by centrifugation ($300 \times g$, 10 min), washed and swollen in a hypotonic sucrose solution (0.1 M sucrose/1 mM $CaCl_2$/0.3 mM NaCl), fixed in glacial acetic acid/methanol, 1:3 (vol/vol), applied to a microscope slide, and exposed to Kodak NTB2 Nuclear Track Emulsion. After a 10-hr exposure, the slides were developed and stained with Giemsa stain.

RESULTS

Influence of Con A Concentration on Lymphocyte Activation. The response of cultures of bovine lymphocytes to various concentrations of Con A was examined by measuring the incorporation of [^{3}H]thymidine into DNA over a 2-hr interval beginning 48 hr after mitogen addition. The dose–response curve generated (Fig. 1*A*) is typical of that noted by a number of investigators (1). With increasing Con A concentration, the level of DNA synthesis increased, reached a peak (≈18 μg of Con A per ml in this system), and subsequently decreased at higher concentrations. Similar results were obtained when cultures were labeled continuously between 24 and 45 hr. At the highest concentration of Con A tested (150 μg/ml), there was no loss of DNA from the cultures (data not shown), implying a lack of cell lysis.

It was of interest to examine the influence of Con A concentration on other parameters of lymphocyte activation. It was reported previously that the accumulation of AdoMet decarboxylase activity was biphasic after activation of lymphocytes with optimal mitogenic concentrations of either phytohemagglutinin (8) or Con A (9). There was an initial induction period beginning at about 3 hr, followed by a second phase that reached a maximum at approximately 28 hr (for example, see the control curve in Fig. 2). The responses of these two phases of AdoMet decarboxylase induction to varying Con A concentrations are shown in Fig. 1*B*. The dose–response curve obtained for AdoMet decarboxylase activity at 28 hr was quite similar to that found for [^{3}H]thymidine incorporation (Fig. 1*A*), with a peak at approximately 20 μg/ml. On the other hand, the initial elevation of AdoMet decarboxylase activity at 9 hr showed a dramatically different dose–response curve. At low doses, the level of AdoMet decarboxylase at 9 hr increased with Con A concentration in a manner similar to that found with [^{3}H]thymidine incorporation and AdoMet decarboxylase at 28 hr. However, unlike the other two parameters, no reduction of the initial peak of AdoMet decarboxylase activity was observed

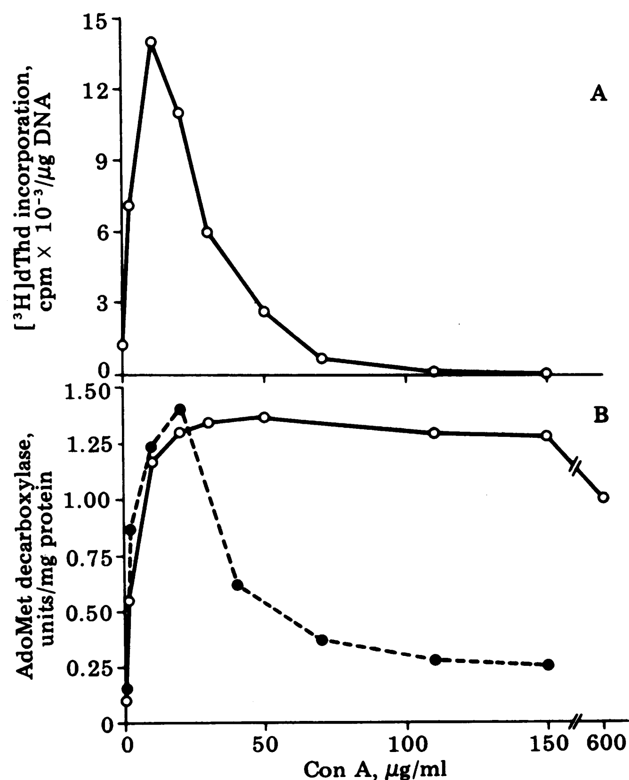

FIG. 1. Influence of Con A concentration on [*methyl*-^{3}H]thymidine incorporation (*A*) and AdoMet decarboxylase activity (*B*). (*A*) Con A was added to 3-ml cultures (9×10^6 cells) at the indicated final concentrations. At 48 hr after Con A addition, all cultures were labeled with [*methyl*-^{3}H]thymidine. Incorporation is expressed as cpm/μg of DNA. (*B*) One-hundred-milliliter cultures (3×10^8 cells) were exposed to Con A at the final concentrations indicated. At 9 (○—○) and 28 (●- - -●) hr after Con A addition, cultures were collected, cell extracts were prepared, and AdoMet decarboxylase activities were measured.

