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Influence of Extracellular Calcium on Cell Permeabilization and Growth Regulation by the Lymphokine Leukoregulin

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Permeablization of human K562 leukemia cells was measured in the presence and absence of extracellular ionic calcium to examine the relationship of ionic calcium to increased membrane permeability and the inhibition of cell proliferation by this lymphokine. In the absence of extracellular calcium, the ability of leukoregulin to permeabilize the cell membrane is diminished but is fully restored by addition of 1 mM extracellular Ca^{++} as shown flow cytometrically by loss of intracellular fluorescein. Membrane permeability is also increased by calcium ionophore A23187 but permeablization is completely blocked in calcium-free medium despite the intramembrane presence of the calcium ionophore. Membrane permeablization by the lectin phytohemagglutinin, in contrast, is independent of extracellular calcium. A similar divergence in cell proliferation activity of the three modulators of calcium flux and membrane permeability occurs in the absence of extracellular calcium. Leukoregulin inhibition of cell proliferation is abolished, inhibition by calcium ionophore A23817 is greatly reduced, and inhibition by phytohemagglutinin is unchanged. Leukoregulin permeabilized K562 cells isolated by fluorescence activated cell sorting resume proliferation after 72 h. In contrast cells permeablized by calcium ionophore A23187 or phytohemagglutinin fail to resume proliferation by 7 days. The membrane permeablizing action of leukoregulin is, therefore, partially dependent upon extracellular calcium. It is also effected through a mechanism other than calcium ionophore transport or lectin type transmembrane signaling, and is accompanied by a reversible inhibition of cell proliferation.

Key words: calcium, leukoregulin, lymphokine, membrane permeablization

Leukoregulin is a lymphokine that interacts directly with the target cell to inhibit the proliferation of and increase the sensitivity of tumor cells to natural killer (NK) lymphocyte cytolysis [1]. The antitumor cell action of leukoregulin is accompanied by a rapid and reversible increase in the plasma membrane permeability (permeablization) of the target cell [1,2]. A similar increase in tumor cell permeablization occurs during

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natural killer lymphocyte cytotoxicity [2] and natural killer lymphocytes secrete leukoregulin [3]. This suggests that leukoregulin is one cytokine responsible for some of the target cell molecular events in the cytotoxic reaction.

Modulators of Ca^{++} flux including calcium ionophores and mitogens have been shown to enhance the membrane permeablizing action of leukoregulin, and intracellular Ca^{++} increases upon incubation of human K562 leukemia cells with leukoregulin [2]. Ca^{++} ions play a significant role in the mechanism of action of a wide range of lymphokines and products of cytotoxic T cells. Extracellular Ca^{++} is required for the cytotoxic action of lymphotoxin [4], NK cells, and cytotoxic T cells [5]. The cytolytic action of cytolysin, which is present in the cytoplasmic granules of cytotoxic large granular lymphocyte tumor cells, also is accompanied by a rapid Ca^{++}-dependent increase in permeability of the target cell [6]. Perforin, a cytolysin-like cytolytic pore-forming protein derived from granules of cloned lymphocytes, is similar to the ninth component of complement since it inserts functional pores in the target cell membrane inducing cytolysis and like cytolysin is dependent on the presence of extracellular Ca^{++} [7–9]. T-cell-dependent target cell lysis is postulated to consist of three major sequential steps of which the second step is dependent on Ca^{++} and is termed "lethal hit" or programming for lysis [4,10]. The action of interferon on the induction of cyclic GMP in L1210 cells has also been shown to be dependent on extracellular Ca^{++} [11]. Other investigators, however, have shown that interferons do not signal cells through phospholipid turnover or increased cytoplasmic free Ca^{++} [12] and cytolysis by murine cytolytic lymphocyte lines is independent of the presence of extracellular Ca^{++} [13]. These reports emphasize the complexities and our incomplete understanding of the role of calcium metabolism in cellular cytotoxicity.

In the present investigation we examine the role of extracellular ionic Ca^{++} in the membrane permeablizing action of leukoregulin to determine the relationship of ionic calcium and increased membrane permeability to the inhibition of cell proliferation by this lymphokine. Comparisons have been made with calcium ionophore A23187 and the mitogen phytohemagglutinin, which also induce a dose-dependent increase in the plasma membrane permeability of K562 cells [2]. Calcium ionophore A23187 was used at 50 μM and phytohemagglutinin at 1.5 μM, concentrations able to increase membrane permeability of more than 50% of K562 cells within 2 h [3]. The relationship of the rapid increase in membrane permeability to cellular proliferation was studied by analyzing the proliferation capacity of leukoregulin, calcium ionophore A23187, and phytohemagglutinin treated K562 cells exhibiting increased membrane permeability that were isolated by fluorescence activated cell sorting.

