Protein kinase C in cytosol and cell membranes of concanavalin A-stimulated rat thymocytes

R. Averdunk and T. Günther*

Institut für Klinische Chemie und Klinische Biochemie, Klinikum Steglitz der Freien Universität Berlin, Hindenburgdamm 30, D-1000 Berlin 45 and **Institut für Molekularbiologie und Biochemie, Freie Universität Berlin, Arnimallee 22, D-1000 Berlin 33, Germany*

Received 5 October 1985; revised version received 21 November 1985

In concanavalin A-treated thymus cells and phytohemagglutinin-treated spleen cells, the distribution of protein kinase C is changed. Shortly after addition of plant lectins, the activity of protein kinase C increased in the cytosol and decreased in the particulate fraction. 2 h later, the activity of protein kinase C decreased in the cytosol and increased in the particulate fraction. Membrane binding of protein kinase C and tion of DNA synthesis showed the same dependency on concanavalin A concentration. A long-term membrane binding of protein kinase C seems to be essential for lymphocyte stimulation.

Protein kinase C *Concanavalin A* *Phytohemagglutinin* *Thymocyte* *Membrane binding*

1. INTRODUCTION

Polyclonal plant mitogens such as Con A stimulate lymphocytes to blast transformation and cell division. The mitogen binds to cell membrane receptors and initiates and maintains a complex program of biochemical and morphological events. The mechanisms of signal transmission have not yet been defined [1]. Activation of phospholipase C and liberation of diacylglycerol may occur, which in turn can activate PK-C by increasing its Ca^{2+} affinity [2,3].

Protein phosphorylation by undefined protein kinase [4] or by PK-C either without [5] or with an increase in $[Ca^{2+}]_i$ [6] was found to be essential for producing cell proliferation. Thus, it has been proposed that phorbol ester – one of its actions is binding of PK-C to the cell membrane [7,8] – and the Ca^{2+} ionophore A23187 together may replace plant lectins to stimulate DNA synthesis in lymphocytes. However, with macrophage-depleted peripheral lymphocytes for the long-term cellular response a low concentration of PHA was still needed in addition to phorbol ester and Ca^{2+} ionophore [6]. We therefore studied the long-term effects of plant lectins on the distribution of PK-C in lymphocytes.

* To whom correspondence should be addressed

Abbreviations: Con A, concanavalin A; PHA, phytohemagglutinin; PK-C, protein kinase C; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; PMSF, phenylmethylsulfonyl fluoride; NP 40, Nonidet P40

2. MATERIALS AND METHODS

Thymocytes and spleen cells were prepared by sieving the tissues of 4–6-week-old Wistar rats in RPMI 1640 medium. Thymocytes ($1–2 \times 10^6$ cells/ml) were incubated in RPMI 1640 medium containing 10% fetal calf serum without or with 4 μg/ml Con A (Seromed, Munich) for 12 h. Thereafter, the cells were washed 3 times in RPMI 1640 medium by centrifuging. According to Kraft et al. [9,10], the cells (3×10^8 cells as a total) were ruptured in buffer A (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA and 2 mM PMSF)

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

with 0.33 M sucrose by homogenisation with 20 strokes in a Potter-Elvehjem homogenisator. After centrifugation at 1500 × *g* for 5 min, the cytosolic fraction was obtained by centrifugation at 150000 × *g* for 60 min. The 150000 × *g* pellet was taken as the membrane fraction. For preparation of cytosolic PK-C, cytosolic fraction, containing 25 mg protein, was applied to columns (0.9 × 5 cm, Pharmacia) filled with DE52-cellulose (Whatman), equilibrated with buffer A, washed with 20 ml buffer A and eluted with a linear (0–0.15 M) NaCl gradient in buffer A. Fractions of 1 ml were collected. For preparation of membrane-associated PK-C, the 150000 × *g* pellet was homogenized in 5 ml buffer A containing 1% NP 40 (30 strokes), shaken for 30 min at 4°C, and centrifuged at 50000 × *g* for 60 min. The detergent-solubilized supernatant fraction was applied to DE52-cellulose columns, washed and eluted as described for the cytosolic fraction. When studying the time and dose dependency of Con A action, the columns were eluted with 0.06 M NaCl.

