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Inhibition of Protein Kinase C Results in Decreased Expression of Bovine Leukemia Virus

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The in vitro expression of bovine leukemia virus (BLV) in short-term cultured bovine peripheral blood mononuclear cells (PBMC) is associated with increased spontaneous lymphocyte blastogenesis. The purpose of this study was to determine whether intracellular pathways responsible for antigen- or mitogen-induced lymphocyte blastogenesis were also responsible for induction of BLV expression. The protein kinase C (PKC) inhibitor 1-(5-isoquinolinylsulfonyl)-3-methylpiperazine dihydrochloride (3-methyl H7) decreased blastogenesis in a dose-dependent manner, as measured by [^{3}H]thymidine incorporation, in unstimulated, lipopolysaccharide-stimulated and phorbol ester (PMA)-stimulated BLV-infected PBMC. Similarly, 3-methyl H7 decreased BLV expression, as measured by production of gp51 envelope antigen or p24^{gag} antigen, in BLV-infected PBMC under the same conditions. Using an RNase protection assay, the inhibition of BLV expression by 3-methyl H7 was shown to be due to decreased transcriptional activity. The cyclic GMP-dependent protein kinase and cyclic AMP-dependent protein kinase inhibitor *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide (HA1004) did not inhibit either BLV expression or blastogenesis of BLV-infected bovine PBMC. Additional evidence for the PKC-dependent expression of BLV was obtained by using a persistently BLV-infected B-lymphocyte cell line, NBC-13. Activation of PKC by PMA in NBC-13 cells increased BLV expression. 3-methyl H7 decreased the PMA-induced expression of BLV in NBC-13 cells in a dose-dependent manner, whereas HA1004 did not inhibit this expression. These results identify a mechanism for the induction of BLV expression through PKC activation and therefore indicate that latency and replication of BLV is controlled by normal B-lymphocyte intracellular signaling pathways.

Bovine leukemia virus (BLV) is a naturally occurring, exogenous, B-lymphocytotropic (2, 42, 47, 48) retrovirus of cattle and the etiologic agent of enzootic bovine leukosis, a neoplastic proliferation of B lymphocytes. BLV is structurally and biologically related to human T-cell leukemia virus types I and II (HTLV-I and HTLV-II). These viruses lack *onc* genes, integrate randomly into the host cell genome (6, 17, 33), and contain several unique open reading frames at the 3′ ends of their genomes which encode the *trans*-acting regulatory proteins Tax and Rex (15, 16, 39, 41, 55). BLV was identified after short-term in vitro incubation of peripheral blood mononuclear cells (PBMC) from cattle affected with persistent lymphocytosis (PL) (43), a nonneoplastic proliferation of B lymphocytes (22, 31, 46, 63). Expression of BLV in vivo is thought to be blocked at the transcriptional level since viral particles, proteins, or RNAs are not readily detected in freshly isolated PBMC or tumor cells (5, 24, 34, 36, 60). However, low-level in vivo expression of BLV does occur since BLV RNA has been detected in freshly isolated PBMC and tumor cells by using the polymerase chain reaction (30), and BLV-infected animals develop a marked and persistent humoral immune response against all viral proteins (18).

Previous studies have identified a possible correlation between cell division or lymphocyte blastogenesis and the in vitro expression of BLV. Mitogens such as concanavalin A and phytohemagglutinin have been reported to increase BLV expression in cultured PBMC from BLV-infected animals (12, 19, 20, 42, 56). Additionally, cultured PBMC from BLV-seropositive cattle affected with PL have been reported to have decreased responses to mitogens because of increased spontaneous [^{3}H]thymidine incorporation (45, 61). The spontaneous [^{3}H]thymidine incorporation in cultured PBMC from cows with PL can be markedly reduced by anti-BLV serum (57, 59), and the inhibitory activity of anti-BLV serum can be reversed with purified BLV (59), which suggests that the spontaneous blastogenic response is viral antigen specific.

