Leukoregulin-increased Plasma Membrane Permeability and Associated Ionic Fluxes

Susan C. Barnett and Charles H. Evans
Tumor Biology Section, Laboratory of Biology, Division of Cancer Etiology, National Cancer Institute, Bethesda, Maryland 20892

ABSTRACT

The role of ion fluxes in the increased plasma membrane permeability of tumor cells exposed to the anticarcinogenic and tumor cell proliferation inhibitory lymphokine, leukoregulin, was examined by flow cytometric analysis of cell surface perturbations indicative of membrane destabilization. The pl 5.3 form of leukoregulin was isolated by ion exchange, isoelectric focusing, and molecular sizing chromatography of lymphokines from phytohemagglutinin-stimulated normal human lymphocytes. Membrane permeability increased within 5 min of leukoregulin exposure of tumor cells and was quantified by following the efflux of fluorescein or influx of propidium iodide. Membrane permeability increased in proportion to leukoregulin concentration. By 2 h, 0.25-30 units/ml induces a 10-90% change in K562 erythroleukemia cell permeability. Similar changes are effected by Ca2+-ionophores A23187 and X-537A but not by the Na+ ionophore monensin or the K+ ionophore valinomycin. The Ca2+ ionophores and the intracellular Ca2+ mobilizers ouabain and amphotericin B enhance, whereas calmodulin inhibits leukoregulin action. Ca2+ channel blockers nifedipine and verapamil and the Na+ and K+ ion transport inhibitors amiloride and atracylsodin, respectively, neither alter membrane permeability nor influence leukoregulin activity. Further evidence for the role of increased Ca2+ flux in leukoregulin's action is provided by detection of increased intracellular free Ca2+ in leukoregulin-treated cells with the Ca2+-sensitive fluorescent probe 2-[2-(bis(carboxymethyl)amino)-5-methylphenoxy]methyl-6-methoxy-8-bis(carboxymethyl)aminoquinoline. Kinetic analysis of cell volume, forward and right angle light scatter, fluorescein efflux, and propidium iodide influx, moreover, reveals that the action of leukoregulin is unique. Membrane perturbations may be critical initial steps in the ability of leukoregulin to directly prevent the development of carcinogenesis as well as inhibit the continued proliferation of neoplastic cells.

INTRODUCTION

Leukoregulin, a recently isolated lymphokine, interacts directly with target cells to prevent the development of carcinogenesis and inhibit the proliferation and increase the sensitivity of tumor cells to natural killer lymphocyte cytolysis (1). Leukoregulin's inhibitory actions on tumor cell growth are predominantly cytostatic. Few target cells are lysed due to interaction with leukoregulin alone. Leukoregulin, however, increases target cell sensitivity to natural killer lymphocyte cytolysis and is secreted by activated natural killer lymphocytes, suggesting that leukoregulin may function as one of the early and pivotal events occurring even within a small percentage of a cell population and establish whether the cell population responds in a homogeneous versus a heterogeneous fashion. Flow cytometry offers an additional dimension to the analysis of viable cell populations by providing the means to simultaneously measure several parameters in an individual cell at the rate of thousands of cells/s. This is in contrast to conventional spectrofluorometric and radionuclide ion flux measurements which are limited to assessing the mean response of the entire cell population under investigation. By measuring changes in membrane permeability flow cytometrically as indicated by the loss of intracellular fluorescein or assimilation of extracellular propidium iodide and the fluorescence of new intracellular Ca2+ binding agents such as Quin 2 in the presence of modulators of ion flux and leukoregulin, it is possible to ascertain whether the increased plasma membrane permeability induced by leukoregulin is associated with an alteration in a specific ion flux.

MATERIALS AND METHODS

Target Cells. K562 erythroleukemia, HT29 colon carcinoma, and RPMI 2650 nasal pharyngeal carcinoma cells were cultured in RPMI 1640 medium containing 10% FBS in a 5% CO2-saturated atmosphere (1).

