Isolation of a Sodium Transport Inhibitory Factor, Inhibitin, from Cultured Leukemic Promyelocytes

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Abstract. Previous studies have shown that leukemic blood contains a factor that has an inhibitory effect on bidirectional sodium transport in erythrocytes. This study was designed to isolate this factor from cultured leukemic promyelocytes. An extract from the promyelocytes reduced significantly ($P < 0.001$) the ouabain-insensitive sodium efflux rate, from 0.096±0.009 to 0.056±0.003 SD. Using the inhibition of ouabain-insensitive sodium transport in erythrocytes as an assay to identify the factor, we ran the crude promyelocyte extract through Sephadex G-25 and G-10, with an intermediate ion-exchange step on DE-32, and finally subjected the active fraction to reverse-phase high-performance liquid chromatography. The specific inhibitory activity of the final fraction was 180-fold higher than that of the crude promyelocyte extract. The inhibitory activity could be destroyed by acid hydrolysis and by enzymatic digestion with several proteases but not by heating at 80°C for 30 min; these characteristics suggest that the active factor, called inhibitin, is a peptide. Inhibitin is released by immature myeloid cells but not by differentiated white cells or by leukemic lymphocytes. It has no effect on potassium influx but inhibits sodium/sodium exchange in erythrocytes.

Introduction

Plasma from patients with acute myeloid leukemia has been shown to exert an inhibitory effect on sodium efflux from erythrocytes (1, 2). In a recent study from this laboratory the supernatant from sonicated leukemic blast cells (which are a mixed cell population) was shown to inhibit sodium efflux through a channel not inhibited by ouabain or furosemide (3). The promyelocyte of the primitive myeloid series is well known for its protein manufacturing capability, and various workers have shown it to be a source of many proteins such as lysozyme (4, 5), vitamin B_{12}-binding protein transcobalamin I (6–8), and myeloperoxidase (9). For this reason an extract from cultured leukemic promyelocytes was tested, and it was found to have a stronger inhibitory effect on ouabain-insensitive sodium transport in erythrocytes than that shown by the extract from myeloid leukemic blast cells (10). In view of this, cultured leukemic promyelocytes were used as a source, and the inhibition of ouabain-insensitive sodium transport across the erythrocyte membrane was used as an assay to isolate the sodium transport inhibitory factor. This report presents a method for isolating the inhibitor, which we have named inhibitin, and some characterization of its chemical and inhibitory properties.

Methods

Reagents

All reagents were prepared in double glass-distilled water and were of Analar grade (BDH Chemicals, Ltd., Poole, Dorset). Sephadex G-25 and G-10 were obtained from Pharmacia Fine Chemicals (Hounslow, Middlesex). The ion-exchange resin DE-32 was purchased from Whatman Ltd. (Maidstone, Kent). Na^{22} and K^{42} were obtained from Amersham International Ltd. (Amersham, Buckinghamshire). Enzymes for proteolytic digestions were obtained from Sigma Chemical Co. (St. Louis, MO). The μBondapak C_{18} column used in reverse-phase high-performance liquid chromatography (HPLC)[1] was obtained from Waters Associates (Milford, MA). Aprotinin (Trasylol) was bought from Bayer UK Ltd., Pharmaceutical Div. (West Sussex).

Solutions

Ringer's solution (pH 7.4) used in sodium transport experiments contained (millimoles per liter): NaCl, 131; KCl, 8; $MgSO_4$, 1; Na_2HPO_4, 7.2; NaH_2PO_4, 1.8; $CaCl_2$, 2; glucose, 10; and bovine serum albumin, 0.05% wt/vol with an osmolality of 290±5 mosmol/kg water. A 10-fold dilution of this buffer (R10 at pH 7.6), minus the glucose and

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1. *Abbreviations used in this paper:* DNP, dinitrophenol; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid.

albumin, was used for the preparation of the promyelocyte extracts (see below). Elution of bound material from DE-32 was accomplished by use of full strength Ringer's solution (pH 8.5) without glucose and albumin. Choline chloride solution used for washing erythrocytes contained (millimoles per liter): choline chloride, 151; $MgCl_2$, 1; and $CaCl_2$, 2.2; the pH was adjusted to 7.4 with Tris. Ouabain (Sigma Chemical Co.) was used throughout at a final concentration of 0.1 mmol/liter. Furosemide (Diuresal; Lagap Pharmaceuticals, Guildford, Surrey) and phloretin (Sigma Chemical Co.) were used at final concentrations of 1 and 0.2 mM, respectively.

Cell culture

Promyelocyte culture. Leukemic promyelocytes (initially obtained from the Chester Beatty Research Institute, Surrey) were cultured by the method of Gallagher et al. (11) and grown in RPMI 1640 (Flow Laboratories, Inc., Irvine, Scotland) supplemented with 20% heat-inactivated fetal calf serum (Gibco Laboratories, Grand Island, NY), 2 mM L-glutamine (Flow Laboratories, Inc.), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco Laboratories). Cells were seeded in plastic flasks at 3×10^5 cells/ml and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were subcultured every 4–5 d, when they had reached a density of 1×10^6/ml, harvested by centrifugation (1,200 *g* for 5 min at 20°C), and stored in 2-ml aliquots at −70°C at 40×10^6 cells/ml. Culture medium was made 5% vol/vol with aprotinin and stored at −70°C in 2-ml aliquots. Culture medium used as control was incubated in an identical manner as the cells (conditioned medium) and stored at −70°C in 2-ml aliquots. The medium from NALM 6 (kindly provided by Dr. P. Bell, Tenovus Institute, Cardiff), a leukemic lymphocyte, was also stored together with the medium from normal bone marrow cells (obtained from the Department of Hematology, Welsh National School of Medicine, Cardiff) which had been maintained in culture (as above) for 4 d.

Myeloid differentiation. To convert the leukemic promyelocytes into more mature myeloid cells, the method of Ghezzo and Pegoraro (12) was used. After the removal of culture medium from leukemic promyelocytes for storage, some cells were cultured in fresh medium containing retinoic acid at a final concentration of 10^{-6} M. Morphological differentiation occurred after 5 d and the culture medium was removed and stored at −70°C.

Isolation and purification steps

The first four steps described below were carried out at 4°C.

Preparation of crude promyelocyte extract. The leukemic promyelocyte cell suspension (2 ml at 40×10^6/ml) was thawed at room temperature and added to 13.2 ml of ice-cold R10 (pH 7.6). The cells were divided into two portions and disrupted with an ultrasonic cell disintegrator (3×1 min bursts; 18 μm peak to peak with cooling on ice between sonications). Immediately after the third sonication 800 μl of aprotinin, a proteinase inhibitor, was added to each tube. The resulting solution was centrifuged at 100,000 *g* for 30 min and the supernatant (crude extract) was stored in 3.5-ml aliquots at −70°C. The results presented have been obtained from 12 separate batches of extract, all prepared as above from this line of promyelocytes.

