Intracellular Regulation of the Production and Release of Human Erythroid-directed Lymphokines

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Abstract

Erythroid burst-promoting activity (BPA) is released from B lymphocytes in soluble (sBPA) and membrane-bound (mBPA) forms. To study intracellular processes involved in production of these physically separable factors, we measured their time course release into serum-free medium from B cells that were pulse-exposed for 5–240 min to nonmitogenic base medium or inhibitors of energy-dependent metabolism (2,4-dinitrophenol, sodium azide, and 2-deoxy-D-glucose), transcription and translation (actinomycin D and cycloheximide), replicative DNA synthesis (cytosine arabinoside), or posttranslational processing (monensin). mBPA and sBPA were initially detectable after 1 and 2 h, respectively. Maximum cumulative levels of 8±0.6 and 9±1.0 U/ml, respectively, were reached after 8 h. In contrast, cumulative mBPA and sBPA levels in medium prepared from cells treated with metabolic inhibitors were reduced by up to 90%. Both surface exfoliation and mBPA expression by intact plasma membranes were diminished. Whereas pulse-exposure to cytosine arabinoside had no effect, treatment with actinomycin D or cycloheximide abolished BPA expression. Exposure to monensin reduced mBPA and sBPA levels to zero in a concentration- and time-dependent fashion. We conclude that production and release of BPA is an energy-dependent process, requiring mRNA synthesis and translation and posttranslational remodeling of the protein but not replicative DNA synthesis. (*J. Clin. Invest.* 1991. 87:213–220.). Key words: erythropoiesis • lymphokines • membrane-bound growth factors

Introduction

Human hematopoietic stem/progenitor cells require cytokines in sufficient concentrations for differentiation and proliferation in vitro (1–3). Many of these factors are produced locally within the bone marrow by neighboring mononuclear cells (4, 5). Normal and transformed B lymphocytes are one source of growth regulators that are required for lineage-specific and panspecific hematopoiesis (6–9). Although the spectrum of B cell–derived biological activities has been characterized, intracellular processes resulting in the production and release of such factors are largely undefined.

In the present study we examine the time course release of erythroid-directed growth regulators into serum-free, liquid culture medium from B lymphocytes that were pulse-treated with specific inhibitors of cell metabolism, protein synthesis, or DNA synthesis. The results indicate that the release of soluble and membrane-associated B lymphokines is an energy-dependent process that requires active cell metabolism. Whereas mRNA synthesis, translation, and vesicular transport through the Golgi apparatus are involved in factor production and/or release, DNA synthesis is not essential for expression of these activities. Thus, erythroid growth factor synthesis and/or release involves cellular pathways that are similar to those required for endocrine hormone production (10, 11).

Methods

Cell collection and separation. Human plateletpheresis residues were collected from healthy volunteers for 2–3 h (model CS3000, Fenwal Inc., Ashland, MA). Informed consent was obtained before pheresis in accordance with Institutional Review Board policies. Cell concentrates were immediately diluted in minimum essential medium (MEM) (Gibco Laboratories, Grand Island, NY) with 20-U preservative-free heparin per ml, layered over Ficoll-Paque (Pharmacia, Inc., Piscataway, NJ), and centrifuged at 800 g for 20 min. Light-density mononuclear cells located at the interface were removed with a Pasteur pipette, resuspended, and depleted of platelets by centrifugation over sucrose (12). Platelets were removed and the washed mononuclear cells were incubated in polystyrene dishes for 60 min at 37°C to remove monocytes, and passed over a nylon wool column, as previously described (12). B lymphocytes that were 92–96% pure by surface immunophenotyping were gently dislodged from the nylon, washed, and incubated with or without inhibitor, as described below. In some experiments, monocyte-depleted, light-density mononuclear cells were rosetted with 2-amino-ethylisothiouronium bromide–treated sheep erythrocytes to remove T lymphocytes, and B cells (≥ 99% pure) were separated by indirect panning with mouse anti-B4 antibody (a gift of Dr. Albert Najman, Faculté de Medicine, Hôpital Saint-Antoine, Paris) as described previously (13). B lymphocytes were > 98% viable as assessed by trypan blue exclusion or by staining with ethidium bromide and acridine orange.

Approximately 1–2 ml of human bone marrow cells was aspirated from the posterior iliac crest of healthy 20–35-yr-old males and females into MEM with Hank's salts, without L-glutamine (Gibco Laboratories) plus preservative-free heparin. Informed consent was given before marrow aspiration. Light-density cells obtained after centrifugation over Ficoll-Paque were washed three times in MEM and sequentially depleted of monocytes and B lymphocytes, as described for peripheral blood cell fractionation. The resultant nonadherent, B cell–depleted marrow cells (consisting of < 1% surface Ig-positive mononuclear cells) were cultured under serum-free conditions, as previously described (14).

