Regulation of G protein-coupled receptor kinase subtypes in activated T lymphocytes. Selective increase of beta-adrenergic receptor kinase 1 and 2.

A De Blasi, … , G Parruti, M Sallese

Research Article

Beta-adrenergic receptor kinase (beta ARK) is a serine-threonine kinase involved in the process of homologous desensitization of G-coupled receptors. beta ARK is a member of a multigene family, consisting of six known subtypes, also named G protein-coupled receptor kinases (GRK 1-6). In this study we investigated the expression of GRKs during the process of T cell activation, which is of fundamental importance in regulating immune responses. T cell activation was induced by exposing mononuclear leukocytes (MNL) to PHA and confirmed by tritiated thymidine incorporation measurement. A substantial increase of GRK activity (as measured by in vitro phosphorylation of rhodopsin) was found after 48 h (331 +/- 80% of controls) and 72 h (347 +/- 86% of controls) of exposure to PHA. A threefold increase of beta ARK1 immunoreactivity was found in MNL exposed to PHA for 72 h. Persistent activation of protein kinase C (PKC) by 10 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) was able to increase beta ARK activity to the same extent as PHA, suggesting a PKC-mediated mechanism. The kinetic of beta-adrenergic-stimulated cAMP production was substantially modified in TPA and PHA-activated cells, indicating that the increased GRK activity resulted in an increased beta-adrenergic homologous desensitization. A three- to fourfold increase in GRK activity was also observed in a population of T cell blasts (> 97% CD3+) exposed to PHA for 48-72 […]

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Regulation of G Protein-coupled Receptor Kinase Subtypes in Activated T Lymphocytes

Selective Increase of β-Adrenergic Receptor Kinase 1 and 2

Antonio De Blasi, Giustino Parruti, and Michele Sallese

Consorzio Mario Negri Sud, Istituto di Ricerche Farmacologiche "Mario Negri," 66030 Santa Maria Imbaro, Italy

Abstract

β-adrenergic receptor kinase (βARK) is a serine–threonine kinase involved in the process of homologous desensitization of G-coupled receptors. βARK is a member of a multigene family, consisting of six known subtypes, also named G protein-coupled receptor kinases (GRK 1–6). In this study we investigated the expression of GRKs during the process of T cell activation, which is of fundamental importance in regulating immune responses. T cell activation was induced by exposing mononuclear leukocytes (MNL) to PHA and confirmed by tritiated thymidine incorporation measurement. A substantial increase of GRK activity (as measured by in vitro phosphorylation of rhodopsin) was found after 48 h (331±80% of controls) and 72 h (347±86% of controls) of exposure to PHA. A threefold increase of βARK1 immunoreactivity was found in MNL exposed to PHA for 72 h. Persistent activation of protein kinase C (PKC) by 10 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was able to increase βARK activity to the same extent as PHA, suggesting a PKC-mediated mechanism. The kinetic of β-adrenergic–stimulated cAMP production was substantially modified in TPA and PHA-activated cells, indicating that the increased GRK activity resulted in an increased β-adrenergic homologous desensitization. A three- to fourfold increase in GRK activity was also observed in a population of T cell blasts (> 97% CD3+) exposed to PHA for 48–72 h. A significant increase in βARK1 and βARK2 mRNA expression was observed 48 h after mitogen stimulation, while mRNA expression of GRK5 and GRK6 was not changed. In conclusion our data show that the expression of GRK subtypes is actively and selectively modulated according to the functional state of T lymphocytes. (*J. Clin. Invest.* 1995. 95:203–210.) Key words: T lymphocytes • G-coupled receptors • receptor regulation • β-adrenergic receptor kinase

Introduction

β-adrenergic receptor kinase (βARK)[1] is a serine–threonine kinase that is involved in the process of homologous desensitization of G protein-coupled receptors (1, 2). Agonist occupancy triggers translocation of βARK from cytosol to plasma membranes, where it phosphorylates agonist-occupied receptors (1, 2). βARK-phosphorylated receptors are only minimally desensitized. An additional cytosolic protein, β-arrestin, is required to induce maximal homologous desensitization (1–3). βARK may regulate a number of G-coupled receptors (1–5), including β2- and α2-adrenergic receptors, muscarinic cholinergic receptors, somatostatin, platelet activating factor (PAF), PGE1 as well as substance P receptors. Known substrate receptors are involved in functions as different as neurotransmission and regulation of immune responses through different intracellular second messengers (5). βARK is a member of a multigene family, consisting of six known subtypes, which have also been named G protein coupled receptor kinases (GRK 1–6) due to the apparently unique functional association of such kinases with this receptor family (1, 4). In this scheme rhodopsin kinase corresponds to GRK1, βARK1 to GRK2, and βARK2 to GRK3. While the expression of rhodopsin kinase is essentially confined to the retina, where it regulates phototransduction, a wide tissue distribution has been reported for many GRKs, the central nervous system being a site of relevant expression for most subtypes (1–3). We have cloned the cDNAs of human βARK1 (5) and βARK2 (6), and demonstrated that PBL, and mononuclear leukocytes (MNL) in particular, represent a site of preferential expression for these kinases. High expression of GRK5 (Chuang and A. De Blasi, unpublished observation) and GRK6 (7) in PBL was also documented, while GRK4 was found not to be expressed in these cells (8).

Several lines of evidence from our work suggested a functional role for βARK1 and possibly for other GRK subtypes, in MNL. We have shown that β-adrenergic receptor (βAR) agonist isoproterenol and PAF can induce βARK translocation in these cells (5). These agents are known to play important roles in modulating immune functions. We have also cloned the cDNA of human β-arrestin1 (9) and shown that MNL express among the highest levels of β-arrestin1. This adds further support to a functional role of the βARK/β-arrestin mechanism of receptor desensitization in immune cells.