Gel filtration (Sephadex G-25 Superfine). The column (1.6×50 cm) was equilibrated with R10 (pH 7.6) and processed by upward flow elution with a flow rate of 10 ml/h. The flow rates in all chromatographic steps were maintained with a peristaltic pump (2120; LKB-Wallac Instruments Ltd., South Croydon, Surrey). The column eluent was monitored at 220 nm (SP8400 spectrophotometer; Pye Unicam Ltd., Cambridge, England), and 5-ml fractions were collected with a fraction collector (model 211; ISCO, Inc., Environmental Div., Lincoln, NE). Both the pH and the conductivity of the column eluent were measured and the sample was only loaded when these were identical to those of the starting buffer; complete equilibration usually took 18 h. During each sample run the pH and conductivity of the fractions were checked to ensure that the system remained stable. The column was calibrated with the following molecular weight markers: lysozyme, 13×10^3 (BDH Chemicals, Ltd.); glucagon, 3.5×10^3 (Novo Laboratories Ltd., Basingstoke, Hampshire, England); bacitracin A, 1.4×10^3 (Sigma Chemical Co.); vasopressin, 1×10^3 (Parke, Davis & Co., Detroit, MI); and glutathione oxidized, 0.61×10^3, and dinitrophenol (DNP)-alanine, 0.29×10^3 (Sigma Chemical Co.).

Ion-exchange chromatography (DEAE-cellulose). The adsorbant (DE-32) was precycled, as recommended in the supplier's literature, and repeatedly suspended in 10 vol of R10, pH 8.5, until the pH stabilized. The column was then poured (2 ml bed volume); the eluent was checked to ensure that the pH remained at 8.5±0.05. Adsorbed material was removed from the column with full strength Ringer's solution (pH 8.5), and after the material eluted the column was given a final wash with 0.5 M NaCl. The flow rate was 60 ml/h; the absorbance was monitored at 220 nm, and 2.5-ml fractions were collected.

Gel filtration (Sephadex G-10). A 1.6×35 cm column packed with Sephadex G10 was equilibrated with 30 mM phosphate buffer (pH 7.6) and eluted at a flow rate of 6 ml/h by upward flow elution, and 2-ml fractions were collected. The void volume of the column was determined with bacitracin A as a marker; the total volume was established with DNP-alanine.

Reverse-phase HPLC. A solvent delivery system (SP 8700; Spectra-Physics, Inc., London) with a μBondapak C_{18} column (250 mm × 4.4 mm) was used. The solvent system employed was: phase A, 0.1% trifluoroacetic acid (TFA) in water; and phase B, 0.1% TFA in MeCN (acetonitrile). The gradient (percentage B in A) was 2% B at time zero, 2% B at time 10 min, and 50% B at time 60 min with a linear gradient from 10 to 60 min. A 500-μl sample from the Sephadex G-10 column void peak was applied and the flow rate was maintained at 1 ml/min with a back pressure of ~1,000 pounds per square inch. The column eluent was monitored at 210 nm and 2-ml fractions were collected. Water was twice distilled before use and all chromatography was performed at room temperature.

Assays for inhibitory activity: erythrocyte sodium transport experiments

These experiments were performed as assays for tracing the inhibitory effect through the purification steps.

Blood samples. Blood samples were taken from the same five normal healthy volunteers throughout the period of this study. Sodium efflux rate constant (see below) did not vary by more than 10% in the blood collected from the same subject on different days. The samples were collected on the day of the assay into chilled heparinized syringes (100 U/ml blood).

Sodium efflux. This method, described fully elsewhere (1), is based on that of Villamil et al. (13). Washed erythrocytes in Ringer's solution were loaded with Na^{22} (3.0 μCi/ml of erythrocytes) and added to prewarmed (37°C) Ringer's solution to give a packed cell volume of <5% (i.e., 0.5 ml loaded erythrocytes in 10 ml total volume). All experiments were carried out in duplicate or triplicate. Samples were taken at 30, 60, and 90 min, and the radioactivity was counted in portions of the suspension and supernatant after centrifugation at 5,000 *g*. The values of 1 − (supernatant counts/suspension counts)

were plotted semilogarithmically against time; the slope was calculated by the method of least-squares and the half-time ($t_{1/2}$) was determined from this slope. The sodium efflux rate constant (i.e., the fraction of intracellular sodium extruded per hour, $^{o}k_{Na}$) was derived from the equation $^{o}k_{Na} = 0.693/t_{1/2}$. The various column fractions were tested for their inhibitory activity by the addition of 100-μl aliquots of these to the prewarmed Ringer's solution. Previous work with leukemic blast cell extract (3), and preliminary experiments with various column fractions during this study had shown that 100-μl aliquots reproducibly exerted detectable inhibitory activity on erythrocyte sodium transport. Ouabain was used throughout at a final concentration of 0.1 mmol/liter.

Sodium influx. This method has been described previously (1). Erythrocytes (1 ml) were incubated in Ringer's solution (20 ml) at a hematocrit of <5% which contained 8 μCi of Na^{22}. After an equilibration period at 37°C for 15 min a 4-ml sample was removed into an ice-cold tube, centrifuged at 4°C for 1 min, and washed three times in 5 vol of unlabeled ice-cold iso-osmotic choline chloride solution. Erythrocytes were then hemolyzed in deionized water to a 2-ml vol and set aside for radioactivity counting. Hemoglobin in the hemolysate was determined and the volume of erythrocytes was estimated under the assumption that 100 ml of cells contained 34 g of hemoglobin. With a known volume of erythrocytes in the two samples, the amount of labeled sodium uptake (U) was calculated from $U = (Na^{t75} - Na^{t15})/SA$, where Na^{t75} and Na^{t15} represent counts per minute per liter of erythrocytes at 75 and 15 min, respectively, and SA is the radioactive counts per millimole of Na^+ in the bathing solution.

Sodium influx in millimoles per liter per hour ($^{i}M_{Na}$) was estimated from the uptake of radioactivity in 60 min from the formula $^{i}M_{Na} = Uk/1 - e^{-kt}$, where U is the amount of labeled sodium taken up in time t and k is the efflux rate constant. The influx rate constant ($^{i}k_{Na}$) was calculated by dividing $^{i}M_{Na}$ by the number of millimoles of Na^+ (140) per liter of Ringer's solution.

