High Expression of β-Adrenergic Receptor Kinase in Human Peripheral Blood Leukocytes

ISOPROTERENOL AND PLATELET ACTIVATING FACTOR CAN INDUCE KINASE TRANSLOCATION*

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Receptor phosphorylation is a key step in the process of desensitization of the β-adrenergic and other related receptors. A selective kinase (called β-adrenergic receptor kinase, βARK) has been identified which phosphorylates the agonist-occupied form of the receptor. Recently the bovine βARK cDNA has been cloned and the highest levels of specific mRNA were found in highly innervated tissues. It was proposed that βARK may be primarily active on synaptic receptors. In the present study, the cDNA of human βARK was cloned and sequenced. The sequence was very similar to that of the bovine βARK (the overall amino acid homology was 98%). Very high levels of βARK mRNA and kinase activity were found in peripheral blood leukocytes and in several myeloid and lymphoid leukemia cell lines. Since agonist-induced βARK translocation is considered the first step involved in βARK-mediated homologous desensitization, we screened a number of G-protein-coupled receptor agonists for their ability to induce βARK translocation. In human mononuclear leukocytes, β-AR agonist isoproterenol and platelet-activating factor were able to induce translocation of βARK from cytosol to membrane. After 20 min of exposure to isoproterenol (10 μM), the cytosolic βARK activity decreased to 61% of control, while membrane-associated βARK activity increased to 170%. 20-min exposure to platelet-activating factor (1 μM) reduced the cytosolic βARK activity to 42% of control with concomitant increase in membrane βARK activity to 214% of control. The high levels of βARK expression in human peripheral blood leukocytes together with the ability of isoproterenol and platelet-activating factor to induce βARK translocation, suggest a role for βARK in modulating some receptor-mediated immune functions.

Exposure of cells containing β-adrenergic receptors (βAR)[1] to β-adrenergic agonists results in rapid and reversible loss of the receptor-mediated response to subsequent stimulation (homologous desensitization) (1). A number of mechanisms underlying homologous desensitization have been well characterized *in vitro*. They include receptor phosphorylation, uncoupling from G_s, and sequestration (1). It is now accepted that receptor phosphorylation is a key step in the process of receptor desensitization (1). A selective kinase (called β-adrenergic receptor kinase, βARK) has been identified (2–8) which phosphorylates the agonist-occupied form of the receptor. Receptor phosphorylation by βARK requires an additional cytosolic factor, called β-arrestin (9), to induce complete homologous desensitization. βARK phosphorylates in an agonist-dependent manner not only βAR but also some other G-coupled receptors: the $α_2$-adrenergic receptor (3), muscarinic cholinergic receptors (4), and, to a lesser extent, rhodopsin (2). Additionally, isoproterenol, prostaglandin E_1 and somatostatin (6, 10) induce translocation of βARK from cytosol to membrane, suggesting that their receptors are also substrates for βARK phosphorylation. Recently, the bovine βARK cDNA has been cloned (5) and the highest levels of specific mRNA were found in highly innervated tissues (5). It was proposed that βARK may be primarily active on synaptic receptors (1).

In the present study, the cDNA for human βARK was cloned and sequenced. High levels of βARK expression were observed in human peripheral blood leukocytes (PBL), which, together with the ability of isoproterenol and platelet-activating factor (PAF) to induce βARK translocation, suggests a role for βARK in modulating some receptor-mediated immune functions.

MATERIAL AND METHODS

Polymerase Chain Reaction Cloning—Forward (F) and reverse (R) primers used for PCR were synthesized using a Beckman System 200A DNA synthesizer. Their positions on the βARK sequence are shown in Fig. 1. With the exception of F1, all were nondegenerate oligonucleotides. Where convenient, the F primers did not include the degenerate base(s) of the 3' codon, so as to minimize 3' end mismatches. F1, F2, F3, F5, R1, R2, R3 were complementary to the bovine sequence (5), while F4 and R4 were complementary to the human sequence obtained from previously sequenced PCR products. The cDNA fragments cloned are: F1–R4; F2–R3; F3–R3; F4–R2; F5–R2; F5–R1. The primer F1 used to clone the 5' end (fragment F1–

