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Ornithine Decarboxylase Induction in Mitogen-Stimulated Lymphocytes is Related to the Specific Activation of Type I Adenosine Cyclic 3',5'-Monophosphate-Dependent Protein Kinase

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SUMMARY

BYUS, CRAIG V., KLIMPEL, GARY R., LUCAS, DAVID O. & RUSSELL, DIANE HADDOCK (1978) Ornithine decarboxylase induction in mitogen-stimulated lymphocytes is related to the specific activation of type I adenosine cyclic 3',5'-monophosphate-dependent protein kinase. *Mol. Pharmacol.*, **14**, 431-441.

The induction of ornithine decarboxylase was studied following the stimulation of human peripheral blood lymphocytes with concanavalin A (ConA) (10 μg/ml). Following treatment with ConA, ornithine decarboxylase activity increased 4-5-fold between 6 and 12 hr of incubation and reached a peak level 10-12-fold above control (unstimulated) values by 48 hr. Increases in incorporation of [^{3}H]uridine into acid-insoluble material followed a similar time course after the addition of ConA to lymphocytes. The rate of incorporation of [^{3}H]thymidine into acid-insoluble material was maximal at 72 hr. The degree of activation of soluble cyclic 3',5'-AMP-dependent protein kinase(s) was determined at various times following ConA stimulation. Between 1 and 2 hr after mitogen administration, the cyclic AMP-dependent protein kinase activity ratio increased markedly and was 0.23 unit above control values by 4 hr. The activity ratio decreased between 4 and 8 hr and returned to higher values after incubation with the mitogen for 12, 24, and 48 hr. Separation of the free catalytic subunit from type I and type II protein kinase holoenzyme isozymes via C_6-aminoalkyl agarose chromatography revealed that only type I protein kinase was activated 4 hr following incubation of lymphocytes with a mitogenic concentration of ConA (10 μg/ml). The addition of dibutyryl cyclic AMP at the same time as ConA (10 μg/ml) resulted in nearly total activation of both type I and type II protein kinases at 4 hr but was inhibitory to the later induction of ornithine decarboxylase and to increased synthesis of RNA and DNA. A high concentration of ConA (100 μg/ml), which also activated both isozyme forms of the kinase at 4 hr, produced only a small increase in ornithine decarboxylase activity and RNA synthesis at later times and no elevation in DNA synthesis. The data suggest that while the early activation of type I cyclic AMP-dependent protein kinase may mediate in a positive manner the induction of ornithine decarboxylase and the mitogenic response of lymphocytes to ConA, concomitant activation of type II protein kinase may inhibit this process.

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INTRODUCTION

The magnitude of the induction of ornithine decarboxylase in tissues stimulated by trophic hormones (1–4) and analogues of cyclic 3′,5′-AMP and/or phosphodiesterase inhibitors (5–7) closely parallels the extent of the activation of cyclic AMP-dependent protein kinase. Therefore we have previously postulated that the induction of ornithine decarboxylase is a mandatory and intermediate step in the trophic response generated by increased cyclic AMP levels and the activation of cyclic AMP-dependent protein kinase (3).

Kuo and Greengard (8) suggested that all the effects of cyclic AMP upon the cell were a result of the activation of cyclic AMP-dependent protein kinases. Studies in many laboratories now suggest that the state of activation of this kinase(s), rather than the actual intracellular level of cyclic AMP, may be a more sensitive and accurate measurement of the degree to which cyclic AMP controls a variety of cellular functions, which include ACTH stimulation of steroidogenesis (9), FSH stimulation of Sertoli cells (10), LH stimulation of testosterone production in Leydig cells (11), and the induction of such enzymes as tyrosine hydroxylase (12) and tyrosine aminotransferase (13, 14), in addition to ornithine decarboxylase (1, 5, 15). This may be due to the rapid turnover rate of cyclic AMP produced by phosphodiesterases and cellular excretion (16–18) or to the ability of ionic fluxes to alter the effect of a given concentration of cyclic AMP on the activation of cyclic AMP-dependent protein kinases (19, 20).

There are two isozymic forms of soluble cyclic AMP-dependent protein kinase in mammalian tissues, remarkably constant in characteristics, and referred to as type I and type II (21, 22). Type I and type II holoenzymes are believed to have different regulatory subunits but identical catalytic subunits (20). The significance of the heterogeneity of the kinase has been difficult to understand in view of the similar biochemical properties of the catalytic subunits. While ornithine decarboxylase induction during cell cycle progression appears to be related to the specific activation of type I protein kinase (23, 24), no such selective intracellular activation has been associated with the action of a hormone or other membrane-active agent believed to exert at least part of its effects through cyclic AMP.