Characterization procedures

CHEMICAL CHARACTERISTICS. *Amino acid analysis.* Amino acid analyses of the pooled active column fractions were carried out with a Chromospek analyzer (Hilger Analytical, Margate, Kent, England). Samples were hydrolyzed with an equal volume of constantly boiling 6 N HCl in evacuated sealed tubes at 120°C for 24 h. The hydrolyzed sample was analyzed after having been evaporated to dryness. Protein concentration was estimated by inclusion of homocystine as an internal standard. Some of the hydrolyzed G-10 active fraction was resuspended in 30 mM phosphate buffer, to the original concentration, and assayed for inhibitory activity on sodium transport.

Estimation of molecular weight of inhibitin. A Sephadex G-25 (Superfine) column was calibrated with markers of known molecular weight and equilibrated with R10 (pH 7.6). A 5-ml aliquot of the active material from the Sephadex G-10 column was loaded, and the absorbance at 220 nm was monitored. The flow rate was maintained at 10 ml/h, 5-ml fractions were collected, and the elution position of inhibitin was determined by testing of the activity of all fractions on ouabain-insensitive sodium transport in normal erythrocytes. This active material was then rerun on the G-25 column equilibrated with R10 pH 7.6 containing 0.1% sodium dodecyl sulfate (SDS) or Triton X-100. The apparent molecular weights were obtained from the plot of log molecular weight versus retention coefficient (Ve/Vo).

Enzymatic procedures. Enzymatic digestions were performed by incubating 500 μl of biologically active material (crude promyelocyte extract) with a 1:50 molar ratio of enzyme (2×10^{-4} M) at 37°C for 2 h in Ringer's buffer at the appropriate pH: pronase, pH 7.6; chymotrypsin, trypsin, and carboxypeptidase A, all pH 7.8. Control solutions consisted of the active material with enzyme that had been boiled for 5 min. The reactions were terminated by heating at 80°C for 10 min, since all of the previous data (3) and our present experience show that the inhibitory activity of inhibitin is not destroyed by this procedure. 100-μl aliquots were then assayed for activity, in the presence of ouabain, on the sodium efflux rate constant. All enzymatic treatments were replicated twice and assayed at least in triplicate.

INHIBITORY CHARACTERISTICS. *Net sodium transport.* Washed erythrocytes were incubated at 37°C in unlabeled Ringer's solution (total volume 20 ml) at a hematocrit of <5% for 5 h. Erythrocyte sodium concentration was determined in the pre- and postincubation samples and the net sodium transport was calculated by subtracting the preincubation value from the postincubation sodium concentration.

Potassium influx. The method of Sachs and Welt (14) was followed in order to estimate potassium influx in ouabain-treated erythrocytes. Cells were washed three times and added at <5% hematocrit to prewarmed (37°C) Ringer's solution containing K^{42} (1 μCi/ml incubating solution) in triplicate. The system was allowed to equilibrate for 30 min; a 4-ml sample was then taken from each flask into ice-cold tubes. The cells were rapidly separated from the supernatant and washed three times in excess ice-cold choline chloride solution. The erythrocytes were hemolyzed, brought to a standard volume with distilled water, and counted in a gamma counter. A second sample was taken 1 h after the first and treated in exactly the same manner. The quantity of cells counted was determined by measuring the hemoglobin, and potassium influx was calculated from the equation: $^{i}M_{K^+} = [(K^{t90} - K^{t30})/K_S](K_o)$, where $^{i}M_{K^+}$ is the influx of potassium in millimoles per liter per hour; K^{t90} and K^{t30} are the activities of the cells in counts per minute per liter of cells at 90 and 30 min, respectively; K_S is the counts per minute per liter of the incubating medium; and K_o is the external potassium concentration in millimoles per liter. This calculation neglects the effect of back diffusion of isotope from cells to medium. The error attributable to this effect has been calculated to be <2%. The derivation of the equation for the calculation of K^+ influx assumes that K_S (the activity of the incubating medium) and Ko (the external potassium concentration) remain constant during the course of the experiment. Since the hematocrit was low (<5%), this can be assumed to be the case.

Site of action. To explore the locus of action of inhibitin on sodium transport pathways across the erythrocyte membrane, sodium efflux experiments were conducted in the presence of inhibitin and various combinations of three other transport inhibitors (ouabain, furosemide, and phloretin). The transport channel inhibited by inhibitin but not by the other agents was thereby determined.

Analytical methods

To determine internal sodium, erythrocytes were washed three times with ice-cold choline chloride solution, and the cell suspension was made up to 2 ml after the final wash. The hematocrit was measured in a counter from Coulter Electronics Inc. (Hialeah, FL). 20 μl of saponin solution (20% wt/vol) and 20 μl of 2 M lithium bromide were added to the sample, and the sodium concentration in the lysate was measured with an integrating flame photometer (model 227; Evans Electroselenium Ltd., Halstead, Essex, England). Standards contained Na^+, K^+, and Li^+ at 2.4, 36, and 20 mmol/liter, respectively. This method gives highly reproducible results (15) and has yielded a coefficient of variance of <10% in the erythrocytes collected under identical conditions from the same subject on different days.

Hemoglobin was measured as cyanmethemoglobin at 541 nm with Drabkin's reagent. Protein in the crude extract was determined by Hartree's modification (16) of Lowry's method (17). The amount of protein in the column fractions was estimated by quantitative amino acid analysis.

Statistical analysis

All results are expressed as a mean±1 SD. Sodium transport in the presence of the inhibitory factor was compared with controls, and the unpaired *t* test was used to determine statistical significance.

Results

Effect of culture media on sodium efflux. To investigate whether leukemic promyelocytes, leukemic lymphocytes, and cells in normal bone marrow secrete inhibitin, we studied the effects of culture media from these cells on the ouabain-insensitive sodium efflux rate constant; the results are summarized in Table I. The culture media from leukemic promyelocytes showed a significant ($P < 0.001$) inhibitory effect, which was lost after these cells had been differentiated into more mature forms by the addition of retinoic acid. The culture media from normal bone marrow cells also showed an inhibitory effect, suggesting that the normal promyelocyte probably secretes inhibitin, whereas the media from leukemic lymphocytes (NALM 6) did not show any effect.

Effect of crude promyelocyte extract on sodium efflux. Supernatant from the sonicated promyelocytes, designated as crude extract (see Methods), had a protein concentration of 4.68 mg/ml; 100 μl of this preparation showed an inhibitory effect of 42% on the ouabain-insensitive sodium efflux rate constant in normal erythrocytes (Table II).

