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Role of Ca^{2+} in the regulation of hormone receptor exposure during lymphocyte activation

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Ca^{2+} is known to be required for mitogen-mediated lymphocyte activation. In order to further define the regulatory role of Ca^{2+}, we have examined the activation events which occur following treatment with ionomycin (a Ca^{2+} ionophore), as compared to those occurring following concanavalin A (Con A) treatment of mouse splenic T-lymphocytes. Our results indicate that ionomycin and Con A induce the exposure of both interleukin-2 (IL-2) and insulin receptors on the surface of the lymphocytes within the first 5 min of treatment. The exposed insulin and IL-2 receptors have the following properties: (1) they consist of both high- and low-affinity receptors; and (2) they appear on the cell surface in small clusters (i.e., patches) or, occasionally, a large aggregate (i.e., cap). c-*myc* gene expression and DNA synthesis occur in both the ionomycin and Con A-treated lymphocytes when either IL-2 or insulin is present in the culture medium. Furthermore, the exposure of both hormone receptors can be inhibited by either EGTA (a Ca^{2+} chelator), bepridil (a Ca^{2+} channel blocker), W-7 (a calmodulin antagonist) or cytochalasin D (a microfilament inhibitor). Treatment with these inhibitors also blocks the expression of c-*myc* gene and DNA synthesis which occur at later times during IL-2 and insulin-induced activation of ionomycin- and Con A-treated lymphocytes. These findings suggest that a Ca^{2+} and calmodulin-mediated contractile system is involved in the exposure of certain hormone receptors which appear to be required for complete lymphocyte activation.

Introduction

One of the requirements for the immune response is the activation of differentiated lymphocytes (i.e., undergo blast formation and mitogenesis) [1–13]. Specifically, T-lymphocytes are activated when they bind with mitogens such as Con A and PHA, or with antibodies such as anti-Thy-1 antibody [1–5]. B-lymphocytes can be activated by binding with the mitogen, lipopolysaccharide (LPA), or with anti-immunoglobulin antibody [2,6–11].

Lymphocytes are known to undergo a cascade of biochemical and morphological changes during mitogenesis [12,13]. In unstimulated mouse splenic lymphocytes, hormone receptors are not detected on the cell surface [14]. One of the earlier changes to occur during mitogenesis is the exposure of certain hormone receptors, such as the IL-2 and insulin receptors [15–17]. IL-2 receptors have been detected within 6–12 h and insulin receptors within 24–48 h, following mitogenic stimulation [15–17]. Recently, however, we have found that Con A can induce the exposure of both IL-2 and insulin receptors as early as 5 min following mitogenic stimulation [18]. The binding of certain growth factors and hormones (e.g., IL-2 and insulin) to their receptors is considered to be essential for lymphocyte proliferation and differentiation [19–25]. However, the regulatory mechanisms responsible for inducing the exposure of lymphocyte hormone receptors during activation are not well-understood.

Calcium ions appear to play an important role during cell activation by transmitting signals from the molecules in the plasma membranes involved in surface recognition to the intracellular components responsible for producing the functional response(s) [26–28]. For example, many hormones exert their effects on target cells in part by raising the Ca^{2+} concentration in the cytoplasm $[Ca^{2+}]_i$, thus activating Ca^{2+}-dependent enzymes that direct cellular responses [18,29]. In par-

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; PHA, phytohemagglutinin; Hepes, 4-(20-hydroxyethyl)-1-piperazineethanesulfonic acid; DPBS, Dulbecco's phosphate-buffered saline; IP_3, inositol 1,4,5-triphosphate.

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ticular, it has been suggested that Ca^{2+} together with inositol lipids [30] and protein kinase C [31] may account for a number of the transient cellular responses that occur during hormone action and cell proliferation.

Calmodulin is an ubiquitous Ca^{2+}-binding protein that regulates a variety of Ca^{2+}-dependent cellular activities [32]. A number of Ca^{2+}/calmodulin-dependent enzymes are involved in the overall processes of cell growth and division [33,34]. In lymphocytes, there is now a great deal of evidence supporting the involvement of Ca^{2+} and calmodulin in receptor capping [35–37] and mitogenesis [18,33,34]. The present study was designed to further examine the regulatory roles that Ca^{2+} and calmodulin may play in the exposure of hormone receptors and the subsequent activation of lymphocytes.