The role of cyclic AMP as a possible mediator of lymphocyte mitogenesis in response to plant lectins has been particularly difficult to evaluate. Slight increases in cyclic AMP have been reported in lymphocytes shortly after stimulation with concanavalin A (25, 26). However, the administration of dibutyryl cyclic AMP simultaneously with the lectin inhibited both increased nucleic acid synthesis and mitosis (25, 26). Using a technique which allows for the rapid determination of the activation state of each type of cyclic AMP-dependent protein kinase, we have assessed the involvement of the kinase isozymes concerning these apparently conflicting roles of cyclic AMP (27). We report that the early activation of type I cyclic AMP-dependent protein kinase and the induction of ornithine decarboxylase appear to be integral parts of the mitogenic response of lymphocytes to plant lectins, while activation of both types of cyclic AMP-dependent protein kinases within 4 hr inhibits subsequent ornithine decarboxylase induction, increased RNA and DNA synthesis, and mitogenesis.

MATERIALS AND METHODS

Peripheral blood lymphocyte culture. Fifty to 100 ml of venous blood from healthy male donors were collected in heparinized tubes. Lymphocytes were isolated under sterile conditions by flotation in Ficoll-Hypaque (Litton Bionetic Laboratory Products, Kensington, Md.), and were washed three times using phosphate-buffered NaCl. The resulting lymphocytes were cultured in disposable plastic culture tubes at a density of 2×10^6/ml in Eagle's minimal essential medium with Earle's salts (Flow Laboratories, Los Angeles) supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and Fungizone (0.25 μg/ml). Lymphocytes were routinely placed into culture in the afternoon and incubated overnight, and mitogen was added on the following morning. Cultures were maintained at 37° for 72

hr in a humidified atmosphere containing 5% CO_2 in air. ConA[3] was obtained from Sigma Chemical Company.

Assay for ornithine decarboxylase. Tubes containing 40×10^6 cells were centrifuged at $500 \times g$ for 10 min. The culture medium was gently decanted, the tube was wiped free of remaining medium, and the cells were suspended in 350 μl of ice-cold homogenizing buffer consisting of 50 mM potassium phosphate (pH 7.2), 1 mM dithiothreitol, 0.1 mM EDTA, and 40 μM pyridoxal phosphate. The lymphocytes were then sonicated for 5 sec using an E/MC sonicator (Kontes, Berkeley, Calif.) fitted with a 5-in. microprobe at a power setting of 6. Microscopic examination of the cells revealed that greater than 95% were broken following this procedure. The sonic extract was centrifuged at $10,000 \times g$ for 10 min at 0°, and the resulting supernatant was used as the source of enzyme activity. Three 100-μl aliquots were removed from each sample and assayed individually for ornithine decarboxylase activity in a total volume of 200 μl. Ornithine decarboxylase activity was determined as the amount of $^{14}CO_2$ released from 1.0 μCi of DL-[^{14}C]ornithine (4.0 mCi/mmole; New England Nuclear) at 37° during a 60-min assay. The assay was performed in 15-ml tapered centrifuge tubes fitted with special stoppers and center wells (Kontes). The $^{14}CO_2$ was trapped in 0.2 ml of ethanolamine–methoxyethanol (2:1) contained in the center well, following injection of 0.5 ml of 1 M citric acid into the mixture after the 60-min incubation. The stoppers were then removed, and the center wells were counted in 10 ml of toluene–ethanol scintillation fluid.

Estimation of RNA and DNA syntheses. RNA and DNA syntheses were estimated by the amounts of [^{3}H]uridine (10 mCi/mmole) and [^{3}H]thymidine (12 mCi/mmole; New England Nuclear), respectively, incorporated into acid-insoluble material. Control and mitogen-stimulated cultures in 96-well microtiter plates containing 4×10^5 cells/well in 0.2 ml of culture medium were pulsed with 2.0 μCi of [^{3}H]uridine for 30 min (RNA) or 0.2 μCi of [^{3}H]thymidine for 4 hr (DNA) per well. Cells from microcultures were harvested onto glass filter paper with a multiple-sample harvester, followed by precipitation of incorporated label with cold 10% trichloracetic acid. The glass paper discs were dried in air and counted in 15 ml of a toluene-based scintillation fluid to a previously set error of 2%. Incubation of lymphocytes with ConA or dibutyryl cyclic AMP did not alter the uptake of [^{3}H]thymidine or [^{3}H]uridine by the cells, since these cultures had the same acid-soluble tritium label as control cultures.

Supernatant protein kinase activity. Lymphocytes (20×10^6) in culture were centrifuged at $1000 \times g$ for 5 min. The culture medium was rapidly decanted, and the pelleted cells were suspended in 350 μl of 25 mM potassium phosphate (pH 6.8), 0.5 mM isobutylmethylxanthine, 5 mM EDTA, 20 mM NaF, 125 mM KCl, and 5 mM 2-mercaptoethanol and sonicated at 0° as described above. The sonic extract was rapidly transferred to a minicentrifuge tube and centrifuged at 4° for 30 sec in a Beckman model B Microfuge. Then 25-μl aliquots of the supernatants were assayed for protein kinase activity in the presence and absence of saturating amounts of cyclic AMP (5 μM) in a total volume of 75 μl of the same buffer with 25 mM magnesium acetate, 200 μg of mixed calf thymus histone (Sigma), and 0.5–1.0 μCi of [γ-^{32}P]ATP (7 mCi/mmole; New England Nuclear), plus sufficient nonradioactive ATP to bring the total substrate concentration to 0.1 mM. The assay was initiated by the addition of the supernatant and allowed to proceed for 5 min at 30°, and then 50 μl of reaction mixture were spotted on Whatman No. 3 MM paper filters. The filters were washed for 20 min in cold 15% trichloracetic acid, followed by two 15-min washes in 5% trichloracetic acid and a 2-min wash in 95% ethanol. The discs were then air-dried and counted in 5 ml of a toluene-based scintillation fluid. The ratio of protein kinase activity in the absence of cyclic AMP to activity in the presence of cyclic AMP was determined as described by Corbin and Reimann (28) from the amount of ^{32}P incorporated into acid-insoluble material without and with cyclic AMP. Under these conditions the assay was linear for 5 min in the

