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Substrate-dependent inhibition of protein kinase C by specific inhibitors

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Protein kinase C (PKC) and its proteolysis-derived protein kinase independent of Ca^{2+} and phospholipids (PKM), were purified from rat brain. By using histone H1 and protamine as substrates, we assayed the effect of several inhibitors of PKC and PKM. The inhibition turned out to be dependent on both the nature of the kinase and the type of substrate assayed. These results may help to interpret the different responses elicited by PKC inhibitors in vivo.

Protein kinase C; Staurosporine; H7; Phorbol ester

1. INTRODUCTION

The involvement of protein kinase C (PKC) in the transduction of extracellular signals ranging from cell proliferation to the control of major metabolic pathways in several tissues has stimulated the search for activators and inhibitors of the enzyme in order to test the participation of this kinase in different biological functions [1,2]. The use of phorbol diesters as activators of PKC has proved to be a very useful tool by which to study the activation of the enzyme in vivo [3,4]. However, regarding the inhibitors, the specificity for the inhibition of PKC is not so evident since other kinases also seem to be affected [5,6]. This lack of specificity may be overcome by using the inhibitors in counteracting the action of specific activators of PKC.

The study of the effect of the inhibitors on PKC is important because in many cases the involvement of PKC in a process should be deduced by the effect of its inhibitors [6–9]. In the present work, we provide evidence suggesting that the results obtained by using this approach may be ambiguous since the action of the inhibitors may depend on the nature of the enzyme (the native PKC or the proteolysis-derived protein kinase M, PKM; [10,11]) and on the physiological substrate of phosphorylation in vivo.

2. MATERIALS AND METHODS

2.1. *Chemicals*

[γ-^{32}P]ATP (3000 Ci/mol) was from New England Nuclear. Sphingosine, H7, gossypol, quercetin and protamine-agarose were from Sigma. DE52 was from Whatman. Phenylsepharose was from Pharmacia. Other chemicals were from Merck or Boehringer.

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2.2. *Purification of PKC and PKM*

PKC was purified from rat brain by DE52, phenylsepharose and protamine-agarose chromatography as described elsewhere [12,13]. The specific activity was 950 units/mg protein. PKM was purified either by the endogenous activity isolated by DE52 chromatography or by in vitro proteolysis of purified PKC. In both cases the enzyme was submitted to further purification by phenylsepharose and protamine-agarose chromatography, in order to authenticate its identity regardless of the origin. To produce PKM in vitro, purified PKC (200 units) was incubated with 40 μl/ml of rat brain cytosol (1:3, w/v) at 30°C for 30 min in the presence of 0.5 mM $CaCl_2$ and 100 ng/ml of phorbol dibutyrate. The reaction mixture was then chilled on ice and applied to a DE52 column (0.5 × 5 cm). After washing with buffer A (in mM: 100 NaCl, 10 β-mercaptoethanol, 1 EGTA, 1 EDTA and 10 Hepes, pH 7.5) supplemented with 0.05% Nonidet P-40, PKM was eluted in a gradient from 100 to 500 mM NaCl in buffer A. PKM activity emerged at 250 mM salt, and the purification was continued by phenylsepharose and protamine-agarose chromatography. PKM was applied to the phenylsepharose column (0.5 × 5 cm) in buffer A containing 1 M NaCl. The enzyme was eluted with buffer A supplemented by 10% glycerol and lacking NaCl. Fractions containing activity were pooled and applied to a protamine-agarose column and eluted with a NaCl gradient in buffer A.