Materials and Methods

Cells

Mouse splenic T-lymphocytes were obtained from Balb C/J mice (Jackson Laboratory, Bar Harbor, Maine) and were prepared by passing the spleen cells over a nylon wool column as described previously [38].

Immunocytochemical localization of ionomycin-induced, newly exposed IL-2 or insulin receptors

Mouse splenic T-lymphocytes were treated with ionomycin (1 μM) for 20 min at room temperature in order to achieve the maximal exposure of their hormone receptors. Ionomycin-treated cells were then fixed in 1% paraformaldehyde (in Dulbecco's phosphate-buffered saline, DPBS) for 20 min at 4°C. Subsequently, either IL-2 (500 U/ml) or insulin ($1 \cdot 10^{-8}$ M) was added to their respective receptors in a buffer containing 100 mM Hepes/120 mM NaCl/1.2 mM $MgSO_4$/2.5 mM KCl/1 mM EDTA/10 mM glucose/1.5 mM sodium acetate (pH 7.8) at 15°C for 15–30 min. The controls received RPMI 1640 medium only, followed by fixation in 1% paraformaldehyde. All fixed cells were washed in DPBS plus 0.1 M glycine followed by incubation with fluorescein-conjugated or colloidal gold-conjugated anti-IL-2 antibodies (an affinity purified fraction) (obtained from Dr. Thomas Malek, University of Miami, Miami, FL) or fluorescein-conjugated or colloidal gold-conjugated anti-insulin antibodies (an affinity purified fraction). To verify the specificity of immunolabeling, an excess amount of IL-2 or insulin was added to the cells to compete for fluorescein-conjugated or colloidal gold-conjugated insulin or IL-2 binding.

The fluorescein-labeled samples were examined with a Zeiss photomicroscope using a 63 × oil immersion lens and epiillumination. Cells were photographed with Kodak plus-X film (Eastman Kodak, Rochester, NY).

The colloidal gold-labeled samples were further fixed with 2% glutaraldehyde, postfixed with 2% OsO_4, dehydrated through ethanol and embedded in Epon 812. Ultrathin sections were obtained on a Sorvall MT2-B microtome using a diamond knife, then stained with uranyl acetate and lead citrate. In some cases, ultrathin sections were not treated with any staining reagents such as acetate and lead citrate in order to obtain a better resolution and contrast of colloidal gold labeling. These materials were then observed under a JEOL electron microscope.

Insulin or IL-2 induced-receptor capping in ionomycin-treated lymphocytes

Mouse splenic T-lymphocytes were treated with ionomycin (1 μM) for 20 min at room temperature in order to achieve the maximal exposure of their hormone receptors. After binding with either IL-2 (100 U/ml), insulin ($1 \cdot 10^{-8}$ M) or to their respective receptors in a buffer containing 100 mM Hepes/120 mM NaCl/1.2 mM $MgSO_4$/2.5 mM KCl/1 mM EDTA/10 mM glucose/1.5 mM sodium acetate (pH 7.8) [39] (with or without treatment with various metabolic inhibitors, such as EGTA (3 mM), Bepridil (20 μM), W-7 (20 μM), cytochalasin D (50 μg/ml), colchicine ($1 \cdot 10^{-5}$ M) and cycloheximide (50 μM)) at 15°C for 15–30 min, the cells were washed three times with the same buffer and fixed by incubation in 0.5% paraformaldehyde (in DPBS) for 20 min at 0°C. The controls received RPMI 1640 medium only, followed by fixation in 1% paraformaldehyde. All fixed cells were washed in DPBS plus 0.1 M glycine followed by incubation with (a) fluorescein-conjugated rabbit anti-IL-2 receptor antibodies (an affinity purified fraction) (obtained from Dr. Thomas Malek, University of Miami, Miami, FL) and (b) fluorescein-conjugated human anti-insulin receptor antibodies (an affinity purified fraction) (a gift from Dr. Guenther Baden, University of Pennsylvania, Philadelphia, PA). To verify the specificity of immunolabeling, some samples received fluorescein-conjugated pre-immune serum or pre-absorbed serum (anti-insulin or IL-2 receptor-free serum). The anti-IL-2 or insulin receptor-free serum was prepared by (1) incubating anti-IL-2 receptor antibody with IL-2 receptor containing CTLL T-lymphocytes to remove specific anti-IL-2 antibody from the serum and (2) incubating anti-insulin receptor antibody with insulin receptor-containing IM-9 B-lymphoblasts to remove specific anti-insulin receptor antibody from the serum. After the CTLL and IM-9 cells had been centrifuged down, the supernatant containing non-specific serum (so-called pre-absorbed serum or anti-IL-2 or anti-insulin-free serum) was used as a control. Both pre-absorbed serum and pre-immune serum showed no obvious fluorescent staining.