[1] Abbreviations used are: βAR, β-adrenergic receptors; βARK, β-adrenergic receptor kinase; PCR, polymerase chain reaction (F, forward; R, reverse primers); PBL, peripheral blood leukocytes (granulocytes + lymphocytes + monocytes); MNL, mononuclear leukocytes (lymphocytes + monocytes); SDS, sodium dodecyl sulfate; ROS, rod outer segments; PKI, protein kinase A inhibitor; H7, 1,5-(isoquinolinesulfonyl)-2-methylpiperazine; PAF, platelet-activating factor; bp, base pair(s); PBS, phosphate-buffered saline; kb, kilobase(s).
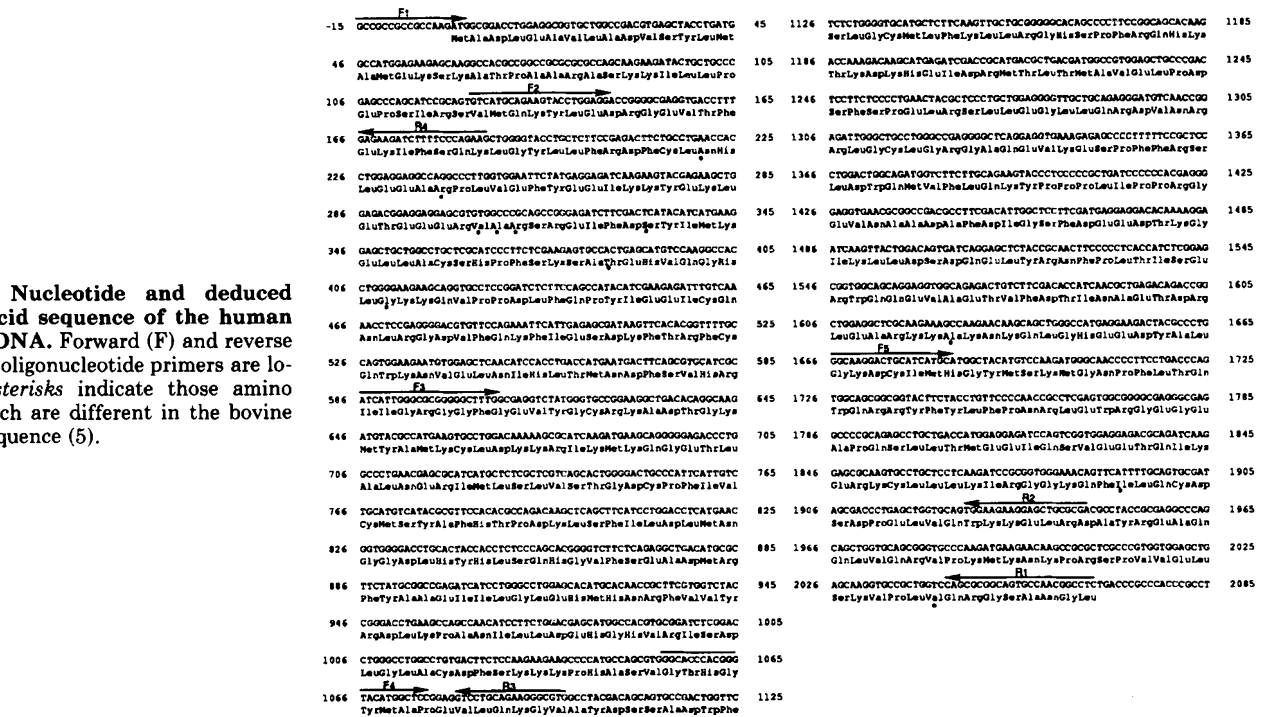
FIG. 1. **Nucleotide and deduced amino acid sequence of the human βARK cDNA.** Forward (F) and reverse (R) PCR oligonucleotide primers are located. *Asterisks* indicate those amino acids which are different in the bovine βARK sequence (5).

R4), was degenerated as follows: GCC *X* CC GCC *X* CC AAG ATG (*X* = A,C,G,T). It corresponds to bp −15 to 3 of bovine βARK, with the ATG at the F1 3′ end corresponding to the first methionine of the coding sequence.

PCR reactions were carried out according to Ref. 11 with minor modifications. To obtain the first cDNA strand, 1 μg of poly(A)⁺ RNA (in a few cases total RNA) from mononuclear leukocytes (MNL) was reverse-transcribed using random hexamers. The cDNA was amplified in 100 μl of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin), 0.8 μg each of F and R primers, 200 μM each of the dNTPs, 2.5 units of *Thermus acquaticus* DNA polymerase (Amplitaq, Perkin-Elmer/Cetus). PCR cycles were modified for our cloning strategy. PCR was performed for 36 cycles, with 1 min of denaturation at 94 °C, annealing for 1 min at various temperatures (see below), and 4 min extension at 72 °C. The annealing was done at 42 °C for the first three cycles (low stringency PCR) and increased stepwise to 47 °C (three cycles) and then to 55 °C (30 cycles, high stringency PCR). This protocol drastically reduced background products when compared with annealing performed at 42 °C for all the cycles (not shown). The amplification finished with 10 min at 72 °C to generate blunt-end products. PCR products were subcloned blunt-end in PTZ18R and used for sequencing.

cDNA Library Screening—A human pituitary cDNA library in λ-bluemid (cloning site *Eco* RI and *Hind*III; Clontech, Palo Alto, CA), containing 1.5 × 10⁶ recombinants was screened (12). Two PCR products from bp 1666 to 2067 (F5-R1 in Fig. 1) and bp 1 to 705, labeled by random priming (Amersham International kit), were used as a probe for two independent screenings. Screenings were done under high stringency conditions (12).

DNA Sequencing—Nucleotide sequence analysis of both strands was performed by the dideoxynucleotide chain termination method, by primer extensions with T7 DNA polymerase (United States Biochemicals or Pharmacia LKB Biotechnology), using as template plasmid DNA prepared according to Maniatis *et al.* (12). Each clone was sequenced in both directions.