Reagents. Phytohemagglutinin (leukoagglutinin isomer), calcium ionophore A23187, ouabain, amphotericin B, calmodulin, nifedpine, verapamil, diphenylhydantoin, atracylsodin, valinomycin, and Quin 2-AM were purchased from Sigma Chemical Company (St. Louis, MO). Monesin and phospholipase C were obtained from Calbiochem (San Diego, CA). Amiloride was a gift from Dr. George Fanelli (Merck, Sharp and Dohme, West Point, PA), and the calcium ionophore X-537A was a gift from Dr. Peter F. Sorreter (Hoffmann LaRoche, Nutley, NJ).

Received 11/20/85; revised 2/12/86; accepted 2/14/86.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom requests for reprints should be addressed, at Building 37, Room 2A03, NIH, Bethesda, MD 20892.

1 The abbreviations used are: Quin 2, 2-[2-(bis(carboxymethyl)amino)-5-methylphenoxy]methyl-6-methoxy-8-bis(carboxymethyl)aminoquinoline; FBS, fetal bovine serum; Quin 2-AM, 2-[2-(bis(carboxymethyl)amino)-5-methylphenoxy]methyl-6-methoxy-8-bis(carboxymethyl)aminoquinoline-tetrakis(acetoxy methyl)ester; FACS, fluorescence-activated cell sorter.

2686
LEUKOREGULIN ION FLUX AND MEMBRANE PERMEABILITY

NJ). Stock solutions of 10^{-1}—10^{-4} M were made at room temperature. The calcium ionophores were dissolved in dimethyl sulfoxide. Ouabain, amphotericin B, calmodulin, atraclytoside, and amiloride were dissolved in RPMI 1640 medium-10% FBS. Nifedipine, verapamil, monestin, and Quin 2-AM were dissolved in acetone. Diphenylhydantoin was dissolved in deionized water adjusted to pH 11 with 1 M NaOH. Stock solutions were stored at 4°C for 2 wk except for the ionophores which were maintained at room temperature for up to 1 mo. Quin 2-AM was stored as a 1.5 × 10^{-2} M stock solution in 500-μl aliquots at −75°C. Immediately prior to use, stock solutions were diluted in RPMI 1640 medium-10% FBS at 37°C.

Leukoregulin was isolated from the lymphokines produced by phytohemagglutinin-stimulated normal human lymphocytes using sequential dialfiltration, isoelectric focusing, and molecular sieving high-performance liquid chromatography (1). Briefly lymphokine was concentrated 50- to 100-fold over an Amicon YM10 membrane (Amicon, Inc., Danvers, MA) and focused for 16–18 h at 4°C at 15 W within a 4.5–6.0 pH ampholine gradient. Lymphokines with pIs of 4.5–6.0 were then further purified by isoelectric elution from a preparative Toyasoda G3000 SWG high-pressure liquid chromatography column at a flow rate of 4 ml/min with 0.02 M sodium phosphate buffer, pH 7.4, containing 0.1% Mr 3500 polyethylene glycol. Fractions within the Mr 40,000–60,000 range containing the leukoregulin activity were pooled and dialyzed over a YM10 membrane against phosphate-buffered saline-0.1% polyethylene glycol. Using this procedure, greater than 50% of the total leukoregulin activity in the original lymphokine was obtained possessing a pI of 5.3 and a high-pressure liquid chromatography molecular weight average of 50,000. Leukoregulin was also isolated by substituting DEAE-ion exchange chromatography for the initial diadfiltration step. Lymphokine was diluted 1:2–1:4 with 20 mM Tris-HCl:0.1% polyethylene glycol, pH 7.4, and 7.5 ml of Fractogel TSK-DEAE 650M (EM Science, Gibbstown, NJ) previously equilibrated with the Tris buffer were added to each 4 liters of the diluted lymphokine. After stirring at 4°C for 1 h, the resin was allowed to settle and was transferred to a 0.9- x 15-cm inner diameter column. The column was washed at 1 ml/min with 10 column volumes of the diluting buffer, and leukoregulin was eluted with 20 column volumes of 0.1 M NaCl:20 mM Tris-HCl:0.1% polyethylene glycol, pH 7.4, at 4°C and concentrated by dialfiltration for isoelectric focusing and molecular sieving chromatography.

