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Isozymic forms of protein kinase C in regenerating rat liver

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

Laboratory of Veterinary Biochemistry, State University of Utrecht, PO Box 80176, 3508 TD Utrecht, The Netherlands

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The expression of multiple forms of protein kinase C (PK-C) was studied in regenerating rat liver using hydroxyapatite column chromatography. Two forms of the enzyme were found in the cytosolic as well as membrane fraction of livers from partially hepatectomized rats. The kinetic variation in the activation of these two liver isozymes by fatty acids, phosphatidylserine and diacylglycerol was similar to that reported for the PK-C subspecies from rat brain, designated types II and III. Intracellular redistribution of PK-C caused by phorbol 12-myristate 13-acetate (PMA) was concentration-dependent and was due to translocation of isozyme III, because type II was insensitive to 5×10^{-8} M PMA. The activity ratio of the two isozymes in either the particulate or cytosolic fraction was the same at 22 h as compared to 4 h after partial hepatectomy.

Protein kinase C; Isozyme; Liver regeneration; Hepatectomy, partial; Phorbol ester

1. INTRODUCTION

Protein kinase C (PK-C) has become widely accepted as a major transmembrane signalling device in the mechanism of action of various hormones and biologically active compounds (review [1]). Recently PK-C turned out to be encoded by a multigene family giving rise to multiple forms of PK-C [2-5] that display subtle individual differences in enzymological behavior [6-8]. As discussed by Nishizuka [9] the existence of 7 PK-C subspecies has now been firmly established. Some tissues such as heart, lung and adrenal cortex may also contain other as yet undefined PK-C isozymes ([6,10], cf. [11]). The existence of multiple forms of PK-C, their different developmental expression [12], dissimilar down-regulation in KM_3 cells [13], different enzymatic characteristics [6-8] and tissue-specific [8,10,14] or even cell-type-specific [15] expression in mammalian tissues, suggest specialized functions for these isozymic forms of PK-C [16].

Adult rat liver is normally in a quiescent state. However, after partial hepatectomy (PH) a synchronous wave of DNA synthesis and subsequent mitosis develops in the liver remnant, thereby allowing investigation of regenerating rat liver. In the course of such studies, we also examined the occurrence and enzymatic properties of PK-C subspecies in an attempt to establish a potential relationship between the isozymic forms of PK-C and the proliferative state of the liver. PK-C has been attributed an important role in tumorigenesis and cellular growth control [1]. Furthermore, it has been proposed that the amount and intracellular distribution of PK-C subspecies may depend on the state of proliferation (cf. [17]).

Correspondence address: W.J. Vaartjes, Laboratory of Veterinary Biochemistry, State University of Utrecht, PO Box 80176, 3508 TD Utrecht, The Netherlands

Abbreviations: DG, 1,2-*sn*-diolein; PH, partial hepatectomy; PK-C, protein kinase C; PMA, 4β-phorbol 12β-myristate 13α-acetate; PS, L-phosphatidylserine

2. MATERIALS AND METHODS

2.1. *Materials and chemicals*

Two-thirds partial hepatectomies as described by Higgins and Anderson [18] were routinely performed between 10:00 and 11:00 h on male Wistar rats (150-200 g) under light ether anesthesia. The animals, which had free access to water and pelleted standard diet in both pre- and post-surgical periods,

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had been kept under an inverted light-dark cycle (dark between 07:00 and 19:00 h) for at least 2 weeks.

Hydroxyapatite was obtained from Bio-Rad (Richmond, USA), DE-52 (DEAE-cellulose) from Whatman (Springfield, UK) and all other chemicals from Sigma (St. Louis, USA). Radioactive compounds were supplied by Amersham International (Amersham, England).