Table I. Effects of Cell Culture Media on the Ouabain-insensitive Component of the Sodium Efflux Rate Constant in Normal Erythrocytes

	Ouabain-insensitive $^{o}k_{Na}$	Percent change	Significance
Ouabain control ($n = 24$)	0.1417±0.016	—	
RPMI 1640 conditioned medium ($n = 15$)	0.1467±0.018	4↑	NS
Leukemic promyelocyte medium ($n = 12$)	0.1034±0.017	27↓	$P < 0.001$
Leukemic promyelocyte medium ($n = 9$) after transformation with 1 μM retinoic acid	0.1304±0.028	8↓	NS
NALM 6 medium ($n = 8$) (pre-B leukemic lymphocyte)	0.1474±0.020	4↑	NS
Normal bone marrow culture medium ($n = 9$)	0.1077±0.019	24↓	$P < 0.001$

The culture media from leukemic promyelocytes and from normal bone marrow reduced significantly the ouabain-insensitive sodium efflux rate constant, whereas the conditioned media (identically incubated as the cells), and the media from leukemic lymphocytes and from differentiated leukemic promyelocytes (due to the presence of retinoic acid [12]) did not show any significant effect (NS).

Table II. Effect of Crude Promyelocyte Extract on Erythrocyte Sodium Efflux Rate Constant

	Sodium efflux rate constant ($^{o}k_{Na}$)		
	Total	Ouabain sensitive	Ouabain insensitive
Control (Ringer's) ($n = 8$)	0.216±0.012	0.120±0.010	0.096±0.009
Promyelocyte extract (4.68 mg protein/ml) ($n = 10$)	0.190±0.006*	0.134±0.005	0.056±0.003*

Sodium efflux experiments were carried out in Ringer's solution alone and in the presence of ouabain both with and without promyelocyte extract. The extract showed a significant inhibitory effect (*$P < 0.001$) on the total and ouabain-insensitive sodium efflux rate constant.
All data are mean±1 SD

Isolation of inhibitin

Gel filtration on Sephadex G-25 Superfine. A sample (3.5 ml) of crude promyelocyte extract was thawed at room temperature and applied to the G-25 column, and aliquots from fractions 3, 17, 20, 30, and 35, and from the two major peaks (Fig. 1, void peak and Peak *A*) were tested. Fractions 3 and 30 were tested individually and the efflux rates obtained with these were averaged and used as the control value; this value (0.091±0.009; $n = 6$) was not significantly different from that obtained in Ringer's solution alone (Table II). The peak inhibitory activity (mean of 34% depression in the ouabain-insensitive component) was caused by fractions 22–28. (Fig. 1 shows individual values obtained with fractions 24, 25, and 26.) These fractions were pooled, designated as Peak *A* (estimated protein, 255 μg/ml), and stored at −70°C. Heat treatment (80°C for 30 min) failed to abolish the inhibitory effect (ouabain-insensitive $^{o}k_{Na}$ of 0.060±0.008, $n = 4$; $P < 0.001$).

Experiments to investigate the effects of Peak *A* on the influx of Na^+ into ouabain-treated erythrocytes showed a fall in the sodium influx rate constant of 24%, from 0.025±0.004 to 0.019±0.002 ($n = 6$; $P < 0.01$). The fractions used in these experiments were not the same as those used in Fig. 1, but the chromatograms were identical, and these fractions also showed a strong inhibitory effect on the sodium efflux rate constant. As would be expected from these results, Peak *A* did not show any effect on the net sodium transport; the erythrocyte sodium concentration in the cells incubated with Peak *A* (10.74±0.7 mmol/l; $n = 6$) was not significantly different from that in the control cells incubated in Ringer's solution alone (10.49±2.5 mmol/l; $n = 6$), and Peak *A* had no effect on the sodium content in addition to that caused by ouabain (14.09±1.7 compared with 14.49±0.6 mmol/liter; $n = 6$).

Ion-exchange chromatography. Peak *A* (10 ml) was titrated to pH 8.5 with Tris and loaded onto the DE-32 column. The

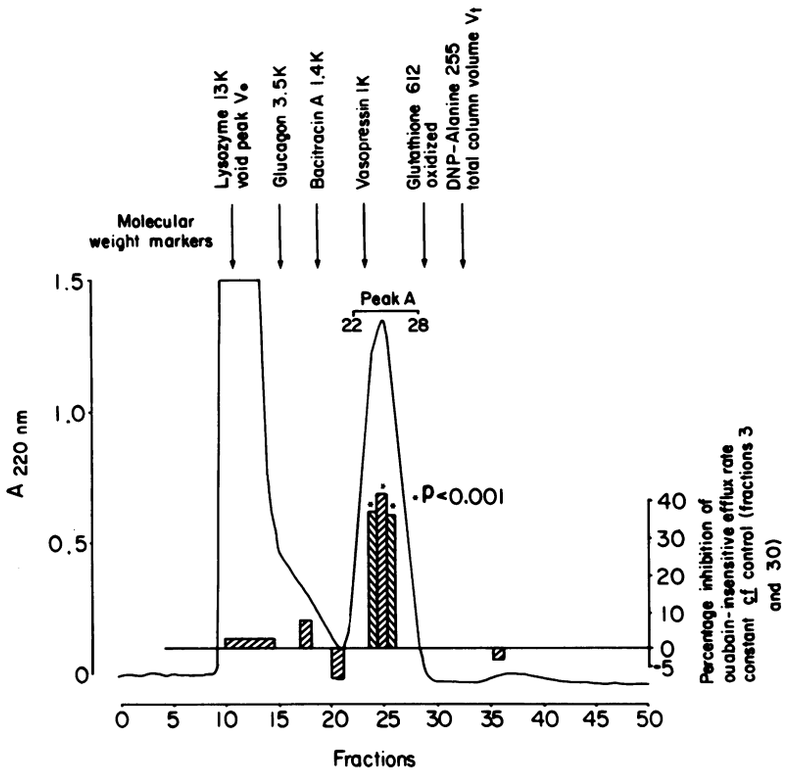

Figure 1. Sephadex G-25 chromatogram of crude extract and the effect of the column fractions on the ouabain-insensitive sodium efflux rate constant in erythrocytes. The passive efflux rate constants obtained with 100 μl of fractions 3 and 30 were averaged and used as a control value (0.091±0.009); the other fractions were compared with this value and the percentage inhibition was calculated (shown as hatched bars). The peak of inhibitory activity (mean, 34% inhibition) occurred at fractions 22–28 (estimated protein, 255 μg/ml). These fractions were pooled (designated Peak A) and stored at −70°C. The column was calibrated with a series of molecular weight markers shown (arrows) at the top of the figure. *K*, × 1,000. cf, compare.

effect of the fractions from the three areas (DE-32 I, II, and III; Fig. 2) was tested on sodium influx into erythrocytes. Of these, only the fractions from DE-32 II (estimated protein, 88 μg/ml) caused a significant drop (18%) in the sodium influx rate constant (0.0233±0.002, n = 6, to 0.0191±0.001, n = 8; $P < 0.001$). This material (DE-32 II) also significantly ($P < 0.005$) reduced the ouabain-insensitive $^{o}k_{Na}$, from 0.1399±0.017 to 0.0941±0.002; n = 4. These fractions were pooled and stored in 5-ml aliquots at −70°C.