Assessment of Cell Membrane Integrity. Cell size, surface conformation, and plasma membrane permeability were measured by forward light scatter, right angle light scatter, and the fluorescence of retained fluorescein or of assimilated propidium iodide, respectively, using a FACS IV fluorescence-activated cell sorting flow cytometer or FACS analyzer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). FACS IV measurements were performed using 488-nm argon laser excitation as previously described (1) except that in the present investigation, fluorescein diacetate and propidium iodide were added directly to the cells, and the fluorescence of the intracellular dyes was measured without centrifugation and washing of the cells (see below). Results using this procedure are equivalent to the previously described method with one permeability unit of leukoregulin defined as that amount of lymphokine which, when incubated with 1 × 10^{5} K562 cells for 2 h, produces a 50% decrease in the fluorescence of the fluorescein-labeled K562 cells. In this method, 10^{5} K562 cells in 500 μl of medium containing 0–200 leukoregulin units/ml or 10^{-2}–10^{-4} M ionophore, phytohemaggglutinin, or other regent are incubated in 12- x 75-mm polystyrene tubes (Falcon Plastics, Oxnard, CA) at 37°C for 2 h on a rocker platform. After incubation, 500 μl of fluorescein diacetate or propidium iodide at 12.5 or 40 μg/ml, respectively, in RPMI 1640 medium-10% FBS are added directly to the tubes without centrifugation or washing of the cells. The cells are incubated at room temperature for 5 min and analyzed directly on the flow cytometer. The filters in the FACS IV flow cytometer include a 488/20-nm band pass for right angle scatter, a 530/30-nm band pass for fluorescein fluorescence, and a 757/26-nm band pass filter for propidium iodide fluorescence. Measurements were taken using 200- to 400-nm argon laser excitation at 488 nm, and 20,000 cells are analyzed for each determination. Cell volume, right angle light scatter, and fluorescence can also be measured using a FACS analyzer with mercury arc lamp excitation with results, unless otherwise indicated, identical to those obtained with the FACS IV sorting flow cytometer. The FACS analyzer is set up with similar filters for fluorescence emissions as the FACS IV using the standard fluorescein-propidium iodide FACS analyzer filter pack.

Both the FACS IV flow cytometer and the FACS analyzer were interfaced in this investigation with a Consort 40 data management system (Becton Dickinson Immunocytometry Systems) and produce comparable results from list mode data (except that the FACS IV flow cytometer provides forward scatter and the FACS analyzer provides cell volume measurements). For volume calibration, nonfluorescent polystyrene beads ranging from 1.08- to 10-μm diameter (Coulter Electronics, Inc., Hialeah, FL) were analyzed at 0.71 mA using a 75-μm orifice and the mean channel recorded. Cell volume was calculated by linear regression analysis. For fluorescein, forward light scatter, and propidium iodide, the control cell fluorescence signal was placed in the lower 10% of the signal channel range. Right angle scatter of the control cells was placed in the middle of the signal channel range by adjusting the amplification.

Kinetic measurements were obtained during a 180-min period of target cell exposure. Individual tubes were analyzed for each time point and for each fluorescent dye, i.e. fluorescein diacetate and propidium iodide, to eliminate spill over of the fluorescein signal into the propidium iodide channel as a result of the overlap in the emission wavelengths of the two dyes. Analyses were performed at 37°C at 0, 2, 5, 10, 20, 30, 40, 60, 90, 120, 150, and 180 min after effector addition. Fluorescein diacetate- and propidium iodide-treated control cells were examined at the start and end of the analysis period to ensure that no laser or other form of fluorescence drift had occurred. Effector concentrations were selected to produce a 50% loss in intracellular fluorescein fluorescence after 2 h incubation with the target cells. The first four time points were read from the same tube which was maintained at 37°C. For the remaining intervals individual sample tubes were kept at 37°C in a 5% CO_2-95% air water-saturated incubator on a rocker platform until 5 min before analysis, when 500 μl of 12.5 μg of fluorescein diacetate or 40 μg of propidium iodide per ml were added to the cells. Three parameters were simultaneously analyzed for each treated cell population: (a) forward light scatter or cell volume; (b) right angle light scatter; and (c) fluorescein or propidium iodide fluorescence.