[3] The abbreviation used is: ConA, concanavalin A.

presence and absence of cyclic AMP, and the addition of activated charcoal to the cells prior to sonication did not alter the activity ratio from control or stimulated lymphocytes.

C_6-aminoalkyl agarose chromatography. C_6-aminoalkyl agarose chromatography was performed as described by Rangel-Aldao and Rosen (29) with minor modifications. The resin was prepared according to Shaltiel and Er-El (30) from CNBr-activated Sepharose 4B (Pharmacia) and 1,6-diaminohexane (Aldrich). Each batch of resin was calibrated in terms of the amount of supernatant protein that could be bound per milliliter of resin and for the initial NaCl concentration required to separate the free catalytic subunit in the flow-through fractions from type I holoenzyme. For lymphocytes, an initial concentration of 50 mM NaCl and up to 15 mg of protein per milliliter of resin gave excellent separation without any reassociation of catalytic and regulatory subunits on the column. Catalytic subunit purified by Sephadex G-100 chromatography (7) and type I kinase holoenzyme purified approximately 20-fold from rat heart via DEAE-cellulose chromatography were used to calibrate the column (22) (Fig. 1). Chromatography of type I and type II holoenzymes incubated with cyclic AMP (Fig. 1D and E) had only one peak, which chromatographed in the same fraction as purified catalytic subunit (Fig. 1C). Since each C_6 resin preparation differed in its capacity to bind the catalytic subunit at low ionic strength, determination of the appropriate initial NaCl concentration for each batch was critical to allow for rapid elution of the catalytic unit in the flow-through fractions and retention of type I holoenzyme by the resin and to prevent reassociation of the catalytic and regulatory subunits during chromatography. Type II holoenzyme, purified from beef heart (28), was found to bind well to C_6-agarose and was easily separable from type I enzyme with a linear NaCl gradient (eluting at 0.25 M NaCl) (Fig. 1).

Resin (1.5 ml) was packed in a disposable plastic syringe and equilibrated with 15 mM potassium phosphate (pH 6.8), 0.5 mM isobutylmethylxanthine, 20 mM NaF, 5 mM 2-mercaptoethanol, 1 mM EDTA, and 30–75 mM NaCl (depending upon the particular batch of resin). Then 50×10^6 lymphocytes were sonicated in 300 μl of the above buffer and centrifuged for 30 sec as described previously. The supernatant (250 μl) was applied to the column and washed with 5–10 ml of buffer. A linear gradient of 10 ml each of 0.05–0.35 M NaCl was used, and fractions were collected; 50 μl of each fraction were assayed for protein kinase activity in the presence of 5 μM cyclic AMP in a total volume of 75 μl as described above. Experiments in which semipurified catalytic and regulatory subunits from type I or type II kinase were mixed revealed minimal reassociation under these conditions. Recovery of activity was greater than 85% in all instances.

RESULTS

Ornithine decarboxylase activity and nucleic acid synthesis in lymphocytes after administration of ConA. Temporal alterations in the activity of ornithine decarboxylase and rates of RNA and DNA synthesis following the addition of ConA (10 μg/ml) to resting lymphocytes in culture are illustrated in Fig. 2. These parameters were dependent upon the concentration of ConA utilized in the experiment (data not shown). ConA (10 μg/ml) produced maximal increases in the activity of ornithine decarboxylase and in the incorporation of [^{3}H]uridine and [^{3}H]thymidine. No detectable increase in ornithine decarboxylase activity occurred in lymphocytes during the first 6 hr after initiation of lectin treatment (Fig. 2). The activity of ornithine decarboxylase was elevated within 12 hr and reached a level 16–18-fold above control values within 48 hr. Increases in the incorporation of [^{3}H]uridine into acid-insoluble material always occurred after the increase in ornithine decarboxylase activity. The changes in RNA synthesis between 12 and 72 hr after the addition of ConA paralleled the alteration in ornithine decarboxylase activity. The rate of DNA synthesis was maximal at 72 hr (Fig. 2).