In this study we investigated the expression of GRKs during the process of T lymphocyte activation, which is of fundamental importance in regulating immune responses to foreign and self antigens. We found that the expression of GRK subtypes is actively and selectively modulated according to the functional

1. *Abbreviations used in this paper:* βARK, β-adrenergic receptor kinase; GRK, G protein-coupled receptor kinase; HrIL-2; human recombinant IL-2; MNL, mononuclear leukocytes; PAF, platelet activating factor; PKC, protein kinase C; ROS, rod outer segments; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

state of T lymphocytes. In particular βARK1 and βARK2 expression was preferentially increased and seemed to be regulated through a protein kinase C (PKC)-mediated intracellular pathway.

Methods

Cell sources and treatments. MNL from healthy volunteers were fractionated using a Ficoll gradient as previously described (5). 12-O-tetradecanoylphorbol-13-acetate (TPA) and ionomycin used for treatments were dissolved in DMSO (0.01% DMSO final concentration). T cell blasts were prepared as described by Rosoff and Mohan (10). Briefly, MNL were stimulated with 5 μg/ml PHA and human recombinant interleukin 2 (HrIL-2, 10 U/ml) was added to the flasks on the third day. After an additional 3 d, cells were washed and cultured for 48–72 h in the absence of PHA or HrIL2. In all experimental conditions, cell viability, as assessed by trypan blue exclusion, was > 90%.

Bovine rod outer segments (ROS) phosphorylation assay. Four GRK subtypes, namely βARK1, βARK2, GRK5, and GRK6, are abundantly expressed in PBL. It has been shown that each of these kinases, transiently expressed in COS cells, is able to phosphorylate ROS, with the following relative order of potency: βARK1 ≫ βARK2 = GRK5 ≫ GRK6. The ability of βARK2 to phosphorylate ROS is ~ 20–40% that of βARK1 (6). Therefore, we will refer to our ROS phosphorylation assay as GRK activity, although it mostly measures βARK1, for which ROS represent a much better substrate (5). Cytosolic GRK activity was assayed as described (5). Briefly, cells were lysed in cell lysis buffer (10 mM Tris, 5 mM EDTA, 7.5 mM MgCl₂, 0, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml benzamidine, 5 μg/ml pepstatin A, at pH 7.4) using a polytron tissue disruptor (Janke and Kunde, Staufen, Germany) and fractioned by centrifuging at 300,000 g for 30 min at 4°C. In each reaction 50 μg of cytosolic protein were used to phosphorylate 300 pmol of urea-treated ROS. The reaction was carried out at 30°C in the presence of light for 30 min. To assay membrane-associated GRK activity, membranes were washed once in cell lysis buffer, centrifuged at 48,000 g for 15 min at 4°C, the resultant membrane pellet resuspended in cell lysis buffer with 200 mM NaCl to detach membrane-bound GRKs, and 2–5 μg of membrane-detached proteins were used in the phosphorylation assay. For DEAE-Sephacel chromatography, 200-μl fractions of crude cytosolic preparations (with a protein content of ~ 1 mg/ml) were loaded onto a small column (300 μl) of DEAE-Sephacel equilibrated with cell lysis buffer. The column was washed with ~ 1 ml of the same buffer and the eluate was used for kinase assay. Bovine urea-treated ROS were purified as previously described (5).

Northern blot analysis. Northern blot analysis was performed as previously described (5). Total RNA (20 μg) isolated by the guanidinium isothiocyanate/cesium chloride method was fractioned on 1% agarose-formaldehyde gel and transferred to a GeneScreen Plus membrane (Du Pont-New England Nuclear, Branschweig, Germany). Northern blot analysis of βARK1, βARK2, and GRK6 were performed using the random-primed cDNA fragments, bp 1055–1946 for βARK1, bp 609–1068 for βARK2 and bp 1413–1822 for GRK6, as probes. For GRK5 an antisense single stranded cDNA (bp 1328–2029) generated as extensively described in reference 5, was used as a probe. RNA blots were hybridized and washed as described (5) and subjected to autoradiography at −80°C for 1–4 d. All results were confirmed at least in two separate experiments, using cells obtained from different individuals.

Western blot analysis of βARK1. βARK1 specific antibody were prepared as in reference 11. Briefly, antisera were raised by immunization in New Zealand White rabbit with the synthetic peptide RDAYRE-AQQLVQRVPKMK corresponding to aminoacids 628–646 of the βARK1 sequence (5). Samples containing 30 μg of proteins were suspended in Laemmli buffer by gentle shaking for 5–10 min and electrophoresed on 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes with a tank transfer system. Blots were

blocked with 6% bovine serum albumin in TBS (100 mM Tris-HCl pH 7.5, 0.9% NaCl) and subsequently with PEG buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.1% polyethylene glycol mol wt 15–20,000) containing 8% fetal calf serum and then TBS with 0.1% Tween 20. βARK1 was detected with the specific antibody diluted in PEG buffer containing 5% of fetal calf serum, and developed with alkaline phosphatase-conjugate goat anti-rabbit igG and 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium. The resulting blots were scanned four times with an LKB Ultroscan XL laser densitometer.