Dual parameter analysis of the list mode data was performed using the standard Version 3 Becton Dickinson Consort 40 software to evaluate the coordinate expression of any of the parameters.

Measurement of Intracellular Calcium. The presence of intracellular free calcium in individual cells was measured using the fluorescent calcium probe Quin 2 by a modification of the spectrofluorometric bulk measurement method of Tsien et al. (5, 6). Briefly, 2–3 × 10^{5} cells in 1 ml of RPMI 1640 medium-10% FBS were incubated with 20 μM Quin 2-AM for 20–30 min in a 37°C water-saturated 5% CO_2-95% air atmosphere on a rocker platform at 8 cycles/min. The cell suspension was then diluted 10-fold with culture medium and incubated for an additional 60–90 min to permit completion of the uptake of Quin 2-AM and hydrolysis to intracellular Quin 2. The cells were then centrifuged at 2000 × g for 3 min and resuspended at 4 × 10^{5} cells/ml. Aliquots of 250 μl or 500 μl were placed into 12- × 75-mm tubes, and an equal volume of medium was added. Cells were either treated with 4 units of leukoregulin or with 3 × 10^{-3} M calcium ionophore A23187, and the fluorescence of the cells with evaluated using the FACS analyzer. The instrument was aligned using 9.98-m-diameter fluorescent fluorospheres (Coulter Electronics, Hialeah, FL), so that the mean channel number of their fluorescence was near the middle of the 256-channel range using the factory supplied UV filter pack to subsequently capture as much of the Quin 2 emission as possible at 500 nm. Excitation filters consisted of two 375-nm short pass and a 360-nm band pass filter. Emission filters were two 400-nm long pass filters, set at an angle of 45° to one another, and a 490-nm band pass filter. Cell volume was used to trigger the analysis with logarithmic amplification, current set at 0.71 mA, and the photomultipliers set at 250 and 450 V, respectively.

Downloaded from cancerres.aacrjournals.org on July 20, 2017. © 1986 American Association for Cancer Research.
for fluorescence and right angle light scatter. These amplification settings placed the autofluorescence peak of the control cells (not labeled with Quin 2) between 70 and 90 on the 256-channel scale. The tube containing the cells being analyzed was maintained at 37°C, and the fluorescence was measured each minute. After 6 min, the tube was placed back in the incubator and at 10 and 30 min reanalyzed on the FACS analyzer.

RESULTS

Leukoregulin induces a highly reproducible (2 SEs, < ± 5%) change in plasma membrane permeability and forward light scatter measurable with a laser-excited flow cytometer (Fig. 1). The alterations in membrane permeability indicated by either the percentage of cells remaining in the peak fluorescence channel or in the channels containing the total peak of fluorescence (as defined by the nontreated cells) are identical. This demonstrates that the homogeneous response of the cells is independent on the initial intracellular fluorescein concentration. Dual parameter analysis, furthermore, shows that the change in plasma membrane permeability and forward light scatter 2 h after leukoregulin treatment is both coordinate and homogeneous throughout the cell population; i.e., the cell response is all or none with the degree of population response increasing directly with the concentration of leukoregulin.