Gel filtration on Sephadex G-10. Active material (10 ml)

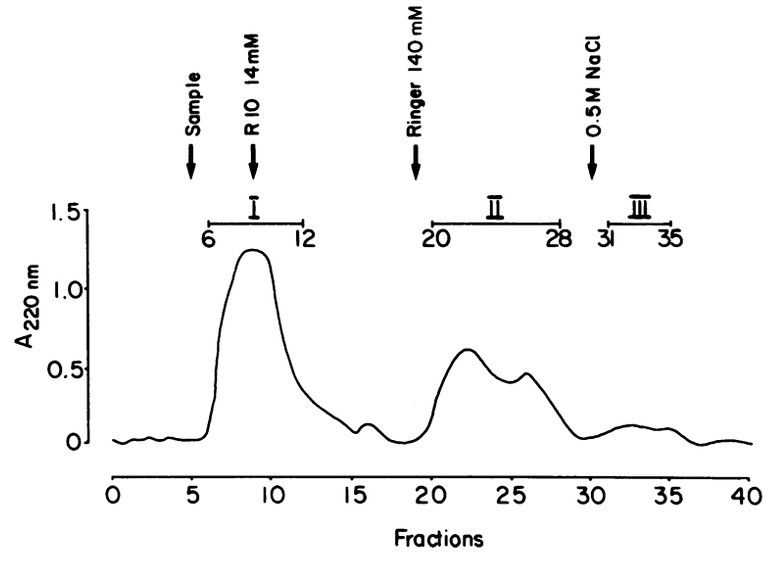

Figure 2. Typical elution profile of Peak A (from G-25 column) on DE-32 ion-exchange resin. Buffer changes were carried out as indicated by the arrows. Fractions were collected and pooled as indicated. Only DE-32 II (protein concentration, 88 μg/ml) showed significant inhibition of influx (decrease in influx rate of 18%, $P < 0.001$; see Results). Ringer, Ringer's solution.

from the ion-exchange column (DE-32 II) was loaded onto the Sephadex G-10 column (Fig. 3). The material eluted from the column in a major symmetrical peak at the void volume (molecular weight > 700) followed by several minor peaks. The elution of the protein in the void peak (protein concentration, 25 μg/ml) was congruent with the elution of inhibitory activity. Aliquots of the pooled fractions (18–26) reduced the sodium influx rate constant from 0.0170±0.001 to 0.0135±0.002 ($n = 4$; $P < 0.001$). When part of the void peak was rechromatographed on the DE-32 column, the elution pattern was identical to that of the initial ion-exchange column. An aliquot (1 ml) of the pooled fractions was subjected to acid hydrolysis (6 N HCl) for 24 h at 120°C. The hydrolysate was then evaporated to dryness and resuspended to 1 ml with the column buffer (30 mM phosphate pH 7.6). Acid digestion completely abolished the inhibitory effect. ($^i k_{Na}$, 0.0164±0.002; $n = 4$).

Reverse-phase HPLC. The active material from the G-10 peak (Fig. 3) resolved into two distinct peaks; a major component at fraction 13 (protein concentration 5 μg/ml) and a minor peak at fraction 15 (Fig. 4). These fractions were lyophilized, concentrated threefold in distilled water containing 0.1% bovine serum albumin, and assayed on the ouabain-insensitive $^o k_{Na}$. Fraction 13 reduced the rate constant by 24% ($P < 0.005$), whereas fraction 15 had no effect.

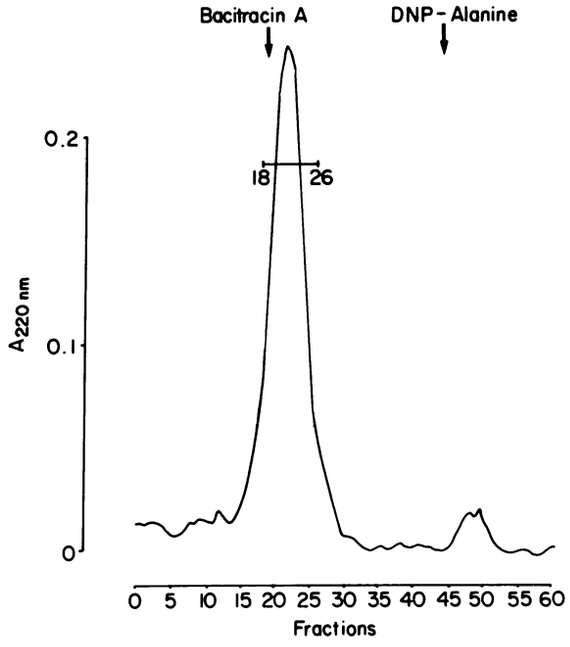

Figure 3. Chromatographic distribution of 10 ml of Peak II from the ion-exchange column (Fig. 2) on a Sephadex G-10 column. The fractions under the bar (18–26) were found to exhibit inhibitory activity (estimated protein, 25 μg/ml) on sodium influx into erythrocytes (see Results). This peak was freeze dried and used for the dose-response experiments and for probing the site of action.

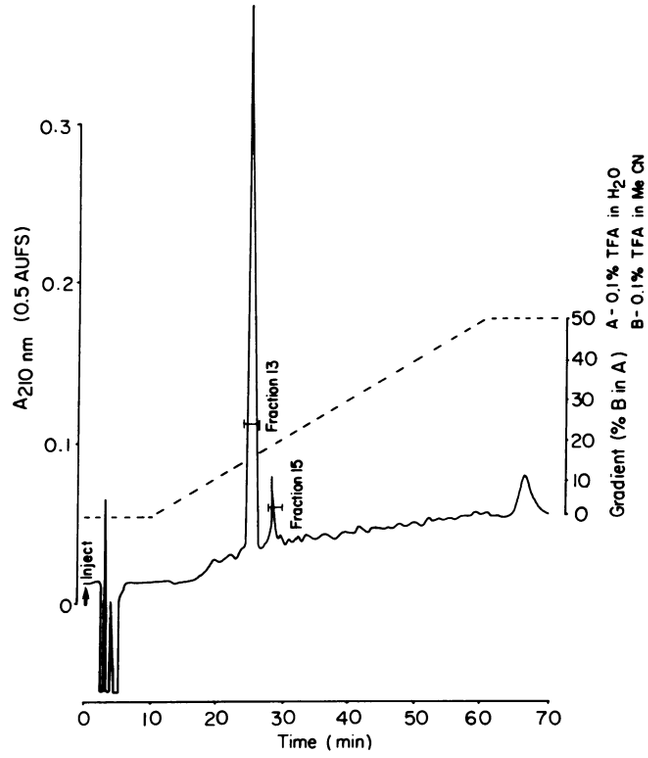

Figure 4. Reverse-phase HPLC profile of 500 μl of G-10 product on μBondapak C_{18}. A MeCN gradient in 0.1% TFA (- - -) was used to process the column. The flow rate was 1 ml/min; 2-ml fractions were collected and the absorbance at 210 nm was monitored (0.5 absorbance units, full scale [AUFS]). A single peak of inhibitory activity was detected at fraction 13 (protein concentration 5 μg/ml); this (after threefold concentration) reduced the ouabain-insensitive sodium efflux rate constant from 0.1566±0.016 to 0.1197±0.016 ($n = 6$; $P < 0.005$).

