FEBS Letters 352 (1994) 109–112

FEBS 14542

The regulation of sodium-dependent transport of anionic amino acids in cultured human fibroblasts

Renata Franchi-Gazzola, Rossana Visigalli, Ovidio Bussolati, Gian C. Gazzola*

Istituto di Patologia Generale, Università degli Studi di Parma, Via Gramsci 14, 43100 Parma, Italy

Received 2 August 1994; revised version received 12 August 1994

Abstract In cultured human fibroblasts the transport of anionic amino acids through the sodium-dependent system X_{AG}^- is stimulated rapidly and transiently by phorbol 12,13-dibutyrate. Transport stimulation is consistent with an effect due to the activation of protein kinase C. Bradykinin (1 μM) and PDGF-AA (100 ng/ml) also stimulate the activity of system X_{AG}^-. The bradykinin effect appears to be fully dependent upon PKC activation whereas the stimulation of aspartate transport by PDGF-AA is also due to PKC-independent mechanisms.

Key words: Growth factor; Proliferation; PKC; L-Glutamate; L-Aspartate; Human fibroblast

1. Introduction

System X_{AG}^- is a Na^+-dependent, high-affinity transport agency for L-aspartate and L-glutamate operative in mesenchymal cells [1–3]; the system is sensitive to membrane potential [4] and interacts efficiently with D-aspartate but not with D-glutamate [1]. Results from our group have indicated that phorbol esters stimulate the Na^+-dependent transport of anionic amino acids through system X_{AG}^- in cultured human fibroblasts [5]. However, preliminary observations from other authors have not confirmed this finding [6]. In this report we have further characterized the effect of phorbol esters on the activity of system X_{AG}^- and demonstrate here that the transport enhancement is fully consistent with an effect mediated by PKC activation. Other activators of PKC have been also evaluated in the attempt to assess whether the stimulation of anionic amino acid transport can be considered an effect of physiological PKC activation.

2. Materials and methods

Human foreskin fibroblasts were derived from a 15-year-old donor. Cells were routinely grown in DMEM supplemented with 10% FBS. All measurements were made with subcultures resulting from $5 \cdot 10^4$ cells seeded into 2-cm^2 wells of disposable 24-well multidish trays (Nunc) and grown for 3 days. Unless otherwise stated, the growth medium was replaced with DMEM containing 0.5% FBS on the day before the experiment. The treatment with PDBu and the other compounds was carried out in this medium. After the treatments specified for each experiment, the transport activity of system X_{AG}^- was evaluated, as described [7], by measuring the initial velocity of entry of [^{3}H]L-aspartate (10 μM; 3 μCi/ml) over 1-min periods in the same conditions adopted for cell culture. At the concentration employed for the assay L-aspartate is a specific substrate of system X_{AG}^- [1,2]. The experiments were stopped with three rapid washes in 3 ml ice-cold 0.1 M $MgCl_2$; ethanol extracts of cells were added to 2.5 ml of scintillation fluid and counted for radioactivity with a Packard 460 liquid scintillation spectrometer. Cell monolayers were then dissolved with 0.5% sodium deoxycholate in 1 M NaOH and protein content was determined using a modified Lowry procedure, as described [7].

For PDBu binding assay, human fibroblasts were washed five times with DMEM and then incubated for 30 min in ice-cold EBSS in the presence of increasing concentrations of [^{3}H]PDBu (2.5–200 nM; 0.05–4 μCi/ml). Non-specific [^{3}H]PDBu binding was assessed in parallel cultures incubated in the presence of 10 μM unlabelled PDBu and the specific binding was calculated by difference. At the end of incubation fibroblasts were washed six times with 0.1 M $MgCl_2$ and extracted with 0.2 ml ethanol; cell extracts were added to 2.5 ml of scintillation fluid and counted for radioactivity. The estimation of binding parameters was performed with a non-linear regression analysis of specific binding data following the equation

$$B = \frac{B_{max} \cdot [PDBu]}{K_S + [PDBu]} \quad (1)$$

Membrane potential has been monitored from the *trans*- membrane distribution ratio of L-arginine, employed as an indicator of membrane potential [8,9], following the equation

$$\Delta\psi = \frac{RT}{F} \cdot \ln \frac{[Arg]_{in}}{[Arg]_{out}} \quad (2)$$

To calculate the intracellular concentration of arginine, the cell water content was calculated from the difference between total water (urea space) and extracellular fluid (inulin space) determined according to the procedure described by Dall'Asta et al. [9].