Identical results are obtained with the mercury arc lamp-excited FACS analyzer and the laser-excited FACS IV cell sorter, when the changes in fluorescence measured by the instruments are compared and when the changes in cell volume (as measured by the FACS analyzer) and forward light scatter (as measured by the FACS IV flow cytometer) are compared. Differences in the measurements by the two instruments are only seen when intensities of the right angle scatter from fluorescein- and propidium iodide-labeled cells are compared. Both instruments demonstrate a decrease in total right angle scatter in K562 cells exposed to leukoregulin. The FACS analyzer, however, reveals that the intensity of right angle scatter from fluorescein-labeled cells is greater than the intensity of right angle scatter from propidium iodide-labeled cells. A similar difference is detected by the FACS IV flow cytometer but only when the mean channel numbers of the right angle scatter peaks are compared. These differences in right angle scatter measured by the two instruments result from the polychromatic broadband wavelength excitation and scatter collection system in the FACS analyzer compared to the monochromatic 488-nm laser excitation and narrow band width right angle light scatter collection in the FACS IV flow cytometer. Despite the differences in the mean intensity, the total pattern of change in right angle light scatter following leukoregulin treatment is the same whether measured by the FACS analyzer or the FACS IV flow cytometer.

The decrease in forward light scatter of leukoregulin-treated K562 cells coincides with a decrease in cell volume. For example, following 2 h of exposure to 2 units of leukoregulin, 55% of K562 cells exhibit a decrease in cell volume. The volume of 37% of the cells remains unchanged (12-μm diameter), and 8% of the cells are slightly larger (14 μm). The K562 cells exhibiting a decrease in cell volume are distributed within several populations: 19% with a decrease to 9 μm and 22% with a decrease to 4-μm diameter. The remaining 14% range from 4-8.5 μm in diameter. Decreases in forward light scatter, cell volume, and fluorescence fluorescence also occur in HT29 colon carcinoma and RPMI 2650 nasal pharyngeal carcinoma cells exposed to leukoregulin. The extent of the changes in general correlates with the sensitivity of the cells to the cytostatic action of leukoregulin; e.g., RPMI 2650 cells exhibit approximately 20-fold more changes in these membrane parameters and in cell proliferation than do K562 cells treated with the same concentration of leukoregulin.

Flow cytometric comparison of K562 cells treated with one of a variety of agents able to modulate monoclonal and divalent plasma membrane and/or intracellular cation flux demonstrates that only those agents able to increase intracellular Ca²⁺ are able to directly increase membrane permeability or increase the membrane permeability induced by leukoregulin (Table 1; Fig. 2). These agents include phospholipase C, phytohemagglutinin, and calcium ionophores A23187 and X-537A, which possess relative activities that vary nearly 10,000-fold (Table 2). Agents that directly increase intracellular Ca²⁺ including the calcium ionophores, phytohemagglutinin, phospholipase C, and two that alone are unable to alter permeability, i.e., ouabain and amphoterin B, also synergistically enhance the permeability changes induced by leukoregulin (Table 2; Fig. 3). This indicates that leukoregulin has its own specific molecular action and that several pathways of Ca²⁺ metabolism may influence leukoregulin-induced membrane destabilization.

Four-parameter flow cytometric kinetic analysis further demonstrates the unique mode of action of leukoregulin in stabilizing the plasma membrane. Each of the four Ca²⁺ modulators (calcium ionophores A23187 and X-537A, phytohemagglutinin, and phospholipase C) mimics the development of leukoregulin-induced changes in cell surface conformation and membrane permeability. Simultaneous analysis of forward scatter or cell volume, right angle scatter, fluorescence retention, and propidium iodide uptake reveals that no Ca²⁺ modulator produces a pattern of membrane change identical to leukoregulin. For example, Fig. 4 illustrates the kinetics of membrane change due to leukoregulin (Fig. 4, top) compared to X-537A (Fig. 4, bottom), the calcium modulator most closely mimicking the membrane action of leukoregulin. While small changes were
Table 1: Modulators of monovalent and divalent cation flux and their effect upon the ability of leukoregulin to increase plasma membrane permeability

