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Calcium-Activated, Phospholipid-Dependent Protein Kinase Activity in Calf Platelets*

J. Font, A. Marino[1], N. Ravelingien[2], J. P. Dehaye[3], M. Trueba, and J. M. Macarulla

Departamento de Bioquímica y Biología Molecular
Facultad de Ciencias
Universidad del País Vasco
48080 Bilbao (Spain)

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Protein kinase C (PKC) has been widely studied from different tissues of mammals. Human platelets display higher levels of PKC activity, if compared with other sources. The PKC activity from calf platelets crude extract was determined in the presence of various protease inhibitors such as PMSF, Leupeptin or Trypsin inhibitor, and the Ca^{2+}-chelators EGTA and EDTA. The free calcium requirement was 0.25 mM, calculated with the help of the Solgas-water computer program, which represents 1 mM $CaCl_2$, in these assay conditions. Optimum PKC activity was obtained at 4 min in the presence of PS plus DAG or TPA, using H1 type III-S histone as substrate. Phospholipid-interacting drugs, such as trifluoperazine, chlorpromazine and tetracaine, inhibited the PKC activity in a dose-dependent manner. Triton X-100, a non-ionic detergent, which is usually employed to solubilize the membrane fraction, in different translocation assays, inhibited PKC activity at concentrations higher than 0.01 %. In these conditions, non-proteolytic PKC activity from calf platelets was easily determined, and shares similar activity levels with those described in human platelets.

Key words: Calf platelets, Protein Kinase C, Free Calcium, Phospholipid, Protease inhibitors.

The protein kinase C (PKC) is a calcium-activated and phospholipid-dependent protein kinase. This ubiquitous enzyme is present in a wide variety of tissues and organs of mammals and other organisms. It is usually present in the cytosol in an inactive form, but in the presence of diacylglycerol (DAG) associates with the plasma membrane where it comes into contact with the phospholipids [namely phosphatidylserine (PS)], needed to elicit its functions (8, 21). Human platelets display higher levels of PKC activity in response to agonists, as assessed by the phos-

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[1] To whom all correspondence should be addressed.

[2] Laboratory of Experimental and Gastro-enterological Surgery, Medicine Faculty, and [3] Laboratory of General and Human Biochemistry, Institute of Pharmacy, Free University of Brussels, B-1000 Brussels, Belgium.

phorylation of a specific 47 KDa protein, which has also been referred, in many reports, as 40 KDa protein (9, 15).

PKC is isolated from the cytosol as an apoenzyme that requires the addition of PS, DAG and Ca^{2+} to form a fully active quaternary complex. DAG alone did not activate PKC, but in the presence of PS, DAG lowered the Ca^{2+} requirement from approximately 100 μM to less than 1 μM (13).

The phorbol esters [like 12-*o*-tetradecanoylphorbol-13-acetate (TPA)], potent tumor promoters, share DAG similar structure and usually act in the same way as DAG. TPA interacts directly with the regulatory domain of the PKC as described previously (11, 21). Several phospholipid-interacting drugs (anaesthetics) such as: chlorpromazine, imipramine, tetracaine and the tranquilizer trifluoperazine cause inhibition in the PKC activation process, by competing with the phospholipid centre, but these drugs do not interact with the enzyme catalytic centre (21).

The aim of this report is to make a characterization of the calf platelets PKC activity *in vitro*, using different specific protease inhibitors (22). The ability of calcium to activate PKC in intact platelets was determined using the computer program Solgaswater. In these conditions the calf platelets PKC in the crude extract shows an optimum of specific activity. This PKC activity was highly reproducible from different platelets preparations. The possible interactions of Triton X-100 over the enzyme activation were also considered, as this detergent is usually employed to solubilize the membranes to which the activated PKC is associated (10).

Materials and Methods

Materials and Chemicals. — Calf platelets-rich plasma and washed platelets were prepared by the method of BAENZIGER and MAJERUS (1). Platelets (approximately 2×10^{10} cells/ml) free of contaminating blood cells were resuspended in a buffer containing 20 mM Tris-HCl pH 7.5, 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 1 mM PMSF, 0.01 % trypsin inhibitor and 0.01 % leupeptin to give a cell density of 2×10^9 cells/ml. The cells were disrupted by sonication in a Soniprep 150 MSE sonicator at 4 microns during 5 min; the sonified medium was centrifuged in a L8-50M/E Beckman ultracentrifuge at 100.000 × g for 60 min at 4 °C. The supernatant was used as PKC crude extract.