MATERIALS AND METHODS

Chemicals

Calcium ionophore A23187 (Sigma Chemical Co., St. Louis, Mo.) was dissolved in dimethyl sulfoxide at a concentration of 5 mM and stored at 25°C. Phytohemagglutinin (leukoagglutinin isomer) was purchased from Sigma and dissolved in RPMI 1640 medium at a stock concentration of 70 μM. Fluorescein diacetate (Polysciences, Inc., Warrington, PA.) was dissolved in acetone to 12 mM and stored at 4°C. Propidium iodide (Polysciences, Inc., Warrington, PA.) was dissolved in phosphate buffered saline to 0.15 mM and stored at 4°C. All solutions were used within 1 month.

Cells and Culture Conditions

K562 human myelocytic leukemia cells were cultured in RPMI 1640 medium containing 10% FBS (medium) in a 5% CO_2-95% air water saturated atmosphere [1]. Medium free of ionic calcium was prepared by adding EGTA to a concentration of 2 mM to calcium-free RPMI 1640 prepared from a select amine kit (Gibco, Chagrin Falls, OH.) containing 10% FBS. The EGTA-treated medium is referred to as Ca^{++}-free medium in this study. The concentration of Ca^{++} in the medium was monitored using a Ca^{++} ion-selective electrode (Radiometer F2112Ca) with a sensitivity range for Ca^{++} from 10^0 to 10^{-8} M and a Radiometer ION85 Analyzer (Radiometer, Copenhagen, Denmark). After addition of 2 mM EGTA no detectable Ca^{++} was present in the EGTA-treated RPMI 1640-10% FBS medium.

Leukoregulin Production

Human leukoregulin was produced by phytohemagglutinin stimulation of mononuclear leukocytes isolated by discontinuous Ficoll-Hypaque gradient centrifugation from normal peripheral blood [3]. Leukoregulin with a pI of 5.1 [14] and a HPLC molecular weight of 50 kDa [1] was isolated from the lymphokine preparations by sequential molecular sizing high performance liquid chromatography on a TSK 3000 SWG spherical silica matrix column followed by column isoelectric focusing within a pH 4–6 1% ampholine gradient as previously described [2]. All buffers used in the isolation procedures were calcium free.

Flow Cytometric Analysis of Target Cell Membrane Permeability

Leukoregulin was quantitated by fluorometric measurement of membrane permeablization of K562 cells using a FACS IV fluorescence-activated cell sorting flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA) as previously described [2]. Briefly, 10^6 K562 cells in 500 μl of medium or Ca^{++}-free medium were incubated with 0-200 leukoregulin units/ml in 12 × 75 mm polystyrene tubes (Falcon Plastics, Oxnard, CA) at 37°C for 2 h on a rocker platform in a 5% CO_2-95% air water saturated atmosphere after which they were washed in medium or Ca^{++}-free medium and resuspended in 1 ml medium containing 30 μM fluorescein diacetate. After 5 min at room temperature, for hydrolysis of the diacetate, the cells were excited at 488 nm and the fluorescence at 530 nm measured. One permeablization unit is defined as the amount of leukoregulin producing a decrease in the fluorescence of 50% of the fluorescein-labeled K562 cells [1]. It is important to note that any decrease in the fluorescence intensity is recorded as a change in membrane permeability in this assay [2]. The membrane permeability of K562 cells after treatment with calcium ionophore A23187 or phytohemagglutinin was assessed by the same procedure.

Cells with decreased fluorescein fluorescence and forward angle light scatter were electronically sorted at 37°C [3] according to the boundaries defined by the FACS IV left sort window, whereas cells with unaltered permeability as demonstrated by high fluorescence and forward scatter were sorted within the boundaries defined by the right sort window. The proliferation of sorted and control nontreated K562 cells was analyzed in a 7 day cell proliferation assay. Cells (1×10^4) in 1 ml of RPMI 1640-10% FBS were aliquoted in triplicate in a 24 well culture plate (Costar, Cambridge, MA) and cell numbers were enumerated on day 1, 3, 5, and 7 using a Coulter Counter as previously described [1].

Evaluation of Dependence on Extracellular Calcium

K562 cells (10^6) were aliquoted in 24 12 × 75 mm polystyrene tubes (Falcon Plastics) as described above in Ca^{++}-free medium with 1 unit of leukoregulin/ml, 50 μM calcium ionophore A23187, or 1.5 μM phytohemagglutinin. All tubes were incubated at 37°C for 2 h on a rocker platform in a 5% CO_2-95% air atmosphere after which 1 mM $CaCl_2$ was added to all but the control tubes and at 0, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150, and 180 min and equal volume of 30 μM fluorescein diacetate was added to one of duplicate tubes and 60 μM propidium iodide to the other. Control tubes were set up in quadruplicate: medium with Ca^{++} in the presence or absence of the effector and Ca^{++}-free medium in the presence and the absence of the effector. Control tubes were analyzed at 0, 45, 90, and 180 min after the addition of Ca^{++} to the experimental tubes. Duplicate tubes, one for fluorescein and one for propidium iodide, were analyzed for each time point to eliminate the spill over of the fluorescein signal into the propidium iodide channel since there is extensive overlap in the emission wavelengths of these two dyes.