Hormone receptor binding assays

Mouse splenic T-lymphocytes were treated with Con A (6 μg) or ionomycin (1 μM) at room temperature for

various time intervals ($t = 1, 2, 3, 5, 10, 20$ and 30 min). To measure the exposed IL-2 or insulin receptors, these Con A-treated or ionomycin-treated T-lymphocytes ($1 \cdot 10^6$ cells per assay point) were further incubated with ^{125}I-IL-2 (specific activity, 50 μCi/μg; concentration ranging from $1 \cdot 10^{-9}$ to 10^{-12} M) or ^{125}I-insulin (specific activity, 100 μCi/μg; concentration ranging from 10^{-9} to 10^{-12} M) in 1 ml of binding buffer containing 100 mM Hepes/120 mM NaCl/1.2 mM $MgSO_4$/2.5 mM KCl/1 mM EDTA/10 mM glucose/1 mg · ml^{-1} bovine serum albumin/1.5 mM sodium acetate (pH 7.8) [39] at 15°C for 15–30 min or 60 min followed by washing extensively at 4°C. In this binding assay, we measured the amount of both IL-2 and insulin receptors expressed after Con A or ionomycin treatment. The level of hormone binding was the same regardless of whether the cells were incubated for 15–30 min or 60 min at 15°C. To confirm the specificity of IL-2 and insulin binding assays, excess amounts of unlabeled IL-2 or insulin were added to compete for the binding of ^{125}I-IL-2 or ^{125}I-insulin; less than 5% of the binding was nonspecific. The amount of ^{125}I-IL-2 and ^{125}I-insulin binding was measured by an LKB Gamma counter.

To obtain the Scatchard plot, ^{125}I-IL-2 and ^{125}I-insulin binding assays were done using ligand concentrations ranging from $1 \cdot 10^{-9}$ to $1 \cdot 10^{-12}$ M for IL-2 and $1 \cdot 10^{-9}$ to $1 \cdot 10^{-12}$ M for insulin. ^{125}I-IL-2 or ^{125}I-insulin were diluted with unlabeled IL-2 or insulin at a fixed ratio of 1:9. Nonspecific binding was determined by performing identical assays in the presence of $1 \cdot 10^{-6}$ M of unlabeled IL-2 or insulin. The level of specific binding was then measured by subtracting nonspecific binding from total binding. Data points from the Scatchard plot were extrapolated and fitted manually following the principles described previously [40]. The experimental points expressed were mean values of at least three to five experiments with a standard deviation less than ± 5%.

Measurement of $^{45}Ca^{2+}$ influx

Mouse splenic T-lymphocytes were first incubated for 30 min in RPMI 1640 medium at room temperature before adding $^{45}Ca^{2+}$ (5–10 μCi/ml; 50 Ci/μg) and beginning Con A (6 μg/ml) treatment. At various time intervals ($t = 1, 2, 3, 5, 10, 20$ and 30 min), Con A-treated samples were rapidly transferred into 10 ml of ice-cold PBS (pH 7.3) plus 2 mM EGTA in order to terminate Ca^{2+} uptake. After washing with an additional 20 ml of cold PBS plus EGTA, the cells were placed in scintillation fluid (Miniblend, ICN) and counted in an LKB Mini Beta Scintillation Counter. Control samples were treated with RPMI 1640 only and no Con A was added. $^{45}Ca^{2+}$ influx measurements were made with both unstimulated cells and cells stimulated by ionomycin or Con A. The data points presented in Fig. 2 are net cpm counts for $^{45}Ca^{2+}$ influx obtained by taking the ^{45}Ca influx (cpm) of stimulated cells (cpm) minus $^{45}Ca^{2+}$ influx (cpm) of unstimulated cells.

