Journal of Cellular Biochemistry 33:151–160 (1987)

Inhibition of T-Lymphocyte Activation by Amiloride

B. Surendra Baliga, Lawrence J. Sindel, Lucy D. Jenkins, Subash Rashatwar, and Michael Artman

Departments of Biochemistry and Pediatrics, University of South Alabama College of Medicine, Mobile, Alabama 36617

The T-lymphocyte activation process involves a series of coordinately coupled biochemical events occuring in response to antigen or mitogen. These events have not been completely characterized. The present studies investigate the mechanism of protein synthesis during the initial phase of T-cell activation. Among the early biochemical changes, induction of protein synthesis was observed as early as 10 minutes after mitogen stimulation of T-lymphocytes. This early protein synthesis was inhibited by cycloheximide but was insensitive to actinomycin-D, indicating the presence of preformed mRNA in resting lymphocytes. Since early protein synthesis parallels the increase in protein kinase C activity in activated T-lymphocytes, these two biochemical events may be related. In the present report, amiloride, an inhibitor of Na^+/H^+ antiport and protein kinase C, significantly inhibited [^{3}H-]leucine and [^{3}H-]thymidine incorporation in a dose-dependent manner into phytohemagglutinin (PHA)-stimulated T-lymphocytes. Furthermore, when T-lymphocytes were stimulated by phorbol myristate acetate, a known activator of protein kinase C, a similar inhibition of protein and DNA synthesis by amiloride was observed. The partially purified cytosol fraction isolated from PHA-activated T-lymphocytes showed a 75% decrease in protein kinase C-mediated [^{32}P] incorporation from ATP in the presence of 100 μM amiloride. These results suggest that the T-cell activation process following exposure to mitogens involves early protein synthesis, which may be mediated by protein kinase C.

Key words: T-cell activation, protein kinase C, amiloride, T-lymphocyte and protein synthesis

The early biochemical events leading to the blastogenesis of T-lymphocytes in response to polyclonal mitogens are not completely understood. Although several alterations in metabolism and the transport of molecules have been characterized during activation, considerably less attention has been focused on specific mitogen-dependent biochemical events. The translation of preformed mRNA appears to be one of the early biochemical events during the activation process [1–3]. Although the mechanism by which the preformed mRNA is translated is not clear, it is speculated that phosphorylation of some components of the protein synthesis system triggers the

Received May 5, 1986; revised and accepted October 13, 1986.

initiation of translation of preformed mRNA. Nishizuka and coworkers have shown that the calcium- and phospholipid-dependent protein kinase (protein kinase C) is activated in several cells as a transmembrane signal for growth and proliferation [4,5]. The activation of protein kinase C has also been demonstrated in mitogen-stimulated T-lymphocytes [6,7]. Protein kinase C has been shown to catalyze in vitro phosphorylation of vinculin [8], ribosomal protein S_6 [9], myelin basic protein [10,11], lysine-rich histone [12], and eIF_2 [13]. In the present report the effects of amiloride, which has been used to inhibit Na^+/H^+ antiport and protein kinase C [14], and polymyxin-B, which has been shown to inhibit protein kinase C through membrane alteration [15,16], on some of the early biochemical reactions in mitogen activated T-lymphocytes is investigated. Amiloride inhibited ^3H-thymidine incorporation and translation of preformed mRNA. When T-lymphocytes were stimulated to proliferate by phorbol myristate acetate (PMA), a known activator of protein kinase C, a similar inhibition of early protein and DNA synthesis by amiloride was observed. Furthermore, in phytohemagglutinin (PHA)-activated T-lymphocytes pretreated with amiloride, protein kinase C activity was only 25% of the activity observed in untreated lymphocytes.

MATERIALS AND METHODS

Purified human transferrin and insulin were obtained from Collaborative Research, Inc. (Bedford, MA). Amiloride hydrochloride was a gift from Merck, Sharp and Dohme (Dr. C.A. Stone). Polymyxin-B sulphate was purchased from Sigma Biochemicals. Leucine, L [3,4,5-^3H(N)] specific activity 140 Ci/mmol, and [methyl-^3H-]thymidine, specific activity 2 Ci/mmol, were obtained from New England Nuclear (Boston, MA). Adenosine-5′[γ-^{32}P]triphosphate, > 2,000 Ci/mmol, was purchased from Amersham (Arlington Heights, IL). Phytohemagglutinin-P (PHA) was obtained from DIFCO Labs (Detroit, MI). All other chemicals used were reagent grade. The incubation medium used to culture the T-lymphocytes was RPMI-1640 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml human diferric transferrin, 10 μg/ml insulin, selenium 6 ng/ml, bovine serum albumin 800 μg/ml, linoleic acid 5 μg/ml and IL-2 20 units/ml.