The objective of this study was to investigate the mechanism responsible for the in vitro induction of BLV expression, specifically, to determine whether virus expression was influenced by intracellular signaling pathways activated during B-lymphocyte blastogenesis (for reviews, see references 7 and 13). B-lymphocyte activation results in the production of diacylglycerol (DAG) and the release of Ca^{2+} from intracellular stores within the endoplasmic reticulum. DAG and Ca^{2+} mobilization stimulate the translocation of protein kinase C (PKC) to an activated membrane-associated state. Phorbol-12-myristate-13-acetate (PMA) and bacterial lipopolysaccharide (LPS) also induce translocation of PKC without Ca^{2+} mobilization, probably by acting as DAG analogs (8, 23, 44, 62). Through unknown mechanisms, PKC mediates intracellular alkalinization through activation of Na^+/H^+ exchange, which in turn leads to increased expression of c-*myc*, c-*fos*, and Ia genes and to lymphocyte blastogenesis (40, 53). PKC can be inhibited by 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7); however, H7 also inhibits cyclic GMP-dependent protein kinase (PKG) and cyclic AMP-dependent protein kinase (PKA) (27). PKG and PKA are selectively inhibited by *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide (HA1004), which minimally inhibits PKC (27) and therefore serves as a control for H7 inhibition

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of PKC. H7 has been shown to inhibit proliferation, interleukin-2 (IL-2) production, and PKC activation in phytohemagglutinin-stimulated bovine PBMC (4), and it has been shown to inhibit proliferation, IL-2 production, and IL-2 receptor expression in mitogen-stimulated human T cells (3).

Several studies have reported an effect of PKC on expression of retroviruses. The transcriptional activation of the HTLV-I promoter by Tax and the accumulation of unspliced HTLV-I mRNA have been shown to be decreased by PKC inhibitors (1, 58). In cell lines chronically infected with human immunodeficiency virus (HIV), PKC activators have been shown to enhance HIV expression and this increased expression can be blocked with PKC inhibitors (10, 25, 37). Additionally, transactivation of HIV type 1 long terminal repeat-directed gene expression by Tat has been shown to require PKC (28). In the current study, experiments were designed to determine whether activation of PKC stimulated BLV expression and whether inhibition of PKC blocked expression of BLV in cultured bovine PBMC and in the persistently BLV-infected B-lymphocyte cell line NBC-13 (21).

MATERIALS AND METHODS

Chemicals. LPS (L4516; Sigma Chemical Co., St. Louis, Mo.) was dissolved in culture medium at 1 mg/ml immediately before use. PMA (P8139; Sigma Chemical Co.) was dissolved in dimethyl sulfoxide at 1 mM and stored at −20°C. The protein kinase inhibitor H7 was obtained from Sigma Chemical Co. (I5262); however, after completion of the experiments it was determined that this compound was actually 1-(5-isoquinolinylsulfonyl)-3-methylpiperazine dihydrochloride (3-methyl H7). 3-methyl H7 has also been shown to inhibit PKC but only 25% as effectively as H7 (38). The PKA and PKG inhibitor HA1004 was also obtained from Sigma Chemical Co. (G1274). 3-methyl H7 and HA1004 were dissolved in distilled water at 10 mM and stored at −20°C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; thiazolyl blue) (M2128; Sigma Chemical Co.) was dissolved at 5 mg/ml in phosphate-buffered saline (PBS) and stored at 4°C in the dark.

Cell isolations and culture conditions. Four clinically normal BLV-seropositive adult female Holstein cattle affected with PL were studied. Three BLV-seronegative cattle were used as controls. BLV serological status was determined by using a competitive enzyme-linked immunosorbent assay (ELISA) as previously described (51), with reagents kindly supplied by Daniel Portetelle (Faculty of Agronomy, Gembloux, Belgium). Venous blood was collected by using EDTA as an anticoagulant. PBMC were isolated by Ficoll-Hypaque (1.077 g/ml) density gradient centrifugation. The mononuclear cell layers were harvested, washed three times with PBS containing 1% bovine serum albumin, and suspended at a concentration of 4×10^6/ml in culture medium (RPMI 1640 with 10% heat-inactivated fetal bovine serum [Hyclone Laboratories, Inc., Logan, Utah], 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml). NBC-13 cells (21) (kindly supplied by Jorge Ferrer, University of Pennsylvania, Kennett Square, Pa.) were suspended at a concentration of 10^6/ml in MEM culture medium (Eagle's minimal essential medium containing 1× nonessential amino acids [Irvine Scientific, Santa Ana, Calif.] and the same supplements as above). All cultures were incubated at 37°C with 5% CO_2 for either 24 or 48 h. LPS and PMA were added to the culture medium at 5 μg/ml and 1 nM, respectively, where indicated.