at high doses of Con A. The level of AdoMet decarboxylase activity at 9 hr remained constant up to 150 μg of Con A per ml and was only slightly reduced at 600 μg/ml (Fig. 1*B*). Thus, the initial peak of AdoMet decarboxylase activity was induced optimally even at high, nonmitogenic doses of Con A.

In light of the above results, it was of interest to examine in detail the time course of AdoMet decarboxylase induction at high doses of Con A. The results of such an experiment, with 108 μg of Con A per ml, are shown in Fig. 2. The initial accumulation of AdoMet decarboxylase from 0 to 9 hr essentially followed the control (18 μg of Con A per ml). However, after 9 hr, AdoMet decarboxylase activity decreased and the second phase of induction was not observed. These results strengthen the data from the Con A dose–response experiments and lead to the conclusion that high doses of Con A block the mitogenic program sometime after the initial 9 hr and prior to the second phase of AdoMet decarboxylase accumulation and progression of cells through S phase.

Reversibility of the High Con A Block. The reversibility of the inhibitory effect of high doses of Con A was examined with a competitive inhibitor of Con A binding, α-methyl-D-mannopyranoside. Cultures were activated with 108 μg of Con A per ml. After 25 hr, α-methylmannopyranoside was added to a final concentration of 0.1 M. The behavior of AdoMet decarboxylase in cells collected at various times after addition of α-methylmannopyranoside is shown in Fig. 2. Addition of the saccharide resulted in a second increase of AdoMet decarboxylase activity, suggesting that the cells were released from the inhibitory effect(s) of the high dose of Con A. As a control, the influence of the saccharide on cells activated with phytohemagglutinin was tested. The binding of phytohemagglutinin to cells is not inhibited by α-methylmannopyranoside (3) and the saccharide could be added to cultures together with mitogen at concentrations as high as 0.14 M with no adverse effect, as judged by the rate of [^{3}H]thymidine incorporation between 42 and 44 hr (data not shown).

Reversal of the Con A-mediated inhibition of cells by α-methylmannopyranoside was further examined by [^{3}H]thymidine incorporation and autoradiography (Fig. 3). In control cultures activated with 18 μg of Con A per ml, the cells began to enter S phase at approximately 24 hr and the rate of [^{3}H]thymidine incorporation increased coincidently with the increase in percentage of cells in S phase. In cultures that were activated with 108 μg of Con A per ml, progression through S phase was dependent on addition of α-methylmannopyranoside. Cultures not receiving the saccharide showed essentially no [^{3}H]thymidine incorporation (see Fig. 1*A*). When the saccharide was added to cultures at 25 hr, the cells began to traverse S phase after a lag of approximately 6–8 hr. Under these conditions, the kinetics of cell labeling with [^{3}H]thymidine (Fig. 3, ■), although delayed, were similar to those in control cultures (Fig. 3, ●). Thus, one simple interpretation of these data is that high doses of Con A activate lymphocytes, but create a re-