Isolation of Cells

Human peripheral blood lymphocytes (PBL) were obtained from Ficoll-hypaque separation of heparinized venous blood drawn from healthy human volunteers. For macrophage depletion and cell synchronization, the mononuclear cell fraction was incubated overnight in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The nonadherent cells were separated by centrifugation, washed twice, and suspended in RPMI 1640 medium containing 10% FCS. The percentage of nonspecific esterase-positive cells was approximately 3% after overnight incubation. For additional depletion of macrophages and B lymphocytes, the cell fraction was further passed through a nylon wool column that was previously equilibrated and preincubated at 37°C for 30 min in RPMI 1640 containing 10% FCS. The T-lymphocyte-enriched fraction showed less than 0.5% macrophage contamination. For some experiments, lymphocytes were positively selected by density gradient centrifugation of spontaneous rosettes formed by T-lymphocytes and sheep red blood cells.

Cell Extract

After cells were incubated (approximately 10×10^6 cells for each experiment tube at 2×10^6/ml concentration) for 1 to 2 hr at 37°C, with or without mitogen, they were isolated by low-speed centrifugation, washed three times with RPMI-1640, and finally suspended in buffer containing Tris-HCl 20 mM pH 7.4, DTT 20 mM, phenylmethanesulfonyl fluoride (PMSF) 1 mM, and 2% Triton X-100 [15–17]. The cells were homogenized with a tight-fitting pestle and centrifuged for 10 min at 10,000g. The pellet was discharged, and the supernatant was centrifuged at 105,000g for 1 hr. This detergent-treated high-speed supernatant containing soluble and membrane-bound protein kinase C enzymes was partially purified by passing through a Sephadex G-25 column followed by anion exchange on a DE-52 column. Briefly, the total enzyme fractions were initially purified on sephadex G-25 (1 × 10 cm). The fractions containing protein kinase C activity were pooled and concentrated. The concentrated sample was then layered on an anion exchange DE-52 column (1 × 10 cm), which was previously equilibrated with buffer (Tris-HCl pH 7.5, EGTA 2 mM, PMSF 1 mM, and DTT 2 mM). The column was washed with 10 ml of above-described buffer and then eluted by 20 ml of buffer containing 70 mM NaCl. Fractions of 0.5 ml were collected. Two protein kinase C peaks were observed. The protein kinase C-containing fractions were pooled, dialysed, and concentrated. The concentrated fractions were stored at −120°C in small aliquots.

Protein Kinase C Enzyme Assay

Protein kinase C activity in T-cell supernatants was assayed by measuring incorporation of ^{32}P from [γ-^{32}P-]ATP into lysine-rich histone III-S [16]. The reaction mixture in 100 μl contained 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 250 μM EGTA, 1 mM $CaCl_2$, 80 μg/ml phosphotidylserine, 250 μg/ml histone III-S, 5 μM [γ-^{32}P]ATP (sp. act. 10,000 CPM/pmol), and 5–10 μl of partially purified protein kinase C (approximately 0.5–2 μg of protein) as indicated. Incubation was carried out at 25°C for 10 min and stopped by the addition of equal volumes of 10% cold trichloroacetic acid (TCA). The TCA precipitates were collected on Whatman GF/C filters, washed with cold 5% TCA, dried, and counted on a liquid scintillation counter using 5 ml of aqueous counting scintillation (ACS, Amersham).