Assay of erythroid burst-promoting activity (BPA)[1] in serum-free bone marrow culture. Cultures were established in Iscove's modified

1. *Abbreviations used in this paper:* BPA, burst-promoting activity (similarly, mBPA, sBPA, and tBPA, membrane-bound, soluble, and total BPA, respectively); CM, conditioned medium; DNP, 2,4-dinitrophenol; IMDM, Iscore's modified Dulbecco's medium; P, shed membrane-derived vesicles; PM, plasma membrane vesicles; S, supernatants; TLCK, tosyl-L-lysine chloromethyl ketone; TPCK, L-1-tosylamide 2-phenylethyl chloromethyl ketone.

Dulbecco's medium (IMDM, Gibco Laboratories), purified human serum albumin, transferrin, ferric chloride, and 2.0 U recombinant human erythropoietin (70–80,000 U/mg, Amgen Biologicals, Thousand Oaks, CA) per milliliter (14). Test plates contained 9% (vol/vol) serum-free, B cell–conditioned medium (CM) or fractions thereof (see below), octylglucoside-extracted B lymphocyte plasma membranes or exfoliated surface membrane-derived vesicles (see below), or nonmitogenic base medium (IMDM). They were maintained for 10–12 d, 37°C, 5% CO_2 in humidified air. Fibrin clots were removed from incubation, fixed to glass slides with glutaraldehyde, and stained with benzidine and hematoxylin. The number of benzidine-positive erythroid bursts (≥ 50 cells) in each 125 μl fibrin clot was scored. Burst-forming unit erythroid (BFU-E)–derived colony formation in cultures of 6 $\times 10^5$ panned marrow cells containing nonmitogenic base medium varied from 16 to 40 bursts per 1.0 ml. One unit (U) of erythroid BPA gave 100% (or twofold) stimulation above burst formation in control cultures plated with an additional 9% IMDM.

Pulse exposure of B lymphocytes to inhibitors of cell function. B lymphocytes (5×10^5/ml) were pulse-exposed for 5–240 min, 37°C, to various concentrations of test inhibitors in serum-free IMDM or base medium alone. Inhibitors of energy-dependent cell metabolism were tested at 10^{-4} to 10^{-2} M. They included 2,4-dinitrophenol (DNP; Sigma Chemical Co., St. Louis, MO), an uncoupler of oxidative phosphorylation (15), sodium azide (Sigma Chemical Co.), a cytochrome oxidase blocker (16), and 2-deoxy-D-glucose (Sigma Chemical Co.). Transcription and translation inhibitors were tested at 10–100 μg/ml, and included actinomycin D (Sigma Chemical Co.), an inhibitor of messenger RNA synthesis, and cycloheximide (Sigma Chemical Co.), an antibiotic that inhibits peptidyl transferase activity (17). Monensin (Sigma Chemical Co.), a carboxylic ionophore which interferes with vesicular traffic of newly synthesized peptides through the Golgi apparatus and terminal glycosylation (18), was employed at 10^{-9} to 10^{-6} M as an inhibitor of posttranslational processing. To determine whether monensin alters BPA expression after its release from lymphocytes, 10^{-9} or 10^{-8} M of the drug was incubated with CM for up to 14 h and residual activity was assayed in the CM after exhaustive dialysis against PBS (see below). Cytosine arabinoside (Sigma Chemical Co.), an analogue of 2'-deoxycytidine, was used as an inhibitor of replicative DNA synthesis at concentrations of 10^{-8} to 10^{-6} M.

Extraction of BPA from plasma membranes and shed vesicles. Plasma membranes and membrane vesicles exfoliated into CM from the surface of control and test B lymphocytes were purified, detergent treated, and assayed for extractable BPA, as previously described (19, 20). Briefly, 2 vol of control or test whole cells were loaded with 1 vol 90% wt/vol glycerol in PBS, disrupted in a Dounce homogenizer, and subjected to isopycnic centrifugation and sucrose centrifugation. Resultant plasma membrane vesicles (PM) and shed membrane-derived vesicles (P) were sequentially extracted with 0.1 N NaOH and 30 mmol/liter n-octyl β-D-glucopyranoside (Sigma Chemical Co.). Solubilized PM and P membranes were stored at 4°C until use in culture for assay of membrane-associated BPA (mBPA).

Preparation and fractionation of conditioned medium. After treatment, B cells were washed three times in IMDM, resuspended to a density of 5×10^6/ml IMDM containing L-glutamine, penicillin, and streptomycin (Gibco Laboratories) in 25-cm² tissue culture flasks (Corning Glass Works, Corning, NY) and incubated for 1, 2, 4, 8, or 14 h at 37°C, 5% CO_2 in humidified air. In some cases, the serine and thiol protease inhibitors leupeptin (10^{-4} M; Sigma Chemical Co.), aprotinin (35 U/ml; Sigma Chemical Co.), phenylmethylsulfonyl fluoride (PMSF, 10^{-5} M; Sigma Chemical Co.), L-1-tosylamide 2-phenylethyl chloromethyl ketone (TPCK, 10^{-5} M; Sigma Chemical Co.) and/or tosyl-L-lysine chloromethyl ketone (TLCK, 10^{-5} M; Sigma Chemical Co.) were also added. CM was freed of cells by centrifugation at 800 g for 20 min, and dialyzed, 48–72 h, against PBS (5 mmol/liter sodium phosphate, 150 mmol/liter sodium chloride, pH 7.6) to remove residual inhibitors. CM was separated into supernatants or CM(S) and plasma membrane-derived vesicle containing pellets or CM(P) by centrifugation at 40,000 g for 40 min, as previously described (19). Super-

natants were filter-sterilized (Millipore Corp., Bedford, MA), and pellets were resuspended in a minimal volume of PBS. Unseparated CM, CM (S), and CM(P) were stored at 4°C until use in culture for assay of total BPA (tBPA), soluble BPA (sBPA), and mBPA, respectively.

