

Characterization of Calcium and Phospholipid Dependent Protein Kinase in Isolated Rat Adipocytes

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Calcium and phospholipid-dependent protein kinase (protein kinase C) from isolated rat adipocytes has been partially purified using DEAE-Sepharose CL-6B and characterized. The enzyme was shown to have similar properties as the kinase isolated from brain or spleen. When histone was used as substrate, an equal amount of cAMP-dependent and calcium and phospholipid-dependent kinase activity was detected from the DEAE Sepharose CL-6B fractions. The major part of protein kinase C (72 %) was isolated from the soluble adipocyte fraction. Of the membranous fractions, the plasma membrane exhibited the highest specific activity. The protein kinase preparations bound [³H]-phorbol-12,13-dibutyrate (PDBU) with high affinity ($K_d=2$ nM) and the number of PDBU binding sites per cell was calculated to 63 000.

The existence of a calcium and phospholipid-dependent protein kinase (protein kinase C) was first reported by Takai *et al.*¹ It has been purified from brain,² spleen³ and heart⁴ and found to be a monomolecular protein species with an apparent molecular weight of 77 000. It is remarkably activated upon binding of either phorbol esters⁴ or diacylglycerols⁵ and distributed in several body tissues,⁶ although the highest specific activity is registered in brain and spleen.⁶ Candidates as endogenous substrates for this kinase have been found in *e.g.* leukemic cells,⁷ lymphocytes,⁸ platelets^{9,10} and in the brain.¹¹ Among the substrates in *in vitro* systems are vinculin¹² and ribosomal 40 S complex protein S 6.¹³ The best evidence for a role *in vivo* comes from experiments performed with platelets.^{9,10} Treatment of the cells with thrombin causes an immediate release of diacylglycerols by the enhanced rate of polyphosphoinositide and phosphatidylinositol breakdown. As a consequence, the sensitivity of protein kinase C towards Ca^{2+} is increased, the enzyme is activated and probably participates in the subsequent phosphorylation of a protein with an apparent molecular weight of 40 000, that in turn might participate in the events leading to the resulting release of serotonin from the platelets. Addition of phorbol esters to the platelets as well as addition of the synthetic diacylglycerol 1-oleyl-2-acetyl-glycerol (OAG) results in a direct phosphorylation of the 40 000 D protein and serotonin release.

The adipocyte is remarkably sensitive towards several hormones and the hormonal responses are rather well characterized. Furthermore, isolated adipocytes represents a homogeneous cell system easy to prepare. We considered it interesting to evaluate a role of protein kinase C as a potential mediator of hormonal signals in the adipocyte. In this communication we describe the presence, subcellular distribution and some characteristics of adipocyte protein kinase C.

EXPERIMENTAL

Abbreviations. KRH, Krebs-Ringer Hepes; PCV, packed cell volume; protein kinase C (calcium- and phospholipid-dependent protein kinase); protein kinase A (cAMP-stimulated protein kinase); PS, phosphatidylserine; DG, 1,3-dioleoylglycerol; TPA, 12-*O*-tetradecanoylphorbol 13-acetate (4- β -phorbol 12-myristate 13-acetate); PDBU, phorbol-12,13-dibutyrate; TCA, trichloroacetic acid; DCIP, dichlorophenolindophenol.

Materials. Collagenase, type CLS, lot 42 N-083, was purchased from Millipore. Bovine serum albumin, Cohn fraction V, lot 42 F-0400, 12-*O*-tetradecanoylphorbol 13-acetate, bovine brain phosphatidylserine, 1,3-dioleoylglycerol and histone type III-S was supplied by Sigma. [γ - 32 P] ATP and [3 H]-PDBU (specific activity 450 GBq/mmol) were obtained from New England Nuclear. Male Sprague Dawley rats weighing 120–160 g were delivered from a local farm. The rats were fed *ad libitum*.