Tritiated thymidine ([³H]TdR) incorporation and flow cytometry analysis of cell cycle. For analysis of [³H]TdR incorporation into DNA, cells were harvested in 96-well plates (100 μl at the cell density of 1 × 10⁶/ml) in RPMI 1640 with 10% of fetal calf serum. After the appropriate incubation time with 1 μCi/well of [³H]TdR labeling was stopped by aspirating the medium and washing the cells twice with cold PBS and once with ice-cold 5% TCA for 10 min at 4°C. TCA was removed, the plates allowed to dry down by air and then resuspended for 30 min in 200 μl of sodium hydroxide. Cell-associated radioactivity was then counted. For cytofluorimetric analysis, at the appropriate time intervals, cells were centrifuged down and washed twice with PBS and counted. 1 × 10⁶ cells were suspended in 2 ml of propidium iodide (50 μg/ml) solution in 0.1% sodium citrate, containing 25 μl RNAse (0.5 mg/ml) and 25 μl of 0.1% Nonidet P 40. Flow cytometry analysis was performed on at least 10,000 cells with a FACStar (Becton Dickinson, Milan, Italy). Human leukocytes from freshly collected blood were used as a DNA standard. Cell cycle analysis was performed using the Baisch method, with a Hewlett-Packard Co. (Palo Alto, CA) 300 computer program (12).

βAR binding and cAMP accumulation. βAR binding and isoproterenol-stimulated cAMP accumulation were performed as previously described (13). βAR were measured on intact cells using ¹²⁵I-pindolol as ligand and CGP 12177 to define nonspecific binding. Incubations were for 45 min at 37°C, followed by rapid filtration and counting. To measure cAMP accumulation, unstimulated and TPA or PHA-stimulated MNL were incubated in RPMI containing 1 mM isobutyl-methylxanthine (IBMX; to inhibit phosphodiesterase) for different times with 1 μm isoproterenol. The reaction was rapidly stopped and cells were centrifuged and resuspended in Tris-EDTA (50 mM) buffer at pH 7.5. After boiling and sonication the proteins were precipitated and two 50-μl aliquots were assayed for cAMP, using cAMP assay kit from the radiochemical center (Amersham International, Buckhinghamshire, UK).

Materials. RPMI 1640 medium was obtained from Biochrom (Berlin, Germany) Tris, SDS, bromophenol blue, acrylamide, and bisacrylamide were purchased from Bio Rad Laboratories (Richmond, CA); EDTA, urea and magnesium chloride from E. Merck (Darmstadt, Germany); DEAE-Sephacel from Pharmacia Fine Chemicals (Piscataway, NJ); DMSO from Research Industries Corporations (Salt Lake City, Utah); [³²P]dCTP and [γ-³²P]ATP from Amersham International; ionomycin and H7 (1-5(isoquinoline sulfonyl)-2-methylpiperazine) from Calbiochem Corp. (La Jolla, CA). All the other materials, including PHA, were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

In vitro exposure of freshly isolated MNL to PHA is a well established procedure for T lymphocyte activation (14). Various methods can be used to monitor this response. These include evaluation of the proliferative response of T lymphocytes by [³H]TdR incorporation, cell cycle DNA measurements, IL-2 secretion, and IL-2 receptor expression measurement (14). To validate our model of T lymphocyte activation, the proliferative response of T cells upon exposure to PHA (5 μg/ml) was monitored by [³H]TdR incorporation (Fig. 1 *a*). MNL stimulated with PHA for 4 or 24 h did not show significant incorporation as compared to the unstimulated controls (500–700 cpm,
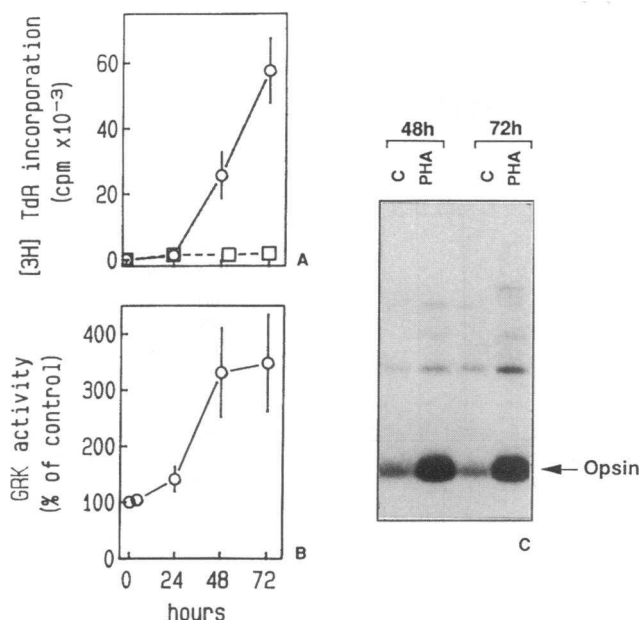
Figure 1. Increased GRK activity in mitogen-stimulated MNL. (A) [³H]TdR incorporation by untreated MNL (squares) or MNL treated with PHA up to 72 h (circles). Data are means±SEM of three independent experiments. (B) Graphical representation of cytosolic GRK activity in mitogen-stimulated MNL. At each time-point, GRK activity in PHA-stimulated MNL was expressed as a percent of that in the respective unstimulated control, which was considered as 100%. Data are means±SEM of three independent experiments. (C) Opsin phosphorylation by cytosolic GRK obtained from untreated MNL (control, C) and MNL exposed to PHA for 48 h and 72 h. Arrow indicates bands of phosphorylated rhodopsin (Opsin), as revealed by autoradiography after polyacrylamide gel electrophoresis. Autoradiography of dried gels was for 14 h at −70°C.