^{3}H-Thymidine Incorporation

In most experiments, a serum-free medium containing RPMI-1640 with 2 mM L-glutamine, 25 mM HEPES, penicillin 100 units/ml, streptomycin 100 μg/ml, 10 μl/ml CR-ITS (insulin, transferrin, selenium, bovine serum albumin, and linoleic acid mixture supplied by Collaborative Research Inc.) was used. To obtain maximum ^{3}H-thymidine incorporation, the cell concentration was increased to 2×10^6 cells/ml. The stimulation assays were initiated in a flat-bottom microtiter plate (Corning 25860) using phytohemagglutinin (PHA) 10 μg/ml (this concentration was found optimal in serum-free media). Cultures were established in triplicate and incubated at 37°C in a humidifed atmosphere of 5% CO_2 and 95% air. Cells were pulsed with ^{3}H-thymidine during the last 12 hr of the 72-hr incubation period [18]. Cells were harvested on glass fiber strips using an automatic sample harvester. These experiments were performed in the absence and presence of amiloride or polymyxin-B. The incorporation of ^{3}H-thymidine was determined by a liquid scintillation spectrometry.

Measurement of ^{3}H-Leucine Incorporation

T-lymphocytes were suspended in leucine-free RPMI-1640 medium. An aliquot of cell suspension containing 4×10^5 cells in a total volume of 200 μl containing 1 μCi of ^{3}H-leucine (sp. act. 140 Ci/mmol) and 10 μg/ml PHA was incubated at 37°C for 1 hr in the absence and presence of amiloride or polymyxin-B as indicated. The reaction was terminated by adding equal amounts of cold trichloroacetic acid containing unlabeled leucine. The resultant precipitate was heated at 90°C for 15 min. The hot TCA precipitates were collected onto GF/A glass fiber filters, washed, dried, and the radioactivity was determined by liquid scintillation counter.

Protein concentrations were determined using Biorad reagent with BSA as a standard.

RESULTS

The kinetics of ^{3}H-leucine incorporation into unstimulated and PHA-activated lymphocytes incubated at 10-, 30-, 60-, 120-, and 180-min intervals are shown in Table I. Protein synthesis gradually increased with time in PHA-activated T-lymphocytes, and ^{3}H-leucine incorporation was up to 20-fold greater in PHA-activated lymphocytes than in unstimulated lymphocytes. The synthesis of these new proteins was completely inhibited by 50–100 μg/ml cycloheximide, but was insensitive to actinomycin D (1–5 μg/ml), which indicated that protein synthesis did not require the synthesis of new mRNA. The analysis of early translational protein products using sodium dodecyl sulfate (SDS) gel electrophoresis showed several radioactive protein peaks of different molecular weights, but the major protein peak was at approximately 12–11 .5K. These results suggest that among the many biochemical events occuring shortly after exposure of T-lymphocytes to mitogens, an increase in the synthesis of proteins is a prominent response. The inhibition of early protein synthesis by cyclo-

TABLE I. Comparison of ^{3}H-Leucine Incorporation by Unstimulated and PHA-Stimulated Lymphocytes*

Incubation time (min)	Inhibitors	^{3}H-Leucine incorporated (cpm)	
		Unstimulated	PHA stimulated
10	None	35 ± 23.8	1,117 ± 189.3
30	None	128 ± 10.4	2,433 ± 305.5
60	None	650 ± 61.1	4,100 ± 360.6
120	None	866 ± 28.9	12,000 ± 2,291
180	None	1,200 ± 200	24,833 ± 3,329
120	+ Cycloheximide (50–100 μg/ml)	83 ± 38.1	182 ± 63.3
120	+ Actinomycin-D (1–5 μg/ml)	1,233 ± 230.9	28,666 ± 5,507

*Comparison of ^{3}H-leucine incorporation by unstimulated and PHA-stimulated lymphocytes. Human T-lymphcytes (2×10^6 cells/ml) in serum and leucine-free RPMI-1640 media were incubated at different time intervals at 37°C in an atmosphere of 5% CO_2 and 95% air. The amount of PHA used was 10 μg/ml, which was found to be optimal for ^{3}H-thymidine incorporation in serum-free medium. The inhibitors were added at the start of reaction. The reaction was terminated by adding equal amounts of cold 10% trichloroacetic acid, and precipitates were collected as described in "Materials and Methods". Radioactivity associated with precipitate was determined by liquid by scintillation counter in ACS (Amersham).

heximide completely abolishes subsequent DNA synthesis and cell division [19–21]. It is thus possible that initiation of protein synthesis in response to mitogen may be a critical proliferation signal. However, the mechanism of mitogen-induced stimulation of early protein synthesis is not understood.