For evaluation of cell proliferation, 10^6 K562 cells were treated for 2 h in medium or calcium-free medium with 4 units of leukoregulin/ml, 50 μM A23187, or 1.5 μM phytohemagglutinin, concentrations that produce an increase in permeability in greater than 50% of the cells when incubated in medium containing extracellular Ca^{++} [2]. 10^4 K562 cells were then incubated in 1 ml of RPMI 1640 + 10% FBS for a 7 day growth assay as described above.

RESULTS

Leukoregulin permeablization of K562 cells as measured by the loss of intracellular fluorescein is partially dependent on extracellular Ca^{++} (Table I; Fig. 1). The increase in membrane permeability resulting from concentrations of leukoregulin producing a 20–50% change in permeability is reduced in the absence of extracellular Ca^{++}. At higher leukoregulin concentrations the membrane permeablizing action of leukoregulin is independent of the presence of extracellular Ca^{++}. The partial dependence of leukoregulin on extracellular Ca^{++} is further demonstrated when Ca^{++} is added to the K562 cells after they have been treated with leukoregulin for 2 h in Ca^{++}-free medium (Fig. 2). The addition of 1 mM extracellular Ca^{++} is followed by a progressive increase

TABLE I. Extracellular Ca^{++} Dependency of Leukoregulin, Calcium Ionophore A23187, or Phytohemagglutinin Induced Membrane Permeability Changes in Human K562 Leukemia Cells

	Percent loss of intracellular fluorescein in the presence of:	
Treatment[a]	1 mM extracellular Ca^{++}	No extracellular Ca^{++}
Leukoregulin, 1 unit/ml	50	16
A23187, 50 μM	62	0
Phytohemagglutinin, 1.5 μM	65	63

[a] 10^6 K562 cells were treated with leukoregulin, calcium ionophore A23187, or phytohemagglutinin for 2 h and labeled with fluorescein as described in Figure 1 but in a separate experiment. Fluorescein loss was assessed by 488 nm excitation of the cells in a FACS IV flow cytometer and compared to fluorescein labeled cells that had been treated with culture medium in the absence of leukoregulin, calcium ionophore A23187, or phytohemagglutinin.

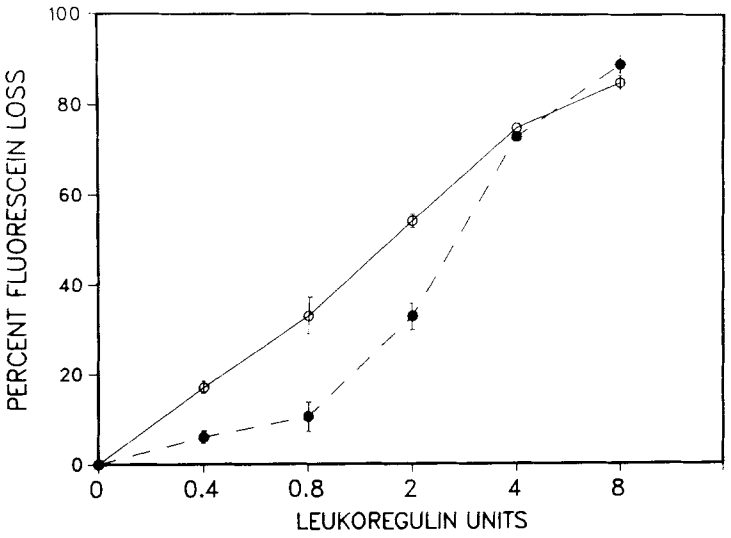

Fig. 1. Dependence of leukoregulin membrane permeablization upon extracellular Ca^{++}. 10^6 K562 cells in 1 ml of medium were treated with 0.4–8 units of leukoregulin for 2 h at 37°C. Each experiment was carried out in duplicate with one tube (open circles) containing 1 mM extracellular Ca^{++} and the other tube (solid circles) containing no extracellular Ca^{++}. Ca^{++} was removed from the medium by adding 2 mM EGTA to chelate the Ca^{++}. After 2 h the cells were incubated for 5 min with 15 μM fluorescein diacetate/ml and their ability to retain fluorescein quantified flow cytometrically. The values shown are the mean of five experiments ±S.E. A similar response was observed with HL60 promyelocytic leukemia cells, e.g., the percent fluorescein loss in calcium-free medium was 15% less at 0.4–2, 5% less at 4, and the same in the presence of 8 units of leukoregulin/ml.

in fluorescein loss and propidium iodide uptake. The net increase in membrane permeability is approximately 22% as measured by intracellular fluorescein loss at 45–180 min and by propidium iodide uptake at 90–180 min following addition of extracellular Ca^{++}. This is in good agreement with the diminution of membrane permeability activity of leukoregulin observed with K562 cells in the absence of extracellular Ca^{++} (Table I). Moreover, if the K562 cells treated with leukoregulin in the absence of extracellular Ca^{++} (Fig. 2) are washed before the addition of fresh medium containing ionic Ca^{++} but no leukoregulin there is no additional increase in membrane permeability.

