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Altered β-adrenoceptor function associated to protein kinase C activation in hyperproliferative T lymphocytes

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Abstract

β-Adrenoceptor (βAR) expression and function as well as its modulation via intracellular transduction signals, were analyzed on the T cell lymphoma BW5147. Independently to the kinetic of proliferation and relative to the number of receptors displayed in normal T lymphocytes, BW5147 cells displayed a decreased number of βAR, uncoupled to adenylate cyclase, but coupled to protein kinase C stimulation. This last effect was impaired by a β-antagonist and by blockers of the enzymatic pathways involved in T lymphocyte proliferation, inducing a recovery of βAR sites. Down-regulation of βAR would implicate the loss of a negative neuroimmune control mechanism for lymphocyte proliferation. The coupling of the remaining sites to a positive signal for cellular activation, would contribute to establish an hyperproliferative state.

Keywords: T Lymphocytes; Cellular proliferation; Neuroimmunology; Signal transduction; Protein kinase C

1. Introduction

Physiological activation of T lymphocytes, leading to differentiation and proliferation, involves a complex series of intracellular signaling events that is initiated by protein tyrosine kinases (TPK) activation, which phosphorylate numerous proteins including phospholipase Cγ1 (PLCγ1) (Mustelin et al., 1990; Tamir et al., 1996). The activated PLCγ1 hydrolyzes membrane inositol phospholipids giving raise to inositol triphosphate and diacylglycerol, which in turn, increase intracellular Ca^{2+} concentrations and activate PKC, respectively (Isakov et al., 1987). Transduction of signal downstream also includes the activation of guanine nucleotide binding proteins, mitogen-activated protein kinases and several transcription factors (Tamir et al., 1996; Graves et al., 1995).

Over the past several years, evidences have been accumulating indicating that immune system cells are regulated by hormones and neurotransmitters (Weigent and Blalock, 1987; Ader et al., 1995). The control of lymphocyte function by the sympathetic system is supported by several evidences: (1) the presence of direct sympathetic innervation of immune cells (Felten et al., 1987; Madden et al., 1998); (2) the direct contact of noradrenaline-containing nerve terminals with lymphocytes (Felten et al., 1987; Felten and Olschowska, 1987), and (3) at the molecular level, the presence of postsynaptic β-adrenergic receptors (βARs) functionally coupled with cAMP-adenylate cyclase signaling pathway on lymphocytes (Bishopric et al., 1980; Plaut, 1987). Moreover, a selective distribution of βAR was described on lymphocyte subpopulations (Pochet and Delespesse, 1983; Genaro et al., 1993). In fact, it was demonstrated that enriched B cells showed a higher βAR density compared to enriched T cells (Genaro et al., 1993; Galant et al., 1978). On the latter cells, receptors showed a higher coupling to the effector adenylate cyclase (a.c.) system than the coupling observed in B cells (Genaro et al., 1993; Griese et al., 1988). Moreover, cells from T cell subsets displayed similar βAR number, but different cAMP-stimulated levels in response to the β-agonist isoprenaline (Genaro et al., 1993). These effects would probably point to a differential modulatory function of βAR on distinct lymphoid subtypes.

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Despite its well characterization, βAR action upon lymphocyte activation and the consequent intracellular signaling events triggered by this activation have not been fully established yet. The expression of βAR on lymphoid cells has been related to a control function of lymphocyte proliferation by increasing intracellular levels of cyclic AMP (cAMP) (Coffey and Hadden, 1985; Carlson et al., 1989). It was recently demonstrated that the agonist operating via the a.c. pathway or cell-permeable cAMP analogues inhibit T cell activation by interfering with the phosphatidyl inositol turnover (Tamir and Isakov, 1994). In fact, an absence of functional βARs was demonstrated on different hyperproliferative lymphoid cell lines (Cremaschi et al., 1991). Also, the mutant S_{49} T lymphosarcoma cells that show an absence of βAR as a phenotypic alteration, display a higher proliferation rate than that observed in the β_2-adrenoceptor-rich wild-type S49 line (Cremaschi et al., 1994). Furthermore, a decrease in βAR number was demonstrated in concanavalin A (Con A)-stimulated murine lymphocytes at the peak of proliferation. This was accompanied by a diminished response to specific agonist stimulation and was impaired by the previous blockade of proliferative activity with tyrosine kinases (TPK) or protein kinase C (PKC) inhibitors (Cazaux et al., 1995). Also, agonist desensitization of βAR in human lymphocytes, impaired both βAR-mediated increase of cAMP and βAR-mediated inhibition of mitogenic activation of resting cells (Werner et al., 1997).

To further study βAR functional role in lymphocyte activation and proliferation, the aim of the present work was to analyze the expression of βAR and the intracellular events triggered by their activation on a hyperproliferative cell line from T lymphoid origin, namely BW5147 cells. Tumor cells showed a poor βAR expression and uncoupling to the a.c. system, but these receptors were able to activate PKC, a signal directly related to cellular proliferation. We also found a bidirectional modulation of βAR expression via the enzymatic pathways triggered by proliferative events.