Fetal bovine serum (FBS) and growth medium (Dulbecco's modified Eagle medium, DMEM) were from Gibco. Sphingosine and staurosporine were purchased from Boeringher. L-[2,3-^{3}H]Aspartic acid (13.5 Ci/mmol), L-[2,3-^{3}H]arginine (55 Ci/ mmol), [^{14}C]urea (4.2 Ci/mmol), [*methoxy*-^{3}H]inulin (355 mCi/ g), and [20-^{3}H(N)]phorbol 12,13-dibutyrate ([^{3}H]PDBu, 20 Ci/ mmol) were obtained from New England Nuclear. The source of all other chemicals was Sigma.

3. Results

The initial velocity of L-aspartate entry into cultured human fibroblasts was increased after incubation in the presence of PDBu (Fig. 1). The time course of the effect indicates that the maximal stimulation was reached after a 15-min incubation and decreased thereafter. After 12 h of incubation in the presence of PDBu aspartate transport had returned to basal values. 4α-PDD, a phorbol ester that not activates PKC [10], had no

*Corresponding author. Fax: (39) (521) 980 388.

Abbreviations: BK, bradykinin; 8-Br-cAMP, 8-Bromo-adenosine-3′:5′-monophosphate (cyclic); DiC_8, 1,2-dioctanoyl-*sn*-glycerol; DiC_{14}, 1,2-dimyristoyl-*sn*-glycerol; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PDBu, phorbol 12,13-dibutyrate; 4α-PDD, 4α-phorbol 12,13-didecanoate; PDGF, platelet-derived growth factor; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PLC-γ, phospholipase C-γ.

SSDI 0014-5793(94)00929-5

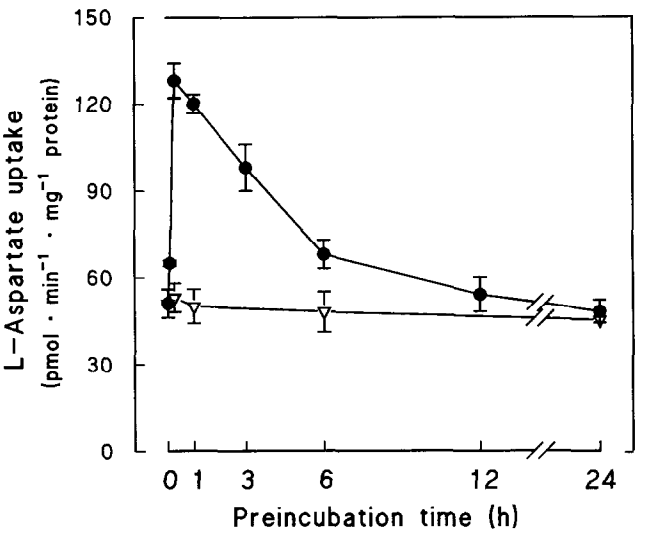

Fig. 1. Effect of PDBu on the transport of L-aspartate in cultured human fibroblasts. The uptake of L-aspartate (assay time 1 min) was determined after the indicated times of pre-incubation in the presence of 100 nM PDBu (●) or 4α-PDD (▽). Note that pre-incubation in the presence of 100 nM 4α-PDD had no effect on L-aspartate uptake, as compared to the untreated controls (0 min pre-incubation). Means of five independent determinations are shown with S.D. indicated.

effect on aspartate transport. Stimulatory effects were also detected after 15-min treatment with 100 nM PMA or with the cell-permeable diacylglycerol DiC_8 [11], employed at a concentration of 200 μM, but not with DiC_{14}, a cell impermeable diacylglycerol [12] (not shown).

Fig. 2 (left panel) shows that a 24-hour incubation of cultured human fibroblasts in a medium supplemented with 1 μM PDBu (a treatment associated with down-regulation of PKC [13]) markedly depressed the specific binding of labelled PDBu (B_{max} = 0.438 ± 0.115 *vs.* 2.19 ± 0.325 pmol/mg protein in untreated cells). After a prolonged treatment with phorbol esters, the transport of aspartate was substantially comparable to that

Table 1
Stimulation of L-aspartate transport of bradykinin: effects of PDBu and staurosporine

	V (pmol/mg prot/min)		Stimulation (%)	*P**
	− BK	+ BK		
None	58 ± 5.1	83 ± 3.2	43	< 0.005
SSP	71 ± 4.9	70 ± 1.7	–	n.s.
PDBu 15 min	144 ± 2.2	140 ± 8.5	–	n.s.
PDBu 24 h	59 ± 3.0	62 ± 3.2	5	n.s.