Diacylglycerol, TPA, phosphatidylserine, Type III-S Histone, Type V-S Histone, phenylmethylsulfonyl fluoride (PMSF), dimethylsulfoxide (DMSO), dithiothreitol, leupeptin, chlorpromazine, tetracaine and trifluoperazine in the hydrochloride form; EGTA, EDTA and Triton X-100 were from Sigma. Trypsin inhibitor and [γ^{-32}P]-ATP, 3000 Ci/mmol, were from Boehringer Manheim and Amersham, respectively. All other chemicals were of reagent grade obtained from various sources.

Enzyme assay. — The assay was performed by a minor modification of the method of KIKKAWA *et al.* (12). In brief, PKC activity was assayed by measuring the incorporation of [γ^{-32}P]-ATP to a protein substrate. Unless otherwise stated, the standard reaction mixture contained a total volume of 250 μl. The reaction was started by adding 50 μl of protein preparation to give a final concentration of 0.5-0.6 mg/ml assay. The reaction was carried out for 4 min at 30 °C and stopped by the addition of 3 ml of 25 % trichloroacetic acid. The protein precipitate was collected over Whatman GF/C fiber filters which were washed four times with 3 ml of 10 % trichloroacetic acid. The radioactivity was determined in a Tri-carb liquid scintillation spectrometer Packard, Model 2000

CA. One unit of protein kinase C is defined as that amount of enzyme which incorporates 1 nmol of phosphate from ATP into Histone per minute at 30 °C; routinely, the specific activity of PKC in the presence of 0.25 mM free Ca^{2+} concentration, PS and DAG was 2.8-3 U/mg protein. The free calcium concentration, in the assay medium, was calculated using a Solgaswater computer program (4). PS and/or DAG were added to glass tubes, dried under N_2 stream and then, the residue was suspended by sonication in a small volume of 20 mM Tris-HCl at pH 7.5 and 0 °C, during 4 min. The basal activity without calcium, PS and DAG was next to 4000 cpm/assay. All reagents were taken up in Ca^{2+}-free double distilled water. The final assay mixture had no effect on PKC activation by itself. Protein concentration was determined by the method of LOWRY *et al.* (16), with bovine serum albumin as a standard.

Results and Discussion

To determine the free calcium concentration in the assay medium, the constants described by NANNINGA *et al* (19, 20) were used; a computer program was used to find out the correlation between the total and the free calcium concentration in the assay mixture (4). Figure 1 shows the calcium molar fraction in function of different $CaCl_2$ concentrations for the major species in the assay medium. Figure 2 depicts the PKC activity at different free calcium (Ca^{2+}) concentrations, in three different assay conditions: in the presence of PS, in the absence of PS, or with PS + DAG; the optimum Ca^{2+} concentration for the PKC activity was between 0.4 to 0.8 mM. The PKC activity obtained here, in the presence of leupeptin and other protease inhibitors, is due to the nonproteolytic activation of the enzyme as described (3). Routinely, 1 mM $CaCl_2$, which corresponds to 0.25 mM free cal-

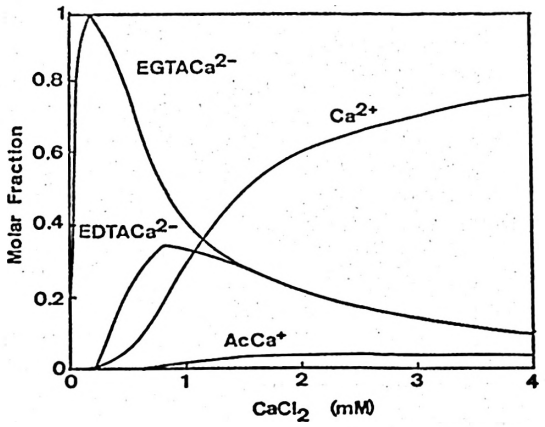

Fig. 1. *Molar fraction representation of the major calcium species in the assay medium, in function of the total calcium concentration.*
Molar fraction values for the different species were obtained with the help of Solgaswater computer program.

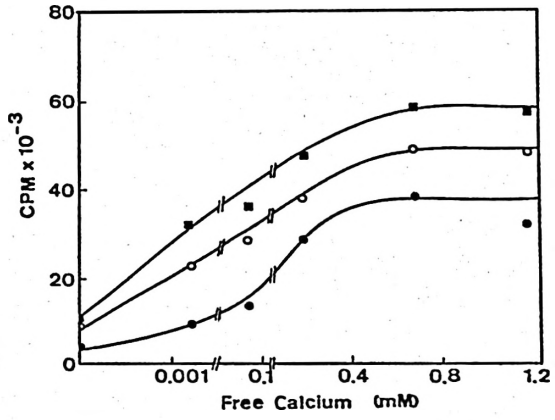

Fig. 2. *Free calcium requirement for PKC activation in calf platelets crude extract.*
PKC activity was measured in the presence of different concentrations (0-1.2 mM) of free calcium, obtained from the computer program data. Enzyme assay was carried out during 4 min at 30 °C using histone H1 type III-S as substrate, in the presence of 40 µg/ml PS (○), both PS and 0.8 µg/ml DAG (■), or in the absence of these effectors (●). In the above conditions, 100 000 cpm represent 2.8-3 U/mg protein. Data are presented as the means of three separate experiments performed in triplicate.