Methods. Rat adipocytes were isolated from epididymal fat pads according to Nilsson and Belfrage¹⁴ with the exception that the albumin solution was freshly prepared each day. The adipocytes were incubated at 37 °C for 30 min in the standard KRH buffer (118 mM NaCl, 5.95 mM KCl, 2.55 mM CaCl₂, 1.19 mM MgSO₄, 1.19 mM KH₂PO₄, 24 mM Hepes, 0.5 mM glucose and 1 % (w/v) bovine serum albumin, pH 7.4) prior to initiation of experiments. Cell concentration was measured as packed cell volume after centrifugation in hematocrite tubes.¹⁵ All adipocyte incubations were performed in polypropylene or polyethylene vessels.

Soluble and particulate fractions of the rat adipocytes were prepared as follows. A 5 ml portion of a 6–14 % (v/v) cell suspension was washed three times with 0.25 M sucrose, 10 mM Tris-HCl pH 7.4, at 37 °C by repeated suspension and flotation. Six ml ice cold 0.33 M sucrose, 2 mM EDTA, 0.5 mM EGTA, 20 mM Tris-HCl, pH 7.5,⁶ was added and the cells were homogenized by ten strokes in a chilled loose-fitting Teflon pestle – glass barrel homogenizer. The fat was removed by a brief centrifugation (30 s, 1000 g) and the infranatant was centrifuged in a Beckman Ti 50.2-rotor at 34 000 rpm (100 000 g) for 60 min, at 4 °C. The resulting supernatant was used as such, and the membrane pellet was solubilized by suspension in 6 ml homogenization buffer containing 1 % Triton X-100. After 30 min on ice the unsolubilized material was removed by high-speed centrifugation as described above.

Subfractionation of membranes was achieved using a slight modification of the method described by Belsham *et al.*¹⁶ Adipocytes, corresponding to 1.5–2 ml of PCV, were isolated as described above and washed once in 10 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose. The cells were disrupted by the addition of 8 ml ice-cold 10 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, 2 mM EGTA, 20 mM NaF, 7.5 mM glutathione (reduced form) and 5 mM EDTA followed by an immediate mixing for 30 s in a Vortex. The fractionation was subsequently performed as described,¹⁶ except that the “supernatant fraction” was centrifuged at 105 000 g for 60 min to yield a pellet of light membranes. The fractions obtained were solubilized in Triton X-100 and further treated by chromatography on DEAE-Sephacel CL-6B as detailed below.

The subcellular fractions were characterized by measuring 5'-nucleotidase,¹⁷ NADPH-Cytochrome C reductase in the presence of 1 mM KCN¹⁸ and Succinate-DCIP-reductase.¹⁹ The specific activities of the enzymes were for plasma membrane, mitochondria and light membrane, NADPH-Cytochrome C reductase 0.13, 1.5 and 3.3 nmol cytochrome C reduced/min and mg of protein, for Succinate-DCIP reductase 5.7, 43.6 and 2.8 nmol of DCIP reduced/min and mg of protein and for 5'-nucleotidase 4.7, 0.79 and 2.5 mU/mg of protein, respectively.

High speed supernatant and solubilized membrane pellet were fractionated by chromatography on DEAE-Sephacel CL-6B (Pharmacia). Each supernatant was applied to a 20 mm high, 6 mm wide ion exchange column equilibrated in 2 mM EDTA, 0.5 mM EGTA, 20 mM Tris-HCl, pH 7.5, and the adsorbed material was then eluted with a stepwise NaCl gradient composed of two column volumes equilibration buffer containing various salt concentrations.