Protein kinase C was assayed by incubation for 3 min at 30°C in 250 μl reaction buffer B containing 20 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 75 mM $MgCl_2$, 100 μg histone H1, 1 μCi [γ-^{32}P]ATP (Amersham) and 100 μM ATP (Boehringer). The protein content of the assays amounted to 50–100 μg, measured according to Lowry et al. [11]. Phospholipid-dependent PK-C activity was determined by adding 20 μg phosphatidylserine and 0.8 μg diolein (Sigma).

The reaction was terminated by addition of 1 ml 25% cold trichloroacetic acid. The precipitate was collected on filters (Sartorius, 0.45 μm, ∅ 2.5 cm) and washed 8 times with 2 ml of 5% trichloroacetic acid. The filters were dissolved in Bray's scintillant and ^{32}P radioactivity was measured in a scintillation counter. All assays were made in duplicate. To determine the Na^+ gradient, the Na^+ content of the PK-C fractions was measured by flame photometry.

3. RESULTS

In unstimulated thymocytes, PK-C was found in the cytosol (approx. 70%) and can be solubilized from the particulate fraction (approx. 30%) (figs 1,2). The enzyme of both sources was eluted under

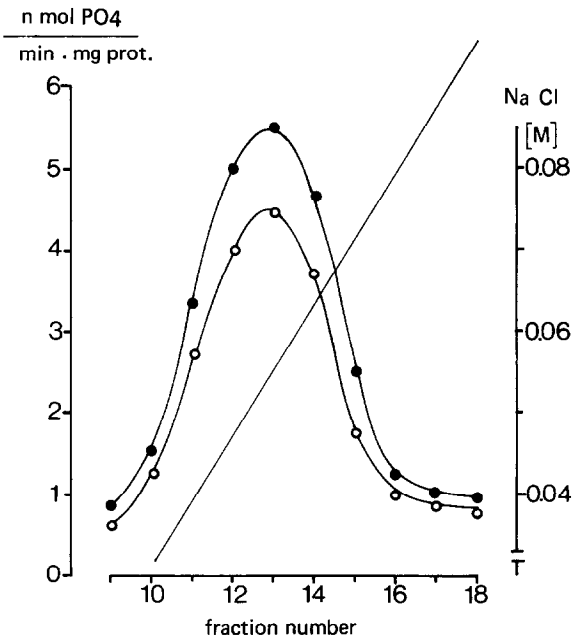

Fig.1. DEAE-cellulose chromatography of cytosolic PK-C. Thymus cells were stimulated with 4 μg/ml Con A for 12 h. The 150000 × *g* supernatant was applied to a column of DE52-cellulose and eluted with a linear gradient of NaCl in buffer A. PK-C activity was measured by $^{32}PO_4$ incorporation in histone H1. (●) Con A-stimulated cells, (○) control cells. Mean of 3 experiments. In fraction 9–16 of unstimulated cells, a total amount of 21 mg protein and 375960 cpm were measured.

identical conditions between 0.04 and 0.08 M NaCl. After 12 h incubation of thymocytes with 4 μg/ml Con A, the amount of PK-C was slightly increased in the cytosol (by 10%) and increased 3-times in the particulate fraction (figs 1,2). As shown in fig.3, the effect of Con A in increasing the particle-bound PK-C was highest at 4 μg/ml, substantiating the experimental approach. Subsequent [^{3}H]thymidine incorporation into DNA resulted in the same bell-shaped curve as in fig.3 (not shown).