2. Materials and methods

2.1. Cell suspensions and culture conditions

The tumor cell line BW5147 (a generous gift from Dr. A. Schimpl, Institute für Virologie und Immunobiologie der Universität Würzburg, Germany) is a T cell lymphoma, that expresses H-2^k haplotype and αβ T cell receptor, as tested by fluorescent activated cell sorter analysis with specific antibodies against the corresponding surface markers. These cells were cultured at an optimal concentration of $1–5\times10^5$ cells/ml, in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and antibiotics, with twice weekly splitting once they have reached exponential growth. Lymphoid cell suspensions from C3H (H-2^k) inbred mice lymph nodes as well as nylon wool purified T cells were used as control and were prepared aseptically as described before (Cremaschi et al., 1989; Genaro et al., 1986). T cell purification was higher than 97% as checked by lysis with anti-Thy plus complement and by indirect immunofluorescence. Cells were cultured in the same medium as tumor cells at a concentration of 1×10^6 cells/ml.

Cells were settled at a final volume of 0.2 ml in 96-well flat-bottom microtiter plates (Nunc™) for microcultures or were kept in T-25 or T-75 culture flasks (Corning, NY) for macrocultures. Cells were cultured for different times and inhibitors were added at the beginning of the culture where indicated.

The proliferation kinetic of BW5147 cells was determined on BW5147 synchronized cells, kept for 24 h in FCS-deprived medium and were then re-cultured in FCS-rich medium. Cultures were pulsed with [^{3}H]thymidine ([^{3}H]TdR, INC, Irvine, CA, USA; 15 Ci/mmol) for 6 h prior to harvest. Cell growth kinetic is depicted in Fig. 1. The peak of proliferation was obtained at 14 h of culture approximately.

2.2. Proliferation assays

Cells were taken from bulk cultures and transferred into 96-well plates for a pulse with [^{3}H]TdR for the last 6 h, as described before (Cazaux et al., 1995). Results are expressed as stimulation index (S.I.) calculated as the rate between dpm values in experimental cultures and dpm from control values obtained with a 3-min lasting-pulse of [^{3}H]TdR. The percentage of inhibition (% inh) for experimental cultures performed in the presence of inhibitors was calculated as:

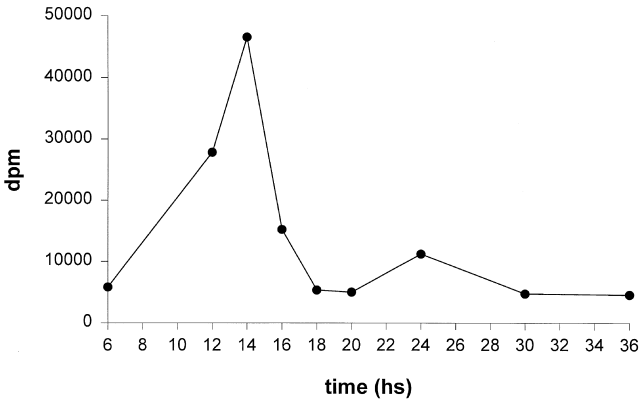


Fig. 1. Kinetic course of synchronized BW5147 cell proliferation. BW5147 cell cultures were synchronized by FCS deprivation. Kinetic of proliferation was determined by culturing 1×10^5 cells in medium with 10% FCS for different times with 6 h lasting pulses of [^{3}H]TdR as explained in Section 2. Results shown are an example of three independent experiments performed by triplicate.

$$\%\ \text{inh} = \frac{[1 - \text{dpm BW5147 cells} + \text{inhibitor} - \text{dpm control}]}{\text{dpm BW5147 cells} - \text{dpm control}} \times 100$$

2.3. [^{125}I]Cyanopindolol ([^{125}I]CYP) binding to intact cells

Cells ($2–3\times10^6$ cells/tube) were incubated with [^{125}I]CYP (New England Nuclear, 2200 Ci/mmol) solutions, 1–300 pM radioligand concentrations in 50 mM phosphate buffer, made isotonic with NaCl, 10 mM $MgCl_2$ and 10^{-4} M phentolamine. After 30 min incubation at 30°C samples were filtered through Whatman GF/C filters as indicated before (Cremaschi et al., 1991) and filters were counted in a Wallac counter. Total binding curves were analyzed by computer program LIGAND as already described (Cremaschi et al., 1991, 1994). The non-specific binding parameter fitted by LIGAND from the total binding curves did not differ from those determined experimentally using 1 μM l-propranolol, as demonstrated by data from tubes containing the β-antagonist that were included in all binding assays. It is worth noting that non-specific binding was always ≤20% of total binding. B_{max} values expressed as sites/cell were calculated from B_{max} pM values determined by LIGAND analysis, according to:

$$B_{max}\ (\text{sites/cell}) = \frac{B_{max}\ (\times 10^{-12}\ \text{M}) \times V_i\ (\text{ml}) \times 6.023 \times 10^{23}\ \text{sites/mol}}{1000\ (\text{ml/l}) \times \text{number cells/assay tube}}$$

where V_i is incubation volume.