Cell viability and blastogenesis. Cell viability was determined with a colorimetric assay as previously described (26). Briefly, quadruplicate samples of 4.0×10^5 PBMC or 1×10^5 NBC-13 in 100 μl of culture medium were incubated in 96-well cell culture plates (Corning Glass Works, Corning, N.Y.). Three hours prior to harvesting, 25 μl of MTT stock solution (5 mg/ml) was added to each well and incubation was continued at 37°C. Cells were lysed and the formazan crystals were dissolved by the addition of 100 μl of lysing solution (20% [wt/vol] sodium dodecyl sulfate, 50% [vol/vol] *N*,*N*-dimethyl formamide, pH 4.7) to each well. After overnight incubation at 37°C, optical densities (OD) at 590 nm were measured by using an ELISA plate reader (Dynatech MR 700; Dyantech Laboratories, Inc., Chantilly, Va.). MTT OD values were used to determine a viability index (OD of untreated control/OD of sample) for each of the parameters measured.

Newly synthesized DNA was measured in bovine PBMC and NBC-13 cells by [*methyl*-^{3}H]thymidine incorporation. Quadruplicate samples of 4.0×10^5 PBMC or 1×10^5 NBC-13 cells in 100 μl of culture medium were incubated in 96-well cell culture plates (Corning Glass Works). Twenty-four hours before harvesting, 5 μCi of [*methyl*-^{3}H]thymidine (Du Pont, NEN Research Products, Boston, Mass.) was added to each well. At the end of the incubation period, cells were transferred onto glass fiber filter paper by using a cell harvester (Titertek model 550; Flow Laboratories Ltd., Mclean, Va.) and the amount of incorporated [*methyl*-^{3}H] thymidine was determined in a liquid scintillation counter (model LS1801; Beckman Instruments, Inc., Irvine, Calif.) and expressed as counts per minute (cpm). Blastogenesis data was expressed as the average [*methyl*-^{3}H]thymidine incorporation (cpm) in PBMC derived from three BLV-seropositive cows affected with PL.

Detection of BLV gp51 and p24 expression. Bovine PBMC or NBC-13 cells were cultured in 24-well cell culture plates (Corning Glass Works) for 24 h. The cell culture plates were subsequently frozen at −20°C and then thawed at room temperature. The freeze-thaw cycle was repeated twice more, and then supernatant was assayed for the presence of BLV gp51 or p24. Expression of the BLV envelope glycoprotein gp51 was determined with an antigen-capture ELISA as previously described (50, 51), using monoclonal antibodies kindly supplied by Daniel Portetelle and with the following modifications. Immulon 2 microtiter plates (Dyantech Laboratories, Inc.) were coated (per well) with 100 ng of monoclonal antibody (MAb) specific for the E epitope of BLV gp51 in 10 mM sodium tetraborate overnight at 4°C and then were washed once with PBST (PBS containing 0.2% Tween 80). Quadruplicate 100-μl samples of cell culture supernatants–50 μl of saturation buffer (PBS containing 2% bovine serum albumin and 0.06% sodium azide)–15 μl of Tween 80 were added to the plates and incubated for 72 h at 4°C. Plates were washed three times with PBST and then were incubated for 2 h at 4°C with (per well) 100 μl of horseradish peroxidase-conjugated MAb specific for either the G epitope (200 ng/ml) or a pool specific for the A, B, B′, D, D′, and E epitopes (100 ng/ml) of BLV gp51. Plates were washed four times with PBST and then were incubated for 15 min at room temperature with 100 μl of TMB solution (0.4 g of tetramethylbenzidine per liter, 0.02% hydrogen peroxide) per well. The colorimetric reaction was stopped by the addition of 100 μl of 1 N H_2SO_4 per well, and OD at 450 nm were measured by using an ELISA plate reader (Dynatech MR 700; Dyantech Laboratories, Inc.).

BLV capsid protein p24 in culture supernatants was

similarly determined, using an antigen-capture ELISA and MAbs kindly supplied by Daniel Portetelle. Briefly, Immulon 2 microtiter plates (Dyantech Laboratories, Inc.) were coated (per well) with 50 ng of MAb 4'G9 specific for BLV p24 in 10 mM sodium tetraborate for 6 h at 4°C and then were washed once with PBST. Quadruplicate 25-μl samples of cell culture supernatants–75 μl of saturation buffer–50 μl of PBS containing 4% Tween 80 were added to the plates and incubated overnight at 4°C. Plates were washed three times with PBST and then were incubated for 2 h at 4°C with (per well) 100 μl of horseradish peroxidase-conjugated MAb 2'C1 and 4'F5 specific for BLV p24 (100 ng/ml). Plates were washed four times with PBST, incubated with substrate, and OD were determined as described above.

