Vol. 158, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 16, 1989 Pages 294-301

METABOLIC RESPONSIVENESS TO PHORBOL ESTER AND ACTIVITY OF PROTEIN KINASE C IN ISOLATED HEPATOCYTES FROM PARTIALLY HEPATECTOMIZED RATS

M. Houweling, W.J. Vaartjes and L.M.G. van Golde

Laboratory of Veterinary Biochemistry, State University of Utrecht, P.O. Box 80176, 3508 TD Utrecht, The Netherlands

Received November 2, 1988

SUMMARY: The ability of isolated rat hepatocytes to respond to phorbol-12-myristate-13-acetate (PMA) with acute stimulation of de novo fatty acid synthesis was markedly depressed at 4, 22 and 48 h after partial hepatectomy (PH). This desensitization was not due to surgical stress as shown by comparison with hepatocytes from sham-operated animals. Moreover, the total activity of protein kinase C (PK-C), the principal phorbol ester receptor, was not down-regulated at 22 h after partial hepatectomy. Partial hepatectomy rather caused a small but distinct shift in subcellular PK-C distribution toward the particulate fraction thereby suggesting a modest activation of PK-C. We conclude that the PH-induced desensitization to PMA occurs at a point beyond PK-C activation.

In recent years it has become increasingly clear that protein kinase C (PK-C) plays an important role in growth control and carcinogenesis. PK-C is activated endogenously by a wide variety of growth factors and hormones and by tumor-promoting phorbol esters such as PMA. It is generally believed that this activation process involves translocation of cytosolic PK-C to the plasma membrane. In turn, activated PK-C has been shown to phosphorylate several intracellular protein substrates including regulators of cellular proliferation (reviewed in 1). The function of PK-C as a signalling device for proliferation is further supported by recent reports showing that overproduction of PK-C leads to altered growth regulation (2,3), and that proliferating cells exhibit higher levels of PK-C activity (4-6) and a higher particulate-to-cytosolic PK-C activity ratio (4-7) than quiescent cells.

As these reports (4-7) dealt with cultured cell lines, we decided to investigate the effect of phorbol diester in hepatocytes freshly isolated from regenerating liver. Adult rat hepatocytes are generally in a quiescent state. Yet, after a two-

Abbreviations: DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; PH, partial hepatectomy; PK-C, protein kinase C; PMA, 4β-phorbol 12β-myristate 13α-acetate; PS, phosphatidylserine; SO, sham operation.

0006-291X/89 $1.50

third partial hepatectomy a synchronous wave of DNA synthesis and subsequent mitosis develops in the residual liver cells within 20-30 h, thus allowing a comparison between proliferating hepatocytes (partial hepatectomy, PH) and non-proliferating hepatocytes (sham-operation, SO).

Previously (8-10) we had already observed a substantial stimulation by PMA of de novo fatty acid synthesis (161 ± 13% of control ; n=11) and of acetyl-CoA carboxylase activity (168 ± 17% of control ; n=7) in isolated quiescent hepatocytes. Here we report a pronounced decrease of this acute metabolic response toward PMA at 4, 22 and 48 h after partial hepatectomy.

As PK-C is the major - if not sole - phorbol ester "receptor" (11), these findings suggested a decreased rather than increased PK-C activity in regenerating liver. Therefore, we measured PK-C activity at a time-point (22 h post surgery) when DNA synthesis is optimal (12,13) in the hepatectomized liver. The results show that there is no difference between the total amount of PK-C activity in hepatocytes isolated after partial hepatectomy and sham-operations, respectively. Moreover, the increased percentage of PK-C associated with the particulate cell fraction after partial hepatectomy points to a modest activation of PK-C in the hepatectomized liver. These data indicate that PH-induced desensitization of fatty acid synthesis to PMA occurs at a point distal to PK-C action.

METHODS AND MATERIALS

Two-thirds partial hepatectomies (14), consisting of excision of the median and left lateral lobes, or sham operations were performed on male Wistar rats (150-200 g) under light ether anaesthesia. The animals had been kept at an inverted light-dark cycle (dark 07.00-19.00 h) for at least two weeks. To avoid the distinct diurnal variation in liver DNA synthesis (15), surgery was routinely performed between 10.00 and 11.00 h. All animals had free access to water and pelleted standard diet in both the pre- and post-surgical period. Hepatocytes were isolated (16) at 4, 22 or 48 h after surgery, and were used in suspension.