In contrast to leukoregulin, calcium ionophore A23187 induces an increase in the plasma membrane permeability of K562 cells, which is totally dependent on the presence of extracellular Ca^{++} at all concentrations of the ionophore (Fig. 3). Calcium ionophore A23187 plasma membrane permeablization, moreover, develops fully following the addition of extracellular 1 mM Ca^{++} (Fig. 4) regardless of whether the cells have been washed after exposure to calcium ionophore A23187 and prior to the addition of extracellular Ca^{++}. Phytohemagglutinin also induces a dose-dependent change in the plasma membrane permeability of K562 cells, but in contrast to both leukoregulin and calcium ionophore A23187 the increase in plasma membrane permeability by phytohemagglutinin is independent of the presence of extracellular Ca^{++} (Fig. 5).

Comparison of leukoregulin, calcium ionophore A23187, and phytohemagglutinin at concentrations that after a 2 h cell exposure produce approximately a half maximal

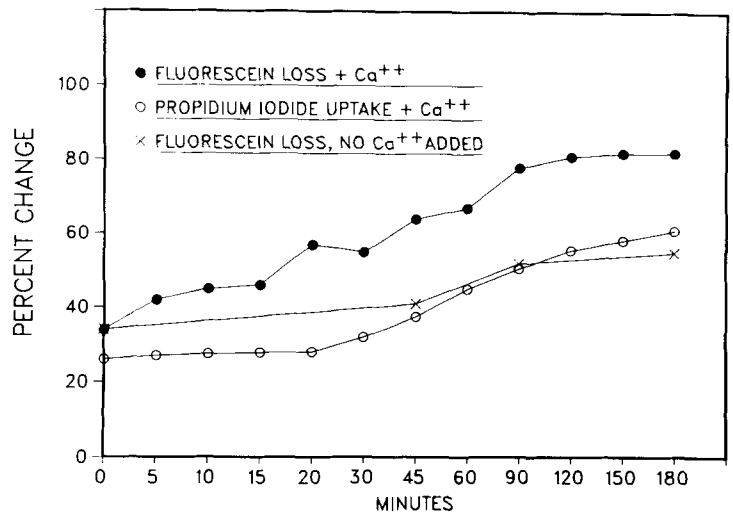

Fig. 2. Effect of extracellular Ca^{++} deprivation and readdition upon leukoregulin-induced membrane permeablization. 10^6 K562 cells were incubated with 1 unit of leukoregulin in Ca^{++}-free medium for 2 h as described for Figure 1 in a separate experiment. Following incubation, 50 μl of 10 mM $CaCl_2$ was added to the medium and the efflux of fluorescein and influx of propidium iodide were measured independently using a flow cytometer. A typical experiment is shown with the standard error between experiments being $\leq$10%.

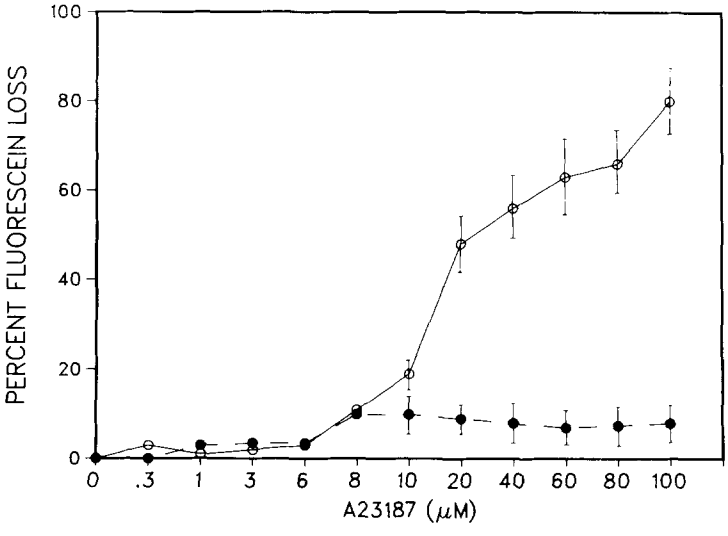

Fig. 3. Dependence of calcium ionophore A23187 membrane permeablization upon extracellular Ca^{++}. 10^6 K562 cells in 1 ml of medium were treated with 0–100 μM calcium ionophore A23187 for 2 h in the presence (open circles) and absence (solid circles) of 1.0 mM extracellular Ca^{++} as described for Figure 1. Each point is the mean of five experiments $\pm$S.E.