2.4. Adenylate cyclase (a.c.) assay

Enzyme activity was measured in a final volume of 150 μl of 50 mM Tris–HCl, 2 mM $MgCl_2$, 1 mM EDTA, pH 7.4 (TME-buffer), containing 0.1 mM ATP, 5 mM theophylline, 0.2 mg/ml creatine phosphate and 2–4 μCi [α-^{32}P]ATP per assay. Direct activation of Gs protein was tested with aluminium tetrafluoride ($AlFl_4$) (10 mM NaF, 20 μM $AlCl_3$ and 6 mM $MgCl_2$) in the presence of 10^{-6} M CGP 12177 (Ciba-Geigy product 12177, 4-(3-*t*-butylamino-2-hydroxypropoxy)benzimidazol-2-one). CGP 12177, a β-adrenergic blocker, blocks the action of remanent endogenous neurotransmitter. Basal samples also contained 10^{-6} M CGP. Hormone stimulation was tested in the presence of 10 μM isoproterenol (ISO) or epinephrine (EPI) and 50 μM GTP. The assay was carried out at 30°C and the [^{32}P]cAMP formed was measured according to Salomon et al. (1974).

2.5. Cyclic-AMP production in intact cells

Cells (10^7 cells/ml) in RPMI 1640, were incubated with 3 isobutyl-*l*-methyl xanthine (MIX, 1 mM) at 37°C for 20 min and were then left alone (basal value) or were incubated with 10 μM ISO or prostaglandin E_1 (PGE_1) for the indicated times. At the end of the incubation time, 2 ml of chilled ethanol were added. The cells were then homogenized and centrifuged at $3500\times g$ at 4°C for 15 min. The supernatants were collected and pellets were re-homogenized in 1 ml of ethanol:water (2:1) and centrifuged. Supernatants were joined and evaporated at 55°C under N_2 stream. The cyclic nucleotide present in the residue was dissolved in 0.5 ml of assay buffer (Tris–HCl 50 mM, pH 7.4, theophylline 8 mM, 2-mercaptoethanol 6 mM, EDTA 1 mM) and stored at −20°C until the assay was carried out. Aliquots of 50 μl were taken for nucleotide determination using the procedure described before (Brown et al., 1971).

2.6. Protein kinase C (PKC) determination

Synchronized cells ($0.5–1\times10^7$ cells/sample) were incubated alone or in the presence of the indicated drugs in FCS-deprived RPMI 1640 medium, for the indicated times (total incubation time, 30 min) and were immediately frozen in liquid N_2. PKC was purified from subcellular fractions as previously described (Genaro and Boscá, 1993). PKC activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into histone H_1 (Genaro and Boscá, 1993). Incubations were conducted in a final volume of 85 μl at 30°C for 30 min. In the final concentrations, the assay mixture contained 25 mM ATP (0.4 mCi), 10 mM Mg acetate, 5 mM β-mercaptoethanol, 50 mg of histone H_1, 20 mM Hepes, pH 7.5, and, unless otherwise indicated, 0.2 mM $CaCl_2$ and 10 mg/ml of phosphatidylserine vesicles. The incorporation of [^{32}P]phosphate into histone was linear for at least 30 min. The reaction was stopped by the addition of 2 ml of ice-cold 5% trichloroacetic acid with 10 mM H_3PO_4. The radioactivity retained on GF/C glass fiber filters after filtration was determined by counting the filters in 2 ml of scintillation fluid. PKC activity was determined after subtracting the ^{32}P-incorporation in the absence of Ca^{2+} and phospholipids. Data were expressed as pmol of phosphate incorporated into the substrate per minute and per 10^7 cells (pmol/min per 10^7 cells). The selective PKC substrate peptide, MBP [4–14] (Yasuda et al., 1990) (Gibco-BRL, Life Tech.) was also used to measure PKC activity purified from subcellular lymphoid fractions, following the instructions of the PKC assay system of Gibco. PKC specificity was confirmed by means of the PKC pseudosubstrate inhibitor peptide PKC [19–36] provided by Gibco.

2.7. Drugs

The following drugs were used in cultures at the final concentrations indicated in Section 3. The protein kinase inhibitors, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine

dihydrochloride (H-7), *N*-(2-guanidinoethyl)-5-isoquinoline sulfonamide hydrochloride (HA 1004), staurosporine, the selective PKC inhibitor GF-109203X (bisindolylmaleimide) and the TPK inhibitor genistein, were purchased from Sigma. H-7 and HA 1004 were dissolved in sterilized water. Staurosporine, GF-109203X and genistein were dissolved in DMSO. All drugs were further diluted (at least 1/1000 or more) in RPMI 1640 medium to achieve the concentrations indicated in Section 3.