Activation of cyclic AMP-dependent protein kinase after administration of ConA. The degree of activation of soluble cyclic AMP-dependent protein kinase was determined at various times following

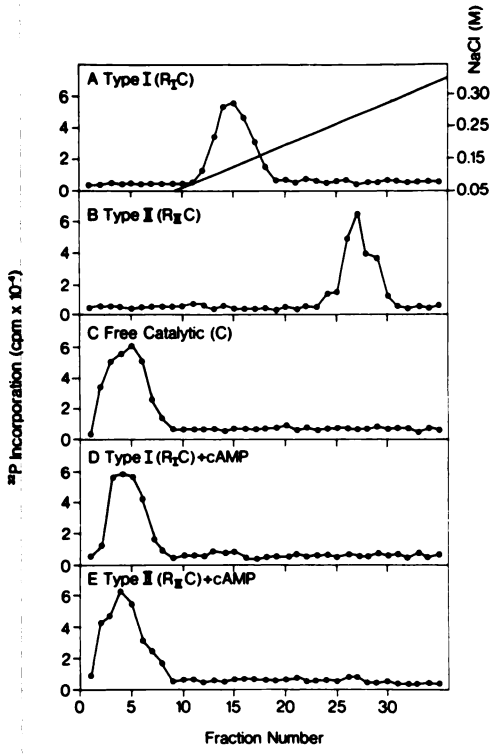

FIG. 1. *C_6-aminoalkyl agarose chromatography of purified type I and type II cyclic AMP-dependent protein kinases*

Columns containing 1.5 ml of resin were prepared and equilibrated at 4° with 5 mM potassium phosphate (pH 6.8), 5 mM 2-mercaptoethanol, 1 mM EDTA, and 50 mM NaCl as described in MATERIALS AND METHODS. The total amount of protein applied to the column was between 0.3 and 0.5 mg. A. Type I holoenzyme (R_IC) purified approximately 20-fold from rat heart via DEAE-cellulose chromatography (22) was applied to the column, which then was washed with 10 ml of equilibration buffer followed by a linear gradient of 10 ml each of 0.05–0.35 M NaCl in the same buffer. Fractions were collected and assayed for protein kinase activity in the presence of saturating levels of cyclic AMP (5 μM) as described in MATERIALS AND METHODS. B. Type II holoenzyme ($R_{II}C$) purified approximately 20-fold from beef heart via DEAE-cellulose chromatography (28) was chromatographed on the C_6-agarose column in a similar manner. C. C_6-aminoalkyl agarose chromatography of the catalytic subunit (C) separated from type I holoenzyme of rat heart by Sephadex G-100 chromatography (7). The catalytic subunit purified in the same manner (7) from type II holoenzyme was also eluted in the flow-through fractions. D. Type I protein kinase from rat heart (as shown in Fig. 1A) was incubated with 50 pmoles of cyclic AMP in a total volume of 250 μl at 30° for 2 min prior to chromatography. E. Type II holoenzyme purified from beef heart was incubated with 50 pmoles of cyclic AMP in a total volume of 250 μl at 30° for 2 min and chromatographed on the C_6-agarose column.

ConA stimulation (Fig. 3). The protein kinase activity ratio of the unstimulated lymphocytes from a variety of donors was 0.36 ± 0.17 (mean ± SE; $n = 17$). Because of the variation between lymphocyte preparations from individual donors, the data in Fig. 3 are represented as the difference in the cyclic AMP-dependent protein kinase activity ratios between control (unstimulated) lymphocytes and cultures from the same donor incubated in the presence of ConA. Unstimulated lymphocytes prepared from a single individual had similar protein kinase activity ratios throughout the 48-hr culture period (SE = ±0.04).

The addition of ConA (10 μg/ml) to lymphocyte cultures resulted in a small increase in the protein kinase activity ratio (less than 0.07) during the initial hour of incubation (Fig. 3). One to two hours after ConA administration, the activity ratio increased markedly and was 0.23 above the control value 4 hr after mitogen stimulation. The activation of protein kinase by ConA appeared to be biphasic, as the activity ratio consistently decreased between 4 and 8 hr and returned to higher values after incubation with the mitogen for 12, 24, and 48 hr (Fig. 3). The increase in the activity ratio of lymphocytes treated with ConA (Fig. 3) was due to an increase in protein kinase activity assayable in the absence of cyclic AMP (Table 1). Total cyclic AMP-dependent protein kinase activity did not change significantly during the 48-hr incubation period (Table 1).