Human Tissue and Cell Sources—Macroscopically normal tissues, obtained from surgically excised samples, were rapidly frozen at −70 °C. Heart samples were papillary muscle from open heart surgery for mitral valve disease; pericardic adipose tissue was from patients undergoing myocardial transplantation for idiopathic congestive cardiomyopathy; lung and liver samples were taken 6–8 cm away from the periphery of tumor tissue, usually in a different lobe. Cultured cells (American Tissue Culture Collection), growing under standard conditions in appropriate media supplemented with 5–10% fetal bovine serum, were harvested directly in guanidinium isothiocyanate.

PBL from healthy volunteers, were fractionated as previously described (13). Briefly, MNL were isolated by Ficoll gradient, and in some cases they were further fractionated into lymphocytes and monocytes by a Percoll gradient. Granulocytes were isolated from erythrocytes by Percoll gradient.

Northern Blot Analysis—Total RNA was isolated by the guanidinium isothiocyanate/cesium chloride method (12). Total RNA (20 μg) was fractionated on a 1% agarose-formaldehyde gel and transferred to a GeneScreen Plus membrane (Du Pont-New England Nuclear). The RNA blot was hybridized with a random primed radioactive cDNA fragment (bp 1055–1946) in 50% formamide, 10% dextran sulphate, 1% SDS, 5.8% NaCl, and denatured salmon sperm DNA (100 μg/ml) for 24 h at 42 °C. The blot was washed in 2 × SSC, 1% SDS at 60 °C, 0.1 × SSC at room temperature and was subjected to autoradiography overnight at −80 °C. All the results were confirmed on RNA from at least two different individuals.

Preparation of Cytosolic and Membrane Fractions for Assay of βARK—The method used was based on that described previously (5). Briefly, cells or tissue fragments were pelleted by centrifugation (800 × g for 5 min), lysed in cell lysis buffer (10 mM Tris, 5 mM EDTA, 7.5 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 10 μg/ml benzamidine, at pH 7.4) using a polytron tissue disruptor (Janke and Kundel) at low speed for 1 min on ice. Unbroken cells and cell nuclei were pelleted by centrifugation (800 × g for 5 min) and discarded. The supernatant was then centrifuged at 48,000 × g for 20 min at 4 °C to separate the plasma membrane and cytosolic fraction, the latter was then centrifuged at 150,000 × g for 60 min at 4 °C. The protein content of the resultant supernatant was measured using Bio-Rad protein assay reagent, and its βARK activity was assayed on the same day (cytosolic βARK assay). Membrane preparation was washed once in cell lysis buffer, recentrifuged at 48,000 × g for 15 min at 4 °C, the resultant membrane pellet resuspended in cell lysis buffer, sonicated for 5 s prior to protein determination.

Bovine Rod Outer Segments Preparation—Previous studies demonstrated that rhodopsin contained in the ROS of bovine retina can be phosphorylated by βARK in a light-dependent manner (2), and that it can be used to investigate βARK activity quantitatively (6). Thus, rhodopsin was used in the present study to measure and compare βARK activity of various cell and tissue preparations. ROS were prepared from bovine retina by stepwise sucrose gradient sedimentation using a method described in Ref. 8. Retinal rhodopsin kinase was degraded by treatment with 5 M urea (14).

Phosphorylation Assay—The phosphorylation reaction was based on that previously described in Ref. 6. Briefly, in each reaction, 100

μg of total cytosolic soluble protein or membrane protein was added to a reaction mixture containing 300 pmol of urea-treated ROS, 50 μM [γ-^{32}P]ATP (2–5 cpm/fmol), 20 mM Tris, 8 mM MgCl$_2$, 3 mM EDTA, 5 mM NaF, 12 mM NaCl, 0.07 mM phenylmethylsulfonyl fluoride, 7 μg/ml leupeptin, 3.5 μg/ml pepstatin, 7 μg/ml benzamidine, at pH 7.4 and a total volume of 150 μl. The reaction was carried out at 30 °C in the presence (or absence) of light for 45 min. The reaction was stopped by the addition of 900 μl of an ice-cold solution containing 10 mM Tris, 100 mM NaCl, 10 mM NaF, 2 mM EDTA, followed by centrifugation at 57,000 × g for 15 min at 4 °C. The resultant pellet was reconstituted in SDS sample buffer and electrophoresed on 10% SDS-polyacrylamide gel.

βARK Translocation Assay—Freshly prepared MNL (2 × 10^6 cells/ml) were incubated in RPMI 1640 with 1% glutamate, 1% penicillin/streptomycin at 37 °C (pH 7.4) without serum for 30 min. Various agonists were then added at different concentrations and incubated at 37 °C for the indicated times. At the end of reaction, ice-cold PBS was added, cells were pelleted, and crude βARK was prepared as described.

