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Differential signal transduction pathways regulating interleukin-2 synthesis and interleukin-2 receptor expression in stimulated human lymphocytes

Marta Szamel [a,*], Hans Leufgen [a], Roland Kurrle [b], Klaus Resch [a]

[a] *Institute of Molecular Pharmacology, Department of Pharmacology and Toxicology, Medical School Hannover, D-30623 Hannover, Germany*
[b] *Behringwerke AG, Marburg, Germany*

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Abstract

In human peripheral blood lymphocytes stimulated via the T-cell antigen receptor/CD3 complex IL-2 synthesis and cellular proliferation were effectively inhibited by a concentration of ouabain as low as 50 nM, whilst the expression of high affinity IL-2 receptors was not influenced. Binding of the monoclonal antibody, BMA 031 to the T-cell antigen receptor/CD3 complex resulted in a bimodal activation of protein kinase C. The activation of protein kinase C-α in the early phase of T-lymphocyte activation was not affected by 50 nM ouabain, in contrast sustained activation of protein kinase C-β, between 90–240 min of stimulation was completely abolished by the cardiac glycoside. When protein kinase C was directly activated by PMA + ionomycin, 50 nM ouabain was ineffective in inhibiting protein kinase C activation, as well as subsequent IL-2 synthesis, suggesting that the glycoside interfered with signal transducing mechanism(s) upstream of the activation of protein kinase C. Ouabain had no influence on the elevation of intracellular calcium concentration in BMA 031 stimulated lymphocytes, ruling out the possibility that it interfered with the T-cell antigen receptor dependent phosphatidylinositol response. In contrast, lysophosphatide acyltransferase catalysed elevated incorporation of polyunsaturated fatty acids was effectively inhibited by low concentrations of ouabain in BMA 031-stimulated T-lymphocytes, whereas stimulation with PMA + ionomycin had no influence on the plasma membrane phospholipid fatty acid metabolism. These results suggest, that differential signal transduction pathways are involved in the activation of protein kinases C-α and -β. They implicate that elevated incorporation of polyunsaturated fatty acids into plasma membrane phospholipids might contribute to sustained activation of protein kinase C-β, and establish a link between activation of protein kinase C-β and induction of IL-2 synthesis in human lymphocytes.

Keywords: Protein kinase C; T lymphocyte; Signal transduction

1. Introduction

The antigen or mitogen induced proliferation of resting T lymphocytes involves at least two major events; the synthesis and secretion of the ultimate mitogen interleukin 2 (IL-2) and the synthesis and surface expression of receptors thereof. Both events are initiated by the binding of activating ligands to the T-cell receptor/CD3 complex [1].

As an immediate consequence of the ligand T-cell receptor interaction the activation of phospholipase C catalysed hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), the so-called 'PI response', has been well established. Generation of the cleaving product inositol 1,4,5-triphosphate (IP_3) is linked to an elevation of the cytosolic free calcium concentration. Besides an increase in cytosolic calcium concentration formation of diacylglycerol second messengers within the plasma membrane have also been shown in response to antigen or mitogen stimulation [1]. Diacylglycerol second messengers activate protein kinases C, a family of enzymes responsible for many cellular functions and growth regulation [2].

Recently it has been shown that short-lived diacylglycerols led to short and transient activation and translocation of protein kinase C. While this was sufficient for the

Abbreviations: CHAPS, 3-((3-cholaminopropyl)dimethylammonio)-1-propanesulfonate; DMSO, dimethylsulfoxide; DTE, dithioerythritol; Hepes, (2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); TCA, trichloroacetic acid; PMA, phorbol 12-myristate 18-acetate; Tricine, *N*-tris(hydroxymethyl)methylglycine; Tris, tris(hydroxymethyl)aminomethane.

* Corresponding author. Fax: +49 511 5324081.

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expression of functionally active IL-2 receptors, synthesis of IL-2 required a sustained activation of the enzyme. These data implicated that an additional molecular mechanism coupled to the T-cell antigen receptor complex is involved in the cellular signalling leading to IL-2 gene expression and subsequent proliferation of T-lymphocytes [3–5].

Recently we have shown that in lymphocytes which received a single dose of short lived diacylglycerol incorporation of polyunsaturated fatty acids into plasma membrane phospholipids prolonged protein kinase C activation, which subsequently led to IL-2 synthesis and proliferation of human lymphocytes. Furthermore, we have shown that monoclonal antibodies raised against the T-cell antigen receptor/CD3 complex caused a bimodal activation of protein kinase C. These data suggested that long-term activation of protein kinase C, most probably involving elevated incorporation of *cis*-polyunsaturated fatty acids into plasma membrane phospholipids, was an absolute requirement of IL-2 synthesis in human lymphocytes [6]. Here we report that in human lymphocytes stimulated via the T-cell antigen receptor/CD3 complex the initial short term activation of protein kinase C is due to the protein kinase C isoenzyme, PKC-α, whereas the sustained activation is related to activation of protein kinase C-β. Inhibition by ouabain of the incorporation of polyunsaturated fatty acids into plasma membrane phospholipids abolishes the long-lasting activation phase of protein kinase C-β and the subsequent synthesis of IL-2 in human peripheral blood lymphocytes.

2. Materials and methods

2.1. Materials

Mouse mAb BMA 031 (IgG_{2b}), raised against monomorphic determinants of the T-cell antigen receptor was a kind gift of Behringwerke AG, Marburg. Ionomycin (Calbiochem) and phorbol 12-myristate 13-acetate (PMA) (Sigma) were dissolved as a stock solution in DMSO at a concentration of 5 mg/ml. For activation of cells aliquots were diluted with RPMI 1640 medium. Fatty acids, i.e., linoleic acid (18:2), linolenic acid (18:3) and arachidonic acid (20:4) (NuCheck Corp.) were dissolved at a concentration of 1 mg/ml in chloroform:methanol (2:1 v/v) and stored at −20° C. Before use aliquots were evaporated under N_2 and suspended in RPMI medium containing 5 mg/ml defatted albumin by sonification (50 w, three times 10 s at 4° C). Human recombinant interleukin-2 (hr-IL-2) and ouabain were from Boehringer, Mannheim.