2.2. *Partial enzyme purification*

All procedures were performed at 0–4°C. Rats were killed at 4 or 22 h after surgery. Livers were removed, homogenized and subfractionated essentially as described by Kosaka et al. [6]. Briefly, approx. 5 g wet wt liver was homogenized in a Teflon-glass homogenizer with 4 vols of 20 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 1 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 83 KIU/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 10 μg/ml soybean trypsin inhibitor, 50 μg/ml *N*-acetyl-Leu-Leu-norleucinal (calpain inhibitor, see [19,20]) and 10 mM β-mercaptoethanol (buffer A).

The homogenate was centrifuged at 100000 × *g* for 60 min and the supernatant was employed as the cytosolic (soluble) fraction. The pellet was rehomogenized in buffer A, supplemented with 1% (v/v) Nonidet NP40 and was stirred for 30 min before centrifugation for 60 min at 100000 × *g*. The resulting supernatant was employed as membrane (particulate) fraction.

Both fractions was applied to a DE-52 column (1.6 × 5 cm) equilibrated with 20 mM Tris-HCl at pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA and 10 mM β-mercaptoethanol (buffer B). The column was washed with buffer B and PK-C was eluted with a linear 0–0.3 M NaCl gradient in buffer B as in [21]. The enzyme fraction obtained was dialysed and subjected to hydroxyapatite column chromatography (0.75 × 12 cm) essentially as in [2] with the exception that we used 10 mM β-mercaptoethanol instead of 1 mM dithiothreitol and a 20–250 mM potassium phosphate gradient. Enzymatically active fractions (1.5 ml) were pooled and dialysed against Hepes buffer (pH 7.5, 30 mM), containing 0.5 mM EGTA, 0.5 mM EDTA, 10 mM β-mercaptoethanol and 10% (v/v) glycerol.

2.3. *Protein kinase C assay*

PK-C activity was assayed with [γ-^{32}P]ATP and histone H-1 (III-S) essentially as in [21], using Hepes (pH 7.5, 30 mM) as assay buffer. L-Phosphatidylserine (PS), 1,2-*sn*-diolein (DG) and fatty acids which were used as activators of the enzyme were stored at −20°C in chloroform under N_2. After evaporation under N_2 they were resuspended in Hepes buffer (pH 7.5, 30 mM) by ultrasonication (6 times for 30 s; 4°C) or, in the case of arachidonic and linoleic acid, by vigorous vortex-mixing under N_2 for 5 min.

2.4. *Other methods*

Rat hepatocytes were isolated and incubated to measure PK-C activity as described [22]. Protein was determined by a modified method of Bensadoun and Weinstein [23].

3. RESULTS AND DISCUSSION

Protein kinase C was partially purified from rat liver homogenates by a two-step purification procedure. The elution profile in the first step (DE-52 column chromatography; not shown), was essentially the same as that observed in the purification of PK-C from isolated hepatocytes [22]. This is not surprising as non-parenchymal cells may represent less than 1% of the liver volume, whereas hepatocytes contribute 72% of the total liver volume [24].

Fig.1 shows a representative elution profile of PK-C in the second step (hydroxyapatite column chromatography), using the cytosolic fraction of regenerating rat liver as enzyme source. The enzyme from the membrane (particulate) fraction showed the same elution profile. In both cases the enzyme could be separated into two distinct fractions, both at 4 and 22 h after surgery. The peak activities eluted at approx. 90 and 145 mM potassium phosphate, respectively, thus corresponding to the type II and III subspecies of rat brain [2,25]. These results are similar to those obtained by Kosaka et al. [6], but contrast with data published by Azhar et al. [26] who found three isozymes. We have no ready explanation for this discrepancy. At any rate, in other experiments using an extensive four-step purification procedure as described by Kikkawa et al. [27], PK-C from rat liver also showed two rather than three subspecies after hydroxyapatite column chromatography (Vaartjes, W.J., unpublished).