Earlier it has been observed that protein kinase C activity is significantly enhanced in mitogen-treated T-lymphocytes [6]. Amiloride has been commonly used to inhibit Na^+/H^+ exchange and Na^+/Ca^{++} exchange. In addition, amiloride has been shown to inhibit protein kinase C mediated phosphorylation in vivo and in vitro [14]. As shown in Figure 1, 25 μM amiloride inhibited ^{3}H-thymidine incorporation at all PHA concentrations used. Although this concentration was at suboptimum level to inhibit DNA synthesis maximally, it maintained the maximum viability (98%) of T-lymphocytes in cell culture. The concentration dependence of the effect of amiloride on ^{3}H-thymidine incorporation was next determined. As shown in Figure 2, amiloride at 100 μM concentration inhibited ^{3}H-thymidine incorporation by more than 90%. Half-maximal inhibition of ^{3}H-thymidine incorporation was obtained with 25 μM amiloride.

The effects of amiloride on early protein synthesis following T-lymphocyte activation was next examined. Amiloride at 100 μM significantly inhibited protein synthesis as measured by ^{3}H-leucine incorporation by PHA-activated lymphocytes (Table II). The effects of amiloride on ^{3}H-leucine and ^{3}H-thymidine incorporation were compared to those of polymyxin-B, another nonspecific inhibitor of protein kinase C [15,16]. The effect of polymyxin-B on ^{3}H-leucine and ^{3}H-thymidine incorporation is shown in Table III. Polymyxin-B at 100 μM inhibited both protein and DNA synthesis, as indexed by reductions in ^{3}H-leucine and ^{3}H-thymidine incorpora-

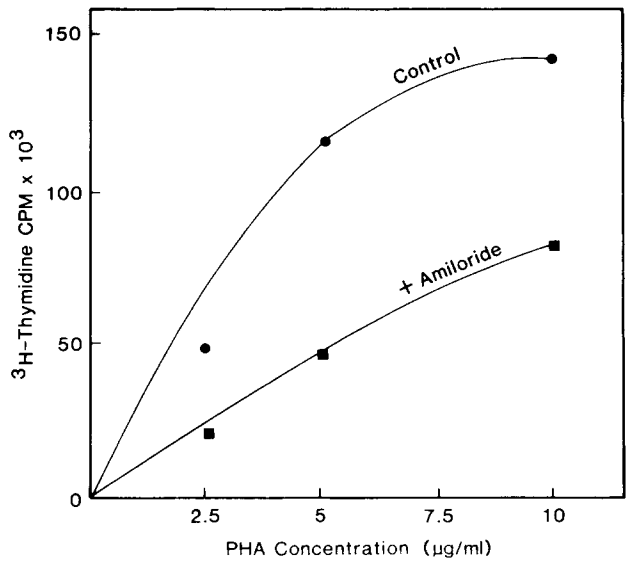

Fig. 1. Effect of amiloride on ^{3}H-thymidine incorporation into DNA of T-lymphocyte at various PHA concentrations. The amiloride concentration was 25 μM. Although this concentration is at suboptimum level, it is used to maintain maximum viability of T-cells in cell culture. Values are means ± SE of triplicate wells.

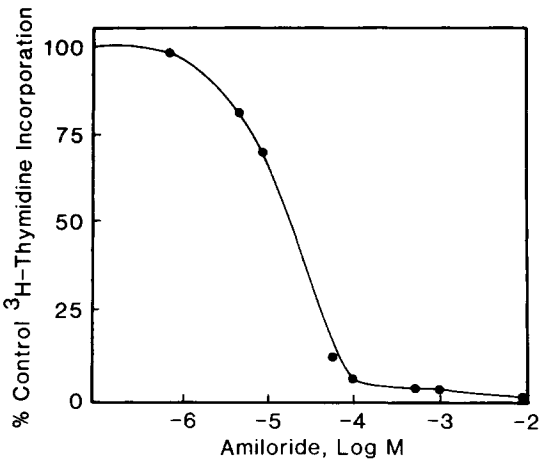

Fig. 2. Quantitation of inhibition of ^{3}H-thymidine incorporation in PHA-stimulated T-lymphocytes by amiloride. Incubation conditions were described in "Materials and Methods." Each data point is the mean of triplicate wells. All results are means of ± SE of triplicate wells.