2.8. Statistical analysis

The Student's *t*-test for unpaired values was used to determine the levels of significance. When multiple comparison were necessary after analysis of variance, the Student–Newman–Keuls test was applied. Differences between means were considered significant if $P \leq 0.05$.

3. Results

3.1. Expression of βAR in hyperproliferative BW5147 cells: comparison with normal T lymphocytes

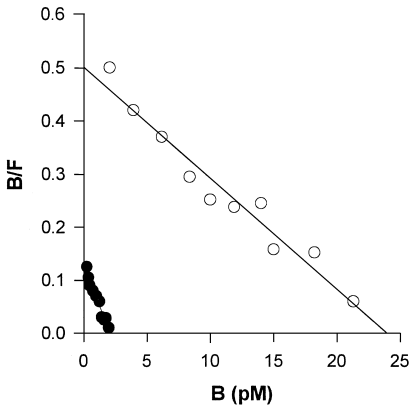
The expression βAR on lymphoid cells was analyzed by binding assays using the specific β-adrenergic radioligand antagonist, [^{125}I]cyanopindolol ([^{125}I]CYP). Table 1 shows that BW5147 cells displayed a significant decrease in the amount of βAR when compared to normal T lymphocytes, as indicated by B_{max} values determined by LIGAND computer program. The affinity constant did not show statistically significant modifications. The decrease in βAR number was not due to culture conditions, as normal cells cultured in similar conditions did not show any reduction in βAR sites. This decrease was observed on synchronized cells and did not show any modification at different times of the proliferative rate.

3.2. Coupling of βAR to the adenylate cyclase system in BW5147 cells

To analyze the functionality of βAR on BW5147 cells, the activity of a.c. in response to β-adrenergic agonists was measured on BW5147 cell membranes. Fig. 2A shows that neither ISO nor EPI were able to stimulate a.c. activity in tumor T lymphoma cells while these drugs induced 6.6- and 4.6-fold increases in normal T lymphocytes, respectively (Fig. 2B). As βARs are coupled to the a.c. system through a Gs regulatory protein, we analyzed if the components of the a.c. system were still preserved in these cells. We found that aluminum fluoride ($AlFl_4$), a direct activator of Gs protein, was able to induce a.c. stimulation on both tumor and normal cells (Fig. 2A,B), pointing to the fact that the remaining receptors on T lymphoma cells were uncoupled to the a.c. system. Moreover, when we measured intracellular cAMP levels on intact BW5147 cells, we did not obtain any response to ISO despite the positive response, i.e., an increment of cAMP levels, to PGE_2 (an autacoid whose receptors are also linked to the a.c. through a GTP binding protein) (Fig. 2C). It is worth noting that both ISO and PGE_2 were able to stimulate intracellular cAMP formation on normal T cells (Fig. 2D). This pointed to the fact that the alteration in receptor

Table 1
[^{125}I]CYP binding in normal and in BW5147 T lymphoma cells

Cell type[a]	Treatment	Time of culture (h)	[^{125}I]CYP binding[b]	
			K_d±S.E. (pM)	B_{max}±S.E (sites/cell)
BW5147 cells	Without FCS	24	26.4±3.2	88.0±10.0*
	With FCS	8	22.3±2.0	50.1±6.0*
		24	17.7±2.0	60.8±6.0*
		Random	28.3±3.5	70.0±12.0*
Normal cells	None	None	76.0±5.3	697.0±27.0
		24	52.0±4.8	723.0±25.0



[a] Lymphoid cell suspensions from BALB/c mice lymph nodes as well as nylon-wood purified T cells were prepared aseptically following the indicated procedures. Cells were kept in culture for 1 day or were analyzed immediately. Results shown in the table are those obtained on total lymph node cells and showed no statistic differences with those obtained from T enriched populations (○) depicted in the Scatchard plot shown. BW5147 cells were cultured, as indicated in Section 2, for the indicated time and Scatchard plot from 24-h cultured tumor cells (●) are illustrated in the adjoining figure. Results from Scatchard plots are representative of at least five independent experiments.

[b] [^{125}I]Cyanopindolol ([^{125}I]CYP) binding was performed on $2–3\times10^6$ cells/tube incubated with increasing concentrations of the radioligand. Total binding curves were analyzed by computer program LIGAND. Results shown are the mean±S.E.M. of at least four independent experiments.

*Differs from normal T lymphocytes with $P<0.001$.