Calcium and phospholipid-dependent protein kinase (protein kinase C) was measured as follows: 50 μ l of enzyme solution in buffer containing 2 mM EDTA and 0.5 mM EGTA was preincubated at 30 °C 2 min after addition of 175 μ l solution containing 1.875 μ mol Mg, 0.2

$\mu\text{mol Ca}$, $3.5 \mu\text{mol Tris}$ and $5 \mu\text{g PS}$ (pH 7.5). The incubation was started by addition of $25 \mu\text{l}$ solution containing $50 \mu\text{g histone}$, $2.5 \text{ nmol } [\gamma\text{-}^{32}\text{P}]\text{-ATP}$ ($0.5 \mu\text{Ci}/2.5 \text{ nmol}$), $0.313 \mu\text{mol Mg}$ and $0.5 \mu\text{mol Tris}$ (pH 7.5). After three minutes at 30°C , the reaction was stopped by addition of $1 \text{ ml } 25\% \text{ (w/v) TCA}$; $0.1 \text{ ml } 0.63\% \text{ BSA}$ solution was added as carrier followed by $3 \text{ ml } 5\% \text{ TCA}$. The precipitate was collected by centrifugation, dissolved in $0.2 \text{ ml } 1 \text{ M NaOH}$, precipitated by addition of $4 \text{ ml } 5\% \text{ TCA}$, centrifuged, dissolved and precipitated again and finally dissolved in $1 \text{ ml } 0.2 \text{ M NaOH}$. An aliquot of 0.5 ml of the solution was measured for radioactivity using Aqualuma plus as scintillator liquid. This standard incubation scheme gives final concentrations of 8.5 mM free Mg and 0.4 mM free Ca (both calculated from dissociation constants given in²⁰ assuming negligible binding of divalent cations to lipids and protein) and $10 \mu\text{M ATP}$. Under these conditions the assay shows linear enzyme and time dependency. Kinase activity in the presence of Ca but absence of PS was routinely subtracted to calculate protein kinase activity. Protein kinase A was measured using $1 \mu\text{M cAMP}$ and histone as substrate under similar conditions, but in the absence of calcium and phospholipids.

Binding of phorbol-ester to crude and partially purified adipocyte supernatant was studied by the method of Ashendel and Boutwell²¹ as detailed below. In order to obtain a lower degree of nonspecific binding the less hydrophobic $[20\text{-}^3\text{H}]\text{-PDBU}$ was used instead of TPA. To the sample ($200 \mu\text{l}$) in buffer containing 2 mM EDTA and 0.5 mM EGTA was added a solution containing $0.8 \mu\text{mol Ca}^{2+}$, $8.75 \mu\text{mol Mg}^{2+}$, $16 \mu\text{mol Tris}$ (pH adjusted to 7.5 with HCl), $20 \mu\text{g PS}$ and the indicated amount of $[^3\text{H}]\text{-labelled}$ and non-labelled phorbol ester to give a final volume of 1.0 ml . The final concentrations were the same as in the standard protein kinase C-assay mixture except for the omission of ATP and histone. After incubation at $4\text{--}6^\circ\text{C}$ for 3 h , 2.5 ml of acetone (-78°C) was added and the precipitate was collected on a 25 mm Whatman GF/C filter aided by a waterjet suction apparatus. The tube and filter was then rinsed with three 2.5 ml portions of chilled acetone. The filter paper was counted for radioactivity in a 25 ml vial with 7 ml Aqualuma plus as scintillator liquid. Analysis of $[^3\text{H}]\text{-PDBU}$ -binding in supernatant according to Scatchard was based on binding data for $[^3\text{H}]\text{-PDBU}$ with constant specific activity corrected for non-specific binding by subtracting binding in the presence of a 300-fold excess of non-labelled PDBU for each $[^3\text{H}]\text{-PDBU}$ -concentration tested.

RESULTS

Identification of calcium and phospholipid-dependent protein kinase in rat adipocytes. Adipocytes, isolated from rat epididymal fat pads, were homogenized and the resulting homogenate was centrifuged at $100\,000 \text{ g}$ for 1 h . Using histone as substrate, no calcium and phospholipid-dependent protein kinase (protein kinase C) activity could be identified in the resulting supernatant. However, when this fraction was subjected to stepwise elution chromatography on DEAE-Sepharose CL-6B, the enzyme activity was eluted from the column at 100 mM NaCl (Fig. 1). From experiments not shown, it appeared that the chromatography step reduced the amount of calcium-stimulated non phospholipid-dependent kinase activity as well as endogenous phosphatases,²³ thus allowing a definite identification of the phospholipid-dependent enzyme.