To determine relative amounts of BLV gp51 or p24 in cell culture supernatants, twofold serial dilutions of a BLV antigen concentrate (Leukassay B; Pitman-Moore Inc., Washington Crossing, N.J.) were assayed in quadruplicate in the BLV gp51 and p24 antigen-capture ELISAs. The initial dilution (1:50) of BLV antigen concentrate was given a relative value of 1.0, and a standard curve of relative antigen concentration versus OD was developed for both BLV gp51 and p24. OD values obtained with culture supernatants were subsequently converted to relative amounts of gp51 or p24 by using the standard curve line equations. To account for variation in cell viability, relative amounts of BLV gp51 and p24 were corrected for cell viability (relative amounts of gp51 or p24/viability index) and expressed as the average percentage of control gp51 or p24 detected in PBMC culture supernatants derived from four BLV-seropositive cows affected with PL.

Detection of BLV transcription. Bovine PBMC and NBC-13 cells were cultured for 6 and 24 h, respectively. Total cellular RNA was subsequently isolated by cell lysis in 4 M acid guanidinium isothiocyanate solution and phenol-chloroform extraction as previously described (9, 52). BLV transcription was measured by using an RNase protection assay (RPA II; Ambion Inc., Austin, Tex.). Total amount of BLV mRNA (unspliced and spliced) was determined by using an antisense riboprobe complementary to 200 bases in the *tax/rex* region (nucleotides 7896 to 8095 according to the system of Sagata et al. [54]) since this sequence is present in all BLV mRNA species. The amount of unspliced BLV mRNA was measured by using an antisense riboprobe complementary to 123 bases in the *gag* region (nucleotides 895 to 1017 according to the system of Sagata et al. [54]). The BLV *gag* and *tax/rex* sequences were subcloned from a proviral clone of BLV, pBLV913 (kindly supplied by James W. Casey, Cornell University, Ithaca, N.Y.), into a plasmid vector containing T7 and SP6 promoters [pGEM-7Zf(+); Promega, Madison, Wis.]. An antisense riboprobe complementary to 299 bases of bovine actin mRNA (nucleotides 41 to 339; GenBank accession no. K00623 [14]) was used to control for various amounts of RNA in each assay. The bovine actin sequence was amplified by using the polymerase chain reaction from PBMC genomic DNA derived from a BLV-negative cow. The promoter sequence for the T7 polymerase was included in the 3' primer as previously described (64). Samples containing 5 to 10 μg of total cellular RNA were hybridized overnight at 50°C with 10^5 cpm each of [α-^{32}P]labeled BLV *tax/rex*-specific, BLV *gag*-specific, and bovine actin-specific probes simultaneously in 20 μl of hybridization solution (RPA II; Ambion Inc.). After hybridization, samples were digested with RNase A and RNase T_1, electrophoresed through 8% acrylamide, and then autoradiographed for 16 to 72 h at −70°C with an intensifying screen.

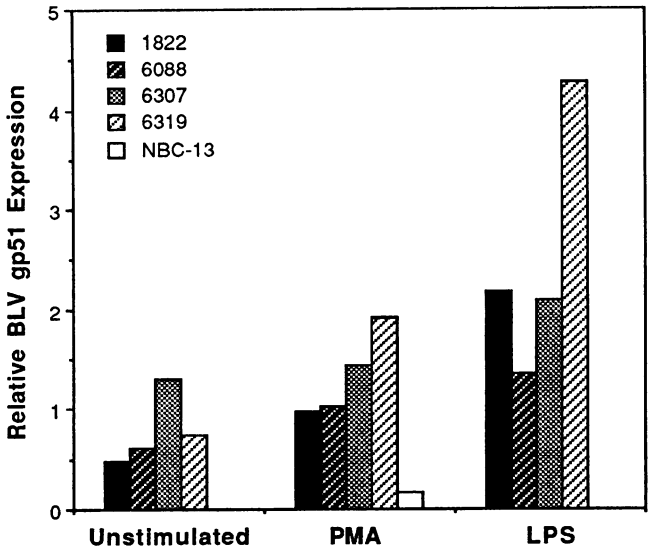

FIG. 1. BLV gp51 expression in unstimulated, LPS-stimulated, and PMA-stimulated PBMC from cattle with PL (no. 1822, 6088, 6307, and 6319) and in unstimulated and PMA-stimulated NBC-13 cells. Cells were cultured for 24 h in medium alone (unstimulated) or in medium containing PMA (1 nM) or LPS (5 μg/ml). Culture supernatants were assayed for BLV gp51 by using an antigen-capture ELISA. OD values were converted to relative values (relative value of 1.0 equals the OD obtained from a 1:200 dilution of stock BLV antigen concentrate) and subsequently corrected for cell viability.