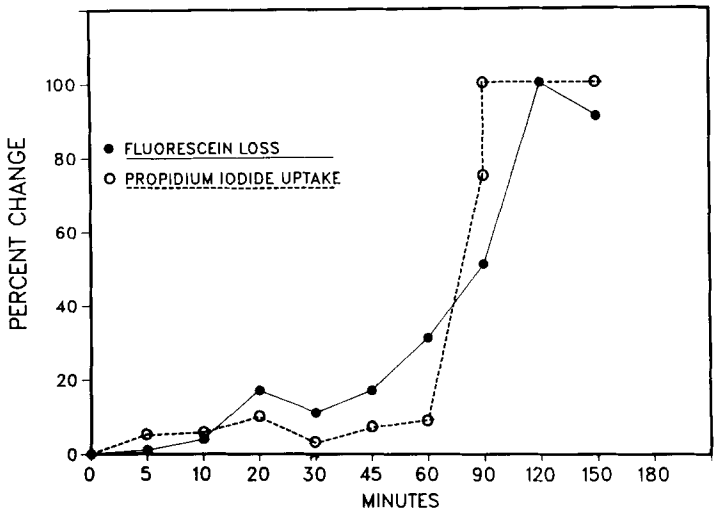

Fig. 4. Restoration of calcium ionophore membrane permeablizing activity by readdition of extracellular Ca^{++} after treatment of K562 cells with calcium ionophore A23187 in Ca^{++}-free medium. 10^6 K562 cells were incubated with 50 μM calcium ionophore A23187 in Ca^{++}-free medium for 2 h after which 50 μl of 10 mM $CaCl_2$ were added to the medium as described for Figure 2. A typical experiment is shown with the standard error between experiments being $\leq$10%. A response similar to that shown was also obtained if the cells were washed after the 2 h incubation period to remove extracellular calcium ionophore A23187 prior to addition of $CaCl_2$.

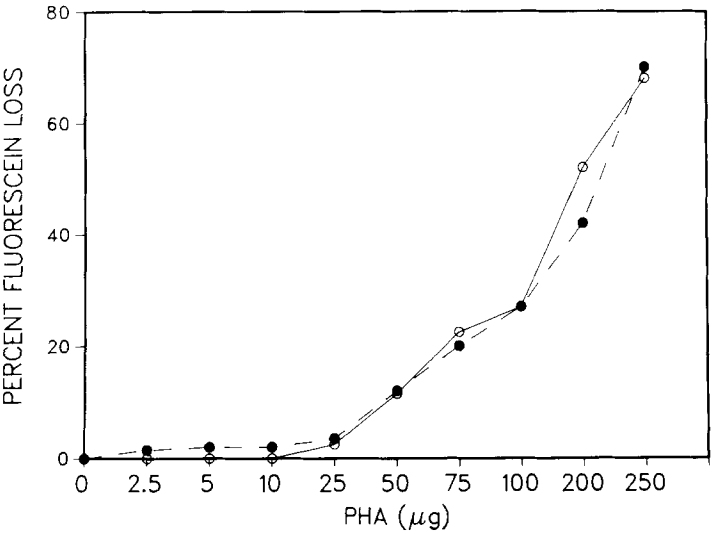

Fig. 5. Independence of phytohemagglutinin membrane permeablization upon extracellular Ca^{++}. 10^6 K562 cells in 1 ml of medium were treated with 1.5 μM phytohemagglutinin for 2 h in the presence (open circles) and absence (solid circles) of 1.0 mM extracellular Ca^{++} as described in the legend to Figure 1. Each point is the mean of five experiments with the standard error between experiments being $\leq$10%.

change in the permeability of K562 cell plasma membrane in the presence of extracellular Ca^{++} shows a similar divergence in their patterns of Ca^{++} dependence (Table I). In the absence of extracellular Ca^{++}, membrane permeability changes induced by leukoregulin are decreased but not eliminated, are eliminated for calcium ionophore A23187, and remain unchanged for phytohemagglutinin. A 2 h exposure to each of the membrane permeablizing agents at a concentration producing half maximal change in the permeability of K562 cell plasma membranes also results in inhibition of the proliferation of K562 cells during the 5 day period after treatment (Fig. 6). The ability of leukoregulin, calcium ionophore A23187, or phytohemagglutinin to inhibit cell proliferation just as observed with respect to their membrane permeability also exhibits a differential dependence on extracellular Ca^{++}. In the absence of extracellular Ca^{++} the proliferation inhibitory action of leukoregulin is eliminated, the inhibition of cell proliferation by calcium ionophore A23187 is decreased and the proliferation inhibitory action of phytohemagglutinin on K562 cells is unchanged.

Cell populations exhibiting alterations in membrane permeability and cell proliferation contain a mixture of affected and nonaffected cells at leukoregulin, calcium ionophore A23187, or phytohemagglutinin concentrations producing less than a maximal change. The affected and nonaffected cells are not generally easy to dissect out from a heterogenous population for further study. Through the use of flow cytometric cell sorting, however, individual populations can be isolated and the cell proliferation capacity of the affected and nonaffected cells examined after exposure to a particular membrane permeablizing agent. The proliferation ability of K562 cells with leukoregu-