cium in the assay medium, was added; this value is similar to that one reported by KLEINE *et al.* (14), in T51B rat liver cells. At free Ca^{2+} concentrations higher than 0.8 mM, a proteolytic degradation of the enzyme has been described (13).

To check the best PKC substrate, two different histone types were assayed. As it is shown in figure 3, the H1 III-S histone was the optimum substrate at the two different calcium concentrations assayed.

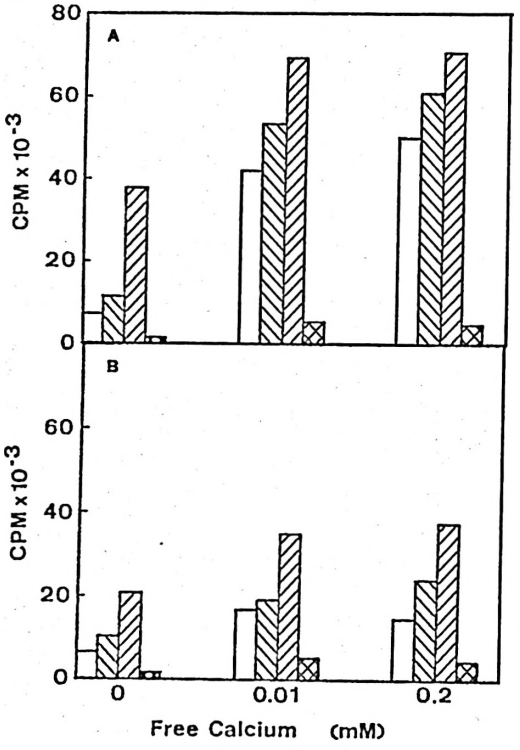

Fig. 3. *Phosporylation of two different histone H1 types by platelets PKC in the presence of two Ca^{2+} concentrations.*
Measurements of PKC activity were determined as described under Materials and Methods, using a) H1 V-S, and b) H1 III-S histone types. The assay was performed in the presence of histone alone □, histone + 40 µg/ml PS ▨, histone + PS + 0.8 µg/ml DAG ▨, or in the absence of any of these substances ▩. Data are presented as the means of two separate experiments performed in triplicate.

Figure 4 shows the time course for PKC activation in calf platelets crude extract. Maximum activity was obtained at 4 min in the presence of PS, or PS plus DAG. The same percentage of PKC activation was shown using PS with either TPA or DAG, but they did not act together as synergistic PKC activators; this finding is in agreement with that described by CASTAGNA *et al.* (3), suggesting that both compounds were at saturating concentration in the assay. In the presence of PS, PKC activity was stimulated 150 % by DAG or TPA, if compared with the basal activity measured with 0.25 mM Ca^{2+} alone. In the absence of PS, DAG or TPA were unable to elicit any PKC activation, and the profiles share the same basal activity for 0.25 mM Ca^{2+}. These results agree with those described by NISHIZUKA *et al.* (21), in rat brain, and NOGUCHI *et al.* (22), in pancreatic acinar cells.

Tetracaine, chlorpromazine and trifluoperazine inhibited PKC activity in a dose-

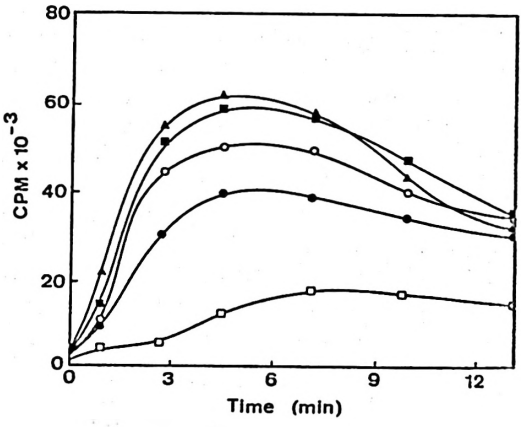

Fig. 4. *Time-course of PKC activation in calf platelets crude extract.*
The PKC activity was determined as described in Materials and Methods, in the presence of 0.25 mM Ca^{2+} (●), Ca^{2+} plus 40 µg/ml PS (○) and 0.8 µg/ml DAG (■) or 10 ng/ml TPA (▲). The basal activity in the absence of these effectors was also measured (□). 100 000 cpm represent 2.8-3 U/mg protein. Data are presented as the means of three separate experiments performed in triplicate.