2.2. Cells and cell culture

Peripheral blood lymphocytes were isolated by Ficoll/Isopaque gradient centrifugation. Cells were washed with RPMI 1640 and cultured in RPMI 1640 supplemented with 10% defatted FCS, 100 U penicillin, 100 μg/ml streptomycin and 2 mM glutamine in flat bottom microtiter plates at a density of $4 \cdot 10^5$ cells/well. Lymphocytes were activated by BMA 031 (5 μg/ml), PMA (100 ng/ml) and ionomycin (0.5 μg/ml) or their combinations as indicated in the experiments. These concentrations proved to be optimal in inducing lymphocyte proliferation [4,5]. After incubating cells for 68 h 0.5 μCi of [^{3}H]thymidine/well (20 Ci/mmol, Amersham) was added for a further 4 h. Cells were then harvested with an automatic cell harvester and [^{3}H]thymidine incorporation determined by liquid scintillation counting.

2.3. Determination of IL-2 synthesis

Cells ($2 \cdot 10^5$ in 0.2 ml of RPMI medium) were stimulated with the monoclonal antibody BMA 031 or with a combination of PMA + ionomycin, as indicated in the experiments, in flat bottom microtiter plates for 20 h. The supernatants were harvested and IL-2 activity quantitated with the help of the murine IL-2 dependent CTLL line as described by Gillis et al. [7]. Control experiments showed that neither DMSO nor any of the substances (i.e., PMA, ionomycin, ouabain) influenced growth and proliferation of the CTLL line, thus showing that proliferation of the CTLL line was really due to the presence of different amounts of IL-2.

2.4. Isolation and identification of IL-2 specific mRNA

Human lymphocytes were stimulated for 20 h in 250 ml Costar flasks (vol, 50 ml), at a cell density of $2 \cdot 10^6$/ml with 5 μg/ml of BMA 031 or with 100 ng/ml PMA + 0.5 μg/ml ionomycin, respectively. Subsequently RNA was isolated as described in Ref. [4]. Briefly, total cellular RNA was prepared by the guanidine isothiocyanate method, size-fractioned on formaldehyde-agarose gels and transferred to nitrocellulose filters. For hybridization, cDNA probes were nick translated with [^{32}P]dCTP (3000 Ci/mmol) to specific activities of $(2–5) \cdot 10^8$ cpm/μg. Hybridization was carried out as described in [8].

2.5. Binding of IL-2

Binding of IL-2 was carried out as described earlier [4]. Briefly, human lymphocytes were stimulated for 24 h. Cells were then washed and $5 \cdot 10^6$ lymphocytes were incubated in 0.2 μl with 0.1 μCi ^{125}I-IL-2 (spec. act. 900 Ci/mmol) and different concentrations of IL-2 ranging from 0.2 pmol to 200 nmol at 4° C for 1 h. Free and bound radioactivity were separated by centrifugation through silicon oil as described [4]. Number and affinity of the receptors were calculated by Scatchard analysis with a computer program.

2.6. Determination of intracellular calcium

Human lymphocytes were preincubated with ouabain for 4 h then loaded in the dark with 1 μM fura-2AM in medium HBSS (Hanks' balanced salt solution). Cells were then washed and allowed to recover for 30 min at 3° C in medium RPMI/10% fetal calf serum. Immediately before use aliquots containing $5 \cdot 10^6$ cells were washed, resuspended in HBSS and stimulated with 5 μg/ml BMA 031. Elevation of intracellular free calcium was detected in a fluorescence photometer with dual excitation at 340 and 380 nm. Emission was monitored at 505 nm. Cells were lysed with 1% Triton X-100 to determine ratios at maximum calcium concentrations, then calcium was chelated with 10 mM EGTA (pH 8.3) to determine ratios at minimum calcium concentrations. Fluorescence intensity ratios were converted into calcium concentrations using a computer program.

2.7. Incorporation of fatty acids into phospholipids

Lymphocytes ($5 \cdot 10^6$ cells) were incubated in 1 ml RPMI 1640 supplemented with 1 mg/ml defatted bovine serum albumin with 10 nmol of [^{14}C]linoleate, [^{14}C]linolenate or [^{14}C]arachidonate, respectively, by mixing ^{14}C-labelled fatty acids (Amersham) and unlabelled fatty acids (NuCheck). The specific activity was kept constant at 5 mCi/mmol.

2.8. Extraction and analysis of lipids

Lipids were extracted as described earlier [9] and separated by thin layer chromatography on TLC plastic sheets (Silica gel 60, layer thickness 0.2 mm) with a mixture of chloroform/methanol/acetic acid/0.9% NaCl (v/v, 50:25:8:4) at 4° C. The individual lipid fractions were visualized by iodine vapor, cut off and counted by liquid