Figure 2. Increased βARK1 immunoreactivity in mitogen-stimulated MNL. (A) βARK1 immunoreactivity was determined in 30-μg cytosolic proteins prepared from control (C) or mitogen-stimulated (PHA) MNL 72 h after stimulation. (B) Cytosolic GRK activity, measured by in vitro rhodopsin phosphorylation (Opsin), was assayed from parallel samples. Results from two individuals are shown.

Fig. 1 a). [³H]TdR incorporation was 20–30-fold higher than in controls 48 h after stimulation, and up to 60-fold higher 72 h after mitogen stimulation (Fig. 1 a). Unstimulated and PHA-stimulated MNL were harvested in parallel at each time-point, and GRK activity was assayed on crude cytosolic preparations from these cells. Cytosolic kinase activity (per mg of protein) was unchanged 4 h after exposure to PHA (Fig. 1 b), and slightly increased 24 h after stimulation (141±23% of controls, Fig. 1 b). Substantial increases were observed after 48 h (331±80% of control) and 72 h (347±86% of controls) of exposure to PHA (Fig. 1, b and c). In our experimental conditions the ROS phosphorylation assay was in the linear range and a similar increase in kinase activity was observed in PHA-stimulated cells relative to untreated controls using amounts of cytosolic proteins ranging from 8 to 100 μg per reaction.

Some experiments using specific kinase inhibitors were performed to validate our phosphorylation assay. Heparin, which is known to selectively inhibit GRK phosphorylation activity both in vivo and in vitro (5) inhibited completely rhodopsin phosphorylation by cytosolic GRK preparations from either control or PHA-treated MNL. By contrast H7, a PKC inhibitor, did not affect rhodopsin phosphorylation at all (not shown). These results also rule out the possibility that the increase in ROS phosphorylation induced by PHA be due to a heparin-
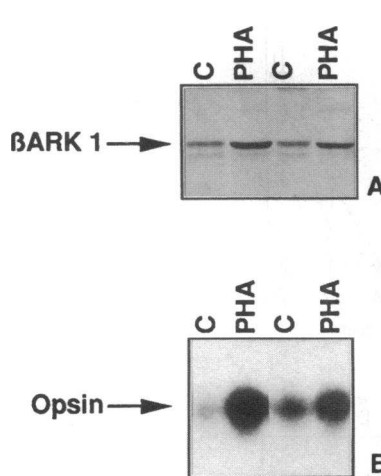
insensitive kinase and in particular to PKC, which is activated in these cells.

βARK1 immunoreactivity was studied in unstimulated and PHA-stimulated MNL (Fig. 2). Polyclonal antibodies were raised against a synthetic peptide derived from the sequence of human βARK1 close to the COOH terminal region (amino acid 628–646) and they recognized βARK1 as a clearly detectable band of an apparent molecular mass of ∼ 80 kD. These antibodies proved to be specific as they recognized βARK1, but not βARK2 (these proteins are 84% identical in their aminoacid sequence) in experiments performed on tranfected COS7 cells expressing either of these kinases (6) (data not shown). The expression of βARK1 in cytosolic proteins from MNL exposed to PHA for 72 h was increased to 287% of the control values (Fig. 2), as measured by immunoblotting. A similar increase in GRK activity was observed in the same cells as assessed in parallel experiments (Fig. 2).

Although GRKs are essentially cytosolic proteins, a substantial amount of kinase activity (∼ 25–30% of total) is associated to plasma membranes of resting cells (T. T. Chuang and A. DeBlasi, unpublished observations). Membrane-associated GRK activity was not affected by PHA treatment (not shown) thus ruling out the possibility that the increase in cytosolic kinase activity found on PHA-treated cells be simply due to translocation of membrane-associated GRKs to the cytosol. Because of the increased size of blast cells and their proliferative state, the amount of proteins obtained from the membrane and cytosolic preparations from PHA-activated MNL was nearly twofold compared to control cells. Therefore, depending on the relative ratio of cytosolic vs membrane proteins as well as on the density of GRK activity in these two compartments, the effect of PHA treatment may appear somewhat different according to the way it is calculated. When GRK activity was expressed per milligram of total proteins (i.e., cytosolic + membrane), there was a > twofold increase induced by 72 h exposure to PHA. Kinase activity was 208±22 and 467±47 fmol phosphate/min per mg of total proteins for untreated and PHA-activated MNL, respectively (n = 3). When expressed as total amount of GRK activity per cell, we found an approximately fivefold increase in PHA-stimulated MNL. In untreated cells GRK activity was 1.66±0.17 fmol/min per 10⁶ cells and was increased up to 8.04±0.80 fmol/min per 10⁶ in cells exposed to PHA for 72 h (n = 3).
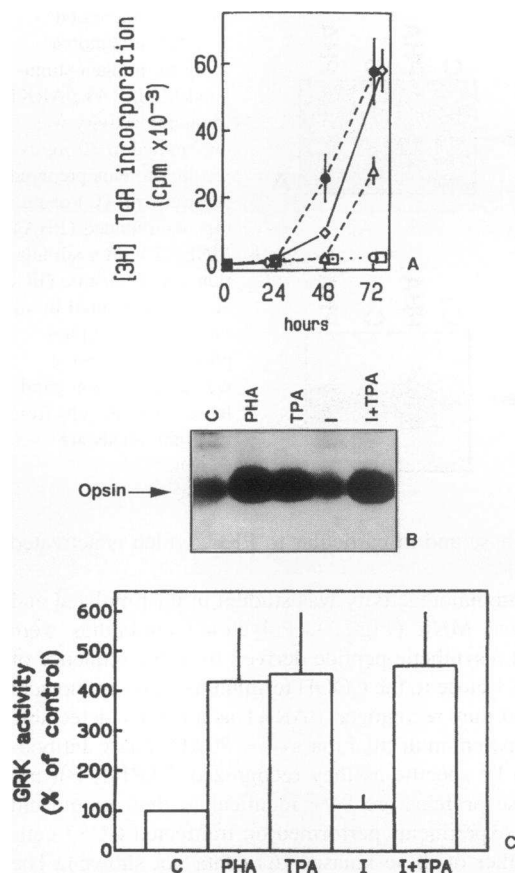
Figure 3. GRK activity in MNL stimulated by various agents. (*A*) The proliferative response as monitored by [³H]thymidine incorporation in control MNL (*squares*) and MNL exposed to PHA (*filled circles*), TPA (*triangles*), ionomycin (*unfilled circles*), TPA plus ionomycin (*diamonds*). Data are means±SEM of three independent experiments. (*B*) Opsin phosphorylation by cytosolic GRK obtained from untreated MNL (control, C) and MNL exposed for 72 h to PHA, TPA, ionomycin (I), and TPA plus ionomycin (I + TPA). Arrow indicates bands of phosphorylated rhodopsin (Opsin), as revealed by autoradiography after polyacrylamide gel electrophoresis. Autoradiography of dried gels was for 24 h at −70°C. (*C*) Graphical representation of cytosolic GRK activity in untreated MNL (control, C) and MNL exposed for 72 h to PHA, TPA, ionomycin (I), and TPA plus ionomycin (I + TPA). GRK activity was expressed as percent of that in the unstimulated control, which was considered as 100%. Data are means±SEM of four independent experiments.