To gain more insight into the mechanism of PK-C changes in cytosol and membranes, the time course of cytosolic and particle-bound PK-C was determined (fig.4). Up to 90 min after addition of Con A, cytosolic PK-C activity increased by 60% and particle-bound PK-C activity decreased by 10%. Between 90 and 120 min particle-bound PK-

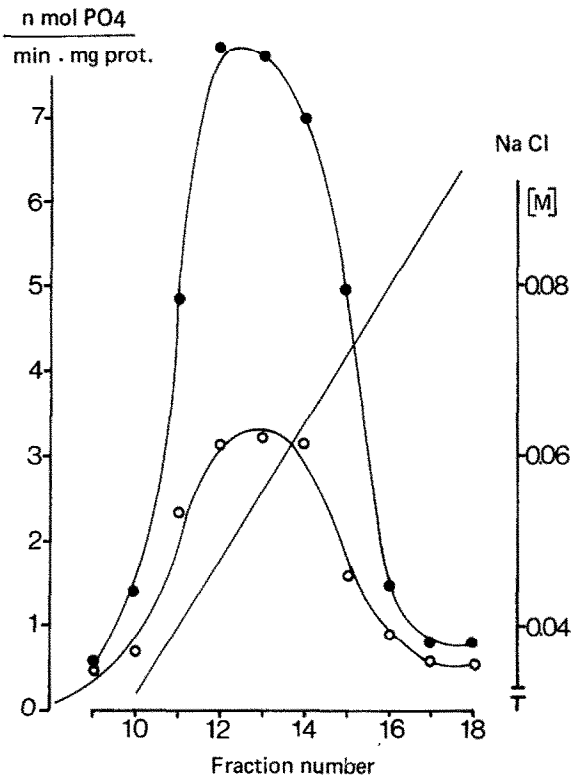

Fig.2. DEAE-cellulose chromatography of detergent-solubilized PK-C. Thymus cells were stimulated with 4 μg/ml Con A for 12 h. The 150000 × *g* pellet was treated with NP 40 and fractionated as in fig.1. (●) Con A-stimulated cells, (○) control cells. Mean of 3 experiments. In fraction 9–16 of unstimulated cells a total amount of 11 mg protein and 159960 cpm were measured.

C increased abruptly 3.7-times and remained constant thereafter up to 48 h. Between 90 and 120 min after the addition of Con A, the cytosolic PK-C decreased to the original value, remained constant up to 3 h and increased thereafter by 50%. The effect of Con A was abolished when 0.1 mM α-methylmannoside was added (not shown). The presence of 1 μg/ml puromycin or 0.1 μg/ml cycloheximide had no effect on the Con A-induced changes in PK-C distribution (table 1). Ouabain (0.1 mM), a potent inhibitor of lymphocyte activation [12], prevented membrane binding of PK-C after 150 min incubation with Con A (table 2).

When rat spleen cells were stimulated with

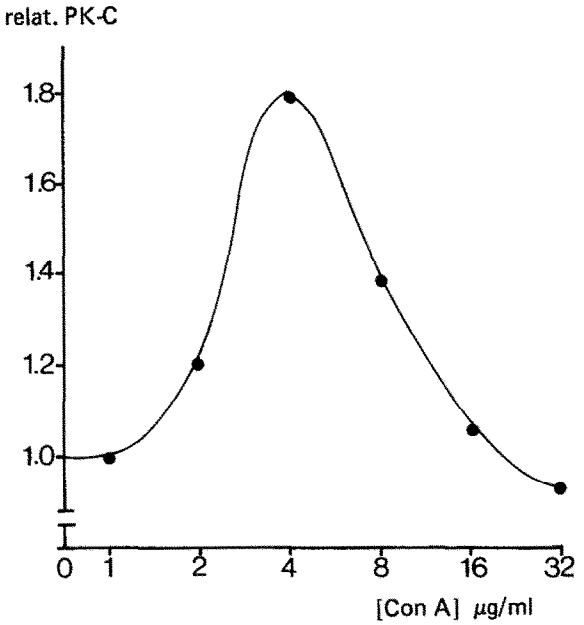

Fig.3. Effect of Con A on particle-binding of PK-C. Thymus cells were stimulated with various Con A concentrations for 3 h. Detergent-solubilized PK-C was applied to a column of DE52-cellulose and eluted with 0.06 M NaCl in buffer A. Mean of 3 experiments.