Purification and yield. Table III summarizes the results of a typical purification procedure starting with 20×10^6 cultured leukemic promyelocytes. The extracted peptide is purified 180-fold and ~18 pg of inhibitin can be obtained per promyelocyte.

Chemical characterization

Composition. Enough material for amino acid analysis of the HPLC peak was obtained by repeated 1-ml injections of the G-10 material, active fractions were pooled, lyophilized, and acid hydrolyzed from these runs. The amino acid analyses of two separate preparations of inhibitin are shown in Table IV. The relative molar ratios of these amino acids, together with the assumed number of residues per molecule of inhibitin, are also given in Table IV.

Estimation of molecular weight of inhibitin. Fig. 5 shows the elution positions of inhibitin relative to a number of other peptides of known molecular weight. In R10 buffer the apparent molecular weight of inhibitin was 955, but its elution position changed when it was rerun on a column equilibrated in R10

Table III. Summary of Purification of Inhibitin from 20 × 10^6 Leukemic Promyelocytes

Step	Protein concentration*	Number inhibitory units recovered/ml‡	Specific activity
			U/mg
Crude extract: sonicated cells	4.68 mg/ml (a)	420	90
Gel chromatography on Sephadex G-25 (Peak *A*)	255 μg/ml (b)	340	1,350
Ion exchange on Whatman DE-32 (DE-32 II)	88 μg/ml (b)	330	3,750
Gel chromatography on Sephadex G-10 (V_o peak)	25 μg/ml (b)	210	8,400
HPLC on μBondapak C_{18} (fraction 13)	5 μg/ml (b)	80§	16,000

V_o, retention coefficient; see Methods.
* Protein concentration: (*a*) determined by Hartree's modification (16) of Lowry's method (17); and (*b*) calculated from quantitative amino acid analyses.
‡ One unit is defined as a 1% drop in passive sodium movement (ouabain insensitive) across the erythrocyte membrane. Peaks were assayed at several dilutions to ensure submaximal concentration and hence avoid saturation.
§ Determined from threefold concentration of HPLC peak.

buffer containing 0.1% detergent (SDS or Triton X-100), to prevent its binding to the gel. This elution position suggests a molecular weight of 2,300. The minimum molecular weight of inhibitin calculated from the amino acid composition of the HPLC peak (Table IV) is 2,658.

Enzymatic studies. Incubation of biologically active material with the relatively nonspecific endopeptidase, pronase and nonspecific exopeptidase, carboxypeptidase A, as well as with trypsin, which has a high substrate specificity, destroyed its ability to reduce the ouabain-insensitive $^ok_{Na}$ in erythrocytes. These results strongly suggest that the biologically active substance is, at least in part, a peptide and that the peptide contains basic (i.e., lysine/arginine) residues. The failure of chymotrypsin to destroy the inhibitory activity suggests the absence of aromatic (i.e., tyrosine/tryptophan) residues. This was confirmed by spectrophotometric analysis of the peptide (HPLC peak), as it failed to absorb at 280 nm. Reduction of the extract with 70 mM mercaptoacetic acid (appropriate controls showed that this treatment alone had no effect) did not destroy activity, indicating the absence of a disulfide bridge in the inhibitory factor. The amino acid composition of inhibitin (Table IV) is consistent with these findings.

Table IV. Amino Acid Analysis of Inhibitin after HPLC

Amino acid	Total nmol in sample	Molar ratio	Assumed residues per mol inhibitin
Aspartic acid	2.34	2.1	2
Threonine	1.41	1.3	1
Serine	3.20	2.9	3
Glutamic acid	3.64	3.3	3
Proline	2.47	2.2	2
Glycine	4.63	4.2	4
Alanine	2.07	1.9	2
Valine	1.10	1.0	1
Leucine	1.33	1.2	1
Lysine	2.07	1.9	2
Arginine	1.38	1.3	1
Total			22

Results shown are mean values for two separate preparations.

Inhibitory characteristics

Dose response curve. The active fractions from the G-10 column were pooled, freeze dried, and redissolved in double glass-distilled water. The material was assayed in a range of concentrations from 10^{-5} to 10^{-9} M on the ouabain-insensitive $^ok_{Na}$. These concentrations were calculated assuming that the molecular weight was 2,600 and that the G-10 peak was 60% pure (the degree of purity was estimated by comparing the amino acid analysis of the G-10 material with that of the pure HPLC peak). There was no significant effect on this process with an inhibitin concentration of 10^{-8} M, but inhibition increased with the higher concentrations, reaching its maximum at 10^{-5} M (Fig. 6). An estimate of the half-maximal inhibitory dose obtained from this curve showed that 1×10^{-7} M inhibitin produced half-maximal inhibition.

Mode of action. Previous studies have shown that the inhibitory factor in leukemic blast cell extract inhibits sodium efflux through a pathway not inhibitable by ouabain or furosemide (3). A series of experiments were carried out to confirm that promyelocyte inhibitin also acted on the nonouabain, nonfurosemide transport pathway. In addition, inhibitin was combined with phloretin to explore whether it acted on the same process as phloretin, which is believed to inhibit Na^+/Li^+ countertransport (18). The results of these experiments are summarized in Table V. Both furosemide and phloretin showed additional, though statistically insignificant, inhibition of sodium efflux rate constant when either was combined with ouabain. However, inhibitin (0.1 μM) showed 26% inhibition when combined with ouabain alone ($P < 0.005$), and 25% inhibition ($P < 0.005$) when combined with all the other standard transport inhibitors. These results suggest that inhibitin inhibits sodium efflux across a pathway that is not inhibited by ouabain, furosemide, or phloretin. Erythrocytes incubated in the presence of inhibitin did not show any significant change in intracellular sodium compared with control cells (data not shown).