TABLE II. Effect of Amiloride on ^{3}H-Leucine Uptake by T-Lymphocytes*

Additions	^{3}H-Leucine incorporated (cpm)
Control	660 ± 40
+ PHA	14,500 ± 1,100
+ PHA + Amiloride (100 μm)	875 ± 65

*Effect of amiloride on ^{3}H-leucine uptake in PHA-stimulated T-lymphocyte cell culture. T-lymphocytes (2×10^6 per ml) were incubated in a total volume 400 μl with 2 μCi of ^{3}H-leucine. 10 μg/ml PHA, and 100 μM amiloride for 2 hr at 37°C. Radioactivity in trichloroacetic acid precipitates was determined as described in the legend to Table I. Data are means ± SE of triplicate determinations.

tion. Thus, amiloride and polymyxin have similar effects on these events after T-lymphocyte stimulation.

The biochemical responses induced by phorbol myristate acetate (PMA) have been attributed to its intercalation into the cell membrane and activation of protein kinase C [5]. Protein kinase C has been implicated as the phorbol ester receptor [9]. In the next experiment the effects of amiloride on T-lymphocyte activation by PHA and PMA were compared. As shown in Table IV, PMA stimulated ^{3}H-leucine and ^{3}H-thymidine uptake by T-lymphocytes in culture was similar to PHA stimulated uptake. Maximum response of T-cells was observed at 250 ng/ml of PMA. Amiloride at 100 μM concentration significantly inhibited both ^{3}H-leucine and ^{3}H-thymidine uptake in T-lymphocytes exposed to PMA or PHA.

In order to determine whether amiloride could inhibit protein kinase C activity in vivo, T-lymphocytes were incubated in serum-free culture medium with PHA and 100 μM amiloride for 4 hr at 37°C. Control tubes were run in parallel with and without PHA. After incubation, cells were lysed, and an extract was prepared as

TABLE III. Effect of Polymyxin-B on ^{3}H-Thymidine and ^{3}H-Leucine Incorporation in PHA-Stimulated T-Lymphocytes*

	Radioactivity (cpm)	
	^{3}H-Thymidine	^{3}H-Leucine
Control	900 ± 40	2,800 ± 50
+ PHA	45,000 ± 1,530	12,000 ± 360
PHA + Polymyxin-B (100 μM)	13,000 ± 290	4,500 ± 250

*Effect of polymyxin-B on ^{3}H-thymidine and ^{3}H-leucine incorporation in PHA-stimulated T-lymphocytes. Partially enriched T-lymphocytes were cultured at a density of 2×10^6 cells/ml in serum-free RPMI-1640 as described under "Materials and Methods." Cells were stimulated by addition of PHA (10 μg/ml) in the presence of 1×10^{-4}M polymyxin-B. Conditions for ^{3}H-thymidine incorporation were the same as described in the legend for Figure 2. ^{3}H-leucine incorporation into total protein was measured essentially as described in Table I. Results represent the mean ± SE of three experiments.

TABLE IV. Comparison of Amiloride Effects on T-Lymphocyte Activation by PHA and PMA*

	Radioactivity incorporated (cpm)	
Mitogen	^{3}H-leucine	^{3}H-thymidine
PHA		
CONTROL	19,017 ± 13.3	2,456 ± 239
1 μg/ml	27,141 ± 1,960	130,563 ± 8,077
2 μg/ml	40,763 ± 5,880	144,443 ± 1,455
5 μg/ml	32,880 ± 6,326	146,396 ± 8,541
2 μg/ml plus amiloride (100 μM)	4,664 ± 96	7,834 ± 76
PMA		
0.25 μg/ml	28,503 ± 2,357	160,570 ± 5,661
0.5 μg/ml	30,333 ± 2,740	151,988 ± 339
1.0 μg/ml	48,700 ± 3,354	143,361 ± 10,617
1.5 μg/ml	29,500 ± 307	134,656 ± 521
1.0 μg/ml plus amiloride (100 μM)	7,148 ± 2,159	15,100 ± 835