Cells were treated, as indicated, with BK (1 μM; 10 min), SSP (100 nM; 20 min), PDBu (100 nM; 15 min or 24 h) before the uptake assay. The results are means of three independent determinations with S.D. indicated; the level of significance was assessed with a two-tail *t*-test for unpaired data.
*Treated with growth factor vs. untreated.

observed in untreated cells. However, the down-regulation of PDBu binding sites completely abolished the short-term stimulation of aspartate transport by a subsequent treatment with phorbol esters (right panel).

At concentrations of 50 nM or higher, the PKC inhibitor staurosporine completely suppressed the stimulatory effect of PDBu on aspartate transport (Fig. 3). The inhibitor selectively affected the PDBu-stimulated portion of aspartate transport; in the absence of the phorbol ester the transport of the amino acid was rather slightly stimulated by staurosporine. A 60% inhibition of PDBu-stimulated aspartate transport was also obtained with sphingosine, another PKC inhibitor, employed at 5 μM (not shown).

Since the activity of system X^-_{AG} is dependent on membrane potential [5,14], the effect of phorbols on membrane potential was investigated. In cultured human fibroblasts PDBu caused a marked and prolonged depolarization of cell membrane while the inactive phorbol ester 4α-PDD had no effect on the membrane potential (Fig. 4).

Growth factors that stimulate phosphatidylinositols hydrolysis and activate PKC (PDGF-AA and BB, bradykinin) were

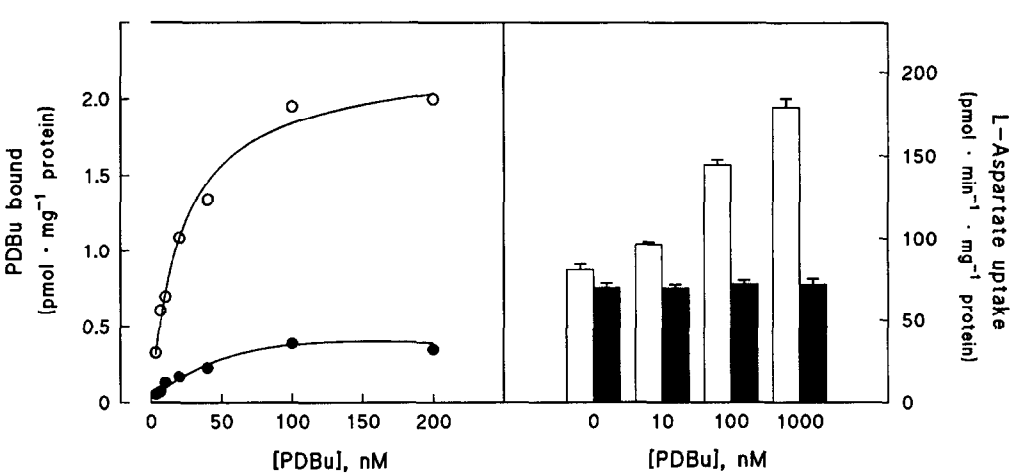

Fig. 2. Down-regulation of PDBu binding and loss of PDBu-dependent stimulation of L-aspartate transport after prolonged exposure of cultured human fibroblasts to PDBu. (Left) Cultured human fibroblasts were incubated for 24 h in DMEM supplemented with 0.5% FBS in the absence (○) or in the presence (●) of 1 μM PDBu. The specific binding of [^{3}H]PDBu was then determined as described in section 2. Lines represent non-linear regression of experimental points to Eqn. 1 of section 2. (Right) The cells were pre-incubated for 24 h in the absence (empty bars) or in the presence (solid bars) of 1 μM PDBu. L-Aspartate transport was measured after a further 15-min incubation in the presence of the indicated concentrations of PDBu. The results are means of three independent determinations with S.D. indicated.