Assay for c-myc gene expression

Isolation of RNA. Cytoplasmic RNA was isolated according to the procedures described previously by White and Bancroft [41] followed by phenol and Sevag (chloroform/isoamylalcohol, 24:1, v/v) extraction. Mouse splenic T-lymphocytes pretreated with ionomycin were incubated with or without IL-2 (100 U) or insulin ($1 \cdot 10^{-8}$ M) in the presence or absence of various metabolic inhibitors (e.g., EGTA (3 mM), Bepridil (20 μM), W-7 (20 μM), cytochalasin D (20 μg/ml), colchicine ($1 \cdot 10^{-5}$ M) and cycloheximide (50 μM)) at 37°C for 30 min. Subsequently, cells were resuspended in 50 μl of ice cold 10 mM Tris-HCl (pH 7.0) and 3 mM EDTA and lysed by 1% NP-40. After centrifuging this NP-40 solubilized material at $15000 \times g_{av}$ for 3 min, the pellet (containing nuclei and unbroken cells) was discarded and the supernatant was extracted with a mixture of phenol/Sevag (1:1, v/v). The amount of RNA obtained by this method was determined by measuring UV absorbance at 260 nm. Isolated RNA was mixed with an equal volume of solution containing 0.15 M NaCl/0.015 M sodium citrate (3 parts) and 37% formaldehyde (2 parts), heated at 65°C for 15 min and stored at −20°C.

Dot blot analysis. Isolated RNA was serially diluted with a solution containing 0.15 M NaCl/0.015 M sodium citrate and manually applied to a pre-wetted nitrocellulose paper (BA 45, 0.45 μm, Schleicher and Schuell) with a capillary pipet. The nitrocellulose papers were then incubated at 80°C for 2 h in vacuo and stored at −20°C.

Hybridization technique. A high specific activity ^{32}P-anti-sense-RNA probe ($1 \cdot 10^9$ cpm/μg) was prepared using SP 6 transcription as described in Ref. 42. The mouse c-*myc* probe consists of 1.5 kb from exons 2 and 3 of c-*myc* cDNA. The blotted paper was treated with 0.15 M NaCl/0.015 M sodium citrate in a sealed bag containing hybridization solution (50% formaldehyde/50 mM sodium phosphate buffer (pH 7.0)/0.15 M NaCl/0.015 M sodium citrate/0.1% SDS/1 mM EDTA/Denhardt's solution/100 μg · ml^{-1} denatured DNA/100 μg · ml^{-1} yeast RNA) at 65°C for 2–4 h. Following the prehybridization, the ^{32}P-labeled probe ($1 \cdot 10^6$ cpm/ml) was added to the blotted paper and hybridized at 65°C for 20 h. The filter was then washed with a solution containing 0.15 M NaCl/0.015 M sodium citrate/0.1% SDS at 50°C. The hybrids were observed by autoradiography using Kodak XAR-5 film with intensifying screen at −70°C.

Measurement of DNA synthesis. Mouse splenic T-lymphocytes ($5 \cdot 10^5$ cells) were dispensed into the wells of microtiter plates containing 0.2 ml of RPMI 1640

medium, in the presence or absence of the Ca^{2+} ionophore, ionomycin (1 μM). Following incubation at room temperature for up to 30 min, the ionomycin-treated cells were supplemented with either IL-2 (100 U/ml) or insulin ($1 \cdot 10^{-8}$ M) in the presence or absence of various metabolic inhibitors (e.g., EGTA (3 mM), Bepridil (20 μM), W-7 (20 μM), cytochalasin D (20 μg/ml) and colchicine ($1 \cdot 10^{-5}$ M)) in 5% CO_2/95% air at 37°C for 12–24 h. Control samples were treated with RPMI 1640 medium only and incubated under the same conditions. Cells were then pulsed with 0.1 μCi ^{3}H-thymidine (ICN Chemical and Radioisotope Division, Irvine, CA) for 4 h at 37°C in 5% CO_2/95% air as described previously [36,37]. Synthesis was stopped by addition of ice-cold 10% trichloroacetic acid (TCA); and the TCA-precipitated materials were washed and collected on Millipore filters, dried and counted in an LKB liquid scintillation counter.