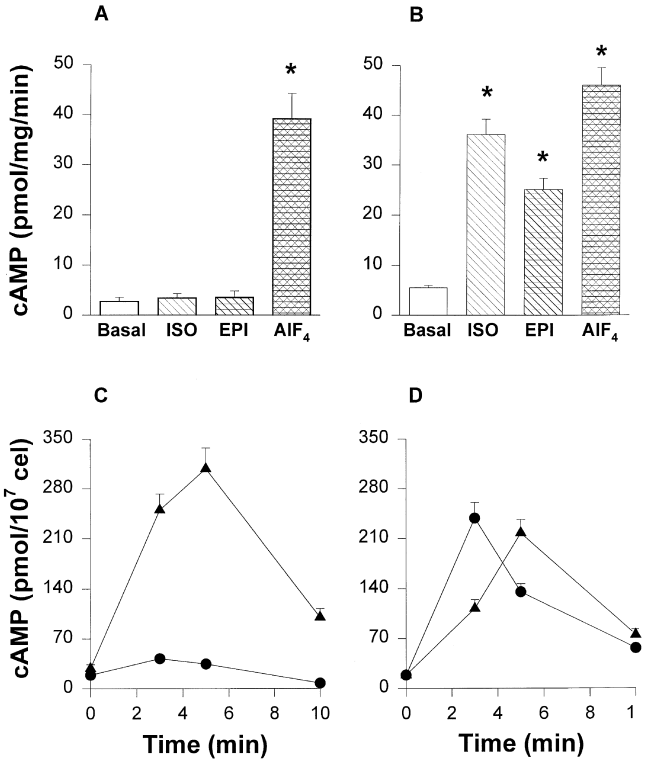


Fig. 2. Functionality of βAR on lymphoma cells in comparison to normal T lymphocytes. (A,B) Stimulation of adenylate cyclase (a.c.) above basal values (Basal), by β-agonists, (−)-isoproterenol (ISO) and (−)-epinephrine (EPI) in concentration 10 μM, and the Gs protein direct activator, aluminum tetrafluoride (AlF_4) were measured on lymphoma (A) or normal T (B) cell membranes as indicated before. Values are the mean±S.E.M. of at least three separate experiments. *Differs significantly from basal values with at least $P \leq 0.01$. (C,D) Kinetics of cAMP production on tumor (C) or normal (D) T lymphocytes; 1×10^6 cells were incubated for the indicated time, in the presence of 10 μM (−)-isoproterenol (●) or 10 μM PGE_1 (▲). Results shown are the mean±S.E. of four independent experiments performed in triplicate.

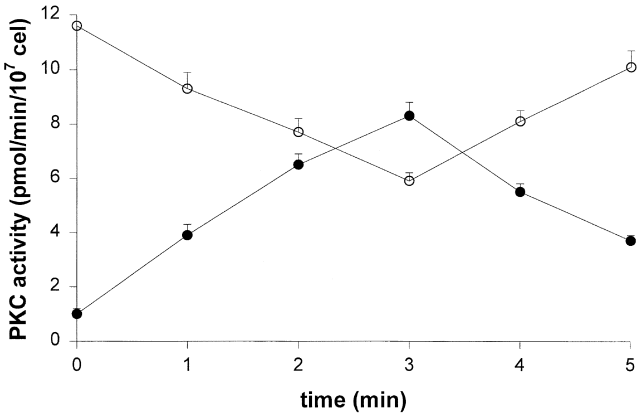


Fig. 3. Kinetic course of (−)-isoproterenol-mediated effect on PKC activity on BW5147 cells. BW5147 synchronized cells were incubated for the indicated times with 10 μM (−)-isoproterenol. PKC activity was purified from subcellular fractions, namely cytosol (○) and membrane (●), and measured as indicated using histone H_1 as substrate. Results are representative of four experiments performed in duplicate. It is worth noting that a 3-min contact of BW5147 cells with the phorbol ester PMA (2 nM), that positively activates PKC, induced approximately a 90% translocation of total (cytosol+membrane) PKC activity to membrane fractions (12.2±0.6 pmol/min per 10^7 cells).

functionality on tumor cells seems to be specific for βAR coupling to the Gs protein.

3.3. PKC stimulation as a consequence of βAR activation

To analyze the possibility of βAR coupling to a positive signal for T lymphocyte proliferation, we studied the ability of the β agonist ISO to induce the activation of PKC. We found that treatment of BW5147 synchronized cells incubated with ISO in RPMI 1640 medium not supplemented with FCS, gave a time-peak increase of PKC activity at 3 min incubation, as measured by histone H_1 phosphorylation. This activation induced the translocation of PKC from cytosol to membrane fractions (Fig. 3).

We performed dose–response curves to ISO, using the specific PKC substrate peptide MBP [4–14] and histone H_1 for comparison, on PKC purified from cytosol and membrane fractions, in order to confirm that this histone kinase activity was indeed PKC activity. Table 2 shows that ISO mediated, in a dose-dependent manner, both the translocation of PKC to membranes and the proliferation of BW5147 cells. The dose of ISO that gave the maximal stimulation of PKC activity and the peak of cellular proliferation was 1×10^{-6} M. It is worth noting that the β-antagonist propranolol (10 μM) inhibited the proliferative action of ISO (0.1 μM) in 97±5%. As it was expected, ISO exerted no effect on both PKC and proliferative activities in normal T lymphocytes (data not shown).