2.3. *Assay of PKC and PKM*

PKC activity was assayed by the incorporation of [^{32}P]P_i into histone H1 in the absence or in the presence of calcium and phospholipids as previously described [14]. The assay (100 μl) consisted of a solution containing in final concentrations 20 μM ATP (0.1–0.3 μCi), 1 mM Mg acetate, 5 mM β-mercaptoethanol, 50 μg histone (type IIIS from Sigma), 20 mM Hepes, pH 7.5, and, except when otherwise indicated, 0.3 mM $CaCl_2$, 10 μg/ml phosphatidylserine and 1 μg/ml dioctanoylglycerol. The reaction was stopped by the addition of 2 ml of ice-cold 5% trichloroacetic acid, 10 mM PO_4H_3. The reaction was linear for at least 20 min. The acid-insoluble radioactivity was retained in GF/C filters and counted after oven-drying in 2 ml of scintillation cocktail. To quantitate PKC activity, the incorporation in the absence of Ca^{2+} and lipids was subtracted from the corresponding samples. When protamine was used as substrate, the assay was similar to that with histone H1, although it contained calcium and lipids considering its relative independence of calcium [15]. PKM activity was assayed as described for PKC, but in the presence of 0.2 mM EGTA and in the absence of calcium and lipids. The use of highly purified PKC and PKM guarantees the

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validity of the results obtained in these assays. The inhibition constant (K_i) for each compound tested was calculated by varying its concentration in the PKC or PKM assay. One unit of PKC or PKM was defined as incorporating one nmol of phosphate into the substrate per min. Proteins were determined according to the method of Bradford [16].

3. RESULTS

The effect of several classical inhibitors of PKC was tested on both PKC and PKM, using histone H1 or protamine as substrates. PKC was purified following standard protocols and was highly dependent on the addition of Ca^{2+} and phospholipids. To obtain PKM, we used two approaches in establishing the PKC-derived origin of this kinase activity: PKM was obtained by DE52 chromatography of cytosols from rat brain (endogenous PKM), or produced in vitro by proteolysis of purified PKC [11]. Fig. 1 shows the purification of PKM from proteolysed PKC, by DE52, phenylsepharose, and by protamine-agarose chromatography. PKM was purified basically as PKC with minor changes in the elution conditions. However, its different nature from PKC is established on the basis of its independence of the activation by Ca^{2+} and lipids and of the different pattern of elution from the DE52 column (250 mM NaCl for PKM and 90 mM for PKC), and from the protamine-agarose, where by contrast to PKM, PKC requires ATP to be eluted [12]. The specific activity of this preparation was 183 units/mg of protein which exhibited exactly the same properties as did the purified endogenous PKM enzyme. Table I summarizes the procedure and the yield for the purification of the endogenous PKM.

Further, to analyze the effect of several PKC inhibitors on both PKC and PKM activities, we used histone H1 and protamine as substrates for both enzymes. The relative incorporation of ^{32}P into substrates was roughly the same for each kinase. The inhibitors used were staurosporine [9], H7 [7], quercetin [17], gossypol [18] and sphingosine [19]. As Table II shows, the K_i values for each inhibitor depended on the nature of both the kinase and the substrate used to incorporate the phosphate. In the case of staurosporine and PKC as kinase, a high inhibition was observed either with histone or protamine as substrates (K_i = 5.5 and 12 nM, respectively). However, PKM was not affected by staurosporine at concentrations up to 1 μM. In contrast to staurosporine, H7 inhibited PKC assayed either with histone or protamine (K_i = 10–20 μM), although it was less efficient in the inhibition of PKM (K_i = 400–500 μM).

A variable pattern of inhibition was observed with sphingosine. It was highly effective in the inhibition of PKC using H1 as substrate, but failed to inhibit the phosphorylation of protamine, and was completely ineffective with PKM. Moreover, the incorporation of ^{32}P into protamine was even stimulated in the presence of this amino alcohol.