Results

Insulin and IL-2 receptor exposure

IL-2 and insulin receptors are generally not found on the surfaces of unstimulated mouse splenic T-lymphocytes (Fig. 1A) [14]. However, treatment of mouse splenic T-lymphocytes with a mitogen such as Con A causes the rapid influx of Ca^{2+} (within 1 min after treatment) (Fig. 2), followed by the exposure of both IL-2 and insulin receptors (within 3–5 min after treatment) (Fig. 2). Scatchard plot analysis, using ^{125}I-IL-2 and ^{125}I-insulin binding assays, indicates that the exposed IL-2 and insulin receptors consist of two populations: (1) high-affinity receptors (approx. 1084 IL-2 binding sites (Fig. 3) and 380 insulin binding sites (Fig. 4) per cell); (2) and low-affinity receptors (approx. 8428 IL-2 binding sites (Fig. 3) and 1523 insulin binding sites (Fig. 4) per cell).

Since the influx of Ca^{2+} occurs prior to hormone receptor exposure during Con A treatment, we have decided to use the Ca^{2+} ionophore, ionomycin, to determine whether Ca^{2+} can directly trigger hormone receptor exposure. Our results indicate that ionomycin itself can induce both Ca^{2+} influx (within 30 s after treatment) (Fig. 2) and the exposure of both IL-2 and insulin receptors within 3–5 min following addition of the ionophore (Figs. 1B–E; Refs. 5 and 6; Fig. 2). Microscopic examination, using both immunofluorescence and immuno-gold labeling techniques, reveals that the ionomycin-induced IL-2 (Figs. 1B and C) and insulin receptors (Figs. 1D and E) appear as aggregates (so-called 'patches') on the lymphocyte surface. Scatchard plot analysis indicates that ionomycin-induced hormone receptors also consist of two populations: (1) high-affinity receptors (approx. 1084 IL-2

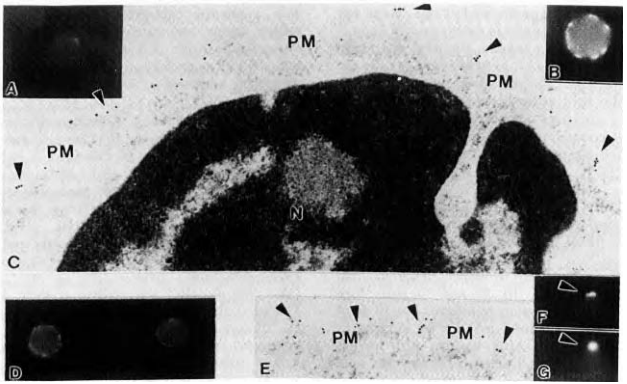


Fig. 1. Immunofluorescence and immuno-gold staining of hormone receptors. (A) Absence of IL-2 receptors on unstimulated (resting) mouse splenic T-lymphocytes. (The same result was observed for insulin receptors in unstimulated cells-data not shown). (B and C) Mouse splenic T-lymphocytes were treated with ionomycin followed by staining with fluorescence-labeled (B) or colloidal gold-labeled (C) anti-IL-2 receptors. (D and E) Mouse splenic T-lymphocytes were treated with ionomycin followed by staining with fluorescence-labeled (D) or colloidal gold-labeled (E) anti-insulin receptors. (F and G) Mouse splenic T-lymphocytes were treated with ionomycin followed by IL-2 or insulin treatment. Capped IL-2 receptors (F) and insulin receptors (G) were detected by staining with fluorescence-labeled anti-IL-2 receptor or anti-insulin receptor, respectively.