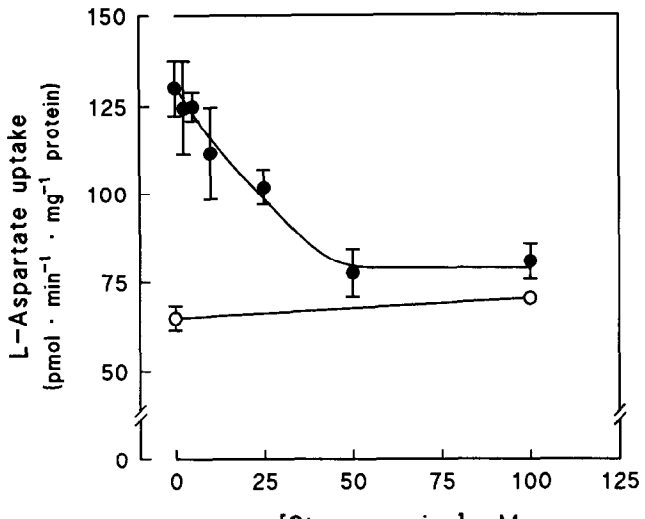

Fig. 3. Inhibition of the PDBu-induced stimulation of L-aspartate transport by staurosporine. L-Aspartate transport was measured after a 20-min pre-incubation in the presence of the indicated concentrations of staurosporine; the last 15 min of incubation were performed in the absence (○) or in the presence (●) of 100 nM PDBu. Data are means of three independent determinations with S.D. indicated.

tested to observe their effects on aspartate transport. Each factor was tested at increasing concentrations for various times of incubation. Both BK and PDGF-AA significantly stimulated transport activity for L-aspartate, although with different time courses. For BK the maximal stimulation was detected at 10 min with a concentration of 1 μM; with PDGF-AA the stimulation was maximal at 100 ng/ml after 2 min of incubation and decreased rapidly thereafter. PDGF-BB did not significantly stimulate the activity of system X_{AG}^-. The stimulatory effects of bradykinin and PDGF-AA were studied in control, untreated cells or in cells incubated with either PDBu (after both short-term and long-term treatments) or staurosporine. The results indicate that the effect of BK (Table 1) was suppressed by staurosporine, not additive to that of PDBu and hindered by the down-regulation of PKC, caused by chronic PDBu treatment. Although not significantly additive to the effect of PDBu, the stimulation of L-aspartate transport by PDGF-AA (Table 2) was neither completely inhibited by staurosporine nor fully suppressed by PKC down-regulation.

Table 2
Stimulation of L-aspartate transport by PDGF-AA: Effects of PDBu and staurosporine

	V (pmol/mg prot/min)		Stimulation (%)	P*
	− PDGF-AA	+ PDGF-AA		
None	65 ± 3.0	102 ± 0.6	57	< 0.002
SSP	73 ± 5.2	90 ± 6.0	23	< 0.05
PDBu 15 min	146 ± 4.7	161 ± 10.2	10	n.s.
PDBu 24 h	71 ± 4.5	86 ± 0.7	21	< 0.05

Cells were treated, as indicated, with PDGF-AA (100 ng/ml; 2 min), SSP (100 nM; 20 min), PDBu (100 nM; 15 min or 24 h) before the uptake assay. The results are means of three independent determinations with S.D. indicated; the level of significance was assessed with a two-tail t-test for unpaired data.
*Treated with growth factor vs. untreated.

4. Discussion

The results presented here indicate that the stimulation of anionic amino acid transport through system X_{AG}^- is a short-term effect of PKC activation in cultured human fibroblasts. It should be stressed that this effect is also detectable with physiological activators of PKC; moreover, the assay of aspartate transport has been performed under conditions similar to those employed for the cultivation of these cells. PDBu effect is rapid and transient; after a 24 h treatment (i.e. under Balcar et al.'s conditions [6]) no stimulation of aspartate transport is detectable, most probably because of PKC down regulation.

PKC activation depolarizes human fibroblasts (Fig. 4); this result indicates that the PKC-dependent enhancement of aspartate transport is not due to an increase in the driving force for transport contributed by membrane potential. The independence from membrane potential changes, the rapid onset of the effect (this paper) and the absence of requirement for an active protein synthesis [5] would suggest that also in cultured human fibroblasts PKC stimulates aspartate transport through a direct interaction with the transporter, as recently described for rat glia [15]. Consensus sites for PKC are indeed present in the cytoplasmic portions of all the predicted models of high affinity, Na^+-dependent glutamate transporters cloned thus far [16].