3.4. Blockade of ISO-induced PKC membrane translocation by βAR antagonist and by different intracellular enzymatic blockers: effects on BW5147 proliferation and βAR expression

To assess the specific involvement of βAR on ISO-induced PKC translocation we studied the effect of the β-antagonist propranolol on membrane PKC activity. Propranolol was able to block ISO-induced PKC activation using both histone and MBP [4–14] as substrates (Fig. 4). Fig. 4 also shows that the effect of ISO on PKC was impaired by different blockers of the enzymatic intracellular pathways involved in T lymphocyte activation, namely the PLC blocker NCDC (Walenga et al., 1980), the TPK blocker genistein (Mustelin et al., 1990) and the calcium blocker verapamil (Zanker et al., 1994).

Moreover, the treatment of BW5147 cells with genistein (20 μg/ml), NCDC (10 μM), with the PK blocker H-7 (5 μM) or with the selective PKC blocker GF-109203X (1 μM), induced both a recovery of βAR sites, as shown by

Table 2
Dose-response stimulation of PKC activity on BW5147 by (−)-isoproterenol

Treatment[a]	PKC (pmol/min per 10^7 cell)[b]				Proliferation[c] (dpm)
	H_1-histone		MBP [4–14]		
	Cytosol	Membrane	Cytosol	Membrane	
None	11.9±0.9 (89%)	1.5±0.3 (11%)	26.7±1.8 (87%)	4.0±0.4 (13%)	5685±503
ISO (10^{-8} M)	9.2±0.8 (77%)	2.7±0.3 (23%)	26.6±2.0 (80%)	6.5±0.5 (20%)	6318±715
ISO (10^{-7} M)	8.4±0.8 (65%)	4.5±0.4 (35%)*	19.2±0.9 (60%)*	12.8±0.5 (40%)*	11607±691**
ISO (10^{-6} M)	7.4±0.6 (55%)*	6.1±0.5 (45%)*	16.6±1.7 (45%)*	20.3±1.9 (55%)*	15098±1267**
ISO (10^{-5} M)	4.2±0.4 (41%)*	6.0±0.3 (59%)*	16.7±0.8 (46%)*	19.6±2.0 (54%)*	6969±825

[a] BW5147 synchronized cells were incubated for 3 min alone (none) or in the presence of increasing concentrations of (−)-isoproterenol (ISO).
[b] PKC activity purified from subcellular cytosol and membrane fractions was assay on both H_1-histone or MBP [4–14] as substrates. Percentages of total (cytosol+membrane) PKC activities on both subcellular fractions are also shown between brackets.
[c] BW5147 cells were synchronized and recultured in FCS-rich medium for 24 h in the absence or presence of the indicated concentrations of ISO. Aliquots (0.2 ml) were taken and pulsed with [^{3}H]TdR as indicated in Section 2. Results shown are the mean dpm values±S.E.M. of three experiments performed in triplicate.
*Differs significantly from basal (none) values with $P<0.001$; **differs significantly from basal (none) values with $P<0.05$.

Table 3
Recovery of β-adrenergic sites on BW5147 cells by blockers of enzymatic-intracellular pathways

Treatment[a]	[^{125}I]CYP binding[b]		Proliferation[c] (% inhibition)
	K_d±S.E. (pM)	B_{max}±S.E. (sites/cell)	
None	20.3±4.7	56.0±6.0	–
Genistein (20 μg/ml)	24.7±3.5	215.8±19.0*	36.0±4.0
H-7 (5 μM)	15.2±2.3	139.7±13.0*	35.0±2.0
HA 1004 (5 μM)	19.6±1.7	63.2±11.0	0.5±0.4
GF-109203X (1 μM)	22.5±2.6	184.8±20.0*	62.0±5.9
NCDC (10 μM)	21.7±3.0	177.2±24.8*	43.0±7.0

[a] BW5147 synchronized cells were incubated for 24 h in FCS-rich medium plus the indicated enzyme blockers.
[b] [^{125}I]CYP binding was performed as described above. Results shown are the mean±S.E.M. of at least three independent experiments.
[c] Proliferation was calculated as percentage of inhibition with respect to non-treated cells (basal values). Results are the mean±S.E.M. of at least three independent experiments performed in triplicate.
*Differ significantly from basal values with $P<0.001$.