Inhibition of ornithine decarboxylase induction and RNA and DNA synthesis by dibutyryl cyclic AMP and high levels of ConA. The effects of dibutyryl cyclic AMP and a high concentration of ConA (100 μg/ml) on protein kinase activation, ornithine decarboxylase activity, and the rates of RNA and DNA synthesis are shown in Table 2. The addition of a high concentration of ConA (100 μg/ml) to unstimulated lymphocytes resulted in a further increase (0.2) in protein kinase activity ratio compared with the values obtained using a low concentration of ConA (10 μg/ml) (Table

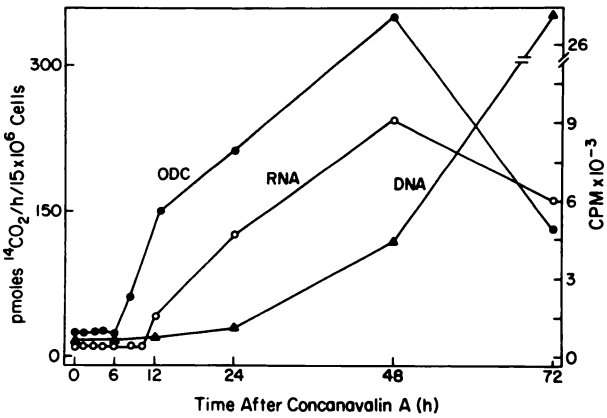

FIG. 2. *Changes in ornithine decarboxylase activity and RNA and DNA synthesis following incubation of lymphocytes with concanavalin A*

Lymphocytes were purified from human peripheral blood and cultured overnight as described in MATERIALS AND METHODS prior to the addition of ConA (10 μg/ml). The activity of ornithine decarboxylase (ODC) (●——●; picomoles of $^{14}CO_2$ per hour per 15 × 10^6 cells) and the incorporation of [^{3}H]uridine (○——○; counts per minute per 30 min per 4 × 10^5 cells) and [^{3}H]thymidine (▲——▲; counts per minute per 2 hr per 4 × 10^5 cells) into acid-insoluble material was determined at the times indicated following the addition of ConA. The values from unstimulated cultures (shown as zero time) did not change throughout the 72-hr incubation period. The data are represented as the averages of duplicate determinations of five separate time courses not differing by more than ±15%.

2). However, this additional activation of cyclic AMP-dependent protein kinase preceded significant inhibition of the induction of ornithine decarboxylase and of the synthesis of RNA and DNA (Table 2) as compared with the mitogenic response elicited by ConA at 10 μg/ml.

Dibutyryl cyclic AMP (0.1 mM) added to lymphocytes resulted in nearly total activation of soluble protein kinase within 4 hr (Table 2). The activity of ornithine decarboxylase and the incorporation of radioactive precursors into RNA and DNA in the cyclic nucleotide-stimulated cultures were unchanged compared with the control cultures. Incubation of lymphocytes with both a mitogenic concentration of ConA (10 μg/ml) and dibutyryl cyclic AMP (0.1 mM) totally activated protein kinase by 4 hr, as evidenced by an activity ratio of 1.0, but inhibited the large increases in ornithine decarboxylase activity and in the synthesis of RNA and DNA that occurred when ConA (10 μg/ml) alone was added to the cultures (Table 2).

C_6-aminoalkyl agarose chromatography of lymphocyte protein kinase. The specific activation of type I and type II cyclic AMP-dependent protein kinase isozymes was determined in lymphocytes incubated for 4 hr with ConA (10 or 100 μg/ml), dibutyryl cyclic AMP (0.1 mM), and ConA (10 μg/ml) plus dibutyryl cyclic AMP (0.1 mM) by rapid chromatography of supernatant preparations on C_6-aminoalkyl agarose columns (Fig. 4). Under the column chromatography conditions in MATERIALS AND METHODS, the free catalytic subunit was eluted from the column in the initial flow-through fractions. Type I (R_IC) and type II ($R_{II}C$) holoenzymes bound to the column and were eluted at NaCl concentrations of 0.12 and 0.27 M, respectively (Fig. 4A). The free regulatory subunits from both type I and type II protein kinases (R_I and R_{II}) were eluted from the column with 0.20 M NaCl (data not shown).

The amount of protein kinase detected as the free catalytic subunit by C_6-aminoalkyl agarose chromatography relative to the total cyclic AMP-dependent protein kinase corresponded closely to the activity ratio calculated from assaying lymphocyte supernatants in the absence and presence of cyclic AMP at all times assayed. C_6-aminoalkyl agarose chromatography of kinase from lymphocytes incubated with ConA (10 μg/ml) for 4 hr (time of maximal protein kinase activation; Fig. 3) indicated a specific decrease in type I holoenzyme concom-

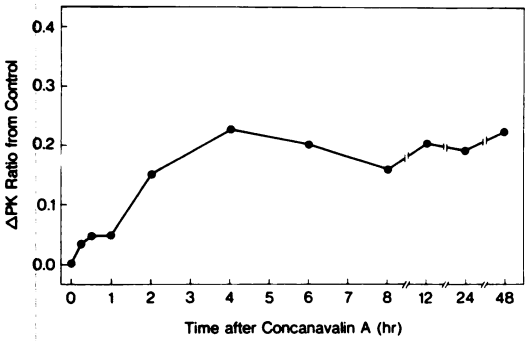

FIG. 3. *Activation of lymphocyte cyclic AMP-dependent protein kinase(s) following incubation with concanavalin A*

ConA (10 μg/ml) was added to lymphocyte cultures after an overnight preliminary incubation period. The cyclic AMP-dependent protein kinase activity ratio was determined in control (unstimulated) and ConA-stimulated cultures as described in MATERIALS AND METHODS at the times indicated following the addition of the mitogen. The data are represented as the difference in the protein kinase (PK) activity ratio between control lymphocytes and cultures incubated with ConA. Each point is the average of at least 10 determinations not differing by more than ±10% in experiments with lymphocytes purified from at least five donors. The activity ratio of unstimulated lymphocytes was 0.36 ± 0.17 (mean ± SEM; n = 17), depending upon the donor. The protein kinase activity ratio of unstimulated lymphocytes from an individual donor did not change significantly during a 48-hr culture period.

itant with increased free catalytic unit (Fig. 4B) in the flow-through fractions. There was no decrease in type II holoenzyme after ConA stimulation.