SDS-Polyacrylamide Gel Electrophoresis—This was carried out using a method previously described (15). Following electrophoresis on a 10% homogeneous slab gel, the gel was stained for protein with Coomassie Blue. Following destaining, the gel was dried and subjected to autoradiography. For a quantitative measurement of βARK activity, two methods were used: (1) rhodopsin bands (molecular mass ~35 kDa) identified by Coomassie Blue staining, were cut and counted for ^{32}P radioactivity; (2) measurement of relative density of rhodopsin bands imprinted on the autoradiographic films by densitometry (RAS, Amersham).

Materials—PCR Amplitaq DNA polymerase was obtained from Perkin-Elmer/Cetus; deoxynucleotides used for PCR were from Pharmacia LKB Biotechnology Inc.; the modifying enzymes end restriction endonucleases were from Bethesda Research Laboratories, from Pharmacia, and from Boehringer Mannheim. The DNA probes for hybridization were prepared with the Amersham random priming kit. The human cDNA library was purchased from Clontech Laboratories Inc. (Palo Alto, CA). The hybridization filters were purchased from Du Pont-New England Nuclear. ^{35}S- or ^{32}P-labeled dATP and dCTP were purchased from Amersham Corp. PKI (P3294), heparin (H-3125) from porcine intestinal mucosa, vasoactive intestinal peptide, and complement 5a were purchased from Sigma; 1,5-(isoquinolinesulfonyl)-2-methylpiperazine (designated H7) and staurosporin were from Calbiochem; PAF was purchased from Bachem (Bubendorf, Switzerland); BN52021 is a gift of Dr. P. Braquet (Institute Henri Beaufour, Le Plesis-Robensir, France); [^{32}P]ATP was from Du Pont-New England Nuclear. All gel electrophoresis materials were purchased from Bio-Rad.

RESULTS

Cloning and Sequencing of Human βARK—The human βARK cDNA was cloned by PCR, with F and R primers complementary to the bovine cDNA sequence (5). To account for possible PCR errors, most of the sequence was confirmed in clones obtained from at least two different PCR amplifications. Most of these clones were also overlapping, so that the sequence of the oligos used for PCR was confirmed in clones spanning these regions. The 3' region was obtained by screening a human pituitary cDNA library using two PCR products as probes. Two βARK cDNA fragments (approximately 1.3 and 2.8 kb in size) were cloned and sequenced from bp 153 to the first 30 bp in the untranslated region (Fig. 1). The human βARK cDNA sequence obtained displayed a very high similarity with the bovine cDNA (93% identity). The overall amino acid identity was 98% (Fig. 1).

βARK mRNA Tissue Distribution—To examine mRNA distribution in human tissues and cells, the 891-bp fragment F4–R2 was used as a probe for Northern blot analysis. Similar to what was observed in bovine tissues, we found a major mRNA species 4 kb in size with, in some tissues, an additional species of 2.4 kb (Fig. 2). As previously reported for bovine, heart and lung showed moderate to low levels of βARK mRNA, while it was not detectable in liver and adipose tissue (Fig. 2). By contrast, βARK-specific transcript was very abundant in
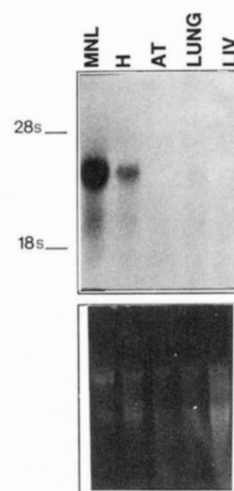

FIG. 2. **Northern blot analysis of human mRNA from various tissues.** Total RNA (20 μg) from MNL, heart (*H*), adipose tissue (*AT*), lung, and liver (*LIV*) was probed with random-primed βARK cDNA fragment bp 1055–1946. Washed filters were exposed at −80 °C for 18–20 h. Data represent three separate experiments. The *lower panel* shows the ribosomal RNA staining in the gel prior to transfer.
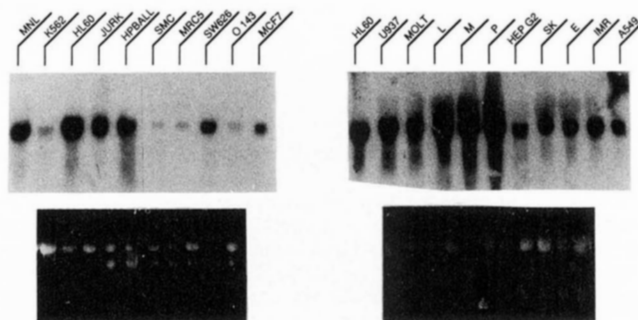

FIG. 3. **Northern blot analysis of mRNA from various human cell types.** Two separate blots are shown. Total cellular RNA (20 μg) from MNL, K562, HL60, Jurkat (*JURK*), HPB-ALL, smooth muscle cells (*SMC*), MRC5, SW626, O 143, MCF7 (*blot on the left*), and U937, MOLT4 (*MOLT*), peripheral blood lymphocytes (*L*), monocytes (*M*), and granulocytes (*P*), HepG2, SK-HEP-1 (*SK*), endothelial cells (*E*), IMR 32 (*IMR*), and A549 (*blot on the right*), was probed with random-primed βARK cDNA fragment bp 1055–1946. Washed filters were exposed at −80 °C for 18–20 h. Data represent three separate experiments. The *lower panel* shows the ribosomal RNA staining in the gel prior to transfer.