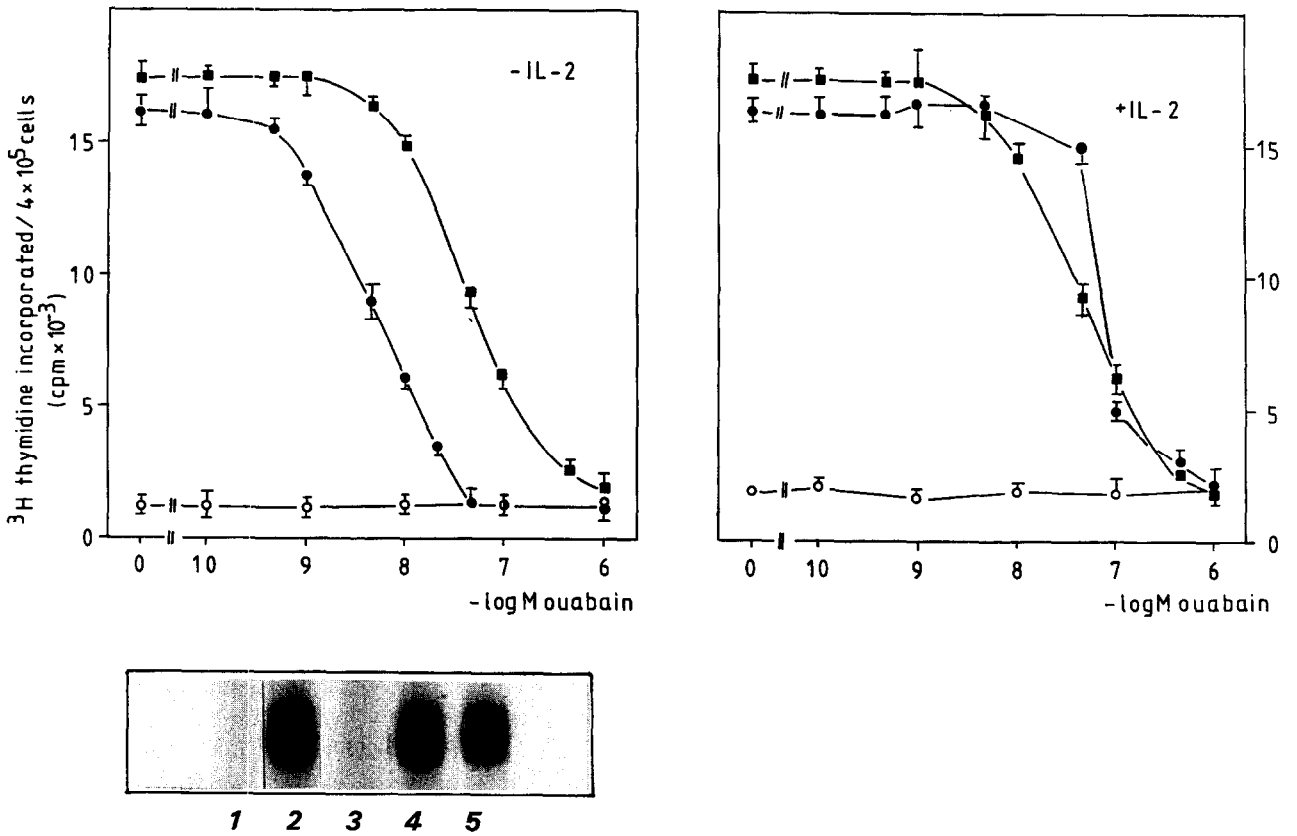

Fig. 1. Inhibition by ouabain of thymidine incorporation in BMA 031 and in PMA + ionomycin stimulated human lymphocytes. Human peripheral blood lymphocytes ($2 \cdot 10^6$ cells/ml) were preincubated with ouabain for 4 h. Cells were then washed and stimulated with BMA 031 (5 μg/ml) or with PMA (100 ng/ml) and ionomycin (0.5 μg/ml), respectively, in the presence or absence of hr-IL-2 (100 U/ml). After 68 h of incubation [^{3}H]thymidine was added for further 4 h and incorporation into DNA measured as described under Materials and methods. Results are means ± S.D. or triplicates of three independent experiments. ○—○, control, ●—●, BMA 031, ■—■, PMA + ionomycin. (Inset) Inhibition by ouabain of IL-2 gene expression of human peripheral blood lymphocytes. Human peripheral blood lymphocytes (10^8 cells) were preincubated with ouabain (50 nM) for 4 h. Cells were then washed and activated with 5 μg/ml BMA 031 or with 100 ng/ml PMA + ionomycin (0.5 μg/ml) for 8 h. Total cellular RNA was then extracted and size-fractionated as described under Materials and methods. Hybridization and autoradiography were performed as described under Materials and methods. 1, Control; 2, BMA 031; 3, BMA 031 + ouabain; 4, PMA + ionomycin; 5, PMA + ionomycin + ouabain.

scintillation counting. Incorporation was measured in fractions containing phosphatidylcholine, phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine.

2.9. Isolation of subcellular fractions from human lymphocytes

Lymphocytes ($5 \cdot 10^6$ cells/ml) were preincubated with $5 \cdot 10^{-8}$ M ouabain for 4 h and stimulated with BMA 031 (5 μg/ml) or PMA (100 ng/ml) for different lengths of time as indicated in the experiments. Cells were then washed and resuspended in isolation buffer (20 mM Hepes (pH 7.4), 1 mM EGTA, 1 mM EDTA, 5 mM DTE and 50 μg/ml leupeptin) at a cell density of 10^8 cells/ml. Cells were disrupted by nitrogen cavitation and cytosolic and plasma membrane fractions isolated as described earlier [4,6,10]. As lymphocytes contain a relatively small amount of endoplasmic reticulum, our preparation can be considered as a crude plasma membrane fraction containing less than 10% contamination by endoplasmic reticulum [10]. Protein was determined as described in [10].

2.10. Protein kinase C assay

Protein kinase C activity was determined as described elsewhere [4,6,10]. Briefly, samples were processed for determination of protein kinase C activity immediately after isolation. Cytosolic protein kinase C was measured in aliquots of the cytosolic fraction without further purification. Plasma membrane bound protein kinase C was solubilized by 1 mM CHAPS (3-((3-chloramidopropyl)dimethylammonio)-1-propanesulfonate) in isolation buffer for 20 min at 4° C setting the protein concentration to 1 mg/ml. The nonsolubilized material was removed by centrifugation (100 000 × *g*, 60 min, 4° C). Protein kinase C was measured in a reaction mixture containing 40 mM Hepes, 10 mM $MgCl_2$, 0.4 mM EGTA, 400 μg/ml histone (type III, Sigma) and 60 μM [^{32}P]ATP (100 cpm/pmol) with or without 2 mM $CaCl_2$, 200 μg/ml phosphatidylserine and 20 μg/ml 1,2-diolein. Lipids were dispersed by sonification. The reaction was started by mixing 100 μl of reaction medium with 10 μg of sample protein in isolation buffer. After an incubation for 3 min at 37° C the reaction was stopped by the addition of 200 μl TCA (10%, w/v) containing 15 mM K_2HPO_4. Precipitated protein was collected on 0.45 μm nitrocellulose filter. The precipitate was washed by 5 × 5 ml of cold TCA (5%, w/v) containing 15 mM K_2HPO_4, the filters were dried and radioactivity was measured by liquid scintillation counting. The enzyme activity is expressed as the difference between the values measured in the presence and absence of phospholipids and calcium in nmol of ^{32}P transferred per min/mg protein of the sample.