Since increased activation of cyclic AMP-dependent protein kinase above that observed with a mitogenic dose of ConA (10 μg/ml) seemed to result in inhibition of the induction of ornithine decarboxylase and of the subsequent increase in RNA and DNA synthesis (Table 2), we wished to determine whether this additional protein kinase activation was associated with a particular isozyme of the kinase. After a high concentration of ConA (100 μg/ml) was added to the lymphocyte cultures, there was almost total activation of type I and type II holoenzymes (Fig. 4C). Similarly, after dibutyryl cyclic AMP (0.1 mM) treatment, both type I and type II isozymes were activated. Essentially all the kinase activity was eluted from the agarose column as the free catalytic subunit (Fig. 4D). Incubation of the lymphocytes with ConA (10 μg/ml) and dibutyryl cyclic AMP (0.1 mM) also activated both type I and type II kinases (Fig. 4E) compared with the cultures incubated with ConA (10 μg/ml) alone, in which only type I isozyme was activated (Fig. 4B).

DISCUSSION

There have been conflicting reports regarding the involvement of cyclic AMP in the regulation of proliferative effects of mitogens on lymphocytes (25, 26). A major argument against a positive role of cyclic AMP in this process has been the observation that while maximal increases in cyclic AMP occur 5–20 min following the addition of ConA or phytohemagglutinin, the presence of the mitogen is required on the cell surface for 8–20 hr to affect DNA synthesis (31–33). The data presented in Fig. 3 show that cyclic AMP-dependent protein kinases remained activated for many hours in the presence of ConA. Sons *et al.* (34) have

TABLE 1

Alterations in supernatant cyclic AMP-dependent protein kinase activity following incubation of lymphocytes with concanavalin A

Peripheral blood lymphocytes were purified on Ficoll-Hypaque gradients from an individual donor. The protein kinase activity from supernatant preparations was determined in the absence and presence of 5 μM cAMP at the times indicated following the addition of ConA (10 μg/ml) as described in MATERIALS AND METHODS. The activity of unstimulated lymphocytes is shown at zero time and did not change significantly throughout a 48-hr culture period. Protein kinase activity is expressed as ^{32}P incorporated into histone in 5 min per 25 μl of supernatant and is the average of three assays not differing by more than ±5%.

Time after ConA	Protein kinase activity		Protein kinase activity ratio (−cAMP / +cAMP)
	−cAMP	+cAMP	
hr	*pmoles/5 min/25 μl*		
0	3.9	11.2	0.35
1	4.5	11.0	0.41
2	5.3	10.8	0.49
4	6.2	11.2	0.55
6	6.0	11.6	0.51
8	5.7	11.5	0.49
12	5.5	11.1	0.50
24	6.2	12.1	0.51
48	6.8	11.8	0.57

TABLE 2

Inhibition of ornithine decarboxylase induction and RNA and DNA synthesis by dibutyryl cyclic AMP and high concentrations of concanavalin A

Lymphocytes were purified and cultured overnight prior to the addition of ConA and dibutyryl cyclic AMP as indicated. The cyclic AMP-dependent protein kinase activity ratio (mean ± standard error) was determined after a 4-hr culture period in supernatants from lymphocytes prepared from three separate donors as described in MATERIALS AND METHODS. Ornithine decarboxylase activity (picomoles of $^{14}CO_2$ per 60 min per 15×10^6 cells; mean ± SEM; $n = 9$) was determined following 24-hr and 48-hr incubations with ConA and dibutyryl cyclic AMP. RNA synthesis is shown as counts per minute of [^{3}H]uridine per 30 min per 4×10^5 cells incorporated into acid-insoluble material (mean ± SEM; $n = 9$) and was measured at 24 and 48 hr. DNA synthesis is represented as counts per minute of [^{3}H]thymidine per 4 hr per 4×10^5 cells incorporated into acid-insoluble material (mean ± SEM; $n = 12$) and was determined after 72 hr as described in MATERIALS AND METHODS.