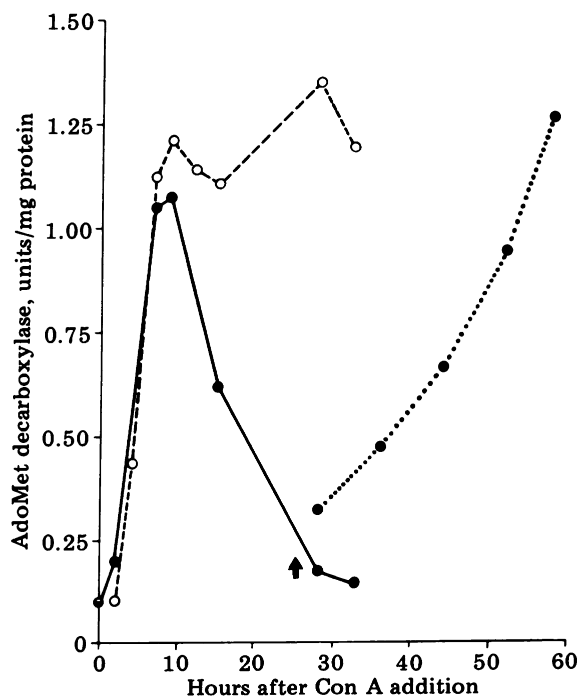

FIG. 2. Changes in AdoMet decarboxylase activity after exposure of lymphocytes to optimally mitogenic (○---○) and high, nonmitogenic (●—●) doses of Con A. Con A was added to 100-ml cultures (3×10^8 cells) to a final concentration of either 18 (○---○) or 108 (●—●) μg per ml. At the indicated times cultures were collected, cell extracts were prepared, and AdoMet decarboxylase activities were measured. The effect of α-methylmannopyranoside addition on AdoMet decarboxylase levels in cultures treated with 108 μg of Con A per ml was examined (●·····●). The saccharide was added as a 10% volume of 1.0 M α-methylmannopyranoside in serum-containing medium 25 hr after Con A addition (arrow).

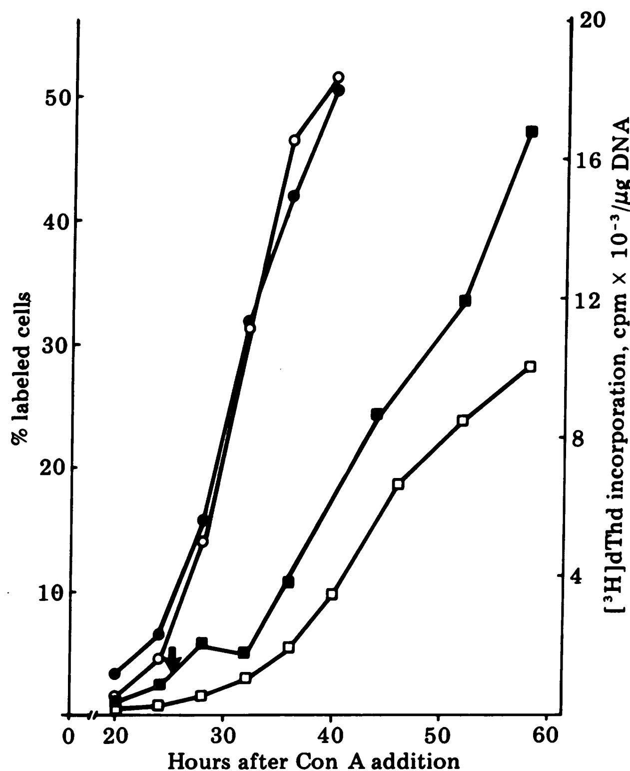

FIG. 3. Influence of α-methylmannopyranoside addition on DNA synthesis in cultures treated with a high concentration of Con A. Con A was added to replicate 3-ml cultures (9×10^6 cells) to a final concentration of either 18 μg/ml (○ and ●) or 108 μg/ml (□ and ■). Two-hour pulses of [*methyl*-^{3}H]thymidine (6.7 Ci/mmol, 5 μCi/ml) were given, ending at the indicated times. Cultures containing 108 μg of Con A per ml were treated with α-methylmannopyranoside 25 hr after lectin addition (arrow) as described in Fig. 2. The percent labeled cells (● and ■) was estimated by autoradiography; at least 300 cells were counted at each time point (SD in triplicate samples was less than 10%). The rate of [*methyl*-^{3}H]thymidine incorporation (○ and □) was determined as described (10) and is expressed as cpm/μg of DNA (SD in triplicate samples was less than 6%). In control cultures not treated with Con A or cultures to which 108 μg of Con A per ml was added but not α-methylmannopyranoside, less than 2% of the cells incorporated [*methyl*-^{3}H]thymidine between 54 and 56 hr. Similarly, the rate of [*methyl*-^{3}H]thymidine incorporation was 0.3% and 2.1%, respectively, of that observed in cultures treated with 18 μg of Con A per ml.

versible arrest of the cells within approximately 8 hr of the G_1/S boundary.