A specific and potent inhibitor of PKM was quercetin, which exhibited K_i values of 4.5 μM for histone and protamine as substrates. However, quercetin failed to inhibit PKC in our conditions. This is despite the fact that quercetin-related flavonoids

Table I

Purification of PKM from rat brain homogenates

Step	Units	U/mg protein	Yield (%)
DE52	180	0.112	100
Phenylsepharose	108	0.321	60
Protamine-agarose	47	182.5	26

Brains were homogenated and centrifuged at 105000 × g for 30 min. The supernatant was incubated at 30°C for 10 min in the presence of 0.5 mM Ca^{2+}, and purified by DE52, phenylsepharose and protamine-agarose chromatography

Table II

Inhibition of PKC and PKM by several ligands

Ligand (K_i)	PKC		PKM	
	Histone	Protamine	Histone	Protamine
Staurosporine (nM)	5.5	12	N.I.	N.I.
H7 (μM)	10	17.5	450	500
Sphingosine (μM)	53	N.I.	N.I.	N.I.
Quercetine (μM)	N.I.	N.I.	4.5	4.5
Gossypol (μM)	30	30	190	N.I.

The enzymes were incubated with the inhibitors prior to starting the reaction with [γ-^{32}P]ATP. N.I., not inhibited

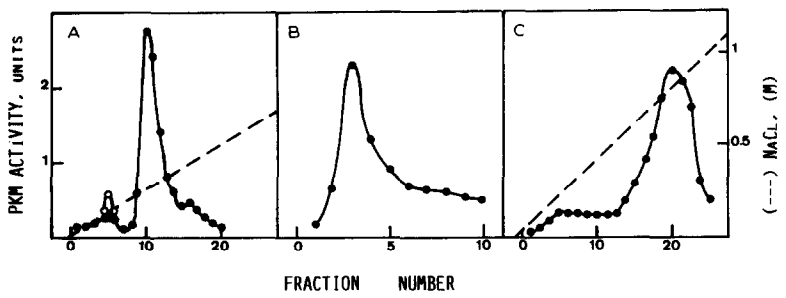

Fig. 1. Purification of PKM from rat brain. PKM was produced in vitro after proteolysis of purified PKC. The steps were DE52 chromatography (A); phenylsepharose (B); and protamine-agarose chromatography, eluted with a gradient of NaCl (0.05–1 M) (C). The activity was measured using histone H1 as substrate and in the absence (filled symbols) or presence (open symbols) of calcium and lipids.

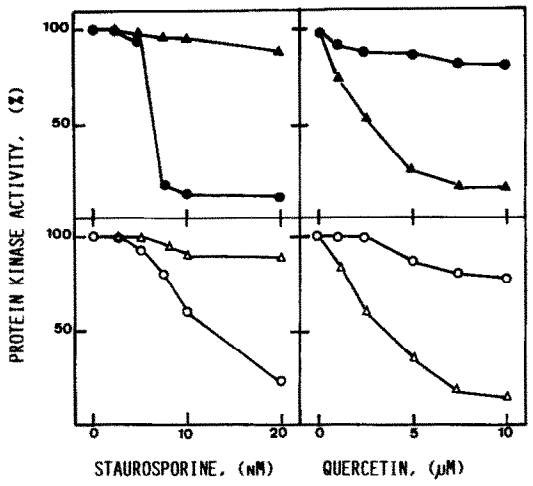

Fig. 2. Inhibition of PKC and PKM by staurosporine and quercetin. Purified PKC (●,○) and PKM (▲, △) were assayed with histone H1 (filled symbols) or with protamine (open symbols) as substrates.

have been reported to be inhibitors of PKC [17]. Fig. 2 shows the kinetics of the inhibition by staurosporine and quercetin of the activity of PKC and PKM purified from rat brain.