*Comparison of amiloride effects on T-lymphocyte activation by PHA and PMA. ^{3}H-leucine uptake was determined by incubating T-cells (2×10^6 cells/ml) in leucine-free RPMI-1640 medium for 4 hr at 37°C. For thymidine incorporation, T-cells were incubated in serum-free media for 72 hr at 37°C. Cells were pulsed during the last 10 hr of incubation with 10 μl/ml ^{3}H-thymidine. Cells were stimulated either by PHA (10 μg/ml) or PMA (200 ng/ml). Amiloride (100 μM) was added as indicated. At the end of incubation, the cells were harvested and counted as described in "Materials and Methods." Results are the average ± SE of triplicate experiments.

described in "Materials and Methods." The protein kinase C in the cell extract was partially purified, by passing through a Sephadex G-25 column followed by a DE-52 anion exchange column as described in "Materials and Methods." The pooled protein kinase C fractions from DE-52 were assayed for Ca^{++} phospholipid-dependent phosphorylation of lysine-rich histone-III. As shown in Table V, protein kinase C activity in PHA-activated T-lymphocytes was fivefold more than untreated cells. Protein kinase C activity was maximal only in the presence of both Ca^{++} and phosphotidylserine. Addition of amiloride (100 μM) to mitogen-stimulated T-lymphocytes caused a significant decrease in the kinase C activity.

DISCUSSION

The activation of T-lymphocytes provides a valuable system for the study of the molecular mechanisms controlling preformed mRNA, since they can be shifted from a translationally inactive quiescent state to active protein synthesis by the addition of mitogen into the culture media. Protein synthesis in T-lymphocytes occurs as early as 10 min after mitogen stimulation and is one of the early biochemical events preceeding initiation of DNA synthesis and cell proliferation [1–3]. The mechanism by which resting lymphocytes maintain this low level of preformed mRNA and how this is translated during transformation remain incompletely defined. Kay and coworkers [19] have reported that there is no change in the rate of polypeptide chain elongation in this early period and that synthesis of new ribosomes is not required [20]. The ribosomes from unstimulated lymphocytes were shown to be less translatable using exogenous mRNA in a heterologous system [22]. These results suggest that initiation of protein synthesis during mitogen activation of T-lymphocytes is due to an unknown control mechanism operative at the translational level.

In the present studies, we have demonstrated that amiloride inhibited T-lymphocyte protein synthesis stimulated by mitogen PHA. Amiloride is a nonspecific agent that has been demonstrated previously to inhibit Na^+/H^+ transport and protein kinase C activity. Recently, Mills et al [23] reported that Na^+/H^+ antiport is not involved in T-lymphocyte activation. Nevertheless, we compared and confirmed our amiloride

TABLE V. Protein Kinase C Activity*

Kinase source	Ca^{++}	PS	Histone	^{32}P incorporation pmol/min/mg
Unstimulated	+	+	+	50
PHA stimulated	+	+	+	300
PHA stimulated	−	+	+	33
PHA stimulated	+	−	+	28
PHA stimulated	+	+	−	10
PHA + amiloride	+	+	+	90

*Inhibition of kinase C activity by amiloride. The cytosol fractions containing protein kinase C activity from unstimulated, PHA-stimulated (10 μg/ml), and PHA-stimulated in the presence of 100 μM amiloride were prepared as described in "Materials and Methods." The amount of protein kinase C (fractions from DE-52 anion exchange column containing 0.5–2.0 μg of protein) used in each experiment were at the optimal level for phosphorylation. Results are the average ± SE of triplicate experiments.