The effects of partial hepatectomy on the isozyme distribution in liver remnants were studied 4 and 22 h after surgery, when ornithine decarboxylase activity [28] and DNA synthesis [29,30] are optimal, respectively. We also studied the time point of 4 h after surgery, because crucial events regulating cell growth are thought to precede DNA synthesis and to occur in the early G_1 phase of the cell cyle [31,32]. However, as mentioned, we could not detect any difference between the hydroxyapatite elution profiles of PK-C of regenerating liver at 4 or 22 h after surgery. The activity ratio of the two PK-C isozymes stayed the same (first vs second peak, pooled fractions, 1:4), in both cytosolic and membrane fractions.

Figs2 and 3 serve to characterize further the isozymes of PK-C from the cytosolic fractions in livers removed at $t = 4$ h after partial hepatectomy. It appeared that the first and second activity peaks (fig.1) indeed behave similarly to the rat brain subspecies designated by Nishizuka and co-workers

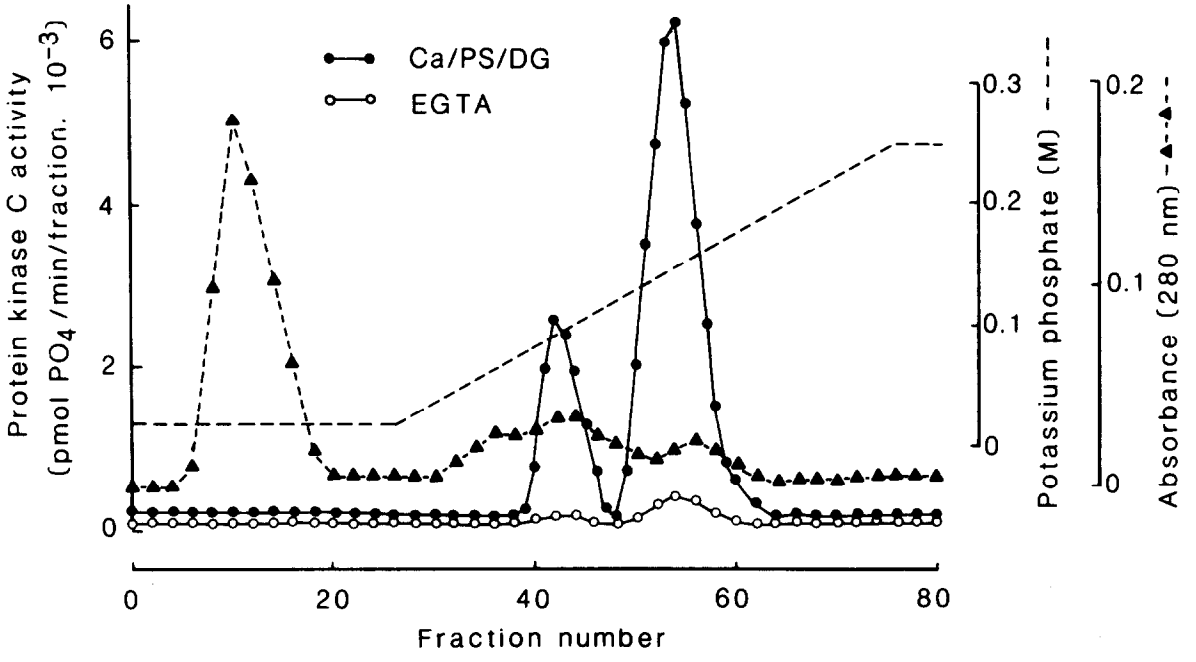

Fig.1. Separation of rat liver protein kinase C into two isozymes by hydroxyapatite column chromatography. PK-C from the cytosolic fraction of regenerating liver obtained 22 h after surgery was partially purified by DE-52 chromatography and subsequently subjected to hydroxyapatite chromatography (see section 2). The fractions of the hydroxyapatite column were assayed for PK-C activity: (●—●) in the presence of L-phosphatidylserine (50 μg/ml), 1,2-*sn*-diolein (DG, 10 μg/ml) and Ca^{2+} (0.3 mM); or (○—○) in the presence of EGTA (0.5 mM).

[7,9,25] as type II (β) and III (α), respectively. Isozymes from the particulate fraction demonstrated the same properties (not shown).