Protein determination. Amounts of protein present in intact plasma membranes and shed, extracellular membrane-derived vesicles were quantitated by the method of Lowry et al. (21) using a BSA standard. Protein measurements were verified by the fluorescamine method (22). Although precise data are unavailable, it is expected that $< 0.1\%$ of total plasma membrane and extracellular membrane vesicle protein is represented by BPA.

Statistical analysis. Mean±SE bursts in quadruplicates were compared by the two-sample ranks test of Wilcoxon and White (23).

Results

Kinetics of BPA release from untreated cells. Release of BPA from highly purified lymphocytes incubated in base medium was determined as a function of time. As shown in Fig. 1 *a*, this lymphokine is released within 1 h, and reaches a maximum cumulative level by 8 h of incubation. The appearance of activ-
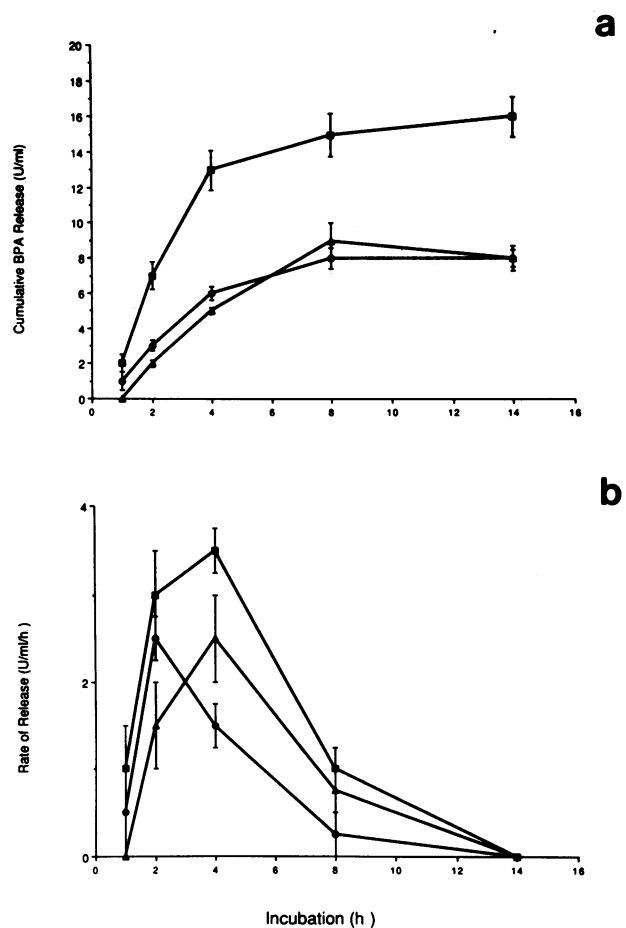

Figure 1. BPA release from untreated B lymphocytes. (*a*) Mean±SE BPA released into unseparated CM (■), CM supernatants (▲), and membrane vesicle-containing pellets (●). At 1 h virtually all BPA present in CM (tBPA) is due to activity associated with shed vesicles (mBPA). Cultures with nonmitogenic base medium contained 24±3 bursts/6 $\times 10^5$ cells per 1.0 ml. B cells were purified to a level of $\geq 99\%$. (*b*) Mean±SE U/ml per h are shown for tBPA (■), mBPA (●), and sBPA (▲). Peak BPA release occurs at 4 h, and no additional BPA release occurs by 14 h of incubation.

Table I. Influence of Protease Inhibitors on Cumulative BPA Levels

Inhibitor	tBPA	mBPA	sBPA
		U/ml	
None	14.5±1.9	6.3±1.0	6.9±1.2
Aprotinin	13.8±0.7	6.5±0.8	6.4±1.1
Leupeptin	14.0±1.3	5.9±0.7	7.1±0.7
Aprotnine + Leupeptin + TPCK + TLCK + PMSF	13.7±0.5	5.8±0.6	6.3±1.2

B cells were incubated in the absence (none) and presence of protease inhibitors for 14 h at 37°C. CM was harvested, fractionated into vesicle-containing pellets and supernatants, and assayed for tBPA (in unseparated CM), mBPA, and sBPA, as described in Methods. Means±SE are displayed.

ity in CM(P) precedes that of CM(S) by ∼ 1 h (see Fig. 1 *a*). The sum of activity in CM(P) and CM(S) approximates total activity present in unseparated CM (tBPA). Addition of non-toxic protease inhibitors during the conditioning process did not alter BPA levels in either unseparated CM or CM fractions (Table I). Similar results were obtained when combinations of protease inhibitors were employed.