Table 1
Inhibition by ouabain of IL-2 secretion in stimulated lymphocytes

Stimulus	Ouabain (nmol/l)	IL-2 secreted (U/ml)
–	–	n.d.
–	1000	n.d.
BMA 031	–	30 ± 2
	1	32 ± 2
	5	16 ± 1
	10	7 ± 3
	50	< 2
PMA + ionomycin	–	212 ± 10
	10	203 ± 12
	50	196 ± 20
	100	185 ± 8
	500	108 ± 4
	1000	10 ± 2

Lymphocytes were preincubated with ouabain for 4 h. Cells were then washed and stimulated for 20 h, IL-2 secretion was determined as described in Materials and methods.
n.d.: not detectable.

2.11. Detection of protein kinase C isotypes by SDS-polyacrylamide gelelectrophoresis and immunoblotting

40 μg membrane protein was processed by SDS-PAGE on 10% polyacrylamide gels as described in [11]. Electroblotting of proteins to Immobilon^R membranes was carried out at a constant current of 200 mA for 2 h. Blots were then incubated with monoclonal antibodies against protein kinase C-α, protein kinase C-β (Seikagaku Kogyo, Tokyo), respectively, for 4 h, followed by incubation with a biotin-streptavidin-alkaline-phosphatase-coupled second antibody. Nitroblue-tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Amersham) were used as substrates to visualize protein kinase C subspecies.

3. Results

3.1. Inhibition by ouabain of [^{3}H]thymidine incorporation in stimulated human peripheral blood lymphocytes

As shown in Fig. 1 human lymphocytes showed a strong proliferative response to the monoclonal antibody, BMA 031, raised against the T-cell antigen receptor, as measured by [^{3}H]thymidine incorporation. Similarly, a combination of PMA and ionomycin also induced cell proliferation, that could not be further enhanced by the addition of exogeneous hr-IL-2. [^{3}H]Thymidine incorporation was inhibited by ouabain in a concentration dependent way. Whereas in lymphocytes which were activated with BMA 031 thymidine incorporation was completely abolished by 50 nM ouabain, DNA synthesis of cells activated with PMA + ionomycin was relatively insensitive to ouabain treatment, and complete inhibition of stimulated thymidine incorporation was achieved only by 1 μM ouabain. In lymphocytes which were activated by BMA 031 inhibition of thymidine incorporation caused by 50

Table 2
Lack of effect of ouabain on the induction of high affinity IL-2 receptors in BMA 031-stimulated human lymphocytes

Stimulus	Ouabain	K_d ($\times 10^{-12}$ M)	Sites/cell
–	–	–	< 50
BMA 031	–	4.15	1430
BMA 031	50 nM	4.11	1340
BMA 031	1 μM	–	n.d.
PMA + ionomycin	–	4.72	1390
PMA + ionomycin	50 nM	4.51	1700
	1 μM	–	n.d.

Lymphocytes ($2 \cdot 10^6$ cells/ml) were preincubated with ouabain for 4 h. Cells were then washed and stimulated with BMA 031 (5 μg/ml) or with PMA (100 ng/ml) and ionomycin (0.5 μg/ml) for 24 h. Cells were then washed again and ^{125}I-IL-2 binding carried out as described under Materials and methods. Results are means of triplicates.
n.d.: not detectable.

nM ouabain was fully reversible by the addition of exogeneous hr-IL-2. In contrast, even very high concentrations of hr-IL-2 were unable to overcome the inhibition of DNA synthesis caused by 1 μM ouabain, the concentration required for complete inhibition of activation by PMA and ionomycin.

As shown in the inset of Fig. 1 IL-2 gene expression was inhibited by 50 nM ouabain in BMA 031-stimulated lymphocytes, whilst this concentration of the glycoside was completely ineffective in inhibiting IL-2 gene expression in cells activated with PMA + ionomycin.

3.2. Effect of ouabain on the IL-2 synthesis in stimulated lymphocytes

Stimulation of human lymphocytes with the monoclonal antibody (BMA 031), or with a combination of PMA and ionomycin, resulted in high amounts of newly synthesized IL-2 (Table 1). In contrast, no IL-2 synthesis was measurable in cells which were preincubated with 50 nM ouabain before activating them with BMA 031. At this concentration ouabain had no effect on IL-2 synthesis in lymphocytes stimulated by PMA + ionomycin. Only when ouabain concentration was raised to 1 μM synthesis of this lymphokine was also suppressed in lymphocytes which were activated with PMA + ionomycin.

3.3. Effect of ouabain in the expression of high affinity IL-2 receptors in BMA 031 and PMA + ionomycin-stimulated human lymphocytes

As shown in Table 2, neither the number nor the affinity of high affinity IL-2 receptors was altered by 50 nM ouabain in BMA 031 or PMA + ionomycin stimulated cells. In contrast, no high affinity IL-2 receptors were detectable in stimulated cells in the presence of 1 μM ouabain.