Two pathways of signaling play a major role in the process of T lymphocyte activation. These are represented by PKC and intracellular calcium (15, 16). Both are activated after T cell receptor stimulation. It is well established that treatment of MNL with a combination of TPA and calcium ionophore can mimic receptor-mediated activation of T cells (15, 16). To analyze the mechanisms of regulation of GRK activity, MNL were treated with a combination of 10 nM TPA, which induces persistent PKC activation in the absence of detectable downregulation of the kinase (15), and 0.5 μM ionomycin, which raises intracellular calcium. In our experiments, the proliferative response of MNL treated with TPA plus ionomycin was approximately of the same extent as that induced by PHA, as demonstrated by [³H]TdR incorporation (Fig. 3 *a*). When given alone,
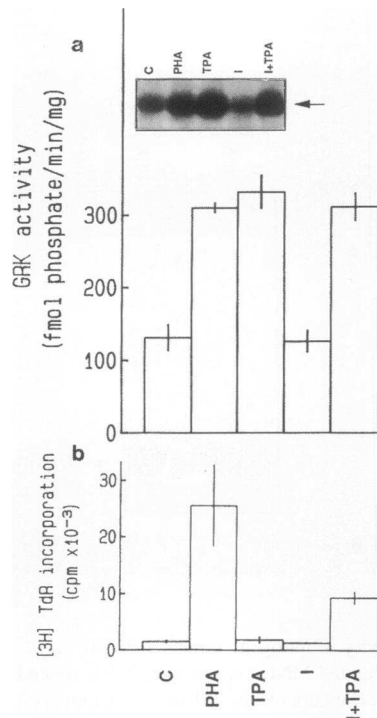

Figure 4. Proliferative response and GRK activity in MNL stimulated by various agents for 48 h. MNL were exposed for 48 h to PHA, TPA, ionomycin (I), and TPA plus ionomycin (I + TPA) and both cytosolic GRK activity (*a*) and [³H]-TdR incorporation measured in parallel experiments. Data are means±-SEM of three independent experiments. Inset shows opsin bands on the autoradiographic film from one representative experiment.

TPA and ionomycin induced partial or negligible proliferative responses, respectively (Fig. 3 *a*). After 72 h of treatment, GRK activity was increased by TPA plus ionomycin to the same level as by PHA (Fig. 3 *b*). Ionomycin alone did not induce any change in GRK activity (Fig. 3 *b*). By contrast TPA alone increased GRK activity to the same extent (three- to fourfold) as in fully activated MNL (i.e., treated either with PHA or with TPA plus ionomycin, Fig. 3 *b*). After 72 h of treatment, TPA induced maximal increase of GRK activity while stimulating cell proliferation by only 50% (Fig. 3, *a* vs *b*). This indicated that GRK activity is increased in response to T cell activation rather than proliferation. This was even clearer when the kinase activity was measured after 48 h of treatment (Fig. 4). Exposure to TPA for 48 h induced a maximal increase of GRK activity, whereas it did not stimulate any proliferative response (Fig. 4), showing that GRK modulation can occur independently of cell proliferation.

Starting with the same number of cells we obtained different yields of cytosolic protein at the end of some treatments. In a typical experimental condition (*n* = 4), after 72 h of treatment one flask of MNL yielded 135±12 μg of cytosolic proteins from untreated cells, 233±39 μg from PHA-treated (*P* < 0.05 vs control), 100±12 μg from TPA-treated, 125±9 μg from ionomycin-treated and 170±13 μg from TPA + ionomycin-treated cells. However, there was no correlation between the effects of treatment on GRK activity and the yield of proteins, as the same increase in kinase activity was found in MNL treated with PHA, TPA, and TPA + ionomycin (Fig. 3), while protein yield from TPA-treated MNL was not significantly different from untreated cells.