The basic reaction medium consisted of Krebs-Ringer bicarbonate buffer (pH 7.4) with 1.3 mM $CaCl_2$, 10 mM glucose and 1% (w/v) defatted and dialysed bovine serum albumin. Triplicate incubations (final volume 2.0 ml; cell concentration, 4-6 mg protein/ml) were carried out at 37°C in a metabolic shaker (82 osc/min) in 25-ml Erlenmeyer flasks under an atmosphere of O_2/CO_2 (19:1). Cell protein was determined by the Lowry method (17), and cellular DNA content as described in (18). Incubation times, 60 min (DNA synthesis and fatty acid synthesis) or 30 min (acetyl-CoA carboxylase activity). DNA synthesis was determined as the incorporation of [^{3}H]thymidine (19). Unless indicated otherwise, incorporation of ^3H_2O (0.5 mCi/ml) into saponifiable fatty acids (8) was used to monitor de novo fatty acid synthesis. Acetyl-CoA carboxylase activity was assayed in digitonin-permeabilized hepatocytes according to (20). Soluble and solubilized particulate fractions of freshly isolated hepatocytes were prepared in the presence of 2 mM EDTA and of 1% (v/v) Nonidet NP40/1 mM EGTA, respectively, exactly as described in (21). After DEAE-52 column chromatography, PK-C activity was measured with [γ-^{32}P] ATP and histone H-1 (III-S) essentially as in (22), using Hepes (pH 7.5, 30 mM) as assay buffer; concentrations of $CaCl_2$, PS and 1,2-sn-diolein in the assays were 0.3 mM, 50 μg/ml and 10 μg/ml, respectively. Data are expressed as mean ± S.D. All statistical analyses were done using unpaired t-testing.

Crystalline porcine insulin was kindly donated by Lilly Laboratories (Indianapolis, USA). All other chemicals were from Sigma Chemical Co. (St Louis, USA). Radioactive compounds were supplied by Amersham International (Amersham, UK).

RESULTS AND DISCUSSION

Whereas sham-operated livers had a normal appearance, the liver remnants resulting from partial hepatectomy showed the visual aspects of a fatty liver. Yet, at 4 and 22 h after surgery both procedures yielded hepatocytes with about the same basal rate of fatty acid synthesis (Fig. 1, closed symbols). Only at 48 h after surgery, partial hepatectomy caused a significant increase ($P<0.005$ as compared to sham operation) in the rate of fatty acid synthesis, in line with earlier observations of Gove and Hems (23). However, when agonist-induced effects are taken into account, there was a remarkable difference between hepatocytes obtained after partial hepatectomy and sham operation, respectively. Whereas the responsiveness of hepatocytes toward PMA tended to be enhanced by the surgical stress of sham-operations (205 ± 11% ; n=12 for sham operation vs 161 ± 13% ; n=11 for non-operated rats), partial hepatectomy caused a marked and prolonged desensitization of the remnant hepatocytes. This difference can be best appreciated when the effect of both procedures on PMA-induced stimulation of fatty acid synthesis is expressed on a relative basis (see Fig. 1, inset).

For the sake of comparison insulin and epidermal growth factor, two other agonists known to stimulate hepatic fatty acid synthesis (24,25), were included in