MNL (approximately 4–5-fold higher than in heart, as quantitated by densitometric analysis of the autoradiogram). Such an unexpected finding suggested a preferential expression of βARK in immunocompetent cells. This idea was further supported by Northern blot analysis of several different cultured cell types (Fig. 3). We compared 16 different human cell types: four lymphoid leukemia cell lines (HPB-ALL, U937, MOLT4, Jurkat), one myeloid leukemia (HL60), one erythroid leukemia (K562), two hepatomas (Hep G2 and SK-HEP-1), and one each: embryonic lung fibroblasts (MRC5), lung carcinoma (A549), neuroblastoma (IMR-32), breast adenocarcinoma (MCF7), ovarian carcinoma (SW626), osteosarcoma (O 143), endothelial cells (4th passage), smooth muscle cells (25th–27th passage, SMC). A detectable βARK mRNA was found in all the cell types, and the highest levels of expression were observed in all the lymphoid and myeloid cell lines (Fig. 3). In these blots, the level of mRNA in PBL, used as internal control, was within the range of lymphomyeloid cell lines (Fig. 3). Direct comparison of βARK expression between human

PBL and brain (these are the two tissues with the highest levels of βARK mRNA) was not possible. In fact, several experiments with fragments from surgically excised brain samples never yielded RNA of quality good enough to be used for Northern blot analysis. To overcome this problem, βARK expression was compared in mRNA from bovine PBL and brain (Fig. 4). The level of βARK expression in bovine PBL was found to be slightly higher than in the brain (Fig. 4). Bovine brain RNA used in these experiments was obtained from frontal cortex.

βARK Activity Tissue Distribution—In order to study whether the high levels of specific mRNA present in MNL are indeed translated into high concentrations of βARK in these cells, a biochemical assay was used to measure the βARK activity in different cells and tissues. According to previous studies (6), bovine rhodopsin was used as specific substrate for phosphorylation. Under our experimental conditions, in the presence of soluble proteins from MNL, rhodopsin was highly phosphorylated (Fig. 5). The kinase activity calculated from four different individuals was 10.0 ± 1.5 fmol/min/0.1 mg soluble proteins (mean $\pm$ S.E.). Rhodopsin phosphorylation was agonist (light) dependent and it was inhibited by heparin in a dose-dependent manner (Fig. 5). It was not blocked by protein kinase C inhibitor staurosporine (10–100

FIG. 4. **Northern blot analysis of bovine tissue mRNA.** Total RNA (20 μg) from PBL and brain, was probed with random-primed βARK cDNA fragment bp 1055–1946. Washed filters were exposed at −80 °C for 16 h. Data represent two separate experiments. The *right panel* shows the ribosomal RNA staining in the gel prior to transfer.

nM, not shown) and protein kinase A inhibitor PKI (1 μM) (Fig. 5). As previously shown (16), the less specific protein kinase C inhibitor H7 (10–100 μM), at the highest concentration used, slightly inhibited rhodopsin phosphorylation (Fig. 5, *lane h*). No such phosphorylation was observed when ROS- or MNL-soluble proteins were incubated separately under identical conditions (Fig. 5). These biochemical features repeat those previously described for bovine brain βARK (7, 16), suggesting that our experimental conditions are suitable for measuring βARK activity. Comparison was then made of the relative expression of βARK activity in MNL, SMC, Jurkat cells, and liver and lung tissues (Fig. 6). The highest activity was found in MNL and Jurkat cells with little or no detectable activity in SMC and liver and lung tissues (Fig. 6). This pattern of βARK activity closely resembles the relative amount of mRNA expression in these cells and tissues.