The rate of BPA release peaked at 3.5±0.3 U/ml per h at 4 h of incubation (see Fig. 1 *b*). Peak rate of release of mBPA (2.5±0.2 U/ml per h) preceded that of sBPA release (2.4±0.3 U/ml per h), as shown in Fig. 1 *b*. Together, the data indicate that the time course release of BPA is similar to that of the release of other lymphokines (24, 25), and is also consistent with BPA release from unseparated lymphocytes (13).

Energy requirement for BPA production. Inhibitors of energy-dependent metabolism (0.1 mM DNP, 10 mM sodium azide, or 10 mM deoxyglucose) were preincubated with B lymphocytes that were washed and, subsequently, used as a source of BPA. As shown in Fig. 2, cumulative tBPA levels in CM

harvested after 14 h of incubation were reduced by ≥ 30 min exposure to each inhibitor (relative to control, $P < 0.05$ or less). Peak rates of tBPA release at 4 h of incubation from treated cells were approximately one-half that of tBPA release from untreated cells: 1.5±0.2, 1.2±0.3, and 1.5±0.3 U/ml per h for cells pulse-exposed for 30 min to DNP, sodium azide, or deoxyglucose, respectively. Both sBPA and mBPA concentrations were similarly diminished (data not shown).

The tBPA level in medium conditioned by cells incubated with base medium alone for 120 min (see Fig. 2) was reduced to a level (from 14.2±0.8 U/ml at 5 min to 9.0±0.8 U/ml at 120 min) that is consistent with production of BPA (see tBPA release at 2 h of 7.0±0.8 U/ml in Fig. 1 *a*) and subsequent removal of BPA after control lymphocytes were pelleted and resuspended in an identical fashion as performed with test lymphocytes. In contrast, tBPA levels of 0.8±0.4, 0.5±0.5, and 1.0±0.7 U/ml, respectively, were measured in test medium prepared from cells pulse-exposed for 120 min to DNP, sodium azide, or deoxyglucose, respectively (relative to control, $P < 0.05$ for each).

Exfoliation of mBPA requires cell metabolism. Although it is well established that normal and malignant mammalian cells exfoliate surface membrane components (26, 27), little is known concerning cellular requirements for this physiologic process. To determine whether metabolic energy is required for exfoliation, membrane protein shedding from cells incubated with medium alone or inhibitors of energy-dependent metabolism was quantified after 2, 4, or 14 h of incubation. Fig. 3 shows that exposure to metabolic inhibitors for 120 min results in dramatic reduction in plasma membrane-derived vesicles present in CM(P) (relative to control, $P < 0.05$ at each time point). The rate of membrane shedding into the CM (P) of treated and control cells was similar with respect to time (peak release at 2 h for all groups) and protein concentration over time (peak rate of 15±2 μg/ml per h for control cells vs. 13±1, 14±2, and 13±2 μg/ml per h for cells pulse-exposed to DNP,
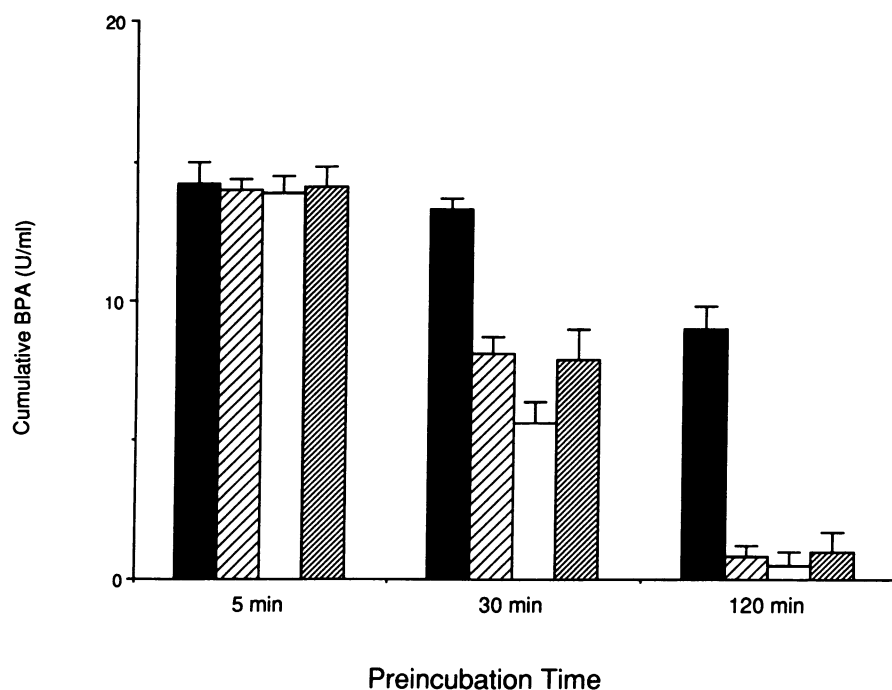