Autoradiographs were digitized for densitometric analysis (Scan Analysis; Biosoft, Ferguson, Mo.). BLV mRNA signals were calculated relative to the bovine actin signal for each sample.

RESULTS

Effect of PKC activation on BLV gp51 expression in PBMC and NBC-13 cells. In 24-h cultured bovine PBMC derived from cattle with PL, activation of PKC with PMA (1 nM) increased BLV gp51 expression approximately 125 to 250% compared with that of unstimulated cultured PBMC (Fig. 1). Similarly, LPS (5 μg/ml) increased BLV gp51 expression approximately 200 to 500% (Fig. 1). In unstimulated NBC-13 cell cultures, BLV gp51 expression was only minimally detectable; however, 24 h after the addition of PMA (1 nM) BLV gp51 was readily detectable in culture supernatants (Fig. 1).

Effect of PKC inhibition on BLV gp51 and p24 expression in unstimulated, LPS-stimulated, and PMA-stimulated PBMC and in PMA-stimulated NBC-13 cells. The PKC inhibitor 3-methyl H7 decreased BLV gp51 expression in a dose-dependent manner in 24-h cultures of unstimulated, LPS-stimulated, and PMA-stimulated PBMC and in PMA-stimulated NBC-13 cells (Fig. 2A through D, respectively). In contrast, the PKG and PKA inhibitor HA1004 had minimal effects on BLV gp51 expression under all culture conditions (Fig. 2A through D). Similar to its effects on gp51, 3-methyl H7 decreased BLV p24 expression in 24-h cultures of unstimulated (27% of control at 125 μM), LPS-stimulated (38% of control at 125 μM), and PMA-stimulated (9% of control at 125 μM) PBMC and in PMA-stimulated NBC-13 cells (31% of control at 100 μM). HA1004 had minimal effects on BLV p24 expression under all culture conditions

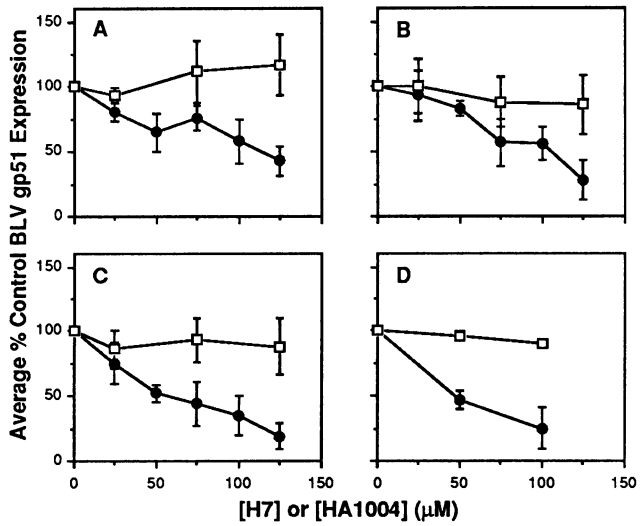

FIG. 2. Effect of 3-methyl H7 (●) and HA1004 (□) on BLV gp51 expression in unstimulated (A), LPS-stimulated (B), and PMA-stimulated (C) bovine PBMC and in PMA-stimulated NBC-13 cells (D). Supernatants from 24-h cultures were assayed for BLV gp51 by using an antigen-capture ELISA. The results with bovine PBMC represent the average percentage of control obtained in separate experiments with four BLV-infected cattle, and the results with NBC-13 cells represent the average percentage of control obtained in two separate experiments.