The kinetic of cAMP accumulation in response to βAR agonist isoproterenol (1 μM) was measured in control MNL and in cells exposed to TPA and PHA for 72 h (Fig. 5). As previously reported (13), in control cells cAMP was rapidly

accumulated and reached a plateau after 3 min. By contrast the kinetic of cAMP accumulation in cells pretreated with TPA and PHA appeared to be drastically modified (a) maximal cAMP accumulation was reached after 1 min isoproterenol treatment; (b) maximal stimulation was reduced to 23 and 6% of the control values in TPA and PHA-treated MNL, respectively; (c) 5–10 min after agonist exposure intracellular cAMP declined to basal levels or below (Fig. 5). Basal levels of intracellular cAMP, which were 77 pmol/mg of proteins in untreated MNL, were slightly (51 pmol/mg) or drastically (19 pmol/mg) reduced in TPA and PHA-treated cells, respectively. Maximal isoproterenol-induced cAMP accumulation over basal, at 5 min, was 139 pmol/mg of protein in control MNL, 32 pmol/mg in TPA-treated MNL, and 8 pmol/mg in PHA-treated cells. The density of βAR was 1,222 sites/cell in unstimulated MNL and 1146 and 1462 sites/cell in TPA and PHA-stimulated MNL, respectively (means of two experiments).

The experiments described so far were performed on freshly isolated human MNL, which in our preparations are 67–72% CD3+ lymphocytes, as demonstrated by cytofluorimetric analysis (data not shown). As PHA is known to selectively activate T lymphocytes, it is likely that activated T cells do account for the increased expression of GRK activity found in MNL exposed to mitogen. To gain further support for this possibility we used a model of enriched T lymphocytes to investigate modulation of GRK activity. A population of T cell blasts (> 97% CD3+) was obtained by sequential stimulation of MNL with PHA and HrIL-2, as described in Methods. Our experiments were carried out on these cells brought to quiescence by a 48–72-h period of HrIL-2 and PHA starvation; in this condition they were > 95% in G_0–G_1, as documented by cytofluorimetric cell cycle DNA measurements (Fig. 6) and [^{3}H]TdR incorporation experiments (not shown). As expected, quiescent T cell blasts showed a significant proliferative response when stimulated with PHA. Cytofluorimetric analysis of cell cycle showed that these cells were 79% in G_0–G_1, 19% in S and 2% in G2 phases after 24 h PHA and 66% in G_0–G_1, 27% in S and 7% in G2 phases 48 h after PHA treatment (Fig. 6). GRK activity in unstimulated T cell blasts was 416±90 fmol phosphate/min per mg protein. A relevant increase in kinase activity was observed 48 h (428±49% of controls, Fig. 7) and 72 h after stimulation with PHA (not shown). In these cells 48 h exposure to TPA induced a maximal increase of GRK
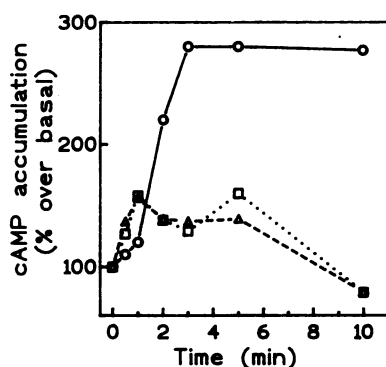

Figure 6. Cell cycle progression in mitogen-stimulated T cell blasts. Cytofluorimetric analysis of cell cycle in T cell blasts untreated (control) or exposed to PHA (PHA) for 24 and 48 h. In the ordinates are the relative cell numbers in the different phases of the cell cycle; in the abscissa is indicated the fluorescence intensity of DNA expressed in arbitrary unit. The experiment shown is representative of three similar.

activity (not shown), further supporting that PKC is involved in regulating GRK activity in T cells. The augmented GRK activity by PHA was still observed after the samples had passed through a DEAE-Sephacel gel, which retains PKC and protein kinase A (5) thereby removing potentially interfering kinases from the phosphorylation assay (Fig. 7). Membrane-associated GRK activity was the same in control and PHA-stimulated T cell blasts (Fig. 7).

To investigate the possible changes of different GRK subtypes in mitogen-stimulated T lymphocytes, we studied the levels of mRNA expression of these kinases in T cell blasts stimulated with PHA for 48 h (Fig. 8 and Table I). A substantial (~ threefold) increase in βARK1 mRNA expression was observed 48 h after mitogen stimulation. The mRNA expression of βARK2 was also affected by PHA stimulation. The two major bands of ~ 8 and ~ 7 kb, which represent the peculiar pattern of hybridization observed in human (6), seemed to be regulated in a different manner; the ~ 8-kb band was unchanged, while the ~ 7-kb band was substantially increased. By contrast PHA-induced T cell activation did not alter the expression of GRK5 and GRK6. No mRNA expression was detectable for GRK4 in control as in PHA-treated T blasts, even when the extremely sensitive RT-PCR assay was used (not shown). This indicates that GRK4 is not expressed in either resting or activated T cells.