Figure 2. Effects of metabolic inactivation on tBPA release. B lymphocytes were pulse-exposed for the indicated periods of time to IMDM alone (*solid bars*), 0.1 mM DNP (*coarse hatched bars*), 10 mM sodium azide (*open bars*), or 10 mM deoxyglucose (*fine hatched bars*). Shown are mean±SE levels measured in CM after 14 h of incubation. Relative to levels in control CM, activity is reduced after 30 or 120 min of exposure to inhibitor ($P < 0.05$ for each).
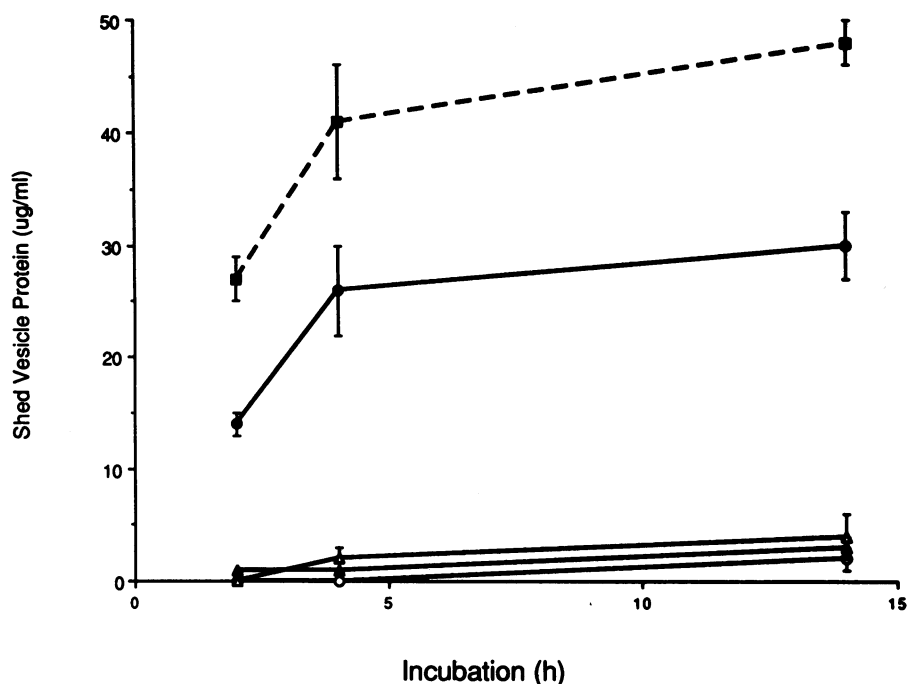
Figure 3. Effect of metabolic inhibitors on cell surface exfoliation. B lymphocytes were pulse-exposed for 120 min, 37°C, to IMDM alone (treated control, ●), 0.1 mM DNP (▲), 10 mM sodium azide (○), or 10 mM deoxyglucose (△), and resuspended in fresh culture medium. Amounts of vesicle protein appearing in CM(P) over time were measured and compared to activity present in untreated control cells (■). Reduction in shed protein in treated control CM(P) relative to untreated control CM(P) is consistent with removal of membrane vesicles that shed early (i.e., after 2 h) into liquid medium (see reference 13).

sodium azide, and deoxyglucose, respectively). Moreover, expression of mBPA per microgram of plasma membrane or shed extracellular membrane vesicles was also reduced (Table II). Taken together, the data suggest that growth factor localization to the plasma membrane and exfoliation from the cell surface require active cell metabolism.

Level of regulation of BPA synthesis. To assess the level of regulation of BPA synthesis, B cells were pulse-exposed to base medium or inhibitors of transcription, translation, or DNA synthesis. As shown in Table III, even brief (5 min) treatment with actinomycin D or cycloheximide reduced the levels of mBPA and sBPA recovered in CM after 14 h of incubation to zero. tBPA, mBPA, and sBPA released into CM after 1, 2, 4, or 8 h of incubation were also undetectable in CM prepared from treated cells. At a concentration of 10 μg of cycloheximide/ml, levels of tBPA, mBPA, and sBPA were barely detectable (relative to control, $P < 0.05$) (data not shown).

In contrast, pulse-exposure of B cells to cytosine arabinoside for as long as 120 min had no effect on the expression of sBPA or mBPA in dialyzed CM harvested after 14 h of incubation (Table IV). This observation is of interest since mitogens are frequently added during incubation of lymphoid cells for the preparation of cytokine-containing CM.