BK stimulation appears to be only dependent upon PKC activation, in agreement with the information available about signal transduction through BK receptor [17]. On the other hand, PKC activation does not entirely account for the stimulation of system X_{AG}^- activity due to PDGF-AA, that is only partially inhibited by PKC down-regulation and staurosporine. PDGF-AA interacts with and activates α subtype of PDGF receptor, which represents a minority of the total number of PDGF receptors of cultured human fibroblasts [18]. However,

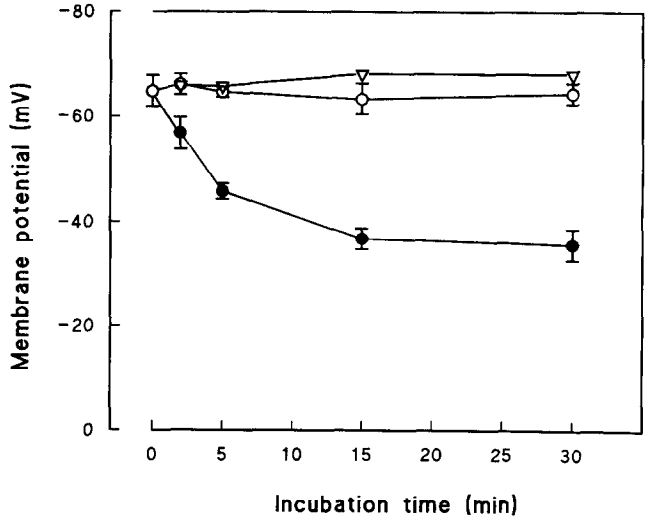

Fig. 4. Effect of phorbol esters on membrane potential of cultured human fibroblasts. Cultured human fibroblasts were incubated for 6 h in DMEM supplemented with FBS (0.5%) and deprived of L-arginine and L-lysine. In the last 90 min of pre-incubation the medium was replaced by DMEM containing [^{3}H]L-arginine (0.02 mM, 1 μCi/ml). At the end of this period, the incubation was prolonged for 30 min in the same medium in the absence (○, control) or in the presence of 100 nM PDBu (●) or 100 nM α-PDD (▽). Cell content of arginine was determined at the indicated times. Membrane potential was determined as described in section 2 according to Eqn. 2. Data are means of three independent determinations with S.D. indicated.

this PDGF isoform is a more potent PKC activator than BB and AB isoforms in models other than human fibroblasts [19]. In various biological models the activation of PDGF receptors is linked not only to PKC activation but also triggers other signal transduction pathways, such as cAMP production [20] and PLC-γ activation [21]. It is therefore possible that one of these additional transduction pathways accounts for the PKC-independent stimulation of aspartate transport since, besides PKC consensus sequences, also a PKA consensus motif appears in the sequence of the cloned high-affinity glutamate transporters [22]. However, in our hands, PKA activation with theophylline and 8-Br-cAMP has failed to cause significant changes in aspartate transport (Franchi-Gazzola and Visigalli, unpublished observation).

In contrast to the stimulation of aspartate transport observed here in PDBu-treated cultured human fibroblasts, the treatment with the phorbol ester is associated with a slow, progressive decrease of system X_{AG}^- activity in NIH3T3 cells [23]. This discrepancy could be referred either to different PKC isoenzymes or to different X_{AG}^- carrier subtypes. The former possibility agrees with available data [24–26] and would suggest that the stimulation of anionic amino acid transport constitutes an effect due to the activation of specific PKC isoenzymes.

Acknowledgements: This study was supported by Target Project 'Applicazioni Cliniche della Ricerca Oncologica', CNR and by MURST, Rome, Italy.