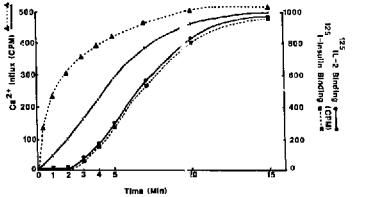


Fig. 2. Con A- and ionomycin-induced Ca^{2+} influx and hormone receptor exposure on mouse splenic T-lymphocytes. Cells were treated with either Con A (6 μg) (×) or ionomycin (1 μM) (▲) followed by Ca^{2+} influx measurement and hormone receptor binding analyses. ▨, exposed insulin binding sites during Con A treatment; ●, exposed IL-2 binding sites during Con A treatment. (Similar insulin and IL-2 receptor exposures were observed in ionomycin-treated cells; data not shown).

TABLE I

Effect of various inhibitors on ionomycin-induced IL-2 and insulin receptor exposure

Ionomycin-treated mouse splenic lymphocytes were preincubated with various drugs at 4°C for 30 min before IL-2 and insulin receptor binding assays were carried out. Each number represents the average of three to five experiments with a standard deviation less than ± 5%.

Treatment	% of control	
	IL-2 receptors	Insulin receptors
Control (ionomycin (1 μM) only)	100	100
EGTA (3 mM)	25	20
Bepridil (20 μM)	31	23
W-7 (20 μM)	9	10
Cytochalasin D (20 μg/ml)	35	25
Colchicine ($1 \cdot 10^{-5}$ M)	98	96
Cycloheximide (50 μM)	88	90

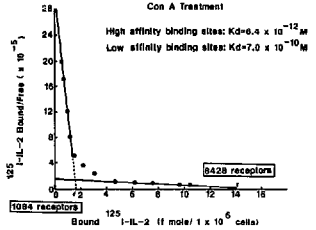
binding sites (Fig. 5) and 820 insulin binding sites (Fig. 6) per cell) and (2) low-affinity receptors (approx. 9632 IL-2 binding sites (Fig. 5) and 2500 insulin binding sites (Fig. 6) per cell).

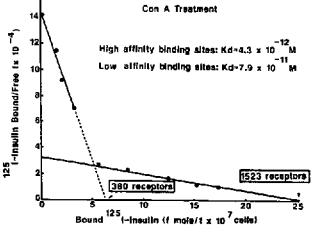
Effects of various inhibitors

Six different reagents – a Ca^{2+} chelator (EGTA), a Ca^{2+} blocker (Bepridil), a calmodulin antagonist (W-7), a microfilament inhibitor (cytochalasin D), a microtubule disrupting agent (colchicine) and a protein-synthesis inhibitor (cycloheximide) – were tested for their effects on ionomycin-induced hormone receptor exposure. Our results indicate that EGTA, Bepridil, W-7 and cytochalasin D can all substantially inhibit ionomycin-induced IL-2 and insulin receptor exposure (Table I). However, colchicine and cycloheximide did not inhibit the exposure of either IL-2 or insulin receptors. These data indicate that Ca^{2+}/calmodulin and microfilaments (but not microtubules and protein synthesis) are probably involved in ionomycin-induced IL-2 and insulin receptor exposure.

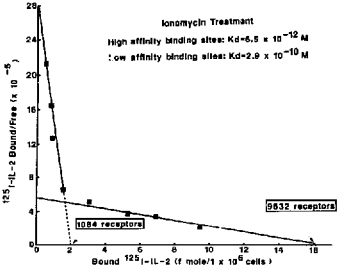
Hormone-induced lymphocyte mitogenesis

Previous studies have demonstrated that hormone-induced cell activation and proliferation are associated with Ca^{2+} mobilization [18,29], receptor patching/capping [18,29] and the expression of proto-oncogenes such as c-*fos* and c-*myc* genes [43,44]. In this study, we have found that lymphocyte mitogenesis (e.g., c-*myc* gene expression and DNA synthesis) did not occur following ionomycin treatment in serum-free medium (Fig. 7A; Table II). However, when IL-2 or insulin was added to the medium following ionomycin treatment, complete lymphocyte activation and mitogenesis ensued. Specifically, the following events occur: (1) IL-2





Figs. 3 and 4. Scatchard plot analyses of IL-2 and insulin binding to Con A-treated mouse splenic T-lymphocytes. The cells were first treated with Con A (6 μg) for 20 min at room temperature and incubated with ^{125}I-IL-2 (Fig. 3) or ^{125}I-insulin (Fig. 4) in the presence of various concentrations of unlabeled IL-2 or insulin as described in Materials and Methods. Data points from the Scatchard plot were extrapolated and fitted manually as described previously [40].