Additions	4 hr	24 hr		48 hr		72 hr
	Protein kinase activity ratio	Ornithine decarboxylase	RNA	Ornithine decarboxylase	RNA	DNA
Control	0.31 ± 0.02	19 ± 3	200 ± 75	23 ± 2	400 ± 70	620 ± 150
ConA (10 μg/ml)	0.55 ± 0.04	124 ± 12	950 ± 83	322 ± 25	8,000 ± 500	27,060 ± 3,000
ConA (100 μg/ml)	0.75 ± 0.06	41 ± 7	390 ± 24	22 ± 4	920 ± 160	540 ± 130
Dibutyryl cyclic AMP (0.1 mM)	0.89 ± 0.10	18 ± 2	173 ± 82	24 ± 5	341 ± 91	545 ± 120
ConA (10 μg/ml) + dibutyryl cyclic AMP (0.1 mM)	1.00 ± 0.05	20 ± 5	185 ± 50	31 ± 2	575 ± 100	1,320 ± 200

recently reported an early peak in the phosphorylation of both histone and nonhistone chromatin proteins occurring 4–8 hr after addition of ConA to cultured lymphocytes. Allfrey, Inoue, and Johnson (35) also have shown that phosphorylation of some cytoplasmic proteins that migrate to the nucleus occurs during this period. We suggest that the mitogen may be required for several hours in order to maintain the activation of protein kinase required for the phosphorylation of these proteins.

In addition, no detectable increase in ornithine decarboxylase activity occurred prior to a 6-hr incubation of lymphocytes with ConA (Fig. 2). The rapid induction of this enzyme has been shown to be mediated by cyclic AMP and cyclic AMP-dependent protein kinase in a wide variety of growth systems (1, 3). A transcriptional mechanism has been implicated in the control of ornithine decarboxylase in most of these instances (1, 3), including mitogen stimulation of lymphocytes (36, 37). The prolonged activation of protein kinase demonstrated here may be required for the regulation of the gene responsible for the increase in ornithine decarboxylase activity as well as for the synthesis of other specific mitogen-induced proteins.

The induction of ornithine decarboxylase has proven to be an early and marked event in the trophic response of many different tissues and cells to hormonal stimulation (1–7). The induction of this enzyme is temporally related to the prior activation of cyclic AMP-dependent protein kinase (1, 3), while the extent of ornithine decarboxylase induction appears to be related to the degree of protein kinase activation (1, 5). The addition of a mitogenic concentration of ConA (10 μg/ml) to cultured lymphocytes led to a large increase in ornithine decarboxylase activity, followed by increased synthesis of RNA and DNA (Fig. 2). The induction of ornithine decarboxylase in this instance was preceded by the specific activation of the type I isozyme of cyclic AMP-dependent protein kinase (Fig. 4A and B). ConA (10 μg/ml) did not activate type II isozyme during this early period. There are numerous reports that the nonspecific elevation of intracellular cyclic AMP levels in mitogen-stimulated lymphocytes by cyclic AMP analogues (38–40), phosphodiesterase inhibitors (38), or pros-

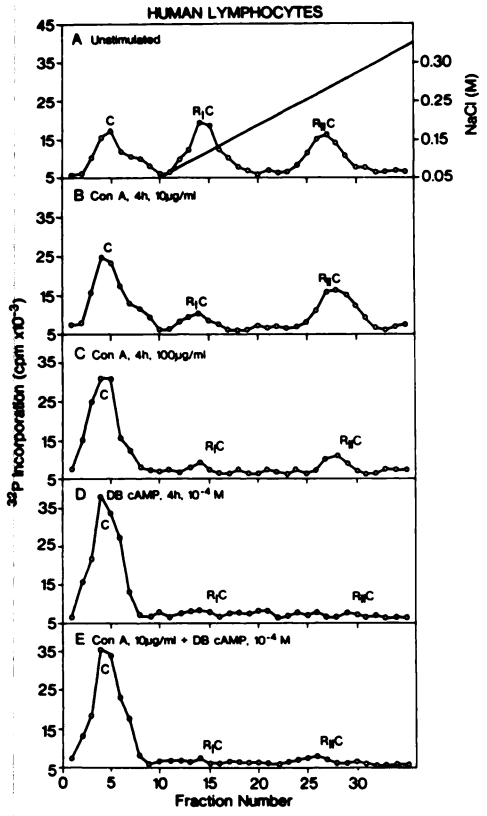

FIG. 4. *C_6-aminoalkyl agarose chromatography of lymphocyte cyclic AMP-dependent protein kinase isozymes*

Columns containing C_6-aminoalkyl agarose were prepared and calibrated for the initial concentration of NaCl required to elute the free catalytic subunit as described in MATERIALS AND METHODS. Lymphocytes (50×10^6) were sonicated in 300 μl of 5 mM potassium phosphate (pH 6.8), 5 mM 2-mercaptoethanol, 1 mM EDTA, and 0.05 M NaCl and centrifuged for 30 sec, and 250 μl of the supernatant were rapidly applied to a C_6-agarose column (1.5 ml) equilibrated with the same buffer. Free catalytic subunit was eluted in the flow-through fractions with 5 ml of buffer. A linear gradient of 10 ml each of 0.05–0.35 M NaCl was applied to elute type I (R_IC) and type II ($R_{II}C$) holoenzymes. A 50-μl aliquot of each fraction was assayed in the presence of 5 μM cyclic AMP. Purified free catalytic subunit from bovine heart (C), type I holoenzyme from rat heart (R_IC), and type II holoenzyme from bovine heart ($R_{II}C$) were eluted from the C_6-agarose column where indicated. Supernatants were applied to the columns from unstimulated lymphocytes cultured for 4 hr (A), lymphocytes stimulated with ConA (10 μg/ml) for 4 hr (B), lymphocytes stimulated with ConA (100 μg/ml) for 4 hr (C), cultures incubated with dibutyryl cyclic AMP (DB cAMP) (0.1 mM) for 4 hr (D), and cultures incubated with ConA (10 μg/ml) and dibutyryl cyclic AMP (0.1 mM) for 4 hr (E).