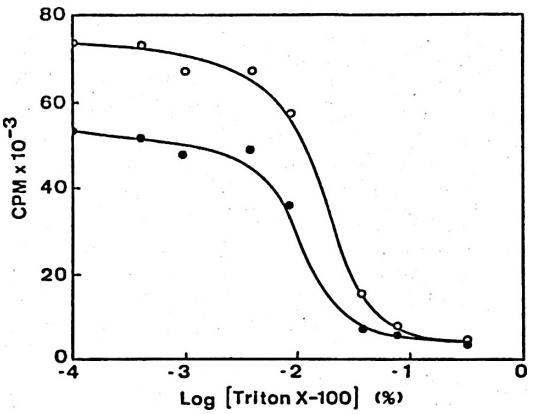

Fig. 5. *Effect of different concentrations of Triton X-100 on the PKC activation.*
Ca^{2+} concentration was 0.25 mM in the presence (o) or absence (•) of 40 µg/ml PS. Data are presented as the means of two separate experiments performed in triplicate.

dependent manner. The IC_{50} (drug concentration that inhibits 50 % of the enzyme activity) was 10^{-3} M for the former and 3×10^{-5} M for the two other drugs. In this way, we have recently demonstrated that chlorpromazine was unable to block the PKC translocation to human platelet plasma membranes stimulated by TPA (5); this finding indicated that in the presence of different phospholipid-interacting drugs, the enzyme could still associate with the plasma membrane, but did not reveal its catalytic activity; the chlorpromazine also caused a deep inhibition of plasma membrane phosphatidylethanolamine methyltransferase, a membrane intrinsecal phospholipid-related enzyme (17). Similar PKC activity inhibition by chlorpromazine was previously described in platelets *in vivo* (23) and in rat brain *in vitro* experimentation (18).

Recently, several studies on PKC requirement *in vitro* have been carried out with phospholipid-Triton X-100 mixed micelles (2, 6, 7) and different translocation assays were made using Triton X-100 to solubilize the membrane bound PKC (24, 25). In order to determine if Triton X-100 has some effect on PKC activity by itself, different detergent concentrations were tested in the calf platelets extract. Figure 5 represents the dose-dependent inhibition of PKC activity in the presence or absence of 40 µg/ml PS; 0.01 % Triton X-100 in the assay medium shows a 30 % of PKC inhibition, while at 0.1 % the inhibition percentage was 100 %.

Although some of the PKC activity requirements tested in this report, such as free calcium concentration or Triton X-100 inhibition, have not been described before in platelets, the PKC activity studied here shares similar characteristics with those previously described from other different sources (15, 23). The specific activity, next to 2-3 U/mg protein, is also similar to that described in different mammalian tissues (human platelets, rat brain, lymphocytes, etc.) (21).

It can be summarized that, in the above conditions, the PKC activity was easily determined in the platelets crude extract with optimum responsiveness (24), and this allows to avoid the previous steps of protein purification, and also to study *in situ* the nonproteolytic enzyme activation pattern.

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Resumen

Se caracteriza la actividad PKC en extractos crudos de plaquetas de ternera, en presencia de inhibidores de proteasas como el PMSF, la leupeptina o el inhibidor de la tripsina, así como en presencia de EGTA y EDTA. La concentración de calcio libre requerida es de 0,25 mM, calculada con la ayuda del programa de ordenador Solgaswater, lo que representa 1 mM de $CaCl_2$. Se obtiene una actividad PKC óptima a los 4 min en presencia de fosfatidilserina más diacilglicerol o el éster de forbol TPA, utilizan-

do el tipo III-S de la histona H1 como sustrato. La trifluoperazina, clorpromazina y tetracaína, fármacos caracterizados por interaccionar con los fosfolípidos de membrana, inhiben de forma dosis-dependiente la actividad PKC. El Triton X-100, un detergente no iónico que se emplea habitualmente para solubilizar fracciones de membrana en los ensayos de translocación, inhibe la actividad PKC a concentraciones superiores a 0,01 %. En estas condiciones, la actividad PKC no proteolítica de plaquetas de ternera se determina fácilmente, mostrando niveles de actividad muy similares a los descritos en plaquetas humanas.

Palabras clave: Plaquetas de ternera, Proteína quinasa C, Calcio libre, Fosfolípidos, Inhibidores de proteasas.

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