Figs. 5 and 6. Scatchard plot analyses of IL-2 and insulin binding to ionomycin-treated splenic T-lymphocytes. The cells were first treated with ionomycin for 20 min at room temperature and incubated with ^{125}I-IL-2 or ^{125}I-insulin in the presence of various concentrations of unlabeled IL-2 or insulin as described in Materials and Methods. Data points from the Scatchard plot were extrapolated and fitted manually following the principles described previously [40].

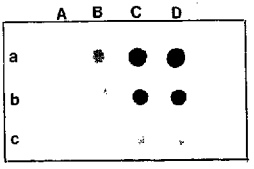


Fig. 7. Analysis of *c-myc* gene expression by dot hybridization. Mouse splenic T-lymphocytes were first treated with ionomycin for 20 min at room temperature. These ionomycin-treated cells were then incubated with or without IL-2 (100 U) or insulin ($1 \cdot 10^{-8}$ M) in the presence or absence of W-7 (20 μM) at 37 °C for 30 min. The expression of *c-myc* gene product was shown as follows: (A) no hormone addition; (B) with IL-2 addition and metabolic inhibitors such as W-7; (C) with IL-2 addition; and (D) with insulin addition. Cytoplasmic RNAs isolated from the aforementioned samples (A–D) were used for cytoplasmic dot hybridization analysis of *c-myc* gene expression according to the procedures described in Materials and Methods. (a) 1 μg RNA; (b) 0.5 μg RNA; (c) 0.25 μg RNA.

TABLE II

Hormone requirement for DNA synthesis in ionomycin-treated lymphocytes

Each number represents the average of three to five experiments with a standard deviation less than ± 5%.

Treatment	DNA synthesis (% of control)
Control (no ionomycin)	100
Ionomycin (1 μM) only	100
IL-2 (100 U/ml) only	102
Insulin ($1 \cdot 10^{-8}$ M) only	101
Ionomycin (1 μM) plus IL-2 (100 U/ml)	400
Ionomycin (1 μM) plus insulin ($1 \cdot 10^{-8}$ M)	350

and insulin receptor capping occurred within 5–10 min after the addition of IL-2 or insulin treatment (Figs. 1F and G); (2) *c-myc* gene was expressed at 30–60 min after IL-2 or insulin treatment (Fig. 7C and D); and (3) DNA synthesis was initiated within 12–24 h following IL-2 or insulin treatment (Table II). Finally, treatment of the lymphocytes with either EGTA, Bepridil, W-7 or cytochalasin D (but not colchicine) blocked both IL-2- and insulin-induced receptor capping (Table III), *c-myc* gene expression (Fig. 7B) and DNA synthesis (Table III). Our data, therefore, suggest that Ca^{2+}/calmodulin-dependent contractile elements are responsible for IL-2 and insulin receptor exposure which, in turn, are required for IL-2 or insulin-dependent lymphocyte activation and proliferation.

TABLE III

Effect of various inhibitors on hormone-induced receptor capping and DNA synthesis in ionomycin-treated lymphocytes

Each number represents the average of three or five experiments with a standard deviation less than ± 5%. Ionomycin (1 μM) was used for the pretreatment of cells.

Treatment	% of control			
	receptor capping		DNA synthesis	
	IL-2	insulin	IL-2	insulin
Control (no inhibitor)	100	100	100	100
EGTA (2 mM)	30	26	18	19
Bepridil (20 μM)	22	25	15	20
W-7 (20 μM)	27	20	16	17
Cytochalasin D (20 μg/ml)	35	30	25	27
Colchicine ($1 \cdot 10^{-5}$ M)	105	102	101	100

Discussion

Recent evidence indicates that the early signal-transduction events leading to mitogenesis include: (a) stimulation of phosphatidylinositide turnover giving rise to IP_3 [30] and diacylglycerol (DAG) [31], which then induce internal Ca^{2+} release [30] and protein kinase C activation [31], respectively; (b) an increase in Na^+/H^+ antiporter activity which causes an increase in cytoplasmic pH [45]; and (c) activation of surface receptor tyrosine kinase activity [46]. In this study, we have examined two early signal-transducing events that occur during lymphocyte activation: influx of Ca^{2+} (Fig. 2) and exposure of two different hormone receptors on the cell surface (Figs. 3–6).