The general characteristics of PK-C as a Ca^{2+}- and phospholipid-dependent protein kinase, the activation of which is further enhanced by diacylglycerol, are displayed by both isozymes (see fig.2); Fig.2 shows that PMA, an agent which directly stimulates PK-C in the same manner as diacylglycerol, activates both isozymes. However, PMA was not able to enhance further, like diacylglycerol (the 100% value in fig.2), the stimulation of PK-C in the presence of Ca^{2+} and PS.

PK-C is markedly stimulated by arachidonic acid and other unsaturated fatty acids, whereas saturated fatty acids are scarcely effective [7,8,33,34]. On the other hand, Morimoto et al. [35] reported stimulation of cerebral PK-C by 100 μM myristic acid (14:0) in the presence of Ca^{2+} which was markedly enhanced by phospholipid. However, fig.3 shows that neither rat liver isozyme was significantly stimulated by 50 μM myristic acid.

The activation of rat liver PK-C subspecies by unsaturated fatty acids is also shown in fig.3. In the mere presence of Ca^{2+}, which is a prerequisite for this activation [7], stimulation of type III is more pronounced than that of type II. Type II only shows an increase in activity in the presence of linoleic acid. The activating effect of unsaturated fatty acids is markedly enhanced in the presence of diacylglycerol or PS. Other reports show that the potency of unsaturated fatty acids to activate PK-C is dependent on their stereochemical configuration (*cis*-oleic acid > *trans*-elaidic acid [34]) and that the number of *cis* double bonds to some degree paralleled the potency of activation (γ-linolenic acid (18:3) > linoleic acid (18:2) > oleic acid (18:1) [33]). It should be noted, however, that these reports did not discriminate between PK-C subspecies. In our hands, in the presence of PS or diacylglycerol, oleic acid was somewhat more effective than linoleic acid or arachidonic acid

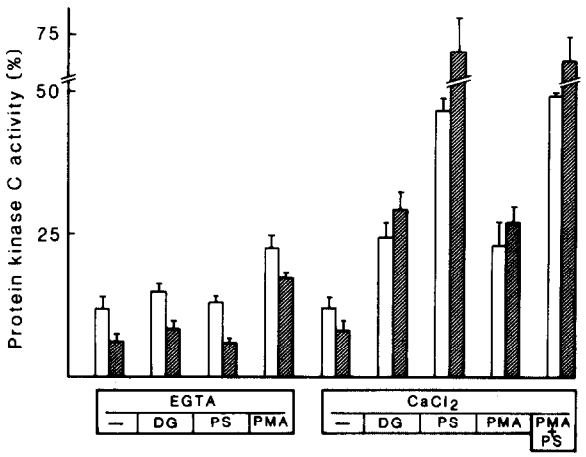

Fig.2. Effect of various activators on two isozymes of protein kinase C obtained after hydroxyapatite column chromatography. Cytosolic PK-C isozymes from regenerating rat liver (4 h after surgery) were assayed in the absence or presence of $CaCl_2$ (0.3 mM) with further additions as indicated. Concentrations of PS, DG and PMA were 50 μg/ml, 10 μg/ml, and 10^{-6} M, respectively. Results were normalized to the activity (100%) obtained in the presence of Ca^{2+}/PS/DG (0.3 mM, 50 μg/ml and 10 μg/ml, respectively). Data are means ± SD of duplicate incubations from three separate experiments. Open bars, type II; hatched bars, type III.