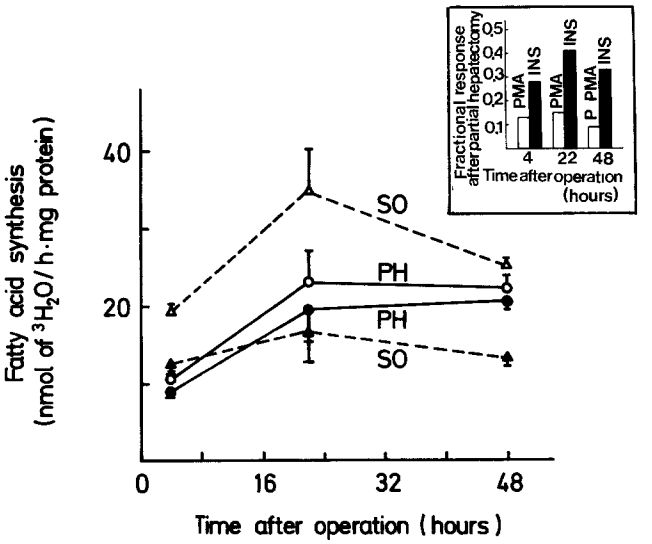

Fig. 1. Effect of PMA on the rate of fatty acid synthesis in rat hepatocytes isolated at different times after partial hepatectomy or sham operation. Partial hepatectomy (PH), solid lines ; sham operation (SO), dashed lines. Fatty acid synthesis is monitored as incorporation of ^3H_2O (0.5 mCi/ml) into total saponifiable fatty acids. Hepatocytes were incubated in the basic medium (see Methods) supplemented with 10^{-6} M PMA (open symbols) or with the solvent (0.1 % v/v) DMSO alone (closed symbols). Shown are means ± S.D. of triplicate incubations from 4 or 2 (48 h) different cell preparations.

Inset: stimulation of fatty acid synthesis by 10^{-6} M PMA and 10^{-7} M insulin (INS) expressed as fractional response, i.e., the agonist-induced increment in the rate of fatty acid synthesis in hepatocytes after PH divided by the same parameter in hepatocytes after SO. Values represent means of triplicate incubations from 4 or 2 (48 h) different hepatocyte preparations.

our experiments as well. Much the same picture as with PMA emerged with epidermal growth factor (not shown), whereas partial hepatectomy caused a less pronounced desensitization to insulin (Fig. 1, inset). The lack of sensitivity of regenerating liver to epidermal growth factor can be easily explained by the reported decrease of its receptors after PH (26), but the insulin data cannot be interpreted in the same manner since plasma membranes from regenerating liver display increased insulin binding according to Caro et al. (27). Insulin desensitization may be due to defective receptor autophosphorylation and/or undefined post-receptor events. At any rate, within the scope of our present study we focussed on the question whether or not the phorbol ester receptor, protein kinase C (11), was down-regulated after PH. The liver remnant remains mitotically quiescent for 12-16 h after partial hepatectomy before it undergoes the first synchronized wave of DNA synthesis between 20 and 24 h after surgery, followed by the mitotic peak approx. 6 h later (12,13). Hence the time-point of 22 h post surgery was selected for further studies.

Table I serves to encounter the objection that if glucose is the sole carbon precursor for fatty acid synthesis, the lack of stimulation of fatty acid synthesis by PMA may merely reflect its inability after partial hepatectomy to stimulate glycolysis. In fact, no matter what isotopic label was used to monitor fatty acid synthesis and whether or not acetate was included in the incubation medium as additional carbon source, PMA consistently failed to enhance fatty acid synthesis after partial hepatectomy to the same extent as it did after sham operations.

Table I. Effect of partial hepatectomy on the stimulation of fatty acid synthesis by PMA with different radioactive precursors and on acetyl-CoA carboxylase activity

	Addition	Rate of fatty acid synthesis (nmol acetyl units/h.mg protein) measured as incorporation of			ACC activity (nmol acetyl-CoA fixed/min.mg protein)
		^3H_2O	^3H_2O*	[1-^{14}C]acetate*	
PH	None	20.4 ± 0.6	32.8 ± 3.2	13.2 ± 0.5	0.20 ± 0.03
	PMA	26.5 ± 0.5	43.8 ± 1.3	17.8 ± 1.9	0.25 ± 0.05
		(130)	(134)	(135)	(125)
SO	None	16.3 ± 0.2	32.2 ± 0.2	9.8 ± 1.1	0.25 ± 0.02
	PMA	31.3 ± 1.0	60.7 ± 0.4	20.5 ± 1.5	0.63 ± 0.05
		(192)	(189)	(209)	(252)

Data shown are means ± S.D. of triplicate incubations from 2 different hepatocyte preparations obtained 22 h after partial hepatectomy (PH) or sham operation (SO), respectively. Incubations were performed in the absence (0.1% (v/v) DMSO added) or presence of 10^{-6} M PMA. In parentheses, values with PMA as % of control. To determine fatty acid synthesis, the basic medium was supplemented with ^3H_2O (0.5 mCi/ml) or [1-^{14}C] acetate (0.5 μCi/ml) and, as indicated by asterisk *, with 10 mM unlabeled acetate. The incorporation of tritium was converted to acetyl equivalents by the factor 1.15 (28). ACC, acetyl-CoA carboxylase.