4. DISCUSSION

The use of inhibitors of PKC has been an effective and useful tool by which to ensure its participation in several biological processes. The mechanism of action of these inhibitors is quite variable, ranging from those like H7 that acts on the ATP catalytic site, to staurosporine that acts (mainly) on the regulatory domain [2]. Activation of PKC by phorbol esters or via enhancement of phosphoinositide turnover in response to agonist binding to membrane receptors may be prevented, in most cases, by incubating the cells with inhibitors such as H7 or staurosporine [2,19,20]. A major criticism of the use of these inhibitors is that its range of specificity in the inhibition of protein kinases is quite broad. However, the unexpected observation has been that, in some cases, these inhibitors failed to block PKC activation. This is in spite of the very potent inhibition of PKC observed in vitro. According to our results, the effectiveness of the inhibitors in preventing PKC-mediated phosphorylation of intracellular substrates may depend on both the nature of these substrates and on the relative participation of PKC and PKM in the phosphorylation process. This is especially relevant in the case of phorbol ester-stimulated cells, since these diacylglycerol analogues promote a rapid proteolysis of PKC and therefore generate important amounts of PKM [21]. Our results may expain the failure of some PKC-dependent processes to be affected by the use of PKC inhibitors. As an example, the recent report may be cited showing a differential pattern of inhibition after topical application of staurosporine on phorbol diester-induced skin inflammation, neutrophil infiltration, epidermal hyperplasia or ornithine decarboxylase induction in mouse skin [22].

An ancillary conclusion from our work is that the selective employment of inhibitors may be useful to analyze the nature of the substrates and the kinase involved in the activation of the PKC system by specific activators such as phorbol esters. The comparison of the results obtained after exposure of the cells to 'selective' inhibitors such as sphingosine, staurosporine and quercetin, may provide data concerning the substrate-type and kinase that participate in a process elicited by PKC activation.

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REFERENCES

[1] Kikkawa, U., Kishimoto, A. and Nishizuka, Y. (1989) Annu. Rev. Biochem. 58, 31–44.
[2] Rüegg, U.T. and Burgess, G.M. (1989) Trends Pharmacol. Sci. 10, 218–220.
[3] Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851.
[4] Nishizuka, Y. (1984) Nature 308, 693–698.
[5] Hidaka, H., Inagaki, M., Kawamoto, S. and Sasaki, Y. (1984) Biochemistry 23, 5036–5041.
[6] Inagaki, M., Kawamoto, S. and Hidaka, H. (1984) J. Biol. Chem. 259, 14321–14323.
[7] Kawamoto, S. and Hidaka, H. (1984) Biochem. Biophys. Res. Commun. 125, 258–264.
[8] Rush, J.S. and Waechter, C.J. (1987) Biochem. Biophys. Res. Commun. 145, 1315–1320.
[9] Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 135, 397–402.
[10] Kishimoto, A., Kajikawa, N., Shiota, M. and Nishizuka, Y. (1983) J. Biol. Chem. 258, 1156–1164.
[11] Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B. and Horecker, B.L. (1986) J. Biol. Chem. 261, 4101–4105.
[12] Woodgett, J.R. and Hunter, T. (1987) J. Biol. Chem. 262, 4836–4843.
[13] Marais, R.M. and Parker, P.J. (1989) Eur. J. Biochem. 182, 129–137.
[14] Díaz-Guerra, M.J.M., Sánchez-Prieto, J., Boscá, L., Pocock, J., Barrie, A. and Nicholls, D. (1988) Biochim. Biophys. Acta 970, 157–165.
[15] Ferrari, S., Marchiori, F., Borin, G. and Pinna, L.A. (1985) FEBS Lett. 184, 72–77.
[16] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[17] Ferriola, P.C., Cody, V. and Middleton, E. (1989) Biochem Pharmacol. 38, 1617–1624.
[18] Kimura, K., Sakurada, K. and Katoh, N. (1985) Biochim. Biophys. Acta 839, 276–280.
[19] Bazzi, M.D. and Nelsestuen, G.L. (1987) Biochem. Biophys. Res. Commun. 146, 203–207.
[20] Schächtele, C., Seifert, R. and Osswald, H. (1988) 151, 542–547.
[21] Tapley, P.M. and Murray, A.W. (1985) Eur. J. Biochem. 151, 419–423.
[22] Yamamoto, S., Kiyoto, I., Aizu, E., Nakadate, T., Hosoda, Y. and Kato, R. (1989) Carcinogenesis 10, 1315–1322.