Potassium influx experiments in ouabain-treated erythrocytes failed to show any inhibitory effect of inhibitin (0.1 μM) on K^{42} influx (Table VI). Furosemide produced a highly

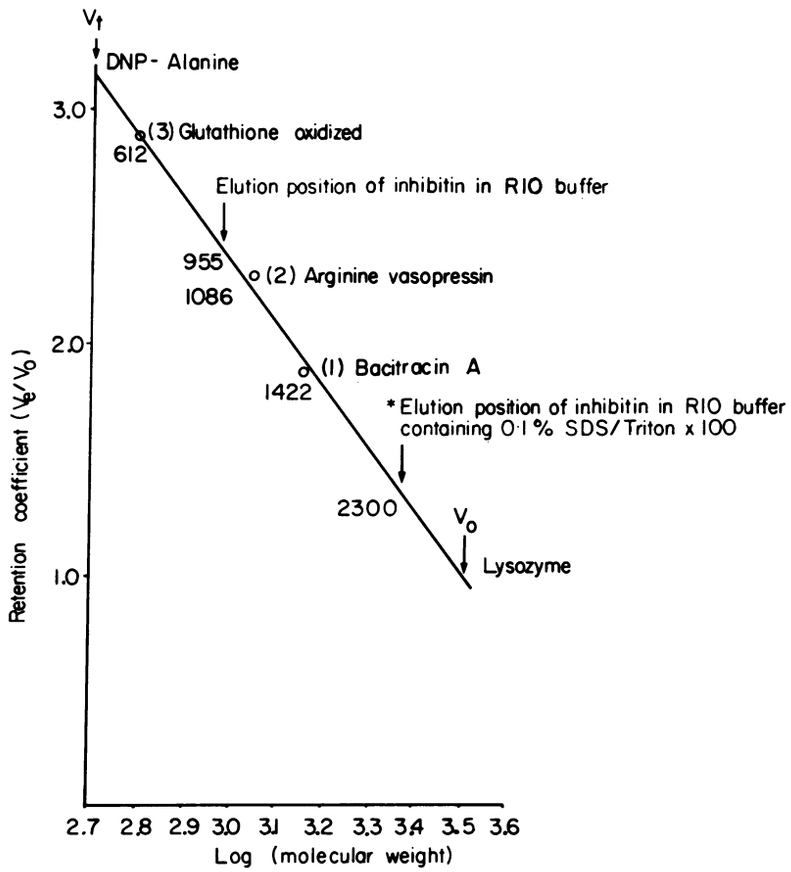

Figure 5. Estimation of the molecular weight of inhibitin by the use of a calibrated column of Sephadex G-25. The void volume (*Vo*) was determined with lysozyme; the elution volume (*Ve*) was chosen at the peak of eluted material. Each point represents the average elution position from three separate experiments with each of the following molecular weight markers: (1) bacitracin A; (2) arginine vasopressin; and (3) glutathione oxidized. The total column volume (V_t) was determined with DNP-alanine. When the column was run in R10 buffer, the inhibitin peak eluted at an apparent molecular weight of 955 (↓). However, in the presence of 0.1% SDS or Triton X-100*, the material elutes at a molecular weight of 2,300 (↓).

significant ($P < 0.001$) 68% inhibition of potassium influx, a value in close agreement with previously published observations (19).

Discussion

Previous studies have shown that plasma from patients with acute myeloid leukemia contains an inhibitory factor which reduces bidirectional sodium flux in leukemic erythrocytes (1). This factor was also found to have some inhibitory effect on sodium transport in normal erythrocytes but a statistical significance had not been achieved (see Table 3 in reference 1). Attempts at purification were made by a "baiting" technique (2); when leukemic erythrocytes were incubated with leukemic plasma and the inhibitory factor bound to the erythrocytes was obtained in subsequent washes, these washes showed an inhibitory effect on sodium efflux from normal erythrocytes. Furthermore, the main effect of this inhibitory factor was found to be on the ouabain-uninhibitable component of sodium efflux. In an effort to find a possible source of this factor, extract from myeloid leukemic blast cells was tested on sodium transport in normal erythrocytes; it reduced sodium efflux at a locus not influenced by ouabain or furosemide, and the effect was not abolished by heating at 80°C for 30 min (3). Similar effects could be demonstrated in a crude extract prepared from leukemic promyelocytes grown in culture (10).

This study was designed to isolate and partially purify an inhibitory factor of sodium transport in normal erythrocytes from cultured leukemic promyelocytes. With its inhibitory effect on the ouabain-insensitive component of sodium transport used as a marker, crude promyelocyte extract was subjected to various chromatographic steps until a fraction free from as much contaminating material as possible was obtained. Acid hydrolysis of the active fraction yielded various amino acids, and digestion with the proteolytic enzymes pronase, trypsin, and carboxypeptidase A abolished the inhibitory activity of promyelocyte cell extract. These results suggest that inhibitin is probably a peptide. The digestion mixture showed no aromatic amino acids, and treatment with chymotrypsin failed to destroy the inhibitory activity, hence the failure to detect the material at 280 nm. It was possible to detect the factor by Lowry's method (17) of protein estimation, but only when the HPLC peak had been concentrated 10-fold. By calibration of the Sephadex G-25 column, in the presence of detergent to prevent the apparently hydrophobic inhibitin from sticking to the gel, the calculated molecular weight of the inhibitory factor

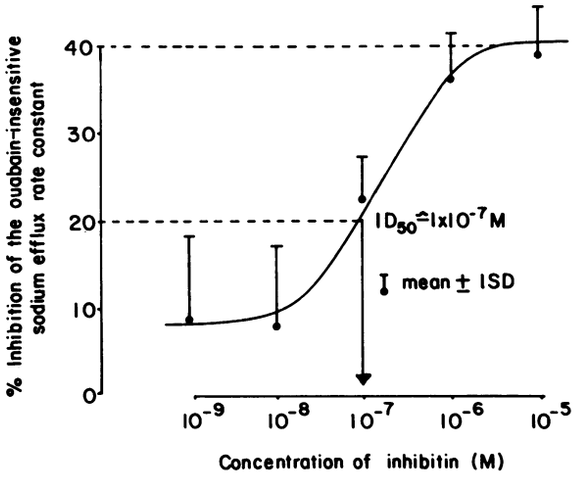

Figure 6. Dose response curve obtained with increasing concentrations of inhibitin. The active material from the G-10 column was freeze dried and redissolved at the molar concentrations shown (assuming a molecular weight of 2,600 and 60% purity) to determine the degree of inhibition on the ouabain-insensitive (passive) sodium efflux rate constant in normal erythrocytes. The inhibitory effect of inhibitin behaved in accordance with Michaelis-Menten saturation kinetics; no significant effect was observed at 10^{-8} M, and maximum inhibition was achieved at 10^{-5} M concentration of inhibitin. The half-maximal inhibitory dose (ID_{50}) is in the region of 1×10^{-7} M, as calculated from this curve. All the data are mean±1 SD of six determinations.

is 2,300. It is possible that inhibitin is derived from a larger protein by proteolysis during the preparation of the crude extract, since aprotinin does not inhibit all proteases. Work to elucidate the precise structure of inhibitin is in progress. Complexing inhibitin with a suitable protein should enable us to raise antibodies so that a radioimmunoassay can be developed.