Table 1

Effect of Con A (4 μg/ml) and puromycin (1.0 μg/ml) or cycloheximide (0.1 μg/ml) on the distribution of PK-C between cytosol and particulate fraction of rat thymocytes

Incubation (min)	Con A	Puromycin	Cycloheximide	PK-C activity[a] (nmol/mg protein per min)	
				Cytosol	Particulate fraction
0	–	–	–	3.3	5.9
30	–	–	–	3.4	5.9
30	+	–	–	4.7	5.3
30	+	+	–	4.8	5.2
30	+	–	+	4.8	5.1
150	–	–	–	3.5	6.1
150	+	–	–	3.0	14.2
150	+	+	–	2.9	14.1
150	+	–	+	3.0	13.9

[a] Mean of 3 experiments

Table 2

Effect of Con A (4 μg/ml) and ouabain (0.1 mM) on the distribution of PK-C between cytosol and particulate fraction of rat thymocytes

Incubation (min)	Con A	Ouabain	PK-C activity[a] (nmol/mg protein per min)	
			Cytosol	Particulate fraction
0	–	–	3.3	6.0
30	–	–	3.3	6.0
30	+	–	4.6	5.4
30	–	+	4.0	5.5
30	+	+	4.8	5.2
150	–	–	3.2	6.5
150	+	–	3.0	13.3
150	–	+	4.5	5.4
150	+	+	5.2	5.9

[a] Mean of 3 experiments

Table 3

Distribution of PK-C of spleen cells between cytosol and particulate fraction after stimulation with PHA

	0	30 min	120 min
Cytosol	2056	2671	600
Particulate fraction	834	431	1290

Isolated spleen cells were incubated with 10 μg/ml PHA in RPMI 1640 medium with 10% fetal calf serum. Before addition of PHA, 30 and 120 min after addition of PHA aliquots were taken. Cytosol and detergent-solubilized particulate fraction were applied to DE52-cellulose columns and eluted with 0.06 M NaCl in buffer A. Values in cpm/mg protein. Mean of 2 experiments

10 μg/ml PHA, first some PK-C was transferred from the particulate fraction to the cytosol. After 120 min, more cytosolic PK-C was bound to cell particles than before (table 3).

4. DISCUSSION

In unstimulated lymphocytes, approx. 30% of PK-C was bound to membranes and the rest was localized in the cytoplasm. During stimulation of

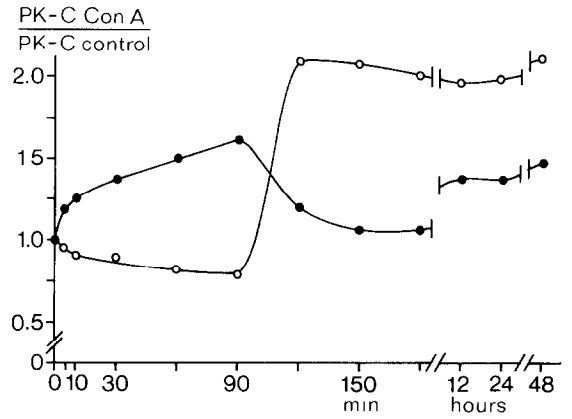

Fig.4. Time course of PK-C distribution between cytosol (●) and particulate fraction (○) after addition of 4 μg/ml Con A to thymus cells. The ratio of PK-C activity of stimulated/unstimulated cells was plotted. The ratio at zero time was taken to be 1.0. Mean of 3 experiments.

thymus and spleen cells with plant lectins, characteristic changes in cytosolic and particle-bound PK-C activity occurred. Three stages can be defined:

(i) Up to 90 min some PK-C may be transferred from membranes to the cytosol. The shift of PK-C from the particulate fraction to the cytosol within the first 5–10 min indicates that an early alteration in phospholipid metabolism by Con A did not cause an increase in membrane binding of PK-C, at least in the majority of thymocytes.