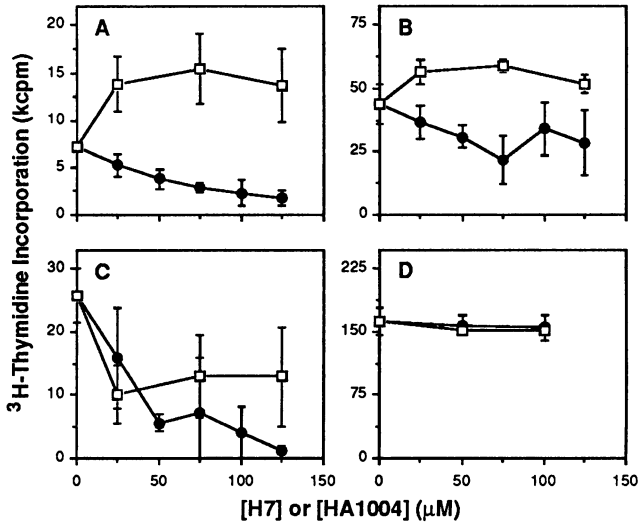

FIG. 3. Effect of 3-methyl H7 (●) and HA1004 (□) on [*methyl*-^{3}H]thymidine incorporation in unstimulated (A), LPS-stimulated (B), and PMA-stimulated (C) bovine PBMC and in PMA-stimulated NBC-13 cells (D). The results with bovine PBMC represent the average cpm obtained from 48-h cultures with three BLV-infected cattle, and results with NBC-13 cells are representative cpm obtained in two separate experiments.

(90 to 109% of control at 125 μM). BLV gp51 and p24 data were derived after adjusting for the effects of 3-methyl H7 and HA1004 on cell viability. In 24-h cultures, 3-methyl H7 decreased PBMC viability in a dose-dependent manner to 50 to 70% at 100 μM 3-methyl H7 but had no effect on NBC-13 cell viability. HA1004 had only minimal effects on cell viability (>90% viable at 125 μM HA1004) in either 24-h PBMC or NBC-13 cell cultures.

Effect of PKC inhibition on [^{3}H]thymidine incorporation in unstimulated, LPS-stimulated, and PMA-stimulated PBMC and in PMA-stimulated NBC-13 cells. Incorporation of [*methyl*-^{3}H]thymidine was not detected in unstimulated, LPS-stimulated, or PMA-stimulated 24-h cultures of PBMC derived from either BLV-positive cattle with PL or BLV-negative cattle. Therefore, [*methyl*-^{3}H]thymidine incorporation was determined in 48-h cultures. In unstimulated 48-h cultures, spontaneous [*methyl*-^{3}H]thymidine incorporation occurred in PBMC from BLV-seropositive cattle with PL compared with BLV-seronegative cattle which remained at background. 3-methyl H7 inhibited spontaneous (unstimulated) blastogenesis of bovine PBMC derived from cattle affected with PL in a dose-dependent manner, whereas HA1004 increased [*methyl*-^{3}H]thymidine incorporation in these cultures (Fig. 3A). Increased incorporation of [*methyl*-^{3}H]thymidine (approximately five times the spontaneous level) occurred in 48-h LPS-stimulated cultures of PBMC from BLV-seropositive cattle with PL (Fig. 3A versus 3B); 3-methyl H7 slightly decreased and HA1004 slightly increased [*methyl*-^{3}H]thymidine incorporation in these cultures (Fig. 3B). PMA increased [*methyl*-^{3}H]thymidine incorporation to approximately three times the spontaneous level in 48-h cultures of BLV-infected PBMC (Fig. 3A versus 3C); 3-methyl H7 dramatically decreased [*methyl*-^{3}H]thymidine incorporation in PMA-stimulated PBMC cultures, and HA1004 had a similar but less dramatic effect (Fig. 3C). PMA increased [*methyl*-^{3}H]thymidine incorporation in NBC-13 cells approximately twofold (data not shown); however, neither 3-methyl H7 nor HA1004 had an observable effect on [*methyl*-^{3}H]thymidine incorporation in NBC-13 cells incubated with 1 nM PMA (Fig. 3D) or without PMA (data not shown).

Effect of PKC inhibition on BLV mRNA expression in bovine PBMC and NBC-13 cells. An RNase protection assay was used to quantitate BLV mRNA expression because of increased sensitivity compared with results of Northern (RNA) blot hybridization. Using this assay, expression of BLV could not be detected in freshly isolated PBMC derived from cattle with PL but could be detected in PBMC after as few as 3 to 4 h of incubation. As shown in a representative autoradiograph of the RNase protection assay (Fig. 4), in 6-h unstimulated PBMC cultures, 3-methyl H7 decreased both total BLV mRNA expression, as evident by a reduction of the *tax/rex* signal, and full-length BLV mRNA, as shown by a decrease in the *gag* signal. Using densitometric analysis, the BLV *tax/rex* signal was calculated relative to the bovine actin signal for each sample and expressed as the average percentage of control. The effect of 3-methyl H7 and HA1004 on total BLV mRNA expression (*tax/rex* signal) in 6-h unstimulated PBMC cultures obtained from four individual BLV-seropositive cows affected with PL is summarized in Fig. 5. 3-methyl H7 decreased total BLV mRNA expression in a dose-dependent manner (36% of control at 100 μM), whereas HA1004 increased total BLV mRNA expression (126% of control at 100 μM). Similar studies with 24-h PMA-stimulated NBC-13 cells showed that 100 μM 3-methyl H7 or HA1004 decreased total BLV mRNA expression to 24 and 75% of control, respectively.