<table>
<thead>
<tr>
<th>Modulator</th>
<th>Mode of action</th>
<th>Effect on K562 plasma membrane permeability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium ionophores A23187 and X-537A</td>
<td>Increase Ca(^{2+}) influx (7, 26)</td>
<td>Increases membrane permeability</td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>Mobilizes intracellular Ca(^{2+}) (13)</td>
<td>Increases membrane permeability</td>
</tr>
<tr>
<td>Ouabain</td>
<td>Inhibits Na(^{+})/K(^{+}) pump, enhances intracellular mobilization of Ca(^{2+}) (27)</td>
<td>Increases membrane permeability</td>
</tr>
<tr>
<td>Amiloride</td>
<td>Increases influx of various ions including Ca(^{2+}) (28)</td>
<td>No effect</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>Binds Ca(^{2+}) (29, 30)</td>
<td>No effect</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>Blocks Ca(^{2+}) channels (31)</td>
<td>No effect</td>
</tr>
<tr>
<td>Verapamil</td>
<td>Blocks Ca(^{2+}) channels (31)</td>
<td>No effect</td>
</tr>
<tr>
<td>Atractyloside</td>
<td>Inhibits Na(^{+}) transport (32)</td>
<td>No effect</td>
</tr>
<tr>
<td>Diphenylhydantoin</td>
<td>Increases Na(^{+}) influx and stimulates Na(^{+})/K(^{+}) transport (33)</td>
<td>No effect</td>
</tr>
<tr>
<td>Sodium ionophore monensin</td>
<td>Increases Na(^{+}) influx (35)</td>
<td>No effect</td>
</tr>
<tr>
<td>Potassium ionophore valinomycin</td>
<td>Increases K(^{+}) influx (36)</td>
<td>No effect</td>
</tr>
</tbody>
</table>

* As measured by flow cytometric forward light scatter and loss of intracellular fluorescein fluorescence after incubation of K562 cells for 2 h with the modulators at \(10^{-6}\) to \(10^{-4}\) M using a FACS IV flow cytometer as described in Fig. 1.

---

**Fig. 3.** Calcium ionophore enhancement of the increase in K562 cell plasma membrane permeability induced by leukoregulin. K562 cells were treated for 2 h with increasing concentrations of leukoregulin (LR) alone (□), with calcium ionophore A23187 alone (×), or with a combination of 0.4 units of leukoregulin able to produce by itself a 19% loss in fluorescein and the indicated concentrations of A23187 (□). † 1/, expected (EXP.) response if the action of the two agents is additive; O, observed (OBS.) response. Similar results are observed in the presence of increasing leukoregulin with a constant concentration of A23187. Analysis was performed as described in Fig. 1.
a rise in intracellular Ca^{2+} after exposure to leukoregulin occurs after 5 min.

**DISCUSSION**

These results establish that the increase in tumor cell plasma membrane permeability after exposure to leukoregulin is accompanied by an increase in intracellular ionized Ca^{2+}. The evidence supporting this conclusion is: (a) only those modulators of monovalent and divalent membrane cation flux which increase intracellular Ca^{2+}, e.g., calcium ionophores, phytohemagglutinin, amphotericin B, and ouabain, produce changes in membrane permeability, cell volume, and cell surface conformation similar to those induced by leukoregulin; and (b) the development of increased membrane permeability detectable by intracellular fluorescein efflux occurs concomitantly with an increase in intracellular Ca^{2+}.

The increase in intracellular Ca^{2+} accompanying the change in membrane permeability produced by leukoregulin may result from Ca^{2+} influx and/or the mobilization of existing non-ionic or bound intracellular calcium. The ability of Ca^{2+} ionophores but neither amphotericin B nor ouabain to closely mimic leukoregulin-induced membrane destabilization suggests that Ca^{2+} influx may be the dominant mechanism underlying the increase in intracellular Ca^{2+} by leukoregulin. The ability of amphotericin B and ouabain as well as the calcium ionophores to synergistically enhance leukoregulin action indicates, however, that the overall increase in intracellular Ca^{2+} and not just the transmembrane influx is important.