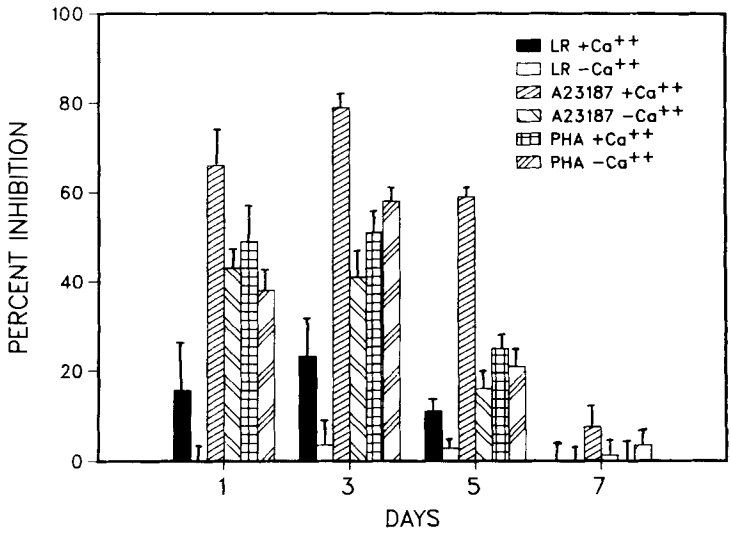

Fig. 6. Proliferation of K562 cells after a 2 h incubation with leukoregulin, calcium ionophore A23187, or phytohemagglutinin in the absence or presence of 1.0 mM extracellular Ca^{++}. 10^6 K562 cells were treated as described for Figure 1 in RPMI 1640-10% FBS medium for 2 h with concentrations of the membrane modulators that produce a permeability change in approximately 50% of the cell population (see Table I). The cells were then washed and 10^4 cells in 1 ml RPMI 1640-10% FBS were plated per well in quadruplicate in a 24 well dish for a 7 day growth assay. Cells were enumerated on day 1, 3, 5, and 7 using a Coulter electronic cell counter. Values shown are the mean ±S.E.

lin induced increased membrane permeability isolated by cell sorting is inhibited for 1–3 days after which it returns to the same rate as exhibited by nontreated or nonaffected cells (Fig. 7A). This ability of leukoregulin treated cells to recover and resume proliferation is in contrast to the irreversible loss of proliferative capacity by calcium ionophore A23187 or phytohemagglutinin treated K562 cells exhibiting increased plasma membrane permeability (Fig. 7B,C). Even calcium ionophore A23187 and phytohemagglutinin treated K562 cells having no demonstrable increase in plasma membrane permeability exhibit some depression in their proliferation rate.

DISCUSSION

This study shows that the cell permeablizing action of leukoregulin is partially dependent on the presence of extracellular Ca^{++} and that the increase in membrane permeability is followed by a reversible inhibition of cell proliferation. This contrasts with the calcium ionophore A23187, which produces a dose-dependent change in the plasma membrane permeability of K562 cells that is totally dependent on the presence of extracellular Ca^{++}, and which results in an irreversible inhibition of cell proliferation. Leukoregulin modulation of membrane permeability and cell proliferation also differs from that induced by phytohemagglutinin transmembrane signaling as the membrane permeablizing action of phytohemagglutinin is independent of extracellular calcium and like calcium ionophore A23187 leads to irreversible inhibition of K562 cell proliferation.

Calcium ionophores equilibrate Ca^{++} across biological membranes [15,16] and also across subcellular organelles, e.g., mitochondria [17], and microsomes [18]. The membrane permeability changes induced by calcium ionophore A23187 that are measured by the efflux of fluorescein from labeled cells are due to the actual transport of Ca^{++} across the membrane and not just to the presence of the ionophore. This is demonstrated by experiments incorporating a wash step after exposure of K562 cells to calcium ionophore A23187 in the absence of extracellular Ca^{++} because membrane permeability changes are only observed following the subsequent addition of extracellular Ca^{++}. Therefore, calcium ionophore A23187 initially equilibrates across the plasma cell membrane and when extracellular Ca^{++} is present is followed by an influx of Ca^{++} into the cell. The resulting increase in intracellular Ca^{++} leads in turn to destabilization of the plasma membrane and an increase in membrane permeability. In contrast, when leukoregulin is washed from the cell prior to the addition of extracellular Ca^{++}, membrane permeability is not increased demonstrating that it is the extracellular nonbound leukoregulin and/or its interaction with the target cell that is calcium dependent.

The membrane destabilizing action of leukoregulin at concentrations of the lymphokine producing a partial increase in membrane permeability of the K562 cell population are more dependent on extracellular Ca^{++} than the increase in membrane permeability at higher concentrations of leukoregulin. The explanation for the shift from calcium dependence to independence may be that at lower concentrations of leukoregulin, membrane destabilization requires extracellular Ca^{++} because the previously described leukoregulin induced increase in intracellular Ca^{++} levels [2] is not high enough to directly affect membrane destabilization without supplementation from an extracellular Ca^{++} source. At high leukoregulin concentrations the increase in intracellular Ca^{++} through mobilization from internal stores may be sufficient so that a contribution from