DISCUSSION

The objective of the current study was to investigate the mechanism by which BLV expression is activated during in

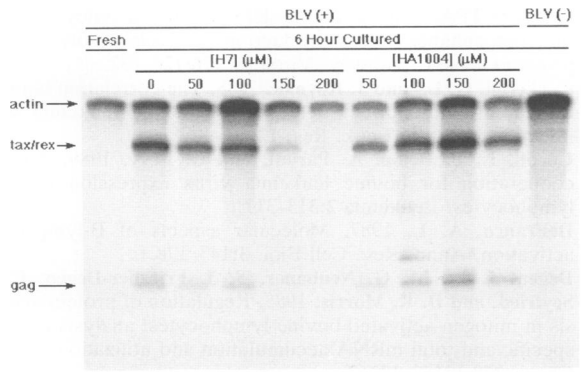

FIG. 4. RNase protection assay showing the effect of 3-methyl H7 (H7) and HA1004 on bovine actin mRNA, total BLV mRNA (*tax/rex* signal), and unspliced BLV mRNA (*gag* signal) expression in a 6-h culture of unstimulated PBMC from a BLV-seropositive cow with PL. Total cellular RNA (10 μg) was simultaneously hybridized with antisense bovine actin, BLV *tax/rex*, and BLV *gag* riboprobes. Uncultured (fresh) PBMC RNA from a BLV-seropositive cow with PL and cultured PBMC RNA from a BLV-seronegative cow [BLV(−)] were included as controls (far left and right lanes, respectively).

vitro incubation of infected PBMC. Specifically, we were interested in determining whether activation of PKC stimulated BLV expression and whether inhibition of PKC blocked expression of BLV. PBMC from BLV-seropositive cattle affected with PL were used in this study because BLV expression is more readily detected in cultured PBMC from these cattle (11). A likely explanation for this fact is that approximately 30% of PBMC from PL cattle are infected with BLV (33) compared with less than 5% in hematologically normal BLV-infected cattle (35, 36). Additionally,

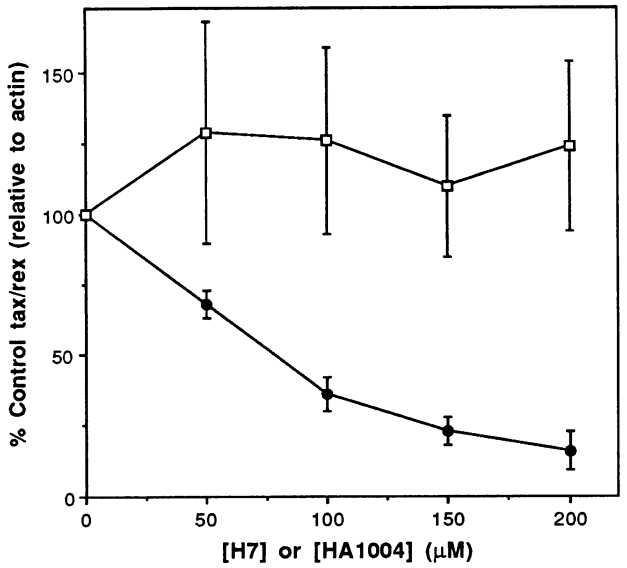

FIG. 5. Effect of 3-methyl H7 (●) and HA1004 (□) on total BLV mRNA expression in unstimulated PBMC from BLV-infected cattle. RNase protection assay autoradiographs were digitized for densitometric analysis. BLV *tax/rex* signals were calculated relative to the bovine actin signal for each sample and graphed as the average percentage of control obtained in separate experiments with four individual BLV-seropositive cattle with PL.

spontaneous blastogenesis is most pronounced in cultured PBMC from cattle with PL (59, 61). This spontaneous blastogenesis is thought to be an antigen-specific proliferative response to BLV antigens produced during short-term culture (57, 59). Our results support this hypothesis, since we demonstrated BLV gp51 and p24 in 24-h culture supernatants before increased spontaneous incorporation of [*methyl*-^{3}H]thymidine was detected.