Leukoregulin may influence Ca^{2+} flux in a manner similar to Ca^{2+} transport by the calcium ionophores, i.e., by direct binding of the ionophore with Ca^{2+} followed by diffusion of the calcium-ionophore complex across the membrane (7). It is interesting,
however, that the Ca\(^{2+}\) channel blockers and the inhibitors of Ca\(^{2+}\) transport, nifedipine and verapamil, fail to inhibit the action of leukoregulin. This suggests that leukoregulin-induced Ca\(^{2+}\) influx occurs via a different type of transmembrane Ca\(^{2+}\) transport channel than that affected by nifedipine or verapamil. Leukoregulin, moreover, has no structural resemblance to the rather small molecular-sized (Mr 400–800) and hydrophobic calcium ionophores A23187 and X-537A. Leukoregulin thus appears to function via a different molecular mechanism, e.g., by receptor-mediated mobilization of intracellular calcium.

The delayed rise in leukoregulin-induced intracellular Ca\(^{2+}\) compared to the rise produced by calcium ionophore A23187 as detected by Quin 2 fluorochromasia is consistent with leukoregulin activation of a receptor-initiated pathway of increased intracellular Ca\(^{2+}\) and resulting membrane destabilization. The molecular pathway by which leukoregulin destabilizes the plasma membrane may be related to the pathway by which the glycosyl binding lectin, phytohemagglutinin, increases intracellular calcium and alters membrane permeability. The data in the present investigation suggest, however, that the pathways are divergent or completely separate as leukoregulin-treated cells but not phytohemagglutinin-treated cells express a delay in propidium iodide influx when compared to intracellular fluorescein efflux. The sequential increase in fluorescein efflux, decrease in cell volume, and influx of propidium iodide indicate, moreover, that leukoregulin-induced membrane destabilization occurs in several steps.

Agonist activation of cell surface receptors is proposed to increase intracellular Ca\(^{2+}\) via the intermediary intracellular second messenger inositol triphosphate (8). This is in contrast to the increase in Ca\(^{2+}\) flux produced by calcium ionophores and the resulting stimulation of cellular responses, e.g., catecholamine release, thyroid stimulation, and lymphocyte transformation, which occurs in the absence of ligand-receptor interaction (9–12). Phytohemagglutinin and phospholipase C, the enzyme that converts phosphatidylinositol bisphosphate to inositol triphosphate (13), produce simultaneous changes in K\(_{\text{v}}\) cell volume, cell conformation, fluorescein efflux, and propidium iodide influx. The difference in the kinetics of K\(_{\text{v}}\) membrane destabilization induced by leukoregulin or by the Ca\(^{2+}\) modulators indicates that leukoregulin does not increase intracellular Ca\(^{2+}\) by acting as a calcium ionophore or by direct hydrolysis of phosphatidylinositol bisphosphate. Leukoregulin may, however, as a result of binding to its receptor increase transmembrane calcium transport and intracellular mobilization of calcium by stimulating one or more points in the pathways of calcium metabolism.

Cells are bathed in a high concentration of Ca\(^{2+}\) (10\(^{-3}\) M), but the intracellular concentration is more than a thousandfold lower (0.1–0.4 x 10\(^{-6}\) M) (14, 15). Cells are relatively impermeable to Ca\(^{2+}\) and generally maintain the large intracellular/extracellular Ca\(^{2+}\) gradient by means of membrane-bound Ca\(^{2+}\)-transport ATPases to pump calcium ions out of the cells or into sequestration sites such as the endoplasmic reticulum (16). There are, however, a variety of ways to perturb intracellular Ca\(^{2+}\) homeostasis. Ca\(^{2+}\) ionophores and channel blockers can directly alter Ca\(^{2+}\) transport, while amphotericin B, ouabain, and related compounds alter the synthesis of energy necessary to maintain the Ca\(^{2+}\) gradient. Stimulation of phospholipid metabolism via a receptor-mediated event leads to the production of diacylglycerol and inositol trisphosphate (13). Diacylglycerol in turn stimulates and regulates protein kinase C, and inositol trisphosphate mediates the mobilization of intracellular Ca\(^{2+}\) stores (17, 18). Protein kinase C and Ca\(^{2+}\) may act synergistically as physiological regulators, modulating a wide range of cellular processes including enzyme, histamine, or insulin release as well as glycolysis and DNA synthesis (19).