Agonist-induced βARK Translocation—Since agonist-induced βARK translocation is considered the first step involved in βARK-mediated homologous desensitization (10), we screened a number of G-protein coupled receptor agonists for their ability to induce βARK translocation in human MNL. Isoproterenol (10 μM), the βAR agonist, induced translocation of cytosolic βARK; after 20 min of exposure to isoproterenol, the cytosolic βARK activity decreased to 60.7 $\pm$ 6.3% ($n = 4$) of control (Fig. 7), while membrane-associated βARK activity increased to 170% ($n = 2$) (not shown). This effect of isoproterenol has previously been observed in S49 and DDT$_1$ MF-2 smooth muscle cells (6, 10). Although this is a novel observation so far as the human immune cell is concerned, we searched for other receptors that were also able to induce βARK translocation. For this purpose, VIP, C5a, and PAF were chosen for having direct effects or their receptors present on MNL. Five and twenty minutes were chosen as incubation times for inducing translocation. Neither VIP (0.5 μM) nor C5a (100 nM) had any observable effect (data not shown), while PAF was able to induce translocation in a time- and dose-dependent manner (Figs. 8–10). At 20 and 40 min, PAF (1 μM) reduced the cytosolic βARK activity to 41.9
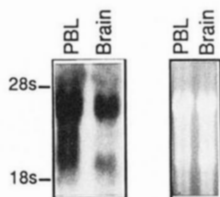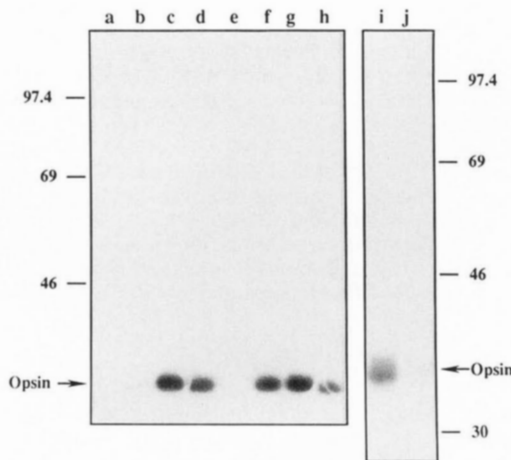

FIG. 5. **Biochemical characterization of MNL βARK activity.** MNL soluble proteins (100 μg) were added to 300 pmol of urea-treated ROS and incubated for 45 min at 30 °C in phosphorylation buffer. Samples were then centrifuged at 57,000 × g for 15 min, and the pellet was resuspended in SDS buffer and electrophoresed on a 10% SDS-polyacrylamide gel. Autoradiography of dried gels was for 1–2 h at −70 °C. Two independent experiments are shown. Different lanes are: reactions with no MNL cell extract (*lane a*) or no ROS (*lane b*) added to phosphorylation mixture; *lane c*: control; *lanes d* and *e*: 1 and 10 μg/ml heparin, respectively; *lane f*: 1 μM PKI; *lanes g* and *h*: 10 and 100 μM H7, respectively; *lanes i* and *j*: phosphorylation reactions carried out in the presence and absence of light, respectively. Data represent at least two separate experiments.
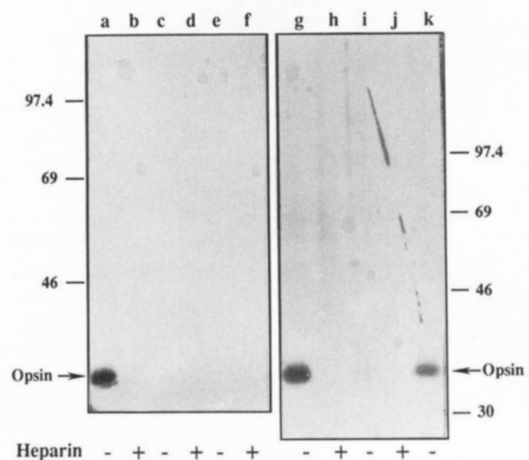

FIG. 6. **Relative tissue and cell distribution of βARK activity.** Soluble proteins (100 μg) from cells or tissues were added to 300 pmol of urea-treated ROS in the presence (+) or absence (−) of 10 μg/ml heparin and incubated for 45 min at 30 °C in phosphorylation buffer. Samples were then centrifuged at 57,000 × g for 15 min and the pellet was resuspended in SDS buffer and electrophoresed on a 10% SDS-polyacrylamide gel. Autoradiography of dried gels was for 1–2 h at −70 °C. Two independent experiments are shown. In the *left* gel, βARK activity was compared in MNL (*lanes a* and *b*), liver (*lanes c* and *d*), and lung tissue (*lanes e* and *f*). In the right gel βARK activity was compared in MNL (*lanes g* and *h*), SMC (*lanes i* and *j*), and Jurkat cells (*lane k*). Data represent at least two separate experiments.
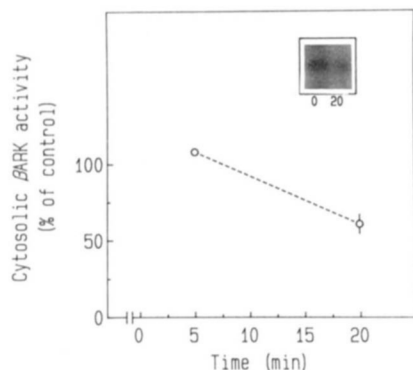
FIG. 7. **Time-dependent reduction of cytosolic βARK activity in MNL exposed to isoproterenol (10 μM).** Freshly prepared MNL were exposed to isoproterenol for 0, 5, and 20 min, and then ice-cold PBS was added and the cells pelleted. Cell pellets were resuspended in cell lysis buffer and were sonicated, and unbroken cells and debris removed by centrifugation. The supernatant was centrifuged at 48,000 × g for 20 min to separate the membrane and the cytosolic fractions. The latter was then centrifuged at 150,000 × g for 60 min at 4 °C; the resultant supernatant contained the crude βARK preparation, which was added to the phosphorylation assay at a quantity of 100 μg of total protein and processed as described. βARK activity is expressed as % of control (100%). Data represent two to five experiments. Insert shows ROS bands on autoradiographic film from one representative experiment.
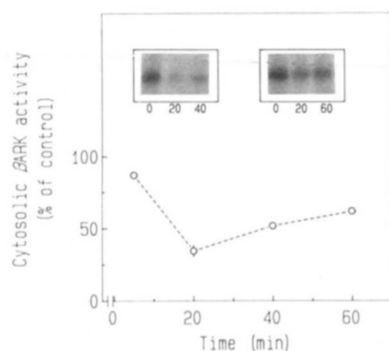