Intracellular processing of BPA. Column-purified BPA migrates as a broad "band" on tightly crosslinked SDS gels, consistent with the charge heterogeneity of glycoproteins (19). Therefore, we assessed the involvement of the Golgi apparatus in the processing of BPA by using monensin, an agent that interferes with transport of proteins through this organelle. Fig. 4 shows that monensin pretreatment results in dose-dependent and time-dependent reduction in tBPA released into CM after 14 h. Whereas 10^{-9} M of the drug showed no effect, 10^{-8} M reduced activity from 12.4 ± 0.9 to 2.1 ± 0.6 U/ml ($P < 0.05$). An equal reduction in mBPA and sBPA was observed (Fig. 4 *a*). In

Table II. Effects of Metabolic Inhibition on Specific Activity of mBPA

Membrane preparation	Incubation time	Base medium	Inhibitors			
			DNP	Na azide	Deoxyglucose	Monensin
	h			*U/μg*		
PM	2	0.31±0.10	0.6±0.02	0.07±0.03	0.06±0.01	—
	4	0.29±0.14	0.04±0.02	0.10±0.05	0.07±0.04	—
	14	0.26±0.11	0.05±0.01	0.06±0.02	0.08±0.05	0.01±0.01
P	2	0.39±0.15	0.10±0.03	0.08±0.01	0.09±0.03	—
	4	0.41±0.09	0.09±0.02	0.10±0.03	0.04±0.01	—
	14	0.34±0.12	0.06±0.02	0.10±0.03	0.07±0.03	0.03±0.02

Highly purified B cells were incubated with nonmitogenic base medium, 0.1 mM DNP, 10 mM sodium azide, 10 mM deoxyglucose, or 10^{-8} M monensin (+ inhibitors) for 120 min at 37°C. They were pelleted, washed, and used to prepare intact plasma membranes (PM) and CM after a 2-, 4-, or 14-h incubation. Shed vesicles were recovered from pellets of CM. The amounts of mBPA and protein sequentially extracted with NaOH and *n*-octyl β-D-glucopyranoside from PM and P were measured, and specific activity was calculated. Results are recorded as mean±SE in quadruplicate experiments. In each case, the value in test PM or P is lower than that in control samples at the ≤ 0.05 level.

Table III. Effects of Transcription Inhibitors on BPA Expression

Preincubation time (min)	Inhibitor concentration	mBPA	sBPA
	μg/ml	U/ml	
Control (5)	0	8.2±0.5	7.5±1.0
Control (30)	0	8.0±0.4	7.0±.07
Control (120)	0	6.5±0.7	6.2±1.1
Actinomycin (5)	10	0.0±0.0	0.0±0.0
Actinomycin (30)	10	0.0±0.0	0.0±0.0
Actinomycin (120)	10	0.0±0.0	0.0±0.0
Cycloheximide (5)	100	0.0±0.0	0.0±0.0
Cycloheximide (30)	100	0.0±0.0	0.0±0.0
Cycloheximide (120)	100	0.0±0.0	0.0±0.0

B cells were incubated with base medium (control) or inhibitor for 120 min at 37°C. Viability was ≥ 95% at end of preincubation by exclusion of Trypan blue and staining with acridine orange and ethidium bromide.

contrast, monensin had no effect on tBPA expression after its release into CM (Fig. 4 b), suggesting that monensin-mediated reduction in BPA levels is due to impaired processing rather than to modification of a fully processed molecule. As shown in Table II, specific activity of the growth factor was reduced in both shed vesicles and intact plasma membranes derived from monensin-treated cells.

Discussion

Hematopoietic-specific growth factors are endocrines and paracrines whose primary action is to control progenitor/stem cell proliferation in a receptor-dependent fashion (2, 3, 5). In this respect, they are similar to other polypeptide growth factors and oncogene products whose hormone-like action is initiated by receptor interaction (28). Many of the latter regulators of cell viability and growth have been found to alter hematopoietic stem/progenitor cell proliferation, including platelet-de-

Table IV. Effects of Cytosine Arabinoside on BPA Expression

Preincubation time (min)	Concentration	mBPA	sBPA
	M	U/ml	
Control (5)	0	8.4±0.9	7.8±2.1
Control (30)	0	8.0±0.6	7.5±0.8
Control (120)	0	5.9±2.0	5.6±1.2
Inhibitor (5)	10^{-8}	8.8±0.4	7.9±0.4
Inhibitor (30)	10^{-8}	8.1±0.7	6.8±0.7
Inhibitor (120)	10^{-8}	5.9±0.8	5.9±0.5
Inhibitor (5)	10^{-7}	8.2±1.0	8.0±1.2
Inhibitor (30)	10^{-7}	7.9±0.9	7.8±0.8
Inhibitor (120)	10^{-7}	5.7±1.5	5.5±0.4

B cells were pretreated for 120 min at 37°C with base medium (control) or cytosine arabinoside (inhibitor) at the indicated concentrations. Cells were washed and resuspended in base medium for 14 h at 37°C. CM was harvested, fractionated, and assayed for BPA, as described in Methods.

rived growth factor (29–31), insulin-like growth factors I and II (32–34), transforming growth factors (35), and a variety of "public" hormones whose actions are diverse and whose target cells are widely distributed throughout the body (36). Peripheral blood, bone marrow, and splenic lymphocytes represent another source of hematopoietic growth factors. Here, we have examined cellular mechanisms involved in the production and release of the human lymphokine BPA, a factor previously purified in this laboratory (20), and found by the investigators to be biochemically and immunologically distinct from other hematopoietic cytokines, including interleukin 3 and erythroid potentiating activity (8, 20, 37). The results indicate that pathways utilized for BPA production are analogous to those involved in peptide hormone synthesis by cells of endocrine glands.