Discussion

PBL express among the highest levels of βARK1 and some other GRK subtypes, as documented by previous work from our laboratory (5, 6). We have shown that high levels of βARK activity in leukocyte subsets are paralleled by high levels of mRNA expression for two subtypes of GRKs, namely βARK1 and βARK2 (5, 6). Our data also indicated that the βARK/β-arrestin machinery may regulate G protein–coupled receptors

Figure 5. Time course of β-agonist-stimulated cAMP production in MNL stimulated by various agents. MNL untreated (circles) or exposed to TPA (squares) or PHA (triangles) for 72 h were incubated for various times with 1 μM isoproterenol. The reaction was then stopped and cAMP was measured. Data are expressed as percentage of the basal cAMP level for each group. For further details Methods and Results. The experiment shown is representative of two similar.
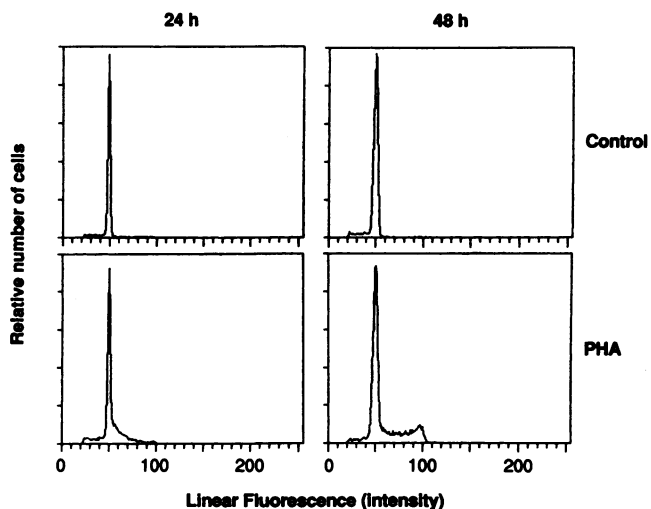
Cytosol   Post-DEAE   Membrane
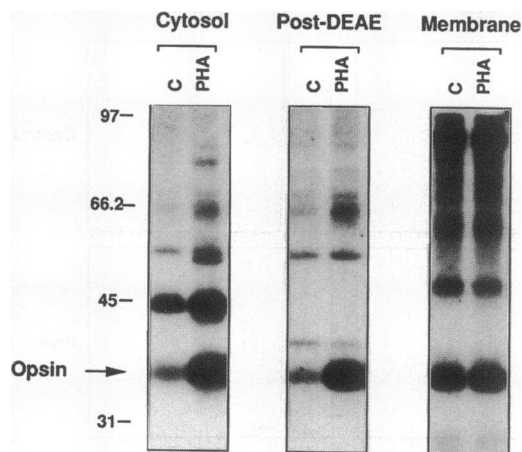
97—

66.2—

45—

Opsin →

31—

Figure 7. Increased GRK activity in mitogen-stimulated T cell blasts. GRK activity was measured by ROS phosphorylation assays on T cell blasts untreated (control, C) or exposed to PHA for 48 h. Arrow indicates bands of phosphorylated rhodopsin (Opsin), as revealed by autoradiography after polyacrylamide gel electrophoresis. Cytosolic GRK preparations were assayed directly (Cytosol) or passed through a DEAE-Sephacel column, the eluate collected and measured for its rhodopsin-phosphorylating activity (post-DEAE). The gel on the right shows ROS phosphorylation by membrane-associated GRK, prepared in parallel experiments. Autoradiography of dried gels was for 24 h at −70°C. Gels are representative of four similar experiments.

mediating immune responses (5, 6, 8). To gain further support for a relevant regulatory role of βARK in immune cells, in this study we have investigated the expression of βARK and of other GRK subtypes in resting and activated T lymphocytes. It was found that GRK activity is modulated during the process of T cell activation, being increased by two- to fourfold after exposure to mitogen. Since the ROS phosphorylation assay mostly measures the activity of βARK1 (5), this finding indi-

Figure 8. mRNA expression of GRK subtypes in mitogen-stimulated T cell blasts. (*A*) mRNA expression for human βARK1, βARK2, GRK5, and GRK6 was studied on total RNA (20 µg) prepared from resting (−) and PHA-stimulated (+) T cell blasts 48 h after stimulation. Molecular weight standards (28S and 18S) are shown. The experiments shown represent subsequent hybridization of the same filter with specific probes for the four genes. (*B*) Ribosomal RNA staining in the membrane after transfer. (*C*) Cytosolic GRK activity, measured by in vitro rhodopsin phosphorylation (Opsin), was assayed from parallel samples. The gels are representative of three similar experiments.

Table I. mRNA Levels of GRK Subtypes in PHA-activated T Cell Blasts Relative to Untreated Controls

		mRNA levels (% of the untreated controls)
βARK1 (GRK2)		382 ± 105
	~8-kb band	109 ± 5
βARK2 (GRK3)*		
	~7-kb band	247 ± 15
GRK5		109 ± 11
GRK6		92 ± 4

mRNA expression, quantified by densitometric analysis of autoradiographic films, is expressed as a percentage of the relative untreated control. Data are means ± SE of three determinations. * For βARK2, the two major mRNA species of ~ 7 and 8 kb in size are regulated in a different manner by PHA treatment.

cates that βARK1 is actively regulated in activated T cells. βARK1 was indeed increased in PHA-activated MNL, as assessed by immunoblotting. The analysis of mRNA expression of different GRK subtypes further supported this possibility, as it showed a substantial and preferential increase of the expression of βARK1 in PHA-activated T cells. The expression of βARK2 was also increased, but to a lower extent, while GRK5 and GRK6 were not affected by these treatments. These data strongly indicate a functional role of βARK in immune cells and describe a physiological process whereby βARK expression is actively regulated.