3.4. Ouabain prevents sustained activation of protein kinase C in BMA 031-stimulated human lymphocytes

Activation of lymphocytes by BMA 031, PMA or PMA + ionomycin, respectively, led to activation of protein kinase C (Fig. 2 and Table 3) as measured by its translocation to the plasma membrane. PMA alone or in combination with ionomycin induced a rapid long-lasting activation and translocation of protein kinase C; after 240 min 90% of the total enzyme activity was still associated with the plasma membrane (Table 3). Preincubation of the cells with ouabain (up to 1 μM) for up to 4 h did not affect the activation and translocation of protein kinase C in PMA

Table 3
Lack of effect of ouabain on the activation and translocation of protein kinase C to plasma membranes of human lymphocytes

Stimulation (min)	Ouabain	Protein kinase C activity (nmol/mg protein per min)	
		activator	
		PMA	PMA + ionomycin
0	–	245 ± 12	218 ± 10
0	50 nM	233 ± 22	207 ± 19
0	1 μM	255 ± 39	226 ± 34
10	–	572 ± 43	509 ± 38
10	50 nM	559 ± 25	497 ± 22
10	1 μM	543 ± 32	483 ± 28
15	–	649 ± 34	577 ± 30
15	50 nM	636 ± 51	566 ± 45
15	1 μM	600 ± 44	534 ± 24
120	–	602 ± 46	535 ± 41
120	50 nM	613 ± 37	545 ± 32
120	1 μM	603 ± 27	536 ± 24
240	–	578 ± 31	514 ± 27
240	50 nM	592 ± 25	526 ± 22
240	1 μM	584 ± 38	519 ± 33

Human lymphocytes were preincubated with ouabain for 4 h. Cells were then washed and stimulated with PMA (100 ng/ml) or with the combination of PMA + ionomycin (0.5 μg/ml), respectively, for different lengths of time. Cells were then disrupted, plasma membranes isolated and protein kinase C activity determined as described under Materials and methods. Results are means ± S.D. of triplicates of four independent experiments.

and/or PMA + ionomycin stimulated cells (Table 3). On the other hand, the monoclonal antibody BMA 031 resulted in activation and translocation of protein kinase C to the plasma membrane after 15 min of stimulation, then the specific activity declined to control levels after 30–60 min. This was followed by a second, long-lasting rise in the enzyme activity (up to 4 h) (Fig. 2A). When lymphocytes were preincubated with 50 nM ouabain before the addition of BMA 031, the initial activation of protein kinase C was still present, the second, long-lasting activation of the enzyme, however, was completely abolished (Fig. 2A). As shown in Fig. 2B cytosolic protein kinase C activity always behaved in a mirror-like fashion.

3.5. Ouabain prevents the translocation of protein kinase C-β to the plasma membrane of BMA 031-stimulated human lymphocytes

The results so far suggested that ouabain might influence different signal transduction pathways leading to activation and translocation of different protein kinase C

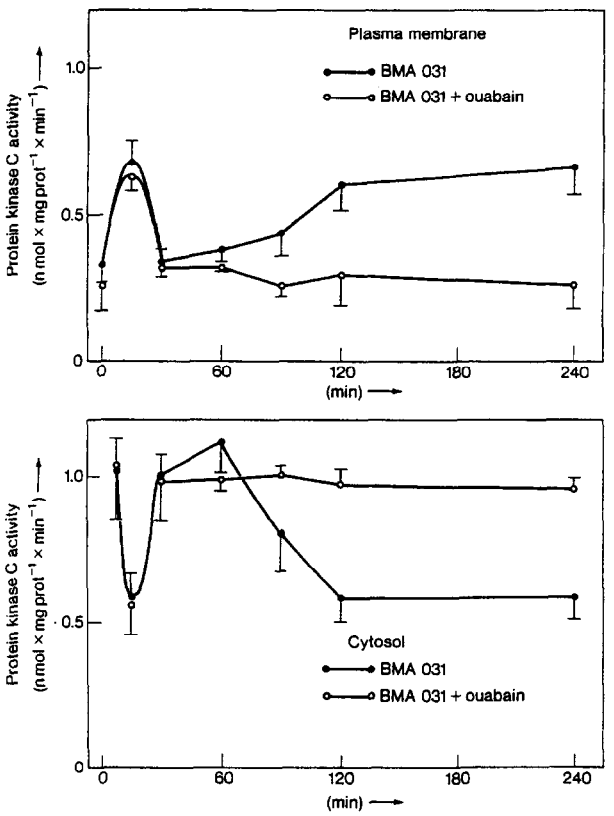

Fig. 2. Inhibition by ouabain of the sustained activation of protein kinase C in human lymphocytes. Peripheral blood lymphocytes were preincubated with 50 nM ouabain for 4 h. Cells were then washed and stimulated with BMA 031 for different lengths of time, as indicated on the abscissa. Cells were then homogenized, cytosolic and plasma membrane fractions prepared as described under Materials and methods. Protein kinase C activity was determined in plasma membrane (upper panel) and cytosolic fractions (lower panel) as described in Materials and methods. Results are means ± S.D. of triplicates of four independent experiments.

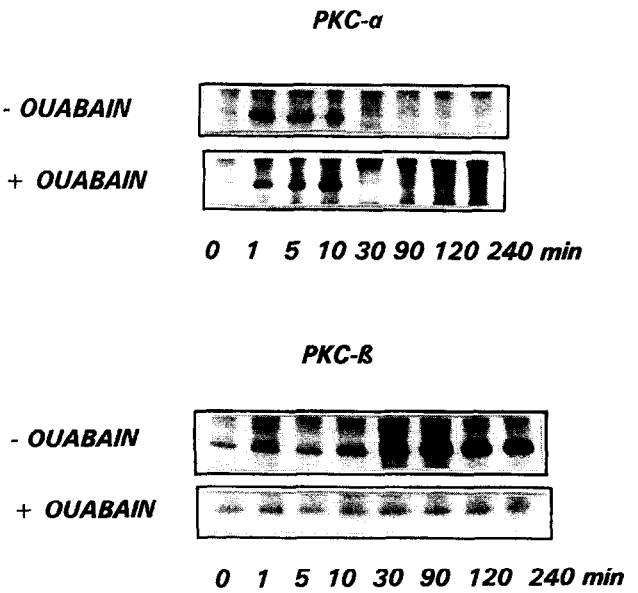

Fig. 3. Inhibition by ouabain of translocation of protein kinase C-β to plasma membranes of human peripheral blood lymphocytes. Human lymphocytes ($2 \cdot 10^6$ cells/ml) were preincubated with 50 nM ouabain for 4 h. Cells were then washed and stimulated with BMA 031 for the times indicated. Cells were then taken up in isolation buffer and subcellular fractions isolated as described under Materials and methods. 40 μg plasma membrane protein was solubilized with CHAPS and processed to SDS-polyacrylamide gelelectrophoresis. Proteins were then transferred to Immobilon^R membranes (200 mA 2 h) and stained with monoclonal antibodies against protein kinase C-α and -β, respectively. Staining was carried out as described under Materials and methods. A single experiment out of four similar ones is shown.