FIG. 8. **Time-dependent reduction of cytosolic βARK activity in MNL exposed to PAF (1 μM).** Freshly prepared MNL were exposed to PAF for the indicated times and then ice-cold PBS was added and the cells were pelleted. Crude βARK preparation was done as described in Fig. 7. βARK activity is expressed as % of control (100%). Data represent two to five experiments. Inserts show ROS bands on autoradiographic film from two independent time courses.

± 5.4% (n = 7) and 52.0% (n = 2) of control, respectively (Fig. 8), with concomitant increases in membrane bound βARK activity to 214 and 171% of control at the same time points (n = 2) (Fig. 9). In addition, 20 μM of the PAF receptor antagonist BN52021 (17) blocked the effects of 1 μM PAF (Fig. 10).

DISCUSSION

The cDNA of human βARK was cloned and sequenced. The sequence is very similar to that of the bovine βARK (the overall amino acid homology is 98%) showing very high interspecies conservation. This feature is common to several other kinases and is likely to be due to the relevant functions of such enzymes. A 891-bp fragment of cDNA was used for Northern blot analysis of mRNA from different tissues and cells (Figs. 2 and 3). The most striking finding of this study was that βARK mRNA is expressed in noninnervated cells, with very high levels of expression in PBL. In parallel, a very high βARK activity was found in MNL (Figs. 5 and 6), suggesting that these high mRNA levels are indeed translated
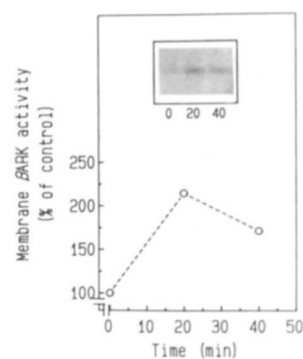

FIG. 9. **Time-dependent increase in membrane associated βARK activity in MNL exposed to PAF (1 μM).** Freshly prepared MNL were exposed to PAF for the indicated times, and then ice-cold PBS was added and the cells were pelleted. Following sonication in cell lysis buffer, unbroken cells and cell debris removed, membrane preparation was obtained by centrifugation at 48,000 × g for 20 min at 4 °C. After washing in cell lysis buffer, 100 μg of total membrane protein was added to the phosphorylation reaction as described. βARK activity is expressed as % of control (100 %). Data represent two experiments. *Insert* shows ROS bands on autoradiographic film from one representative experiment.
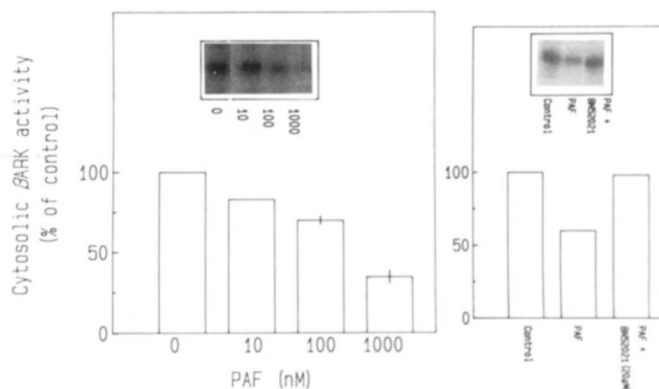

FIG. 10. **Reduction of cytosolic βARK activity in MNL exposed to PAF.** Two independent sets of experiments are shown. *Left*, dose-dependent reduction of cytosolic βARK activity in MNL induced by PAF. Freshly prepared MNL were exposed to different doses of PAF for 20 min, and crude βARK preparation was done as described in Fig. 7. Data are means ± S.E. of two to five separate experiments. *Right*, inhibition of PAF-induced βARK translocation by the inhibitor BN52021. MNL were incubated for 20 min with buffer (control), 1 μM PAF or 1 μM PAF plus 20 μM BN52021. The latter group had been preincubated with BN52021 for 30 min. Data are means of two separate experiments. βARK activity is expressed as % of control (100%). Respective *inserts* show ROS bands on autoradiographic films from respective representative experiments.

into a high concentration of βARK.

The presence of such a high level of βARK expression in PBL suggests a functional role for this kinase in these cells. Indeed, in MNL, βAR agonist isoproterenol and PAF were able to induce translocation of βARK from cytosol to the membrane, which appears to be the first step in homologous desensitization (10). That isoproterenol was able to induce translocation of βARK came as no surprise since βAR has been shown to be a substrate for βARK (10), and its activation induced βARK translocation, albeit in different cell types (6, 10). Since catecholamines have been shown to have numerous modulatory effects on immune cells (18), our observation suggests that βARK is directly involved in the catecholaminergic regulation of immune functions.