Table II. Effect of partial hepatectomy on the total PK-C activity, DNA content and DNA synthesis in hepatocytes isolated 22 h after surgery

	Total PK-C activity (pmol PO_4 incorp./min)		DNA content (μg/mg protein)	DNA synthesis (pmol ^{3}H-thymidine inc./h.mg protein)
	per mg cellular protein	per μg cellular DNA		
PH	104.8 ± 4.4	5.8 ± 0.6	18.2 ± 1.4	1.63 ± 0.23*
SO	106.0 ± 5.4	7.0 ± 0.8	15.1 ± 1.9	0.28 ± 0.02

Total PK-C activity, DNA content and synthesis were determined as described in Materials and Methods. Data shown are means ± S.D. from 4 different hepatocyte preparations after partial hepatectomy or sham operation, respectively.
* $P<0.001$ as compared to sham operation.

Moreover, PMA desensitization was also reflected in its failure to induce an appreciable increase in the activity of acetyl-CoA carboxylase, the key regulatory enzyme of de novo fatty acid synthesis (Table I).

When expressed as a function of cellular protein, the total activity of PK-C in hepatocytes from hepatectomized or sham-operated rats was about the same (see Table II). A similar result was found when PK-C activities were expressed as a function of total cellular DNA. With regard to data from normal (non-operated) rats (not shown) one might even say that neither partial hepatectomy nor sham operation did affect the total PK-C activity at t=22 h post surgery. Table II also demonstrates the effectiveness of PH vs SO in terms of DNA synthesis.

The cellular level of PK-C as determined here in vitro is indicative of its total enzymatic capacity present in hepatocytes. However, PK-C in situ needs a membranous environment for its activation (1). Therefore, even if the overall total PK-C activity is the same in hepatocytes after PH and SO, the actual PK-C activities in the cells may differ dependent on the relative distribution of PK-C between cytosol and membranes. For this reason we also examined the particulate-to-cytosolic PK-C distribution ratio. Fig. 2 illustrates the partial purification by DEAE-cellulose chromatography of PK-C from the soluble fraction of hepatocytes isolated after PH. This elution profile was essentially the same for the particulate fraction and also for the soluble and particulate fractions of hepatocytes from sham-operated rats (not shown). In all cases the activity peak eluted with 0.075 M NaCl and there was no clear sign of a separate activity peak eluting with 0.150 M NaCl as found by Miloszewska et al. (5). The small peak observed at 0.250 M NaCl, which was somewhat larger in the solubilized particulate fraction (not shown) than in the soluble fraction, probably represents proteolytically truncated forms of PK-C (cf. ref 29) which are calcium- and phospholipid-independent.

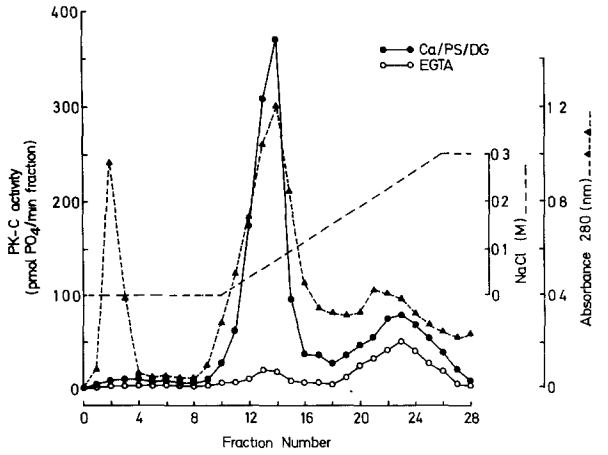

Fig. 2. DEAE-cellulose column chromatography of the soluble fraction of rat hepatocytes isolated 22 h after partial hepatectomy. The column (0.75 x 5.0 cm) was eluted with a linear 0-0.3 M NaCl gradient in a buffer (pH 7.5) composed of 30 mM Hepes/ 2 mM EDTA/ 20 mM β-mercaptoethanol. The total gradient volume was 15 ml and fractions of 0.5 ml were collected and assayed for PK-C activity (see Methods) in the presence of either $CaCl_2$, PS and 1,2-diolein (Ca/PS/DG, ●—●) or 0.5 mM EGTA O—O. Fractions 10 to 16 were pooled for the determination of total PK-C activity.