The rate of [^{3}H]thymidine incorporation into DNA in cultures treated with a high dose of Con A and reversed with α-methylmannopyranoside at 25 hr was *less* than would be expected from the percentage of cells in S phase at a given time (compare the reversed with the control cultures in Fig. 3). That is to say, the [^{3}H]thymidine incorporation per cell active in DNA synthesis was reduced in these cultures. One might postulate from these results that cells released from a Con A-mediated arrest are still in the process of recovery (i.e., accumulation of DNA precursors, enzymes involved in DNA synthesis, polyamines, etc.) upon entrance into or progression through S phase.

Influence of High Con A on Other Enzyme Activities. Ornithine decarboxylase, like AdoMet decarboxylase, is among the earliest enzyme activities known to be elevated in activated lymphocytes (8). It was of interest to determine the influence of high doses of Con A on the induction of ornithine decarboxylase and compare these results with the effect on AdoMet decarboxylase. The time courses of ornithine decarboxylase induction in cultures activated with optimal (18 μg/ml) and inhibitory (108 μg/ml) concentrations of Con A are shown in Fig. 4. At the high concentration of Con A, the induction of ornithine decarboxylase was, if anything, somewhat more rapid than in control cultures. However, between 10 and 15 hr the level of ornithine decarboxylase activity began to fall dramatically. Hence, ornithine decarboxylase behaved similarly to AdoMet decarboxylase in that the initial increase of activity was essentially normal in cells activated with high concentrations

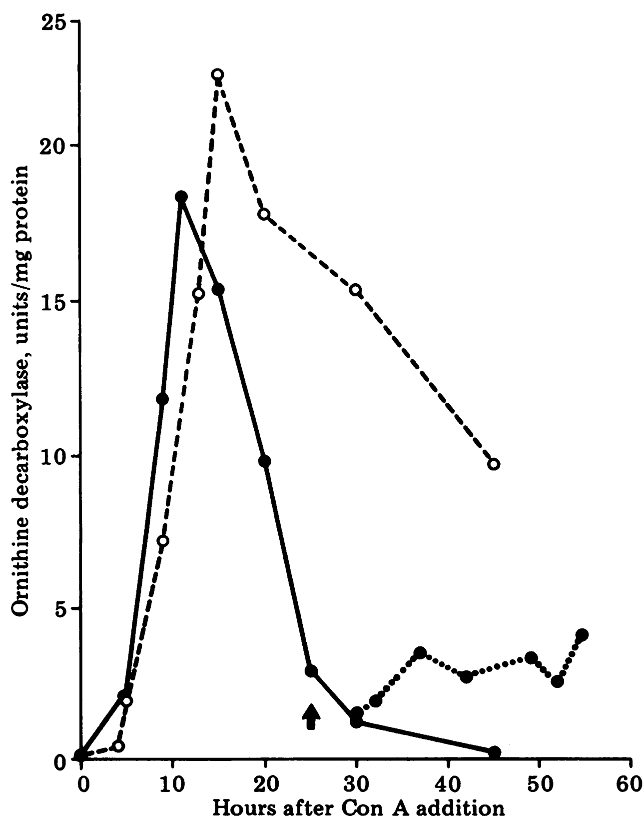

FIG. 4. Changes in ornithine decarboxylase activity after addition of Con A at 18 (○---○) and 108 (●—●) μg/ml to lymphocyte cultures. Con A was added to replicate 50-ml cultures (1.5×10^8 cells); at the indicated times cultures were collected, cell extracts were prepared, and ornithine decarboxylase activities were measured. α-Methylmannopyranoside was added at 25 hr (arrow) to cultures containing 108 μg of Con A per ml (●·····●).