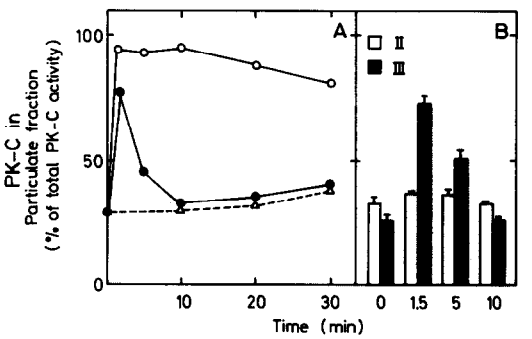

Fig.4. (A) PMA-induced intracellular redistribution of protein kinase C in rat hepatocytes isolated 4 h after partial hepatectomy. Hepatocytes were incubated with 10^{-6} M PMA (○—○), 5 × 10^{-8} M PMA (●—●) or with the solvent dimethyl sulfoxide only (Δ---Δ). PK-C activity was measured in both soluble and particulate fraction as described in section 2 and is expressed as percentage found in the particulate fraction. Data are means of duplicate incubations from two separate experiments. (B) PMA-induced redistribution of two isozymes of protein kinase C in hepatocytes isolated 4 h after partial hepatectomy. Hepatocytes were incubated with 5 × 10^{-8} M PMA and redistribution of isozymes was measured after hydroxyapatite column chromatography as described in the text. Data are means ± SD of duplicate incubations from two separate experiments. Open bars, type II; closed bars, type III.

(fig.3).

The translocation of PK-C induced by the phorbol ester PMA is concentration-dependent as shown in fig.4A. Exposure of intact hepatocytes to

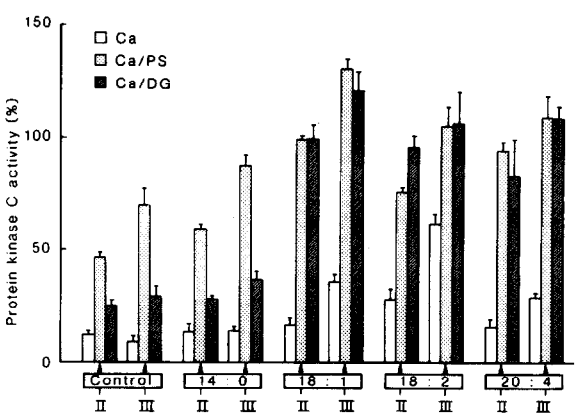

Fig.3. Activation of two isozymes of protein kinase C by various free fatty acids. Cytosolic PK-C subspecies from regenerating rat liver (4 h after surgery) were assayed with 50 μM fatty acid or buffer (control) in the presence of Ca^{2+} (0.3 mM) (open bars), Ca^{2+} (0.3 mM) and PS (50 μg/ml) (stippled bars), Ca^{2+} (0.3 mM) and DG (10 μg/ml) (hatched bars). Results were normalized as described in the legend to fig.2. Data are means ± SD of duplicate incubations from three separate experiments.

10^{-6} M PMA caused a rapid and prolonged (30 min) translocation of PK-C from the cystolic to membrane fraction. The rate of PMA-induced translocation was the same when cells were treated with 5 × 10^{-8} M PMA, but this concentration only caused a short (< 10 min) translocation of PK-C to the membrane fraction. Fig.4B shows that the two isozymes of PK-C (II, III) behaved differently when exposed to 5 × 10^{-8} M PMA. The translocation of PK-C activity with 5 × 10^{-8} M PMA (fig.4A) is exclusively caused by a shift of isozyme III, since isozyme II appears to be insensitive to this PMA concentration. This observation is in contrast with findings of Ase et al. [13], who found that in KM_3-cells isozyme type II was translocated more quickly to the membrane fraction than type III.

In conclusion, we report here the existence of two isozymes of protein kinase C in regenerating rat liver. These two isozymes behave similarly to the rat brain subspecies designated types II and III, as shown by the application of stimuli like Ca^{2+}, PS, diacylglycerol and unsaturated fatty acids. Further we found that these isozymes showed dif-

ferent sensitivity toward 5×10^{-8} M PMA. Type III showed the same translocation profile as found for total PK-C activity, whereas type II was insensitive to this concentration of PMA. The expression of PK-C isozymes was the same at 4 and 22 h after partial hepatectomy. Whether the same holds true for the first 3 h after surgery remains to be seen.

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