The particulate fraction, obtained as described under "Experimental", was solubilized with Triton X-100 and chromatographed in parallel with the supernatant fraction on DEAE Sepharose CL-6B. The fractions collected from the ion exchange column were assayed for the content of cAMP-dependent protein kinase activity (protein kinase A) and protein kinase C using histone as substrate. Both protein kinase C and protein kinase A activities were distributed mainly in the soluble fraction (70% , Table 1). Calculations revealed that by assuming a quantitative elution from the column of both types of kinases, the adipocyte would contain an equal amount of each kinase activity, *i.e.* about 50 pmol of $[^{32}\text{P}]$ incorporated into histone per min and ml of packed cell volume.

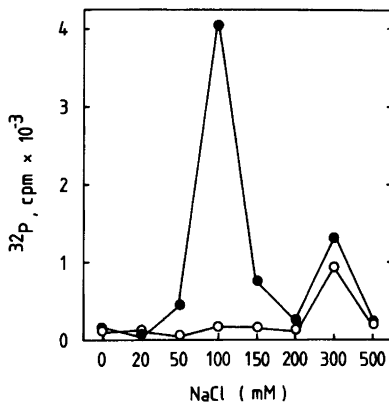


Fig. 1. Protein kinase C detected after chromatography of adipocyte supernatant fraction on DEAE Sepharose CL-6B. The high speed supernatant of adipocyte homogenate, prepared as described under "Experimental", was applied onto a DEAE-Sepharose CL-6B column equilibrated in 20 mM tris-HCl, pH 7.5, containing 2 mM EDTA and 0.5 mM EGTA. Elution was carried out in a stepwise manner using two column volumes of the equilibration buffer containing indicated concentrations of sodium chloride. Aliquots of 50 μl were assayed for protein kinase C activity in the presence (\bullet), or in the absence (\circ) of PS, as described under "Experimental".

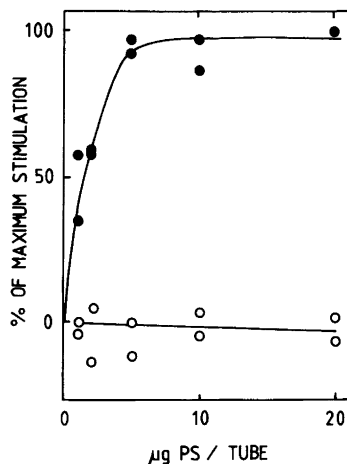


Fig. 2. Activation of adipocyte protein kinase C by phosphatidylserine. The 150 mM sodium chloride fraction recovered from chromatography of the high speed adipocyte supernatant on DEAE Sepharose CL-6B using elution with a stepwise gradient of 0, 50, 150 and 500 mM NaCl was used as enzyme source. Incubations with increasing amounts of PS were carried out in the presence (\bullet) or in the absence (\circ) of 0.4 mM free calcium. Results (from two experiments) are expressed as per cent of maximum kinase activity (10–20 pmol of [^{32}P] incorporated into histone per min and ml).

Subcellular distribution of the protein kinase C activity in the adipocyte. Isolated adipocytes were homogenized in a medium containing EDTA and EGTA and subsequently fractionated according to procedures developed by Denton and collaborators¹⁶ as described under "Experimental". The membranous subcellular fractions thus obtained were solubilized with Triton X-100 and fractionated on DEAE-Sepharose CL-6B. As seen from Table 2, the plasma membrane fraction had the highest specific protein kinase C activity.