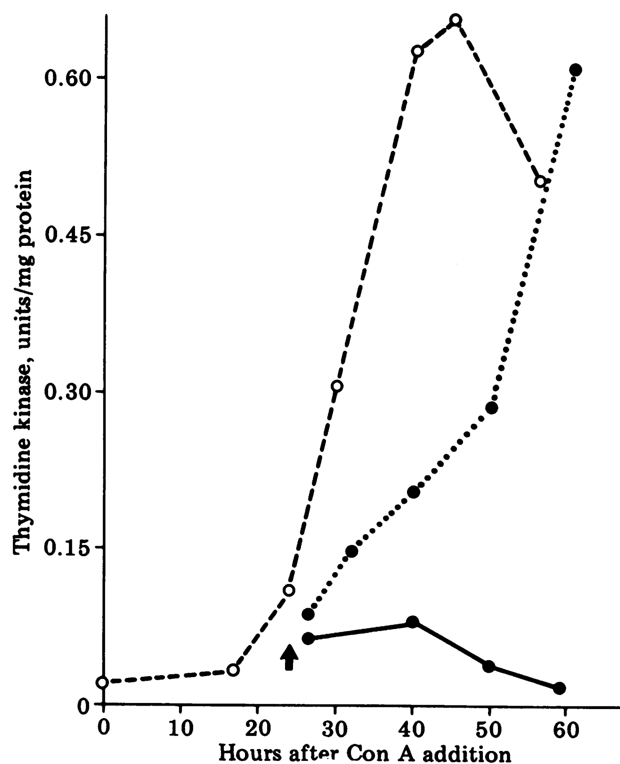

FIG. 5. Changes in thymidine kinase activity as a function of time after addition of Con A at 18 (○---○) and 108 (●—●) μg/ml to lymphocyte cultures. Con A was added to replicate 100-ml cultures (3×10^8 cells); at the indicated times cultures were collected, cell extracts were prepared, and thymidine kinase activities were measured. α-Methylmannopyranoside was added at 25 hr (arrow) to cultures containing 108 μg of Con A per ml (●·····●).

of Con A. An interesting difference between ornithine decarboxylase and AdoMet decarboxylase was observed when the high Con A block was reversed with α-methylmannopyranoside at 25 hr. In contrast to AdoMet decarboxylase, there was very little increase of ornithine decarboxylase activity after reversal of the high Con A block (Fig. 4).

Ornithine decarboxylase and the early phase of AdoMet decarboxylase, which are induced by high doses of Con A, may be representatives of an "early" regulatory class of proteins. However, the second phase of AdoMet decarboxylase accumulation, which is blocked by high doses of Con A, appears to coincide with a group of "late" enzymes involved in DNA replication—e.g., thymidine kinase (16–18). In control cultures, thymidine kinase activity began to rise approximately 24 hr after mitogen addition (Fig. 5), coinciding with the onset of DNA synthesis (compare with [^{3}H]thymidine incorporation in Fig. 3). In contrast, there was essentially no induction of thymidine kinase in the presence of high Con A. Reversal of the high Con A block with α-methylmannopyranoside led to an accumulation of thymidine kinase activity, as was found with AdoMet decarboxylase and [^{3}H]thymidine incorporation.

DISCUSSION

In an earlier report, McClain and Edelman (19) suggested that lymphocytes were responsive to high doses of Con A, but that entry into S phase was inhibited. This conclusion was based on measurements of [^{3}H]thymidine incorporation after α-methylmannopyranoside addition to cultures treated with high doses of Con A. In this study, we have shown directly that high, nonmitogenic doses of Con A activate lymphocytes to undergo some early events of the mitogenic sequence. The initial accumulation of AdoMet decarboxylase and ornithine decarboxylase activities proceeded similarly to that in cultures

stimulated with doses of Con A that were optimally mitogenic. The time courses of accumulation of AdoMet decarboxylase (Fig. 2) and ornithine decarboxylase (Fig. 4) indicated that the normal mitogenic program is first inhibited 10–15 hr after addition of high Con A. After this time, the levels of these enzymes decreased relative to control cultures, and thymidine kinase, an activity that normally increases coincidently with the onset of synthesis, did not accumulate.