(ii) Between 90 and 120 min, there was a transfer of PK-C from cytoplasm to membranes and membrane binding of PK-C. The mechanism remains open. A rise in diacylglycerol and $[Ca^{2+}]_i$ may play a role [13]. However, the sum of both enzyme fractions exceeded the original sum, representing a gain of PK-C.

(iii) After 12 h, there was a second increase in PK-C activity in cytoplasm at constant particle-bound PK-C. Since inhibition of protein synthesis had no effect on the Con A-induced translocation of PK-C, the late membrane binding of PK-C is not secondary to growth response, but seems to be a direct event in lymphocyte stimulation. When the mitogenic effect of Con A was abolished by ouabain [12], there was no late membrane binding of PK-C.

Similar to the result with Con A, in HL60 cells an increase in cytosolic PK-C was found up to 90 h after the addition of retinoic acid [14]. The increase in cytosolic PK-C by retinoic acid also occurred at constant membrane-bound PK-C. This behaviour is in contrast to the effect of phorbol ester which only induced a rapid and transient membrane binding of PK-C at constant total PK-C activity [7,8,15]. A long-term increase in PK-C activity in the $10000 \times g$ supernatant and nuclear fraction was also found in peripheral lymphocytes, stimulated with PHA [16]. Thus, our results indicate that a long-term PK-C activation by membrane binding is involved in the mechanism for promoting DNA synthesis in lymphocytes. This conclusion would agree with the result that stimulation of lymphocytes with phorbol esters, or diacylglycerol and Ca^{2+} ionophore needed a low concentration of plant lectin [6].

REFERENCES

[1] Hume, D.A. and Weidemann, M.J. (1980) Research Monographs in Immunology, vol.2, pp.89–139, Elsevier, Amsterdam, New York.
[2] Ogawa, Y., Takai, Y., Kawahara, Y., Kimura, S. and Nishizuka, Y. (1981) J. Immunol. 127, 1369–1374.
[3] Ku, Y., Kishimoto, A., Takai, Y., Ogawa, Y., Kimura, S. and Nishizuka, Y. (1981) J. Immunol. 127, 1375–1379.
[4] Chaplin, D.D., Wedner, H.J. and Parker, C.W. (1980) J. Immunol. 124, 2390–2398.
[5] Di Virgilio, F., Lew, D.P. and Pozzan, T. (1984) Nature 310, 691–693.
[6] Kaibuchi, K., Takai, Y. and Nishizuka, Y. (1985) J. Biol. Chem. 260, 1366–1369.
[7] Farrar, W.L. and Anderson, W.B. (1985) Nature 315, 233–235.
[8] Farrar, W.L., Thomas, T. and Anderson, W.B. (1985) Nature 315, 235–237.
[9] Kraft, A.S., Anderson, W.B., Cooper, H.L. and Sando, J.J. (1982) J. Biol. Chem. 257, 13193–13196.
[10] Kraft, A.S. and Anderson, W.B. (1983) Nature 301, 621–623.
[11] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[12] Szamel, M., Schneider, S. and Resch, K. (1981) J. Biol. Chem. 256, 9198–9204.
[13] Wolf, M., LeVine, H. iii, Stratford May, W. jr., Cuatrecasas, P. and Sahyoun, N. (1985) Nature 317, 546–551.
[14] Durham, J.P., Emler, C.A., Butcher, F.R. and Fontana, J.A. (1985) FEBS Lett. 185, 157–161.
[15] Durham, J.P., Butcher, F.R. and Fontana, J.A. (1982) Biochem. Biophys. Res. Commun. 108, 840–845.
[16] Horenstein, A., Piras, M.M., Mordoh, J. and Piras, R. (1976) Exptl. Cell. Res. 101, 260–266.