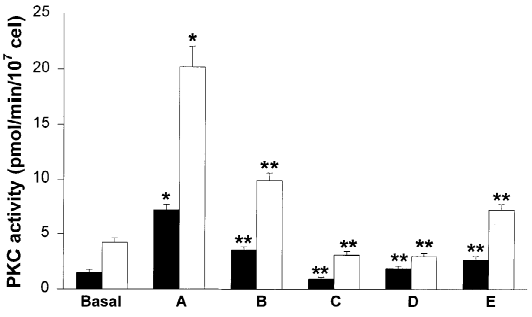


Fig. 4. Blockade of (−)-isoproterenol-induced PKC membrane translocation by the β-antagonist propranolol and by blockers of the enzymatic intracellular pathways involved in T lymphocyte activation. PKC activity on both histone H_1 (■) or MBP [4–14] (□) phosphorylation is shown. BW5147 synchronized cells were incubated for 20 min alone (Basal), with 10 μM propranolol (B), 10 μM NCDC (C), 20 μg/ml genistein (D) or 1 μM verapamil (E) and for an additional 3 min with 1 μM isoproterenol (A–E). It is worth noting that all blockers used have no effect on basal PKC activity (data not shown). Values are the mean±S.E. of at least five independent experiments performed in duplicate. *Differs significantly from the corresponding basal values with $P\leq0.01$. **Differs from isoproterenol stimulation with $P\leq0.005$.

the B_{max} values, as well as an inhibition of cellular proliferation (Table 3). Interestingly, the PK blocker HA1004 (5 μM) neither induced a recovery of these sites nor inhibited cellular proliferation. It is worth noting that both H-7 and HA 1004 inhibit cyclic nucleotide-dependent PK at 5 μM, but at this concentration, only H-7 inhibits PKC (Asano and Hidaka, 1984; Takahashi et al., 1990). Fig. 5 shows that staurosporine (1 nM) that selectively blocked PKC (Tamaoki et al., 1986), increased βAR sites as observed in the Scatchard plots obtained for [^{125}I]CYP binding on BW5147 cells in the presence or in the absence of this blocker. Data obtained from BW5147 cells treated with genistein are given for comparison.

4. Discussion

The interaction between the neuroendocrine and the immune system implies a bidirectional circuit involving shared usage of common signal molecules and their receptors. Here we show an example of these interactions

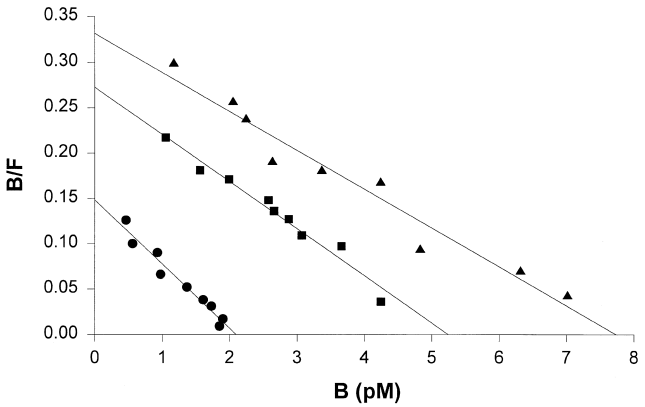


Fig. 5. Scatchard analysis of βAR recovery on BW5147 cells after pretreatment with PKC or TPK blockers. T lymphoma synchronized cells were incubated for 24 h in FCS-rich medium alone (●), or in the presence of 1 nM staurosporine (■), or 20 μg/ml genistein (▲). [^{125}I]CYP binding assays, with increasing concentration of the radioligand, were performed as described in Section 2. Results are representative of four independent experiments performed in triplicate. B_{max} values from staurosporine or genistein treated cells differ significantly from basal values with $P \leq 0.01$.

between the sympathetic system and T lymphocyte function. In fact, the expression and function of βAR are modulated by T lymphocyte activation, as supported by the finding of a reduced number of βARs, functionally uncoupled to the a.c. system, in BW5147 T cells. This R uncoupling is indicated by the inability of β-adrenergic agonists to promote a.c. activation and intracellular cAMP production in BW5147 cells, despite their ability to induce these effects in normal T lymphocytes, and the direct activation of Gs with ALF_4 leading to a.c. system stimulation to a same extent in tumor and normal T cells. Furthermore, PGE_2 increased cAMP formation on both cellular types, thus indicating a specific uncoupling of βAR to the a.c. system in the lymphoma cell type.

Reduced β-adrenoceptor distribution on these cells was unaffected when studied at different times of the proliferative rate or by having the cells in culture randomly for several days. We have previously shown that mitogenic activation of normal T lymphocytes decreased βAR sites at the peak of proliferation (Cazaux et al., 1995), but these receptors remained coupled to the a.c. system. These facts pointed to important differences between the functionality of βARs on both normal and tumoral cells. The inability to demonstrate changes in the expression of βAR in BW5147 proliferative rate could be probably related to the transformed phenotype of this cell line. In fact, the reduction number of βAR was not modified on synchronized BW5147 cells kept in non-FCS supplemented medium for 24 h. Under this experimental condition higher but not-significant B_{max} values were obtained. It is possible that these higher B_{max} values indicate that arrest on cellular growth would increase βAR number. This could not be confirmed by setting the cells in the absence of FCS for longer periods of incubation as this condition results in cell death. Nevertheless, when inhibiting cellular proliferation with blockers of the intracellular enzymatic pathways that are involved in T cell activation, we found a recovery of β-adrenergic binding sites, thus indicating that these events are involved in the down-regulation of βARs.