isotypes in lymphocytes stimulated via the T-cell antigen receptor/CD3 complex.

In BMA 031-stimulated lymphocytes protein kinase C-α protein was translocated to the plasma membrane within 5 min, after 30 min of stimulation the amount of this isoenzyme reverted to control levels in the plasma membranes. Preincubation with 50 nM ouabain did not influence the translocation of protein kinase C-α (Fig. 3A). In contrast translocation of protein kinase C-β protein detectable between 90 min and 4 h in the plasma membrane of BMA 031-stimulated lymphocytes was completely suppressed by pretreatment of cells with 50 nM ouabain (Fig. 3B).

As the activation of the protein kinase C isoenzymes may independently be linked to the T-cell antigen receptor, it was an obvious possibility to investigate the effects of ouabain on different signalling processes in stimulated lymphocytes.

3.6. Lack of effect of ouabain on the elevation of intracellular calcium concentration in BMA 031-stimulated human lymphocytes

Fig. 4 shows that binding of the monoclonal antibody BMA 031 to the T-cell antigen receptor resulted in elevation of intracellular calcium concentration within 1 min.

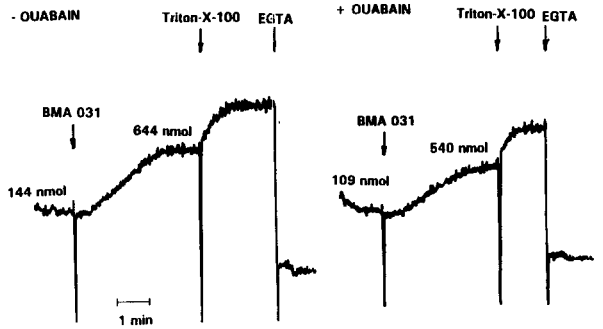

Fig. 4. Lack of effect of ouabain on the elevation intracellular calcium concentration in BMA 031-stimulated human lymphocytes. Peripheral blood lymphocytes were incubated with 50 nM ouabain for 4 h and loaded thereafter with Fura-2 as described in Materials and methods. Cells were stimulated with BMA 031 (5 μg/ml) (first arrow). F_{max} and F_{min} were determined by the addition of 1% Triton X-100 (second arrow) and 10 mM EGTA (third arrow), respectively. One representative experiment out of three similar independent ones is shown.

Pretreatment of lymphocytes for 4 h with 50 nM ouabain did not exert any effect on the receptor-mediated elevation of intracellular calcium concentration.

3.7. Ouabain inhibits the incorporation of polyunsaturated fatty acids into plasma membrane phospholipids of BMA 031-stimulated human lymphocytes

When lymphocytes were activated with the monoclonal antibody BMA 031, the incorporation of different *cis*-polyunsaturated fatty acids, linoleic acid (18:2), linolenic acid (18:3), or arachidonic acid (20:4) into different phospholipid species was stimulated two to three fold (Fig. 5). Upon addition of ouabain the elevated incorporation of the fatty acids into plasma membrane phospholipids was inhibited in a concentration-dependent way, as shown earlier in mitogen stimulated human lymphocytes [12]. Complete inhibition of BMA 031-stimulated fatty acid incorporation was achieved by 50 nM ouabain. In sharp contrast, neither PMA alone, nor the combination of PMA and ionomycin, were able to stimulate the incorporation of saturated or polyunsaturated fatty acids into plasma membrane phospholipids (data not shown). Ouabain (up to 1 μM) had no effect on the incorporation of fatty acids in PMA + ionomycin stimulated cells or into resting cells.

4. Discussion

The induction of proliferation in resting T-lymphocytes requires at least two essential events; the synthesis and secretion of the ultimate mitogen interleukin-2 and the synthesis and surface expression of receptors thereof [1]. Ouabain, a known inhibitor of lymphocyte activation, at concentrations not affecting intracellular ion concentrations, has been shown in several species to selectively interfere with IL-2 synthesis [12–14].

This was corroborated with human peripheral blood lymphocytes stimulated with the monoclonal antibody BMA 031, raised against the monomorphic determinants of the α/β chains of the T-cell antigen receptor. As human being represents the most ouabain sensitive species, a concentration as low as 50 nM completely suppressed