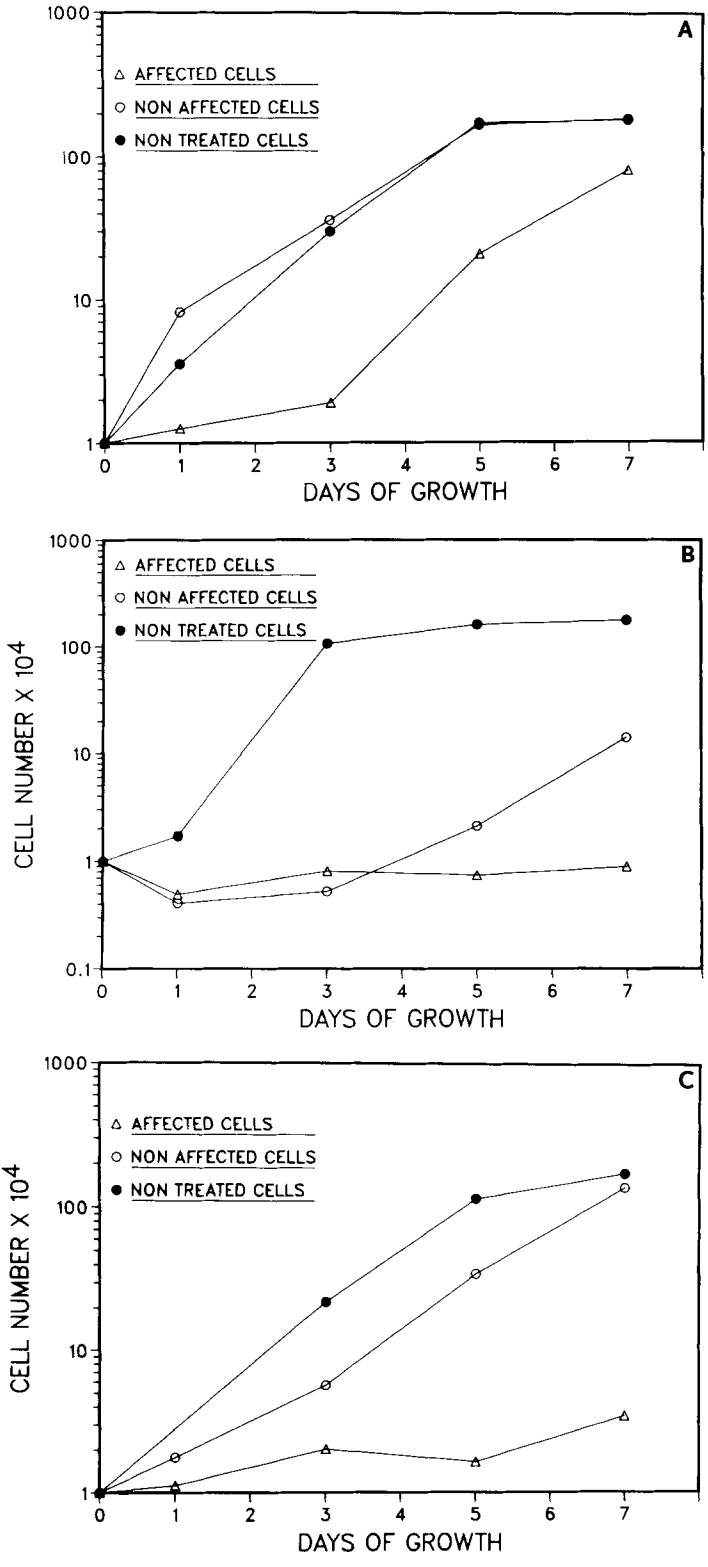
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Figure 7

extracellular Ca^{++} is not required. The kinetics of permeability change [2], the 50 kDa molecular size [1], hydrophilicity, and partial Ca^{++} dependence of leukoregulin are, moreover, inconsistent with leukoregulin itself being a calcium ionophore. The membrane destabilizing action of the mitogen phytohemagglutinin, unlike both leukoregulin and calcium ionophore A23187, is totally independent of the presence of extracellular Ca^{++} showing again the unique nature of leukoregulin's mode of action [2].

This investigation also demonstrates that exposure of K562 leukemia cells to any of the three modulators of membrane stability for 2 h is sufficient to affect the proliferation of the cells. The inhibition of cell proliferation by leukoregulin, calcium ionophore A23187, or phytohemagglutinin, moreover, exhibits a divergent pattern of sensitivity to extracellular Ca^{++} that parallels the divergence in the Ca^{++} dependence of the membrane permeability induced by these modulators of Ca^{++} metabolism. Two hour exposure to leukoregulin in Ca^{++}-free medium abrogates leukoregulin's inhibition of cell proliferation whereas inhibition by calcium ionophore A23187 is greatly reduced and the inhibition by phytohemagglutinin is unchanged. This suggests that the membrane permeability changes are directly related to the inhibition of cell proliferation induced by these modulators of intracellular calcium flux and membrane stability. The cell proliferation inhibition action of calcium ionophore A23187 is not eliminated in Ca^{++}-free medium in contrast to the abrogation of increased membrane permeability by calcium ionophore A23187 in the absence of extracellular Ca^{++}. This is probably because calcium ionophore A23187 is not completely removed from the cells by washing the cells in calcium-free medium because the ionophore has inserted into the plasma membrane. When the cells are resuspended in normal Ca^{++}-containing medium for the cell proliferation assay, the membrane bound ionophore then is able to transport the added extracellular Ca^{++} into the cell increasing intracellular Ca^{++} and inhibiting the growth of the K562 cells. This is consistent with the isolation of membrane permeablized and nonaffected cells by electronic cell sorting where both the affected permeablized and nonaffected calcium ionophore A23187 treated cells exhibit a marked decrease in their proliferative capacity. Cell sorting also reveals that the cells permeablized by phytohemagglutinin display greatly reduced proliferative capacity compared to the nonaffected cells retaining normal membrane permeability isolated from the phytohemagglutinin treated cell population.