The inhibitory effects of Con A at 108 μg/ml were reversible. After addition of α-methylmannopyranoside, the activities of AdoMet decarboxylase and thymidine kinase increased and the cells progressed through S phase. On the other hand, ornithine decarboxylase activity failed to increase substantially after saccharide addition. One interpretation of these results might be that ornithine decarboxylase and thymidine kinase are representatives of enzymes in early and late regulatory classes, respectively, whereas AdoMet decarboxylase, which accumulates biphasically, may be under both regulatory influences. Thus, the increase in AdoMet decarboxylase and thymidine kinase and the lack of ornithine decarboxylase accumulation after addition of α-methylmannopyranoside are consistent with an ordered sequence of events in the mitogenic program. It has been reported that synchronized chinese hamster ovary cells accumulate AdoMet decarboxylase in late G_1/early S phase (20). This probably corresponds to the second phase of AdoMet decarboxylase accumulation observed in the lymphocyte system which is coincident with the entrance of cells into S phase.

One interpretation of our results is that at high doses of Con A the lectin binds initially at mitogenic levels but then, in a slow process, the amount of bound Con A increases to levels that are inhibitory. This seems unlikely in view of the fact that the level of AdoMet decarboxylase at 9 hr remained close to normal even at a Con A concentration of 600 μg/ml (see Fig. 1*B*). Using the above interpretation, it would require approximately 10–15 hr for Con A at a concentration of 108 μg/ml to bind to inhibitory levels (see Fig. 2). If the rate of binding were to show the expected first-order dependence on Con A concentration, one would expect that, at a concentration of 600 μg/ml, Con A would become inhibitory in approximately one-sixth the time or at most 2–3 hr after lectin addition. This was clearly not the case (see Fig. 1*B*).

Any explanation for our results must take into consideration that high, nonmitogenic concentrations of Con A: (*i*) initially stimulate a normal cellular response by the criteria of AdoMet decarboxylase and ornithine decarboxylase activities; (*ii*) interrupt the mitogenic program between 10 and 15 hr after addition in a way that apparently cannot be accounted for by a time dependence of lectin binding or effects on cell viability; and (*iii*) *reversibly* inhibit mitogenesis, as judged by the progression of cells through S phase and the accumulation of AdoMet decarboxylase and thymidine kinase activities after addition of α-methylmannopyranoside. A simple model consistent with these findings is that small lymphocytes are stimulated to progress through the G_1 phase of the cell cycle under the influence of high doses of Con A. Metabolic changes associated with mitogenesis, such as increased enzyme levels, proceed normally for 10–15 hr. However, after this time, high Con A reversibly blocks the progression of cells either in G_1 or at the G_1/S interface of the cell cycle. Although the lag between α-methylmannopyranoside addition to cells treated with high doses of Con A and the progression of cells through S phase (see Fig. 3) is suggestive of a Con A-mediated arrest of cells in G_1, this delay might, alternatively, be the result of the time required for the saccharide to reverse the inhibition of cells arrested at G_1/S. However, considering that the effects of high doses of Con A are first observed 10–15 hr prior to the normal entry of cells into S phase (see Figures 2 and 4), a G_1 arrest seems to be the more likely of the two possibilities.

The nature of the interaction of Con A with cells leading to inhibition remains in question. Correlative evidence has suggested a relationship between the immobilization of cell receptors by Con A and the inhibition of mitogenesis (4). Consistent with this idea, succinyl-Con A, a divalent derivative of Con A, does not inhibit either mitogenesis or receptor mobility at high doses (4, 5). It is clear that if immediate restriction of receptor mobility occurs in the lymphocyte system under the influence of high doses of Con A, then "normal" lymphocyte activation can proceed for at least 10 hr under these conditions. Whether inhibition of cell-cycle progression is due to a restriction of the mobility of surface receptors remains to be determined.

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