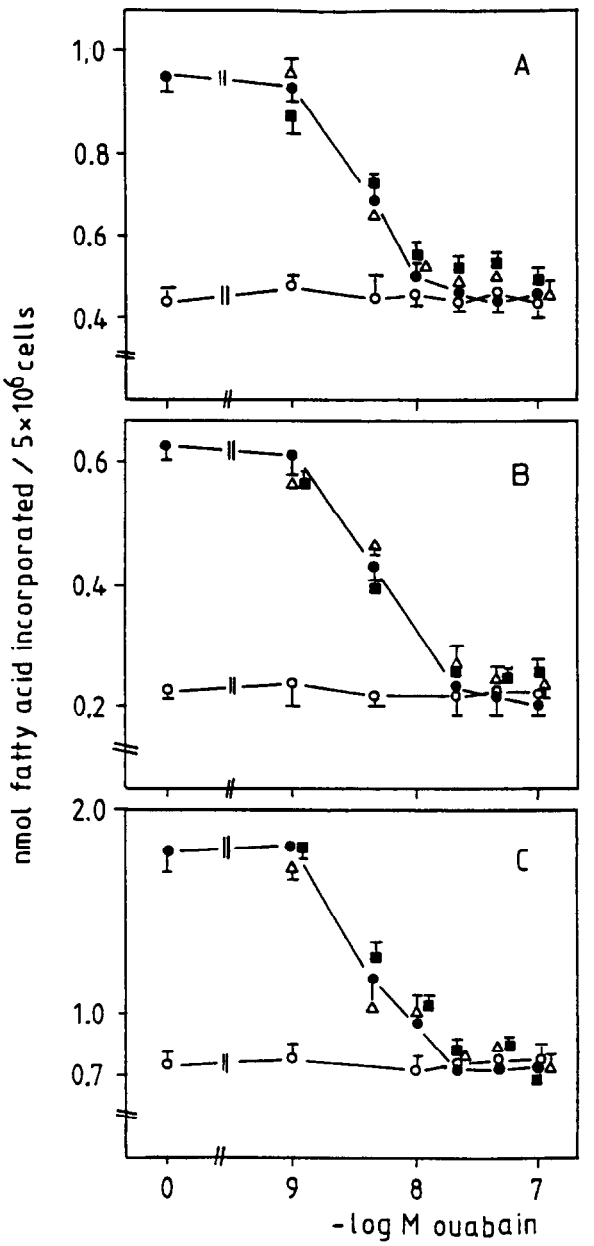

Fig. 5. Inhibition by ouabain of the BMA 031-stimulated incorporation of *cis*-polyunsaturated fatty acids into phospholipids of human lymphocytes. Peripheral blood lymphocytes ($5 \cdot 10^6$ cells/ml) were preincubated with ouabain for 4 h. Cells were then washed and stimulated with BMA 031 in the presence of ^{14}C-labelled polyunsaturated fatty acids for 4 h. Lipids were then extracted and separated by thin-layer chromatography as described under Materials and methods. Results are means ± S.D. of duplicates of three independent experiments. (A) Phosphatidylcholine, (B) phosphatidylinositol, (C) phosphatidylethanolamine, ○—○, control; ●—●, BMA 031 + linoleic acid (18:2); ■—■, BMA 031 + linolenic acid (18:3); △—△, BMA 031 + arachidonic acid (20:4).

BMA 031 induced proliferation, and completely blocked IL-2 synthesis (see Fig. 1 and Table 1), while it had no effect on the expression of high affinity receptors (see Table 2), which represent the functionally active receptor in T-lymphocytes. Proliferation and IL-2 synthesis evoked by the phorbol ester (PMA) in combination with the calcium ionophore, ionomycin, proved to be resistant to 50 nM ouabain. Only much higher concentrations of ouabain (i.e., 1 μM) led to inhibition of proliferation. At this high concentration ouabain prevented both IL-2 and IL-2 receptor synthesis also in PMA + ionomycin-stimulated cells. Ouabain at a concentration of 1 μM leads to drastic changes of ion fluxes in human cells, thus those concentrations also directly interfere with protein synthesis [15] and hence are non-specific.

For both events – IL-2 synthesis and IL-2 receptor expression – it has been well documented that activation of protein kinase(s) C represent a necessary signal transducing step [16]. The requirements for its activation, however, appear to be entirely different. We and others have shown recently that a rapid and transient activation, which can experimentally be induced by the addition of short-lived membrane permeable diacylglycerols in the presence of Ca^{2+}-raising ionophores, is sufficient for inducing the synthesis of the IL-2 receptors [3–6,16,17], whereas under such conditions no IL-2 synthesis can be observed.

In contrast, repetitive addition of diacylglycerols or of the slowly metabolized phorbol ester (PMA) also induce the synthesis of this cytokine ([3,4,18]; see also Fig. 1 and Table 1). From these experiments it has been postulated that IL-2 synthesis requires the maintenance of protein kinase C activation for a sustained period of time [16].

Indeed, PMA alone or in combination with the calcium ionophore, ionomycin, have been shown to evoke prolonged activation and translocation of protein kinase(s) C ([5,16]; see also Table 3). Under these conditions both classical (cPKC) and calcium independent (nPKC) isotypes of PKC are activated and translocated [19]. The lack of effect of ouabain (even at concentrations as high as 1 μM) implicates that the glycoside does not interfere with direct activation of any of the 'classical' or calcium-independent isoenzymes of PKC.

We have shown earlier that BMA 031, as well as other antibodies binding to the T-cell antigen receptor/CD3 complex and able to trigger human peripheral T-lymphocytes to proliferate, lead to a bimodal activation of protein kinase C. A rapid and transient translocation is followed by a second phase of activation which is maintained for at least several hours ([5,6] and Fig. 2).

More specifically, as shown in this paper, activation via the T-cell antigen receptor led within 5 min to a transient translocation of protein kinase C-α to the plasma membranes, followed by a fast dissociation as shown by immunoblot analysis (Fig. 3). After 90 min of stimulation protein kinase C-β was translocated and remained bound to the plasma membranes for up to 4 h (Fig. 3). Incubation of cells with 50 nM ouabain did not influence translocation of protein kinase C-α. In contrast, the sustained activation of protein kinase C-β was specifically and completely abolished by ouabain.

Our results thus suggest, that activation of protein kinase C-α, a ouabain insensitive step of signal transduction, might be responsible for the regulation of IL-2 receptor expression. On the other hand, inhibition of the translocation and activation of protein kinase C-β also abolished IL-2 synthesis, suggesting that sustained activation of this protein kinase C isotype is involved in the regulation of IL-2 gene expression and synthesis in human lymphocytes.