Ca^{2+} mobilization occurs during growth factor or mitogen-induced activation of various cell types including lymphocytes [18,29,47,48] and neutrophils [49]. Con A has been shown to cause IP_3 formation in lymphocytes [50,51]. IP_3 then triggers the release of Ca^{2+} from internal storage sites [52] and, possibly, the influx of external Ca^{2+} [53]. Since changes in Ca^{2+} influx and/or intracellular free Ca^{2+} are considered essential to cell activation, we decided to employ a Ca^{2+} ionophore, ionomycin, to determine whether Ca^{2+} is directly involved in the signal transduction mechanism(s) (e.g., hormone receptor exposure) leading to lymphocyte proliferation.

IL-2 and insulin receptors are generally not present on the surface of unstimulated mouse splenic T-lymphocytes [14]. When lymphocytes are activated by a variety of mitogens, the receptors for these hormones become detectable [15–18]. Similar findings have recently been reported for other cell types [54]. For example, several different growth factors and peptide hormones have been shown to induce a rapid translocation of transferrin receptors and glucose transporter from intracellular pools to the cell surface [54]. Recently, we have reported that both Con A and electric stimulation induce exposure of insulin and IL-2 receptors (within 3–5 min after treatment) in mouse splenic lymphocytes [18]. The molecular mechanisms involved in these mitogen-mediated membrane changes (e.g., receptor exposure and expression) are not fully understood.

In this study, we have determined that, similar to Con A treatment (Figs. 2–4), ionomycin can induce exposure of both IL-2 and insulin receptors on the lymphocyte plasma membrane (Figs. 1B–E, 5 and 6). The observed increase in insulin receptors following ionomycin or Con A treatment may be a requirement of lymphocyte proliferation. In the ionomycin-treated cells, the binding of either insulin or IL-2 to their respective receptor promotes a sequence of cellular activation including hormone receptor clustering (Figs. 1F and G), proto-oncogene expression (Figs. 7C and D) and finally DNA synthesis (Table II). Our data suggest that Ca^{2+} may be directly involved in revealing masked or cryptic forms of hormone receptors. Alternately, Ca^{2+} may trigger the translocation of internal membrane pools of IL-2 and insulin receptors to the plasma membrane. The binding of hormone to ionomycin-induced hormone receptors is required for complete lymphocyte activation.

Another mechanism by which Ca^{2+} may trigger early signal transducing events during cell activation involves its interaction with calmodulin, an ubiquitous Ca^{2+}-binding protein. Ca^{2+}-dependent calmodulin activity is known to be responsible for the activation of a number of important cellular enzymes, including adenylate cyclase, membrane-associated Ca^{2+}, Mg^{2+}-ATPase, myosin light chain kinase, etc. [32,55]. As shown in Figs. 1B–E, the IL-2 and insulin receptors on the surface of ionomycin-treated lymphocytes clustered into patches or caps (Figs. 1F and G). Recent evidence from our laboratory indicates that a number of cytoskeletal protein (e.g., actin, myosin, fodrin, etc.) are either directly or indirectly associated with clustered hormone receptors [55–61]. The fact that various inhibitors such as Ca^{2+}-channel blockers, Ca^{2+} chelators, calmodulin antagonists, and cytoskeletal inhibitors (but not microtubule-disrupting drugs) can prevent ionomycin-induced exposure of clustered hormone receptors (Table I) and also block hormone-mediated receptor capping (Table III), c-*myc* gene expression (Fig. 7B) and DNA synthesis (Table III) suggests that Ca^{2+} and calmodulin-dependent contractile cytoskeletal elements (but not microtubules) are required for early signal transduction, leading eventually to lymphocyte activation and proliferation.

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