Loss of receptors might be a result of receptor internalization and degradation, as a consequence of kinase activation leading to β-adrenergic desensitization (Sibley et al., 1988). Sibley et al. (1984) showed that activation of PKC by phorbol diesters impaired the isoproterenol-stimulation of a.c. with no effect on fluoride activity, in duck erythrocytes. Similarly, we observed no response to β-agonists, but positive action of AlF_4 on a.c. activity, in BW5147 cells. Also, β-adrenergic receptor kinases, that are involved in βAR homologous desensitization, were increased by T lymphocyte activation through a PKC-mediated mechanism (De Blasi et al., 1995). In BW5147 cells, both TPK and PLC blockers interfered with the biochemical events downstream to TPK activation, leading to a recovery of βAR. Additionally, a similar effect was observed by inhibiting PKC, both selectively by the doses used of H-7 (and not HA1004) and staurosporine, or specifically by GF-109203X. The signal transduction capacity of the cells with recovered β-adrenoceptors are now under study. Preliminary results seem to indicate that the recovered receptors are now coupled to the a.c. system.

So we analyzed whether the remaining βARs were able to activate a positive signal for cell proliferation, namely PKC activity. We found that the β-adrenergic agonist isoproterenol was able to induce the specific activation of PKC, in a dose-dependent manner, as shown by the phosphorylation of both the histone H_1 and of the specific PKC-substrate MBP [4–14]. The β-agonist did not induce either PKC activation or cellular proliferation in normal T lymphocytes, but it increased BW5147 cell proliferation in a dose-dependent manner. The effects observed on BW5147 cells could be a function of the transformed phenotype of this cell line and could contribute to its hyperproliferative pattern.

The effect of ISO both on BW5147 cell proliferation and on PKC activation were blocked by the β-antagonist propranolol. The fact that propanolol did not completely reverted ISO-mediated activation of PKC is difficult to explain and could be related to the transformed phenotype of BW5147 cells. The effect of ISO on PKC activity was also inhibited by the blockade of TPK and PLC, thus indicating that the intracellular events involved in PKC activation are involved in βAR-mediated effects as well. Furthermore, the fact that verapamil also inhibited ISO-induced PKC activation, would probably indicate that Ca^{2+}-dependent PKC isoenzymes are stimulated by the β agonist. Supporting this idea, a dose-dependent increase in cytosolic-free Ca^{2+} concentrations by ISO, was demonstrated in the human Jurkat T cell line (Takemura et al., 1995). The exact molecular mechanism of βAR coupling to PKC activation remains to be elucidated. However, a

homology between intracellular receptors for activated PKC and the β subunit of G proteins implicated in membrane anchorage of the β-adrenergic receptor kinase, was described (Ron et al., 1994). In addition, other authors (Daaka et al., 1997) have demonstrated that β_2-adrenoceptors, besides most of their actions being commonly mediated through Gs protein coupling to the a.c. system, are able to stimulate mitogen-activated protein kinases by coupling to a Gi protein and through a pathway involving non-receptor TPK in HEK293 cells.

Modulation of βAR expression on T lymphocytes were involved in cellular function (Ramer-Quinn et al., 1997). The lowering of βAR number could be a consequence of the cascade of intracellular biochemical events that transduces the signal across the outer membrane into the cell nucleus, triggering a genetically predetermined program which would include βAR down-regulation. Evidence of βAR down-regulation as a change in the synthesis of receptors at the gene level was demonstrated in thymocytes after an immunogenic challenge (Morale et al., 1992). Furthermore, the marked decrease in β_2AR number and β_2AR mRNA levels found early after immunization were related to the facilitation of the immune response (Morale et al., 1992).

Taking together our previous results on mitogen-activated T cells (Cazaux et al., 1995) and the present data, we suggest that either the stage of T lymphocyte differentiation or the mode of cell activation might be important for regulating βAR number. Down-regulation of βAR would imply the loss of a mechanism that exerts a negative neuroimmune control of cellular proliferation. Other authors demonstrated that the induction of cAMP-dependent protein kinase activity inhibit anti-CD3 Ab-induced T cell proliferation (Bauman et al., 1994) and that increased activation of PKA contributes to T cell deficiency in common variable immunodeficiency (Aukrust et al., 1999). The fact that the remaining βAR sites are able to couple to a positive signal for T lymphocyte proliferation would point to a differential degree of modulating T cell function, leading to an hyperproliferative state. Our results showing the cross-talk among intracellular biochemical pathways would sound basis for future research in the differential neuroimmune control involved in normal and tumor cell development.

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