The finding that concentrations of 50 nM ouabain do not inhibit IL-2 synthesis when protein kinase(s) C is directly activated by the combination of PMA and ionomycin suggests that ouabain interferes with processes proximal to the activation of protein kinase C, i.e., with signal transduction pathways associated with its activation. Triggering of the T-cell antigen receptor/CD3 complex is associated with an activation of phospholipase C-γ, leading to a breakdown of phosphatidylinositolbisphosphate (PIP_2) within the first minutes of activation [20]. Although not formally proven, the close temporal correlation suggests that the initial transient activation of protein kinase C-α may be related to the 'PI response' and thus to generation of diacylglycerols from PIP_2 and elevation of intracellular calcium by inositoltriphosphate, IP_3. Ouabain did not affect this pathway, as evident from an unchanged rise in the cytosolic calcium concentration in BMA 031 stimulated lymphocytes (see Fig. 4).

The physiologically formed diacylglycerols have recently been shown to have half lives of less than 10 min [21]. The T-cell antigen receptor/CD3 complex associated transient activation of phospholipase C-γ thus cannot explain the sustained activation of protein kinase C-β. Data of several laboratories indicated that the T-cell antigen receptor/CD3 complex regulates IL-2 synthesis by its ability to regulate multiple intracellular signals in T cells [16,21,22]. The involvement of a phospholipase C-γ independent signalling pathway for IL-2 synthesis has been evoked recently by Wegener et al. [23]. They suggested that the T-cell antigen receptor/CD3 complex contains at least two parallel transduction modules [23]. Recently we have shown that incorporation of polyunsaturated fatty acids into plasma membrane phospholipids might be involved in the prolonged activation of protein kinase C [6]. In animal T-lymphocytes mitogen stimulation leads to a rapid preferential incorporation of polyunsaturated fatty acids, and thus to an increase of their content in phospholipids [13,24]. Ouabain at concentrations, which inhibited mitogen induced proliferation, also prevented incorporation of polyunsaturated fatty acids in rabbit lymphocytes by inhibiting the activation of the key enzyme acyl CoA:lysophosphatide acyltransferase [13]. We show here that BMA 031, too, leads to an increased incorporation of polyunsaturated fatty acids into phospholipids of human

lymphocytes; 50 nM ouabain was sufficient to completely inhibit BMA 031-induced increase in fatty acid incorporation (Fig. 5). As the same concentrations of the cardiac glycoside also prevented prolonged activation of protein kinase C-β (and subsequent IL-2 synthesis), it suggests that plasma membrane phospholipid fatty acid metabolism is associated with T-cell antigen receptor-dependent signalling pathways associated with the prolonged activation of this protein kinase C isoenzyme. A plausible molecular mechanism could consist in the generation of diacylglycerols from phosphatidylcholine by a phosphatidylcholine specific phospholipase C/D. Phosphatidylcholine in T-lymphocytes contains predominantly saturated fatty acids [24,25]. Diacylglycerols which are able to activate protein kinase C must, however, contain polyunsaturated fatty acids [26]. Increased incorporation of polyunsaturated fatty acids into phosphatidylcholine thus may be a prerequisite for generating protein kinase C activating diacylglycerols via this pathway. We have recently found a calcium-dependent plasma membrane bound phospholipase D in the plasma membranes of human lymphocytes, resulting in elevated diacylglycerol levels upon stimulation (H. Leufgen, K. Resch and M. Szamel, unpulblished data).

In previous studies we have shown that the cell surface receptor of ouabain, e.g., Na^+/K^+-ATPase and lysophosphatide acyltransferase were spatially and functionally coupled with the high affinity 'mitogen receptors' in calf, rabbit, and human lymphocytes [27–29]. More recently by the means of affinity chromatography on immobilized anti-CD3 monoclonal antibodies we succeeded in isolating plasma membrane subfractions from human Jurkat T-cells; in plasma membrane vesicles bearing the T-cell antigen receptor/CD3 complex high specific activity of ($Na^+ + K^+$)ATPase and lysophosphatide acyltransferase was detected, suggesting an association of both enzymes with the T-cell antigen receptor/CD3 complex (M. Szamel and K. Resch, unpublished data).

The results suggest that the two essential events in T-lymphocytes activation, the synthesis of IL-2 and the expression of its cell surface receptors require different activation signals. While the IL-2 receptor gene expression might be regulated by the activation of protein kinase C-α, IL-2 synthesis and cellular proliferation require sustained activation of protein kinase C-β.

We thus propose that the T-cell antigen receptor/CD3 complex is coupled to phospholipase C-γ, as well as to enzymes of the deacylation-reacylation cycle, phospholipase A_2 and lysophosphatide acyltransferase, both being essential for T-lymphocyte activation [4,5,16]. Activation of receptor-coupled lysophosphatide acyltransferase and thus the incorporation of *cis*-polyunsaturated fatty acids into plasma membrane phospholipids leads to a prolonged activation of protein kinase C-β and thus to IL-2 gene expression and clonal expansion of T-lymphocytes.

The inhibition of the activation of human lymphocytes by ouabain bears similarities to the mechanism of immunosuppression by cyclosporin A. Cyclosporin A too inhibits IL-2 synthesis without affecting IL-2 receptor expression [30]. Recently we found that in human peripheral lymphocytes – as used in this study – cyclosporin A also suppressed activation of protein kinase C-β selectively without affecting activation of protein kinase C-α, which besides the inhibition of the calcineurin phosphatase might represent a novel target for the immunosuppressive agent [31].

Taken together the results suggest that the two key events in the mitogenic activation of T-lymphocytes, the synthesis of IL-2 and the expression of its surface receptors involve different signalling pathways which both originate from the T-cell antigen receptor/CD3 complex. Expression of high affinity receptors for IL-2 is dependent on the transient activation of protein kinase C-α, which is mediated by the activation of phosphatidylinositol-bisphosphate specific phospholipase C-γ. Gene expression of IL-2 requires the sustained activation of protein kinase C-β, which is independent of the activation of phospholipase C-γ. The signal transduction leading to the activation of protein kinase C-β is less clear, but appears to involve changes of the fatty acid moieties of plasma membrane phospholipids.

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