PAF is a phospholipid with diverse potent effects, ranging from regulation of platelets, lymphocytes, monocytes, granu-

locytes, to pathological responses such as in asthma and allergy (for review see Ref. 19). PAF receptor has recently been cloned and shown to be a member of the G-protein-coupled receptor family, with multiple serine and threonine residues in the C-terminal region as potential phosphorylation sites (20), and PAF has been shown to induce homologous desensitization (21, 22). Protein kinase C has been implicated to be only partially responsible for PAF-induced homologous desensitization (23), which is in line with the presence of protein kinase C phosphorylation site sequence motifs in the C-terminal region of PAF receptor. The present observation of PAF-induced βARK translocation in MNL suggests a role for βARK as well. The situation here may be analogous to that of βAR desensitization, *i.e.* βARK-mediated phosphorylation is responsive to receptor activation at high agonist concentrations (24), since we observed βARK translocation only at PAF concentrations of 10–100 nM or more. Thus, the notion that PAF receptor and βAR in MNL act as substrates for βARK, backed by the high levels of βARK expression in these cells, directly supports the claim of a role for βARK in human immune functions.

In the light of the present findings, the working hypothesis that βARK may be active only at the synapses level (1, 5), need to be expanded. We suggest that, in addition to the synaptic receptors, βARK may be a potent modulator of at least some receptor-mediated immune functions.

In this regard, the recent cloning of some peptide hormone receptors (25, 26), thrombin receptor (27), chemotactic receptor for formyl peptide fMet-Leu-Phe (28), interleukin-8 receptor (30, 31) (all seven-membrane spanning domain receptors) provide further evidence that the number of cellular functions which are mediated by G-coupled receptors is far larger than previously expected. These receptors appear to be good candidates for βARK regulation as they do contain C-terminal tails rich in serine and threonine residues, potential phosphorylation sites for βARK (29). Additionally, at least some of them (*i.e.* thrombin and formyl peptide fMet-Leu-Phe receptors) became desensitized after exposure to their respective agonists (homologous desensitization) (27, 32). Therefore, it is possible that βARK may serve to regulate a wider spectrum of immune cell receptors, in addition to PAF and β-adrenergic receptors.

An additional point of interest raised by the present observation of PAF-induced βARK translocation concerns the nature of βARK receptor substrates; PAF-receptor activation is shown to be coupled to the activation of phospholipase C, while all the known receptor substrates for βARK identified so far are coupled to adenylate cyclase, namely βAR, muscarinic acetylcholine receptor, α_2-adrenergic receptor, somatostatin, and prostaglandin E_1 receptors. While it has been suggested that βARK phosphorylates only the adenylate cyclase-related G-protein-coupled receptors, no βARK translocation factor has yet been identified. Thus, it remains possible that the nature of the second messenger system may not necessarily reflect the ability of one receptor to induce translocation of βARK.

The analysis of βARK transcript from different human cells and tissues raises several points of potential interest (Fig. 3). First, a detectable amount of βARK transcript was found in all of the tested cells, whereas it was not observed in several tissues or organs (including liver, muscle, and adipose tissue (Ref. 5 and present study)). Second, βARK transcript, although undetectable in normal liver, was quite abundant in two differentiated hepatoma cell lines (Hep G2 and SK-Hep1). Third, as expected for nonlymphomyeloid cells, the highest levels of expression were found in neuroblastoma cells

(IMR-32). Finally, βARK transcript, highly abundant in all of the lymphoid and myeloid cell lines tested, was poorly expressed in K562 cells, which are highly undifferentiated blasts sharing some characteristics of the erythroid lineage. Several questions raised by these observations are presently under investigation in our laboratory.

This study also demonstrates that MNL contain high levels of βARK activity which can be easily detected by a phosphorylation assay using bovine ROS as a substrate. This assay thus provides a useful tool for further investigation of βARK activity in PBL in physiological and pathological conditions in humans.

During the preparation of this manuscript, two papers were published which are related to the present work (33, 34). The first shows the sequence of human βARK cDNA as obtained by screening a human retinal library. The coding region of the two sequences (Ref. 33 and present paper) differed only in bps 631, 1265, 1394, and 1716. The second paper (34) reported on the cloning of a second bovine subtype of βARK (referred to as βARK 2). Based on the relative homology with the two bovine βARK subtypes (5, 34), the present sequence must be considered as the human form of the βARK 1. Additionally, since βARK 2 was five times less efficient than βARK 1 in phosphorylating rhodopsin (34), we are confident that our kinase assay, based on rhodopsin phosphorylation, mostly measures βARK 1 activity.

In conclusion, we provide here the first evidence for a high level of human βARK expression and activity in PBL. With the support of the data on isoproterenol and PAF induced βARK translocation in MNL, we suggest that βARK plays a role in modulating at least some receptor-mediated immune functions.

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