More importantly, as quantified in Table III, there was a modest shift of PK-C activity to the particulate cell fraction after PH. Further proof in this respect was given by [^{3}H]phorbol 12,13-dibutyrate binding data of the particulate fractions (PH: 6.47 ± 1.43 vs SO: 3.47 ± 0.95 pmol phorbol 12,13- dibutyrate/mg protein). These results which we interprete as a small activation of PK-C at 22 h after PH do not explain, of course, the PH-induced desensitization to PMA (Fig. 1 and Table I). Finally we compared the effect of partial hepatectomy and sham operation on the time-course of intracellular PK-C translocation as induced by

Table III. Subcellular distribution of PK-C activity in rat hepatocytes isolated 22 h after surgery

	PK-C activity		
	soluble	particulate	particulate
	(pmol PO^4 incorp./min.mg cellular protein)		(% of total)
PH	73.2 ± 2.0#	33.6 ± 0.6*	31.5 ± 0.6*
SO	90.7 ± 5.5	16.9 ± 2.4	15.7 ± 2.3

Chromatography fraction nrs. 10 to 16 (see Fig. 2) were pooled to determine the Ca^{2+}- and phospholipid-dependent histone kinase activity present in soluble and particulate hepatocyte fractions. Values shown are means ± S.D. of 3 different preparations from hepatectomized or sham-operated animals, respectively.
* $P<0.001$; # $P<0.01$, as compared to sham operation.

PMA (21). The results (not shown) revealed no clear-cut difference in kinetics between hepatocytes from hepatectomized or sham-operated livers in this respect.

In summary, partial hepatectomy caused a remarkable and persistent dampening of PMA-induced stimulation of fatty acid synthesis and acetyl-CoA carboxylase activity. In search for an explanation we found that this desensitization to PMA is not due to a decreased total activity or intracellular redistribution of PK-C. At 22 h after PH there was neither down-regulation of hepatocyte PK-C nor the massive up-regulation reported for proliferating fibroblasts (4-6) or epithelial cells (7). Therefore, desensitization of fatty acid synthesis to PMA apparently occurs at a step distal to PK-C action. This conclusion implicitly assumes that PK-C does not directly affect acetyl-CoA carboxylase activity (cf. 10).

While this work was in progress, Okamoto et al. (30) published data suggesting considerable activation of PK-C in regenerating rat liver prior to the initiation of DNA synthesis, whereas our results only indicate a small stimulation of PK-C activity. The reason for this discrepancy is not yet clear as comparison of data is hampered by the differences in methodology. Perhaps the time elapsed after PH is a dominant factor in determining the functional role of PK-C. Crucial events regulating cell growth are thought to occur in the early G_1 phase of the cell cycle. During this phase cells either cease proliferation and enter a resting G_0 state or proceed to initiate DNA synthesis (31,32). It could well be then that PK-C promotes the G_0 to G_1 transition of hepatocytes, initially after PH, followed by sequential changes in its role and in its subcellular distribution later during the cell cycle. In fact, anti-proliferative actions of PK-C in the post-G_1 phases have been documented for various cell lines (33-37). Hence further studies at various time-points after PH are needed to learn whether PK-C activity is modulated differently in different phases of the hepatocyte cell cycle.

ACKNOWLEDGEMENTS

These investigations were supported in part by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands organization for scientific research (NWO).

REFERENCES

1. Nishizuka, Y. (1986) Science 233, 305-312.
2. Housey, G.M., Johnson, M.D., Hsiao, W.L.W., O'Brian, C.A., Murphy, J.P., Kirschmeier, P. and Weinstein, I.B. (1988) Cell 52, 343-354.
3. Persons, D.A., Wilkison, W.O., Bell, R.M. and Finn, O.J. (1988) Cell 52, 447-458.
4. Adamo, S., Caporale, C., Aguanno, S., Lazdins, J., Faggioni, A., Belli, L., Cortesi, E., Nervi, C., Gastaldi, R. and Molinaro, M. (1986) FEBS Lett. 195, 352-356.
5. Miloszewska, J., Trawicki, W., Janik, P., Moraczewski, J., Przybyszewska, M. and Szaniawska, B. (1986) FEBS Lett. 206, 283-286.
6. Halsey, D.L., Girard, P.R., Kuo, J.F. and Blackshear, P.J. (1987) J. Biol. Chem. 262, 2234-2243.