Phospholipid metabolism is therefore an important aspect of Ca\(^{2+}\) homeostasis, and phospholipid turnover is thought to regulate the stability and thus the permeability of the plasma membrane through the production of lipids possessing a range of membrane functions. These functions include opening an ion channel, acting as a Ca\(^{2+}\) ionophore as in the case of phosphatidic acid, being a second messenger regulating enzymatic actions and the permeability of the plasma membrane to cations, or the mobilization of intracellular Ca\(^{2+}\) as in the case of inositol triphosphate (18, 20). Phospholipase C once activated may change membrane permeability as it moves in the plane of the membrane to activate protein kinase C (13, 21). Agonists may also be capable of opening Ca\(^{2+}\) channels in the plasma membrane without any changes in phosphoinositide metabolism (13). In addition, any direct damage to the cell membrane may lead to a change in its permeability and result in an influx of Ca\(^{2+}\) (4). The interrelationships of these many aspects of calcium metabolism to one another and to cellular homeostasis are only beginning to be defined and indicate the number of points in phospholipid metabolism where leukoregulin has the potential to affect calcium homeostasis and membrane stability.

How may an increase in intracellular Ca\(^{2+}\) flux and membrane permeability specifically relate to the cytostatic action of leukoregulin? A rise in intracellular calcium has been shown to be a common end point for toxic cell death, although the question whether the rise in intracellular Ca\(^{2+}\) is the causative or a secondary event in the mechanism leading to target cell lysis remains to be defined (4). An increase in intracellular calcium alone is insufficient to cause cell destruction, as increased intracellular calcium is important in the activation of cell responses without cell death and in the maintenance of cell proliferation (22–24). Increased Ca\(^{2+}\) influx also leads to formation of a calcium-dependent regulator protein-Ca\(^{2+}\) complex which stimulates Ca\(^{2+}\)-ATPase, cyclic 3',5'-nucleotide phosphoesterases, and protein kinases which in combination with extracellular Ca\(^{2+}\) are important, although as yet incompletely understood, in regulating cell proliferation and growth (24). In contrast, the antiproliferative action of partially purified lymphotoxin has been shown to involve creation of an ionic imbalance within the target cell including an increase in intracellular Ca\(^{2+}\) associated with lysis of the cell (2, 3). Lysis may, however, be a secondary event of lymphokine target cell interaction resulting from the inability of the target cell to repair the cell membrane destabilization due to lymphokine-target cell interaction.

Leukoregulin destabilization of the cell membrane with its associated change in membrane permeability and in membrane fluidity (25) is a major alteration in cell physiology capable by itself of leading to inhibition of cell proliferation and to conditioning of the cell to cytolytic destruction by direct-acting lytic molecules or cellular effectors such as natural killer lymphocytes. Cells resistant to the direct cytolytic action of leukoregulin (1) may either be able to repair leukoregulin-induced membrane damage or by as yet undefined means counteract the leukoregulin-directed events associated with increased intracellular calcium and cell destruction. Further definition of the intricacies of the pathways of Ca\(^{2+}\) metabolism and their interrelationship with membrane integrity will permit more specific molecular assignment of the role of Ca\(^{2+}\) in the anticancer action of leukoregulin. These are important avenues to pursue in the definition of cellular homeostasis in order to understand
the molecular pathways by which lymphokines and cellular effector and target cell mechanisms operate to maintain and alter plasma membrane integrity.

REFERENCES


Leukoregulin-increased Plasma Membrane Permeability and Associated Ionic Fluxes

Susan C. Barnett and Charles H. Evans


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/6/2686

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.