The inhibitory factor isolated from leukemic promyelocytes has the same characteristics as those previously demonstrated in leukemic plasma (1), in partially purified factor from leukemic plasma (2), and in myeloid leukemic blast cell (3) and promyelocyte extracts (10). It affects mainly the ouabain-uninhibitable component of sodium transport, it does not alter net sodium flux, and its effect survives heating at 80°C for 30 min. The inhibitory activity of inhibitin behaves in accordance with Michaelis-Menton kinetics, with maximal effect at 10^{-5} M concentration (Fig. 6). This fact was, of course, unknown to us during the purification sequence, and, therefore, the concentration of the inhibitory factor was not kept constant in various experiments at each stage.

All of the available evidence from previous work (1–3) and the present data suggest that inhibitin acts on a site that is not inhibited by ouabain, furosemide, or phloretin. It seems that its main inhibitory effect is achieved by reducing sodium transport across the Na^+/Na^+ exchange pathway, since inhibitin reduces both sodium efflux and influx by equivalent amounts and does not alter net sodium transport in erythrocytes. As inhibitin acts on a nonfurosemide pathway it seems unlikely that it would affect furosemide-sensitive Na^+/K^+ cotransport.

Table V. Effects of Inhibitin, in the Presence and Absence of Ouabain, Furosemide, and Phloretin, on the Sodium Efflux Rate Constant in Normal Erythrocytes

	Sodium efflux rate constant ($^{o}k_{Na}$)	Percent inhibition and significance
Ouabain (0.1 mM) (n = 5)	0.1554±0.021	
Ouabain (0.1 mM) + inhibitin (0.1 μM) (n = 8)	0.1146±0.018	26% $P < 0.005$
Ouabain (0.1 mM) + phloretin (0.2 mM) (n = 5)	0.1461±0.013	
Ouabain (0.1 mM) + phloretin (0.2 mM) + inhibitin (0.1 μM) (n = 5)	0.1100±0.009	25% $P < 0.001$
Ouabain (0.1 mM) + furosemide (1 mM) (n = 4)	0.1414±0.007	
Ouabain (0.1 mM) + furosemide (1 mM) + inhibitin (0.1 μM) (n = 4)	0.1057±0.012	25% $P < 0.005$

All data are mean±1 SD. Final concentration of all the inhibitors in the incubation medium is shown in parentheses. Inhibitin (G-10 material, 60% pure) produced additional inhibition when combined with each of these other inhibitors.

Table VI. The Effect of Inhibitin on Potassium Influx into Ouabain-treated Erythrocytes in the Presence and Absence of Furosemide

	K influx	Percent inhibition
	mmol/liter per h	
Ouabain (0.1 mM) (n = 6)	0.7640±0.058	
Ouabain (0.1 mM) + inhibitin (0.1 μM) (n = 6)	0.7082±0.029	7.3 NS
Ouabain (0.1 mM) + furosemide (1 mM) (n = 6)	0.2479±0.008*	
Ouabain (0.1 mM) + furosemide (1 mM) + inhibitin (0.1 μM) (n = 6)	0.2357±0.020*	4.9 NS

All data are mean±1 SD of six individual determinations. The final concentration of the inhibitors in the incubation medium is shown in brackets. Furosemide significantly decreased (*$P < 0.001$) potassium influx into ouabain-treated erythrocytes. Inhibitin (G-10 material, 60% pure) had no significant effect (NS)

This assumption was confirmed in a series of experiments in which inhibitin did not show any significant effect on K^+ influx in ouabain-treated erythrocytes in the presence and absence of furosemide (Table VI). These experiments also suggest that inhibitin is unlikely to have any effect on K^+/K^+ exchange in normal erythrocytes.

The fact that inhibitin acts on Na^+/Na^+ exchange pathway may be relevant to the hyponatremia reported to occur in acute myeloid leukemia (20–22). Like the Na^+/Na^+ exchange in erythrocytes, proximal renal tubular reabsorption is known to operate 1:1 monovalent cation exchange (23). It can transport Na^+ as well as lithium (24–26) and can operate in an Na^+/Na^+ exchange mode (23). Furthermore, some diuretics with a potent natriuretic effect in the kidney have been found to have no effect on net sodium flux in erythrocytes (27, 28). Massive loss of Na^+ in the urine has been known to coincide with the destruction of leukemic blast cells after antileukemic therapy (21, 22). Even though the pathogenesis of various electrolyte disturbances in acute myeloid leukemia is multifactorial (22, 29), it is possible that the inhibitory factor, released from the primitive white cells, may have an etiological role in producing high excretion of Na^+ in the urine in leukemic patients with the consequent hyponatremia. Inhibitory activity was demonstrated in promyelocyte culture media, suggesting that inhibitin is released from the cells. However, this thesis is speculative and a natriuretic effect of inhibitin remains to be established.

The isolation of inhibitin from leukemic promyelocytes has two other interesting implications. First, as it inhibits residual sodium efflux, which is not inhibited by ouabain, furosemide, or phloretin, it may prove to be an important tool for investigating the channel that has been the subject of confusion and investigation for many years. It is outside the scope of this report to discuss the arguments for and against a second pump, but the availability of a specific Na^+/Na^+ inhibitor should help elucidate the various modes of sodium transport in erythrocytes. It already seems clear that Na^+/K^+ cotransport and Na^+/Na^+ exchange may be conducted by two distinct carriers, both inhibitable by two different inhibitors such as furosemide and inhibitin. The second implication is that inhibitin may be secreted by normal promyelocytes and may have a physiological significance. This possibility is supported by the results of the experiments that showed a strong inhibitory activity in the culture media from normal bone marrow. The secretion of inhibitin seems confined to the immature myeloid cells, since no significant activity has been found in normal white cells (3), and myeloid differentiation of leukemic promyelocytes resulted in a loss of the inhibitory activity. Although the culture media from leukemic lymphocytes did not show any inhibitory activity, it is impossible to say from the results of this study that the promyelocyte is the only cell that secretes inhibitin. Further studies are necessary to establish whether other immature cells also secrete inhibitin, since it may be playing an important part in the regulation of membrane transport during the early development of some cells.

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