Intracellular Ca^{++} is proposed to be involved in immunological mediated cell killing and in cell injury caused by toxins [7,19,20]. Cell killing is divided into three stages: recognition, programing for cell death, and disintegration [19,21]. The first and

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Fig. 7. Proliferation of leukoregulin (**panel A**), calcium ionophore A23187 (**panel B**), and phytohemagglutinin (**panel C**) treated K562 cells with normal and increased membrane permeability after isolation of the cells by fluorescence activated cell sorting. 4×10^6 K562 cells in 500 μl of medium were incubated with 4 units of leukoregulin, 50 μM A23187, or 1.5 μM phytohemagglutinin for 2 h as described for Figure 1. Fluorescein labeled cells exhibiting normal membrane permeability, as indicated by the retention of fluorescein fluorescence and those cells with increased membrane permeability as demonstrated by decreased fluorescein fluorescence, were isolated from the leukoregulin treated population by fluorescence activated electronic cell sorting. Control cells incubated with fluorescein diacetate in the absence of leukoregulin that were not sorted and sorted cells were evaluated in a 7 day growth assay. Inoculations of 1×10^4 cells per well of a 24 well plate were enumerated on day 1, 3, 5, and 7 as described for Figure 6. Each panel is a typical experiment with the standard error between experiments being $\leq$10%. Comparison of the proliferation capacity of sorted and nonsorted control cells showed that electronic cell sorting by itself did not affect the rate of K562 cell proliferation during the 7 day period of growth.

second stages are dependent on divalent cations. During programing for death the cytotoxic T lymphocyte inflicts irreversible damage upon the target cell and this continues into the disintegration stage probably being manifested by nonlethal membrane perturbations rather than by cell death [21]. It has been postulated, moreover, that there are two levels of intracellular increase in free Ca^{++}. One level of increase Ca^{++} leads to changes in cell structure and function but not to cell lysis; the second increase, which is greater than 10 μM, potentiates membrane damage and leads to lysis and cell death [21]. Leukoregulin appears to increase intracellular Ca^{++} sufficiently to induce membrane destabilization [2] and cytostasis [23] but not to trigger cytolysis of K562 cells even though the membrane permeability changes produced by leukoregulin and the more lethal calcium ionophore A23187 and lectin phytohemagglutinin are equivalent in terms of intracellular fluorescein loss. In K562 cells, a decrease in extracellular Ca^{++} results in the inhibition of cell proliferation, which accompanied by an increase in free intracellular Ca^{++} as measured by the fluorescent calcium chelator indo-1 [24], provides further evidence in this cell system of an interrelationship between cell proliferation and intracellular Ca^{++}. Inhibition of cell growth and DNA synthesis also result when the extracellular Ca^{++} concentrations are reduced in several other cells including Swiss 3T3 cells [25], BALB/c 3T3 cells [26], epidermal keratinocytes [27], and lymphocytes [28].

Leukoregulin is a unique lymphokine that rapidly induces nonlethal membrane permeablization in K562 cells. This form of membrane destabilization is partially dependent on extracellular Ca^{++} unlike the totally calcium-dependent target cell inhibitory action of many other products of cytotoxic T cells, e.g., cytolysin and perforin [7–9] and some lymphokines, e.g., lymphotoxin [4] and interleukin-2 [29]. The membrane permeability changes induced by leukoregulin correlate closely with subsequent changes in cell proliferation. It is likely, however, that many steps are involved in the mechanism of leukoregulin-induced inhibition of cellular proliferation since calcium ionophore A23187 and phytohemagglutinin produce similar changes in cell membrane permeability to those observed in leukoregulin treated cells, but as opposed to leukoregulin, exert an irreversible action on cell proliferation.

Leukoregulin also increases intracellular Ca^{++} in the target cells [2]. The results of the present investigation suggest that this Ca^{++} increase may be supplemented from extracellular sources and be important in the leukoregulin-induced membrane permeability and growth proliferation changes. Leukoregulin's action is a complex multifactorial process. Leukoregulin is secreted by natural killer type lymphocytes [3,30] and during the interaction of lymphocytes with target cells [23]. This lymphokine has a high specificity for tumor target cells [1], and leukoregulin's membrane permeabilizing and proliferation inhibitory action may be early events conditioning the target cell for natural killer lymphocyte and other forms of immune cytolysis. Recent investigations have shown that the increase in plasma membrane permeability in K562 leukemia cells is preceded by a rapid opening and closing of cation selective channels in the plasma membrane [31] and is accompanied by an elevation in membrane protein kinase-C [32] and increase in intracellular uptake of tumor inhibitory antibiotics [33]. Further examination of the molecular relationships between the transmembrane signals induced by this lymphokine and the resulting increase in plasma membrane permeability and inhibition of cell replication will lead to a more complete understanding of the physiological role and therapeutic potential of leukoregulin.

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