results with polymyxin-B, a cyclic polycationic peptide antibiotic, another nonspecific inhibitor of protein kinase C [15]. Polymyxin-B inhibited protein and DNA synthesis in a dose-dependent manner similar to amiloride in mitogen-activated T-lymphocytes. Thus, these results suggest a link between protein kinase C activity and early T-lymphocyte protein synthesis. The inhibition of early protein synthesis by cycloheximide had no effect on PHA-induced protein kinase C activity, indicating that kinase C activation seems to be independent of early protein synthesis. The effects of phorbol ester have been studied in various cultured cells, and it was demonstrated to induce the differentiation of several myeloid leukemic cells [24–27]. Phorbol esters are known to be membrane-perturbing agents and direct activators of protein kinase C [4,5]. The observation that amiloride also inhibits PMA-induced T-lymphocyte activation is particularly intriguing. Although the mechanism of mitogenesis induced by PHA is unclear, PMA is thought to mediate T-lymphocyte activation by stimulation of protein kinase C. Our results suggest that protein kinase C may play an important role in the early activation of protein synthesis. Although the substrate of protein kinase C phosphorylation is not known at this time, this phosphorylation may be a key event in the mechanism of synthesis of regulatory proteins of T-cell activation. Phosphorylation of ribosomal S_6 protein has been shown to affect both the binding and translation efficiency of reconstituted protein synthesis systems [28]. The measurement of S_6 ribosomal protein phosphorylation in mitogen-activated T-lymphocytes is in progress. Based on our results we speculate that protein C-mediated phosphorylation may be involved in the translation of preformed mRNA during the early phase of T-cell activation process.

REFERENCES

1. Ahern T, Kay JE: Exp Cell Res 92:513–515, 1975.
2. Cooper HL, Braverman R: J Biol Chem 256:7461–7467, 1981.
3. Wettenhall REH, London DR: Biochim Biophys Acta 349:214–225, 1974.
4. Nishizuka Y: Science 225:1365–1370, 1984.
5. Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U, Nishizuka Y: J Biol Chem 257:7847–7851, 1982.
6. Kaibichi K, Takai Y, Nishizuka Y: J Biol Chem 260:1366–1369, 1985.
7. Ashendel CL, Mionor PL: Carcinogenesis 7:517–521, 1986.
8. Werth DK, Niedel JE, Pastan I: J Biol Chem 258:11,423–11,426, 1983.
9. Nishizuka Y: Nature 308:693–698, 1984.
10. Wise BC, Glass DB, Jen-Chou C-H, Raynor RL, Katoh N, Schatzman RC, Turner RS, Kibler RF, Kuo JF: J Biol Chem 257:8489–8495, 1982.
11. Kikkawa U, Takai Y, Minakuchi R, Inohara S, Nishizuka Y: J Biol Chem 257:13,341–13,348, 1982.
12. Takai Y, Kishimoto A, Inoue M, Nishizuka Y: J Biol Chem 252:7603–7609, 1977.
13. de Haro C, de Herreros AG, Ochoa S: Proc Natl Acad Sci USA 80:6843–6847, 1983.
14. Besterman JM, Stratford MW, LeVine H, Cragoe JE, Cuatrecassas P: J Biol Chem 260:1155–1159, 1985.
15. Nel EA, Wooten MW, Gooldschmidt-Clermont PJ, Miller PJ, Stevenson CH, Galbraith RM: Biochem Biophys Res Commun 128:1364–1372, 1984.
16. Baliga BS, Sindel LJ, Jenkins LD, Sachen J: Biochem Biophys Res Commun 135:649–654, 1986.
17. Fabbro D, Jungmann RA, Eppenberger U: Arch Biochem Biophys 239:102–111, 1985.
18. Kuvibidila S, Nauss K, Baliga BS, Suskind RM: Am J Clin Nutr 37:15–25, 1983.
19. Kay JE, Ahern T, Lindsay VJ, Sampson J: Biochim Biophys Acta 378:241–250, 1975.
20. Kay JE, Leventhal BG, Cooper HL: Exp Cell Res 54:94–100, 1969.
21. Veresio L, Holden HT: J Immunol 124:2288, 1980.

22. Lodish HF: Annu Rev Biochem 45:39–61, 1976.
23. Mills GB, Cheung RK, Cragoe EJ, Grinstein S, Gelfand EW: Immunol 136:1150–1154, 1986.
24. Collins SJ, Gallo RC, Gallagher RE: Nature 270:347–349, 1977.
25. Huberman E, Callaham MF: Proc Natl Acad Sci USA 76:1293–1297, 1979.
26. Rovera G, O'Brien TG, Diamond L: Science 204:868–870, 1979.
27. Rovera G, Saratoli D, Damsky C: Proc Natl Acad Sci USA 76:2779–2783, 1979.
28. Buckhard SJ, Traugh JA: J Biol Chem 258:14003–14008, 1983.