7. Craven, P.A. and DeRubertis, F.R. (1987) Cancer Res. 47, 3434-3438.
8. Vaartjes, W.J. and de Haas, C.G.M. (1985) Biochem. Biophys. Res. Commun. 129, 721-726.
9. Vaartjes, W.J., de Haas, C.G.M., Geelen, M.J.H. and Bijleveld, C. (1987) Biochem. Biophys. Res. Commun. 142, 135-140.
10. Bijleveld, C., Geelen, M.J.H., Houweling, M. and Vaartjes, W.J. (1988) Biochem. Biophys. Res. Commun. 151, 193-200.
11. Niedel, J.E., Kuhn, L.J. and Vandenbark, G.R. (1983) Proc. Natl. Acad. Sci. USA 80, 36-40.
12. Grisham, J.W. (1962) Cancer Res. 22, 842-849.
13. McGowan, J.A. and Fausto, N. (1978) Biochem. J. 170, 123-127.
14. Higgins, G.M. and Anderson, R.M. (1931) Arch. Pathol. 12, 186-202.
15. Barbiroli, B. and Potter, V.R. (1971) Science 172, 738-741.
16. Seglen, P.O. (1976) Meth. Cell Biol. 13, 29-83.
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
18. Fiszer-Szafard, B., Szafard, D. and Guevara de Murillo, A. (1981) Anal. Biochem. 110, 165-170.
19. Nakamura, T., Yoshimoto, K., Nakayama, Y., Tomita, Y. and Ichihara, A. (1983) Proc. Natl. Acad. Sci. USA 80, 7229-7233.
20. Bijleveld, C. and Geelen, M.J.H. (1987) Biochim. Biophys. Acta 918, 274-283.
21. Vaartjes, W.J., de Haas, C.G.M. and van den Bergh, S.G. (1986) Biochem. Biophys. Res. Commun. 138, 1328-1333.
22. Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 13341-13348.
23. Gove, C.D. and Hems, D.A.(1978) Biochem. J. 170, 1-8.
24. Geelen, M.J.H., Beynen, A.C., Christiansen, R.Z., Lepreau-Jose, M.J. and Gibson, D.M. (1978) FEBS Lett. 95, 326-330.
25. Vaartjes, W.J., de Haas, C.G.M. and van den Bergh, S.G. (1985) Biochem. Biophys. Res. Commun. 131, 449-455.
26. Earp, H.S. and O'Keefe, E.J. (1981) J. Clin. Invest. 67, 1580-1583.
27. Caro, J.F., Poulos, J., Ittoop, O., Pories, W.J., Flickinger, E.G. and Sinha, M.K. (1988) J. Clin. Invest. 81, 976-981.
28. Jungas, R.L. (1968) Biochemistry 7, 3708-3717.
29. Kishimoto, A., Kajikawa, N., Shiota, M. and Nishizuka, Y. (1983) J. Biol. Chem. 258, 1156-1164.
30. Okamoto, Y., Nishimura, K., Nakayama, M., Nakagawa, M. and Nakano, H. (1988) Biochem. Biophys. Res. Commun. 151, 1144-1149.
31. Pardee, A.B. (1974) Proc. Natl. Acad. Sci. USA 71, 1286-1290.
32. Stiles, C.D., Capone, G.T., Scher, C.D., Antoniades, H.N., Van Wyk, J.J. and Pledger, W.J. (1976) Proc. Natl. Acad. Sci. USA 76, 1279-1283.
33. Kariya, K-I., Fukumoto, Y., Tsuda, T., Yamamoto, T., Kawahara, Y., Fukuzaki, H. and Takai, Y. (1987) Exptl. Cell Res. 173, 504-514.
34. Huang, C-L. and Ives, H.E. (1987) Nature 329, 849-850.
35. Takada, K., Amino, N., Tetsumoto, T. and Miyai, K. (1988) FEBS Lett. 234, 13-16.
36. Kinzel, V., Bonheim, G. and Richards, J. (1988) Cancer Res. 48, 1759-1762.
37. Yamamoto, T., Tsuda, T., Hamamori, Y., Nishimura, N. and Takai, Y. (1988) J. Biochem. 104, 53-56.