To examine the level of regulation of BPA synthesis/release, the effects of various agents known to interfere with replicative DNA, mRNA, and protein synthesis on BPA production was determined. The results demonstrate that the transcription inhibitor actinomycin D and the translation inhibitor cycloheximide both completely block assayable BPA production. Not only sBPA but also membrane-associated BPA was reduced (see Table III), suggesting that the generation of either form of the factor requires active mRNA and protein synthesis. In contrast, DNA synthesis does not appear to be required for elaboration of these activities (see Table IV). This result is consistent with the previous experience that, whereas lymphocyte exposure to lipopolysaccharide or plant lectins augments growth factor levels in CM, it is not essential for their production (19). Accordingly, lymphocyte mitogens may increase growth factor synthesis similar to their selective augmentation of the level of synthesis of other cytosolic proteins (38, 39), without requiring the cells to undergo replicative DNA synthesis, entry into S phase and ultimately, mitotic division.

Preliminary characterization of BPA shows it to be an integral membrane glycoprotein that is similar to other hematopoietic growth factors in molecular mass and charge heterogeneity (2, 3, 19, 20). Since remodeling and terminal glycosylation of oligosaccharide units of many glycoproteins take place in the Golgi apparatus, the effects of monensin on BPA expression were assessed. Marked reductions in both total and specific activities of the factor in CM and plasma membranes prepared from monensin-treated cells (see Table II and Fig. 4) strongly suggest that production of bioactive BPA involves transport through and out of the Golgi complex. The importance of post-translational processing is further supported by the finding that enzymatic deglycosylation of column purified BPA removes > 90% of bioactivity (personal observation).

Normal and neoplastic cells shed surface membrane components (19, 40–42). Exfoliation of plasma membrane constituents may mediate diverse cell functions, including communication with other cells within the proximal environment, selective release of lipids and proteins, activation of the blood coagulation system, and embryonic proteolysis (43). Shedding of gangliosides and other membrane components from the surface of neoplastic cells may be a mechanism by which these cells suppress antigen presentation (44), alter immune effector responses (45), and escape immune detection (46). We have observed that membrane vesicles shed from the surface of normal lymphocytes behave as carriers of signal molecules for hematopoietic progenitor/stem cells (12, 19).

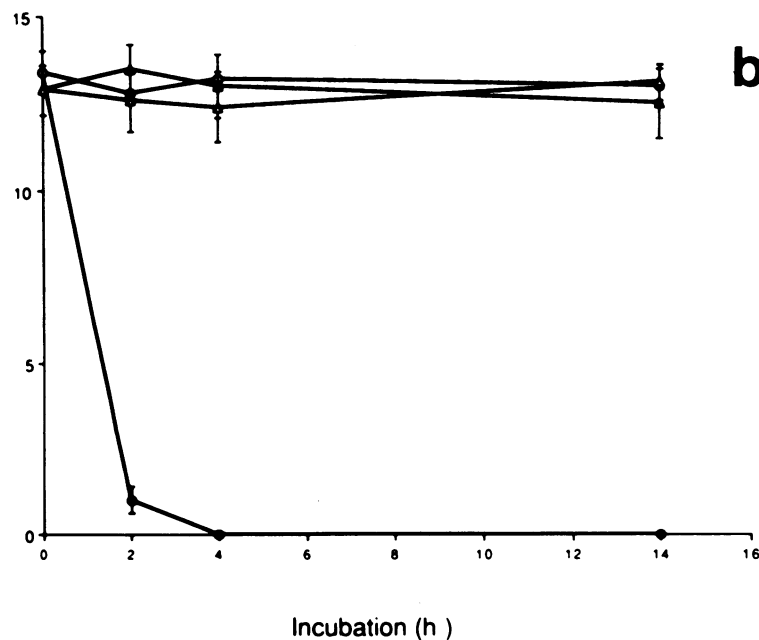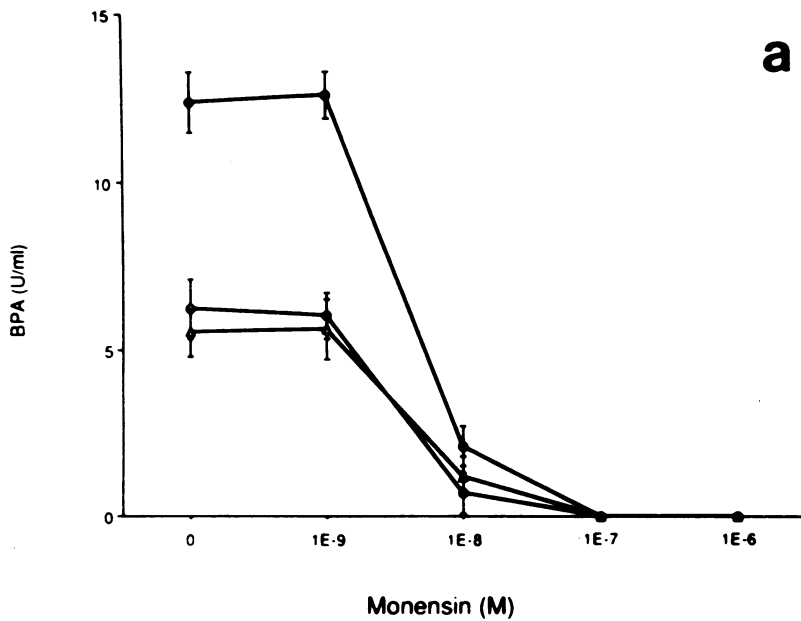Cholesterol, phospholipid, and protein compositions as