The activation of T lymphocytes is a pivotal event in the immune response of all vertebrates to foreign and self antigens (15, 16). It is a multi-step phenomenon, triggered by effective contact of an external antigen with the antigen receptor TCR on the surface of a T lymphocyte. This activates a yet ill-defined cascade of signaling events, leading to persistent activation of PKC as well as to persistent rises of cytoplasmic calcium (15, 16). Within a few hours following TCR stimulation, all of the early events determining the irreversible commitment of T lymphocytes to the activated state take place (16). These include immediate induction of nuclear transcription factors as c-fos, followed by increased levels of mRNA expression for IL-2, IL-2Rα, IL-4, IL-5, IFNγ, and other lymphokines (16). As a consequence of early events, expression of many relevant functional parameters is actively regulated at the molecular level in activated T lymphocytes. Our time-course experiments allocate the increase of βARK activity among late G1-early S phase events following mitogen stimulation of T cells. At this stage, irreversible commitment of T lymphocytes to their activated state has already taken place (15, 16). Increase of βARK expression appears then to be one of the phenotypic changes through which T cells adjust to suit their changed functional tasks. Increased expression of PKC subtypes has been demonstrated at the mRNA level in PHA-stimulated MNL, as well as in T cell blasts, with similar time-courses as for βARK1 in our experiments (17, 18). A similar profile of increase has been recently reported for a number of other kinase activities, regulated upon mitogenic stimulation of T lymphocytes (19). A dramatic increase of PAF receptor has been demonstrated on the surface of T lymphocytes 48–72 h after stimulation with
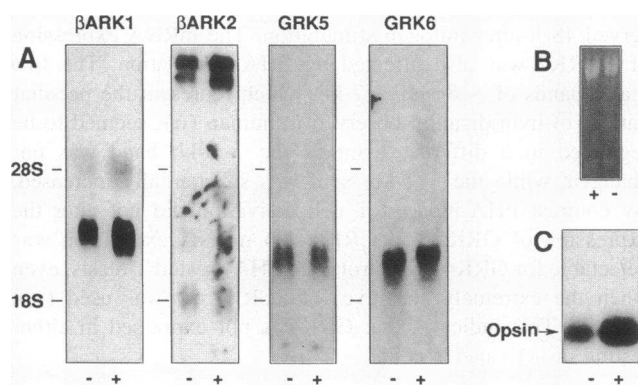
mitogen (20). Higher levels of βARK may be related to higher levels of PAF receptors, for which a functional role of this kinase has already been suggested by our previous work (5).

Although it is conceivable that several G-coupled receptors are functionally regulated by βARK, evidence has accumulated showing that βARK is directly involved in the mechanism of β-adrenergic receptor desensitization. Therefore, some experiments were done to assess whether homologous desensitization of βAR is modified in TPA and PHA-stimulated MNL. It was found that the kinetic of cAMP production in response to isoproterenol was substantially modified in TPA and PHA-activated MNL; the production of cAMP was substantially reduced, while cAMP accumulation reached a plateau more rapidly than in control cells. These changes are indicative of increased homologous desensitization, consistent with the increased levels of GRK activity measured in these cells. Opposite changes (i.e., increased cAMP production and delayed attainment of maximal accumulation) were observed in cells in which βARK-mediated desensitization was blunted, either by mutagenesis of the β-adrenergic receptor or by a dominant negative βARK mutant (21, 22). Other changes in the receptor–G protein cyclase system may occur in activated MNL which could, at least in part, modify the agonist-stimulated cAMP production. This is particularly true for PHA-treated cells where changes in shape, proliferative state, as well as in basal cAMP levels were observed (see Results and reference 23). However, as the kinetics of agonist-stimulated cAMP production were similarly modified in both TPA and PHA-activated MNL, these changes are likely to be due, at least in part, to an increased efficiency of homologous desensitization as a result of increased GRK activity.

An analysis of GRK subtypes based on sequence homology lead to the subdivision of this family into two subfamilies, with βARK1 and βARK2 representing one subfamily (1). Our findings showing that βARK1 and βARK2, but not GRK5 and GRK6, are increased in PHA-treated T cell indicate that one relatively homogeneous subgroup of GRK kinases is actively regulated during the process of T cell activation. A recent study reported that expression of βARK1, and to a lesser extent of βARK2, was increased in the failing human heart (24). It was suggested that this finding may explain, at least in part, the marked desensitization of the β-adrenergic receptor system which has been documented to occur in such a pathological condition (24).

Several lines of research have demonstrated that stimulation of PKC for at least 6 h is necessary for maximal T cell activation (15). At that time the complex series of intracellular events that are initiated by PKC has been committed (15). Our data clearly show that stimulation of PKC, as accomplished by exposing MNL to low doses of TPA, was able to induce an increase of βARK comparable to that obtained by PHA treatment. These findings strongly indicate that the regulation of βARK expression is part of the cascade of events which is initiated by PKC activation. Persistent PKC activation has been shown to mediate a variety of events in the process of T lymphocyte activation (15). Interestingly, PKC is directly responsible for the reduced responsiveness of activated T lymphocytes to further stimulation through TCR, by direct phosphorylation of TCR γ subunits (15). βARK modulation by a PKC-dependent mechanism may then represent another way through which responsiveness of T lymphocytes is tuned according to their functional state by PKC.

A different efficiency of mitogen-activated lymphocytes in desensitizing some type(s) of G-coupled receptor(s) is suggested by the evidence of decreased responsiveness to a variety of hormonal stimuli, including β-AR (Fig. 5), PGE1, and histamine (23). We propose that the increased βARK activity in activated T cells may represent one mechanism by which these cells can turn off some regulatory stimuli, possibly mediated through G-coupled receptors, which could interfere with their essential commitment to respond to a specific antigen.

In conclusion, we document the existence of an active mechanism of regulation for the expression of βARK1 and, to a lesser extent, of βARK2. Our data clearly relate the increased expression of βARK to the process of T cell activation. These findings describe a new mechanism by which the regulation of receptor mediated responses can be modulated.

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