Using the PKC inhibitor 3-methyl H7, we were able to block spontaneous [*methyl*-^{3}H]thymidine incorporation in 48-h cultures of bovine PBMC from PL cattle. In a dose-dependent manner, 3-methyl H7 also decreased expression of BLV gp51 and p24 to less than 50% of control in unstimulated 24-h cultures and reduced total BLV transcription to less than 25% of control in unstimulated 6-h cultures. From these results, it cannot be determined whether the effect of 3-methyl H7 on spontaneous blastogenesis is direct or indirect through decreased expression of BLV antigens. The effects of 3-methyl H7 on spontaneous [*methyl*-^{3}H]thymidine incorporation and BLV expression may occur in separate B-lymphocyte subpopulations, since it has been reported that B lymphocytes which spontaneously incorporate [*methyl*-^{3}H]thymidine can be separated by density gradient centrifugation from B lymphocytes expressing BLV (32). 3-methyl H7 likely inhibits spontaneous blastogenesis as a result of a combination of blocking of BLV expression in infected B lymphocytes with subsequent indirect inhibition of blastogenesis in BLV antigen-specific B lymphocytes and direct inhibition of blastogenesis in BLV antigen-specific lymphocytes exposed to residual antigen.

Effects of the PKC inhibitor 3-methyl H7 were further studied in BLV-infected cells stimulated with known PKC activators, including LPS and PMA, to better understand the relationship between virus expression and lymphocyte blastogenesis. Similar to the results in unstimulated PBMC cultures, 3-methyl H7 caused a dose-dependent decrease in BLV expression in LPS-stimulated 24-h PBMC cultures. In contrast to the effects of 3-methyl H7 on BLV expression, 3-methyl H7 only minimally decreased [*methyl*-^{3}H]thymidine incorporation in LPS-stimulated 48-h PBMC cultures. Although LPS has been shown to directly activate PKC (8, 62), activation of PKC does not explain all the effects of LPS (13, 29). It is probable that 3-methyl H7 only inhibits the LPS-induced responses that are mediated through PKC. In PMA-stimulated cultures, 3-methyl H7 caused a dose-dependent decrease in both [*methyl*-^{3}H]thymidine incorporation and BLV expression. Interestingly, the PKA and PKG inhibitor HA1004 also decreased [*methyl*-^{3}H]thymidine incorporation without decreasing BLV expression in PMA-stimulated PBMC cultures. In addition, our results demonstrate that activation of PKC with PMA induces BLV expression in the persistently BLV-infected B-lymphocyte cell line NBC-13 (21), which can be subsequently inhibited in a dose-dependent manner with 3-methyl H7 without affecting [*methyl*-^{3}H]thymidine incorporation. These results further suggest that the mechanism of 3-methyl H7-mediated inhibition of BLV expression is a direct effect of PKC inhibition rather than an indirect effect of the inhibition of DNA synthesis.

Because BLV mRNA is not readily detected in freshly isolated PBMC (30), activation of BLV expression would likely occur at the transcriptional rather than posttranslational level. Using an RNase protection assay, we have demonstrated that inhibition of PKC results in decreased total BLV transcription. However, these results do not exclude the possibility that PKC is also involved in activa-

tion of the *trans*-regulatory protein Rex and therefore affects mRNA splicing. In an HTLV-I-infected T-cell line, H7 has been reported to block accumulation of unspliced HTLV-I mRNA (1), however, in contrast to our results with BLV, the total amount of viral mRNA did not appear to decrease.

These results identify the involvement of PKC in the initiation of BLV expression in short-term PBMC cultures and PMA-stimulated NBC-13 cells and support the hypothesis that expression of BLV occurs subsequent to activation of PKC when infected B lymphocytes undergo blastogenesis. In vivo, BLV would remain latent until the infected B lymphocyte is exposed to antigen specific for its membrane immunoglobulin. Antigen-induced blastogenesis of the BLV-infected B lymphocyte would activate PKC and initiate expression of BLV. Expression of BLV would subsequently cause production of viral particles, infection of naive B lymphocytes, stimulation of the host immune response, and finally elimination of the BLV-expressing cell because of the presence of cytotoxic anti-BLV antibodies (49) or other yet-to-be-identified effector immune mechanisms. Continual or intermittent rounds of low-level antigen production (below the level of detection) would explain the apparent absence of antigenemia, low levels of mRNA production (30), and persistent high-titered antiviral antibody response in BLV-infected animals. The results of this study have significant implications for the mechanism of induction of BLV expression and for the mechanism of viral latency in BLV infection. The induction of BLV expression by activation of PKC indicates that replication of this oncogenic retrovirus is controlled by normal B-lymphocyte intracellular signaling pathways.

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