References

[1] Gazzola, G.C., Dall'Asta, V., Bussolati, O., Makowske, M. and Christensen, H.N. (1981) J. Biol. Chem. 256, 6054–6059.
[2] Dall'Asta, V., Gazzola, G.C., Franchi-Gazzola, R., Bussolati, O., Longo, N. and Guidotti, G.G. (1983) J. Biol. Chem. 258, 6371–6379.
[3] Balcar, V.J. (1992) FEBS Lett. 300, 203–207.
[4] Bussolati, O., Laris, P.C., Longo, N., Dall'Asta, V., Franchi-Gazzola, R., Guidotti, G.G. and Gazzola, G.C. (1986) Biochim. Biophys. Acta 854, 240–250.
[5] Franchi-Gazzola, R., Bussolati, O., Visigalli, R., Rotoli, B.M., Rossi, P.A., Guidotti, G.G. and Gazzola, G.C. (1990) Biochem. Biophys. Res. Commun. 173, 1304–1310.
[6] Balcar, V.J., Shen, J., Bao, S. and King, N.J.C. (1994) FEBS Lett. 339, 50–54
[7] Gazzola, G.C., Dall'Asta, V., Franchi-Gazzola, R., and White, M.F. (1981) Anal. Biochem. 115, 368–374.
[8] Bussolati, O., Laris, P.C., Nucci, F.A., Dall'Asta, V., Franchi-Gazzola, R., Guidotti, G.G. and Gazzola, G.C. (1989) Am. J. Physiol. 256 (Cell. Physiol. 25), C930–C935.
[9] Dall'Asta, V., Rossi, P.A., Bussolati, O. and Gazzola, G.C. (1994) Biochem. Biophys. Acta 1220, 139–145.
[10] Grinstein, S., Cohen, S., Goetz, J.D., Rothstein, A. and Gelfaud, E.W. (1985) Proc. Natl. Acad. Sci. USA 82, 1429–1433.
[11] Davis, R.J., Ganong, B.R., Bell, R.M. and Czech, M.P. (1985) J. Biol. Chem. 260, 1562–1566.
[12] Strålfors, P. (1988) Nature 335, 554–556.
[13] Collins, M.K.L. and Rozengurt, E. (1984) J. Cell. Physiol. 118, 133–142.
[14] Bussolati, O., Rotoli, B.M., Dall'Asta, V., Guidotti, G.G. and Gazzola, G.C. (1990) Biol. Chem. Hoppe-Seyler 371, 293–294.
[15] Casado, M., Bendahan, A., Zafra, F., Danbolt, N.C., Aragón, C., Giménez, C. and Kanner, B.I. (1993) J. Biol. Chem. 268, 27313–27317.
[16] Kanner, B.I. (1993) FEBS Lett. 325, 95–99.
[17] McAllister, B.S., Lebb-Lundberg, L.M.F., Javors, M.A. and Olson, M.S. (1993) Arch. Biochem. Biophys. 304, 294–301.
[18] Seifert, R.A., Hart, C.E., Phillips, P.E., Forstrom, J.W., Ross, R., Murray, M.J. and Bowen-Pope, D.F. (1989) J. Biol. Chem. 264, 8771–8778.
[19] Kazlauskas, A., Bowen-Pope, D., Seifert, R., Hart, C.E. and Cooper, J.A. (1988) EMBO J. 7, 3727–3735.
[20] Rozengurt, E. (1991) Eur. J. Clin. Invest. 21, 123–134)
[21] Heldin, C.H. and Westermark, B. (1991) CRC Critical Reviews in Oncogenesis 2, 109–124.
[22] Pines, G., Danbolt, N.C., Bjørås, M., Zhang, Y., Bendahan, A., Eide, L., Koepsell, H., Mathisen, J.S., Seeberg, E. and Kanner, B.I. (1992) Nature 360, 464–467.
[23] Longo N., Visigalli, R., Franchi-Gazzola, R., Dall'Asta, V., Bussolati, O., Guidotti, G.G. and Gazzola, G.C. (1989) Eur. J. Cell Biol. 49, 93.
[24] Mischak, H., Goodnight, J., Kolch, W., Baron, G.M., Schaechtle, C., Kazaniets, M.G., Benniberg, P.M., Pierce, J.H and Mushinski, J.F. (1993) J. Biol. Chem. 268, 6090–6096.
[25] McCaffrey, P.G., Rosner, M.R., Kikkawa, V., Sekiguchi, K., Ogita, K., Ase, K. and Nishizuka, Y. (1987) Biochem. Biophys. Res. Commun. 146, 140–146.
[26] Zhang, Q.Y., Hammerberg, C., Baldassare, J.J., Henderson, P.A., Burns, D., Miroslav, C., Voorhees, J.J. and Fisher, G.J. (1992) J. Immunol. 149, 1402–1408.