taglandins (41) results in the inhibition of RNA and DNA synthesis and eventual cell division. Similarly, Hadden *et al.* (42) showed that high concentrations of ConA (100 μg/ml) elevated cyclic AMP levels above those found in lymphocytes incubated with smaller amounts of ConA (10 μg/ml) and also inhibited the mitogenic process. Our data indicate that whereas early activation of type I protein kinase appears to be correlated in a positive manner with the mitogenic response elicited by ConA (10 μg/ml), either dibutyryl cyclic AMP or a high concentration of ConA, which resulted in the early activation of both type I and type II isozymes, was inhibitory to mitogenesis (Fig. 4C–E and Table 2). At 100 μg/ml, ConA produced nearly total activation of type I and type II kinases, a slight increase in ornithine decarboxylase activity and in RNA synthesis, and no change in the basal incorporation of [^{3}H]thymidine into DNA. The stimulatory effects of ConA (10 μg/ml) upon ornithine decarboxylase and on RNA and DNA syntheses, which were preceded by the specific activation of type I kinase, were totally inhibited by the addition of dibutyryl cyclic AMP, which activated both kinases.

The addition of dibutyryl cyclic AMP did not appear to be toxic to lymphocytes, since the recovery of viable cells, determined by trypan blue exclusion, was greater than 90% following 24, 48, and 72 hr of incubation with ConA (10 μg/ml), dibutyryl cyclic AMP (0.1 mM), or ConA (10 μg/ml) plus dibutyryl cyclic AMP (0.1 mM), as well as in control cultures (data not shown). Incubation of lymphocytes with 100 μg/ml of ConA for 24, 48, and 72 hr resulted in recoveries of viable cells of 93%, 72%, and 36%, respectively (data not shown). Therefore the effects of high concentrations of ConA are difficult to interpret because of their toxic effects on the cells. The inhibition of ornithine decarboxylase and RNA synthesis by 100 μg/ml of ConA at 24 hr (Table 2) would appear, however, to be related to the activation of type II cyclic AMP-dependent protein kinase, since the lymphocytes are viable at this time.

The mechanism by which the simultaneous activation of type I and type II cyclic AMP-dependent protein kinases may lead

to this inhibition is not known. It is becoming increasingly evident, however, that the two isozymes of cyclic AMP-dependent protein kinase have different functions in the cell. In addition to being present in different molecular proportions in a wide variety of tissues, type I and type II kinases appear to have different rates of synthesis and turnover during the cell cycle (23), after viral transformation (43), during testicular development (44), and during cardiac hypertrophy (45). Rosen and co-workers (29) have shown that the regulatory subunit of the type II isozyme can be phosphorylated, altering its affinity for cyclic AMP and suggesting a unique cellular role for this isozyme.

A number of early biochemical changes occur following the addition of a mitogen to cultured lymphocytes. These include increased transport of divalent cations, amino acids, carbohydrates, nucleosides, lipid precursors, and serum factors; new protein synthesis and protein phosphorylation; new synthesis of RNA; and increased levels of cyclic GMP (25). All these events may be essential for mitosis and subsequent differentiation of the cells, and any or all of these parameters may be blocked by the activation of type II cyclic AMP-dependent protein kinase at a time during the mitogenic response when only the type I isozyme is normally activated.

The potential for selective activation of the two isozymes of cyclic AMP-dependent protein kinase suggests that analogues of cyclic AMP added exogenously may not always mimic the action on cellular metabolism of the hormone or agent that requires cyclic AMP as a second messenger. The appropriate response would be promoted only if the added cyclic AMP analogue temporally activated the same forms of the kinase as the hormone or specific agent. For example, incubation of lymphocytes with dibutyryl cyclic AMP activated both forms of the kinase and did not mimic the action of the mitogen on ornithine decarboxylase and RNA and DNA synthesis (Table 2).

Cyclic AMP and cyclic AMP-dependent protein kinases have been shown to play an important role in the regulation of new protein synthesis and the induction of specific enzymes (46). The control mechanisms involved in this process may involve regulation at the levels of both transcription (1, 46, 47) and translation (48). Continued investigation of the specific activation patterns of type I and type II cyclic AMP-dependent protein kinases in relation to cyclic AMP-mediated enzyme induction may lead to a better understanding of the mechanisms by which protein kinase(s) regulates protein synthesis and cell growth and differentiation.

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