Figure 4. Effect of monensin on BPA production. (a) Mean±SE tBPA (●), mBPA (○), and sBPA (△) levels in medium condition by B lymphocytes treated for 120 min at 37°C with monensin. Activity is reduced to zero by exposure to of 10^{-7} or 10^{-6} M monensin. (b) Mean±SE tBPA levels in medium conditioned by B cells treated for the various times with 10^{-9} M (▲) or 10^{-8} M (●) monensin. Also shown are BPA levels in medium that was prepared from untreated lymphocytes which was subsequently incubated for various times with 10^{-9} M (△) or 10^{-8} M (○) monensin, and dialyzed exhaustively to remove the drug. Whereas pretreatment of B cells with 10^{-8} M monensin for ≥ 4 h abolishes all activity, exposure to medium conditioned by untreated cells for similar time periods does not alter tBPA levels (relative to control, $P > 0.10$ at each time point).

well as fluidity properties of vesicles shed from murine and human lymphocytes are distinct from those of intact lymphocyte plasma membranes (47, 48). Therefore, rather than being a random process, cell surface exfoliation may be a directed activity (48). If true, then one would predict that surface shedding requires active cell metabolism and consumes energy. The results indicate that this is the case with regard to the release of mBPA from B lymphocytes. Both surface membrane exfoliation per se and mBPA expression by CM (P) were markedly reduced by pulse-exposure to metabolic inhibitors of cell metabolism (see Fig. 3 and Table II). There was no apparent delay in extracellular membrane vesicle formation over the 14-h incubation used in this study, and cell viabilities immediately after drug exposure and at the time of CM harvest were similar to those of untreated cells. Interestingly, a recent report indicates that platelets which are incubated with 2-deoxyglucose and antimycin A fail to shed membrane vesicles in response to collagen or thrombin but show apparently normal vesiculation in response to complement (49), suggesting that in some cases, active metabolism is required for platelet vesiculation as well.

Several mechanisms have been thought to be involved in plasma membrane exfoliation (40). Cell surface proteases (50, 51) and phospholipases (52) may enzymatically modify the plasma membrane, resulting in extracellular vesicle formation. Results showing that the addition during conditioning of protease inhibitors, including leupeptin, aprotinin, TPCK, TLCK, and/or PMSF does not alter BPA levels in CM(P) (see Table I), suggest that release of the activity in association with vesicles is not likely due to proteolysis. Nevertheless, since other surface proteases (such as the acidic and metalloproteases) were not

specifically inhibited, the possibility that proteolysis may participate in mBPA release from the cell membrane has not been completely excluded.

In addition, cytoskeletal proteins may play a role in extracellular vesiculation (53, 54). The investigators have previously reported that actin filament formation is necessary for surface exfoliation and mBPA expression by both intact lymphocyte plasma membranes and shed extracellular vesicles (54). Accordingly, microfilament integrity may be important for mBPA localization as an integral component of the plasma membrane. Since actin polymerization is an energy-consuming process (55), the possibility that cell metabolism is required for mBPA expression by plasma membranes was investigated. The results show that the specific activity of mBPA in extracts of intact plasma membranes is markedly reduced by metabolic inhibitors (see Table II), suggesting that intracellular trafficking and/or insertion of growth factor into the plasma membrane requires metabolic energy. The relationship of soluble and membrane-bound forms of the factor is unknown but kinetic analysis of their appearance in liquid culture medium (see Fig. 1) raises the possibility that sBPA may be derived from mBPA, an hypothesis that is supported by the finding that a monoclonal antibody can be used to immunoadsorb both sBPA and solubilized mBPA from solution (56).

In summary, we report that pulse-treatment of B lymphocytes with specific inhibitors of mRNA synthesis, translation, and posttranslational modification renders the cells incapable of producing and releasing the erythropoietic growth factor BPA. Exposure to inhibitors of energy-dependent cell metabolism results in impaired extracellular vesiculation and reduced activity per ug membrane protein. The data suggest a model wherein BPA production by lymphocytes is similar to hormone synthesis in endocrine cells, involving transcription, translation, and processing of the growth factor. Moreover, release of the growth factor as an integral component of extracellular, membrane-derived vesicles also requires cell metabolism, supporting the concept that exfoliation is a directed cell process that enables neighboring cells to signal each other.

Acknowledgments

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