Table 1. cAMP-dependent protein kinase activity and protein kinase C activity detected after chromatography of solubilized particulate adipocyte fraction and high speed supernatant on DEAE Sepharose CL-6B. The sum of kinase activities in all fractions are given and are expressed as pmol of [^{32}P] incorporated into histone per min and ml of PCV. The data represent mean \pm S.D. of the indicated number of experiments.

	Kinase activity/pmol [^{32}P] per min and ml PCV cAMP-dependent (n=3)	Ca^{2+} , PS-dependent (n=4)
Adipocyte supernatant	31.0 \pm 7.8	46.8 \pm 13.1
Solubilized particulate fraction	8.1 \pm 2.7	16.6 \pm 7.7

Table 2. Subcellular distribution of membranous adipocyte protein kinase C. The adipocyte homogenate was fractionated using sucrose gradient centrifugation as described under "Experimental". The specific kinase activity is expressed in relation to the amount of [^{32}P] incorporated into histone per min and mg of protein in the plasma membrane fraction (5,8–19 pmol/min mg). The results represent mean \pm S.D. out of three experiments.

Membrane fraction	Protein kinase C activity	
	% of total membrane-associated activity	% of maximum specific activity
Plasma membrane	38 \pm 11	100
Mitochondria	49 \pm 18	43 \pm 37
Light membrane	13 \pm 6	63 \pm 39

Characterization of the soluble calcium and phospholipid-dependent protein kinase. The calcium and phospholipid-dependent kinase was characterized as eluted from the DEAE-Sepharose column. Half maximum activation of the enzyme activity was reached when 8 μg of phosphatidylserine/ml was present in the incubation mixture, whereas no kinase activation was registered by PS in the absence of added calcium in the incubation mixture (Fig. 2). The calcium requirement of the enzyme is shown in Fig. 3. In the presence of PS, half maximum activation was evident when the free calcium concentration was 20 μM . Using 1 μM calcium and 20 μg of PS/ml, diolein (40 $\mu\text{g}/\text{ml}$) or TPA (40 ng/ml) stimulated the protein kinase C activity by 10–20 % (data not shown).

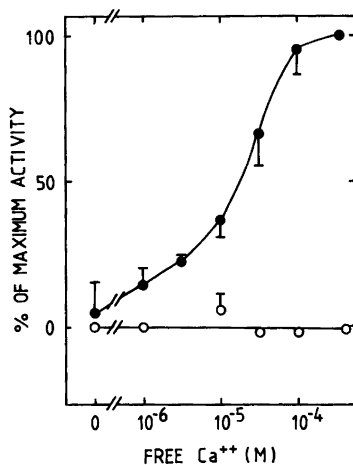


Fig. 3. Activation of adipocyte protein kinase C by calcium. The DEAE-Sepharose CL-6B fraction of the adipocyte high speed supernatant was used in the incubations with indicated amounts of free calcium in the presence (●) or in the absence (○) of 5 μg PS. Other conditions as described in legend to Fig. 2 and under "Experimental". Results are expressed as per cent of maximum kinase activity and constitute the mean \pm S.D. of three experiments.

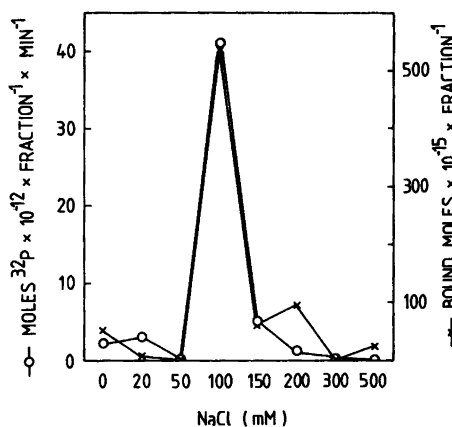


Fig. 4. [^3H]-PDBU binding to DEAE Sepharose CL-6B fractions obtained by chromatography of adipocyte high speed supernatant in comparison to the activity of protein kinase C. Binding experiments were carried out with 200 μl aliquots of the fractions as described under "Experimental". One out of three experiments is shown.

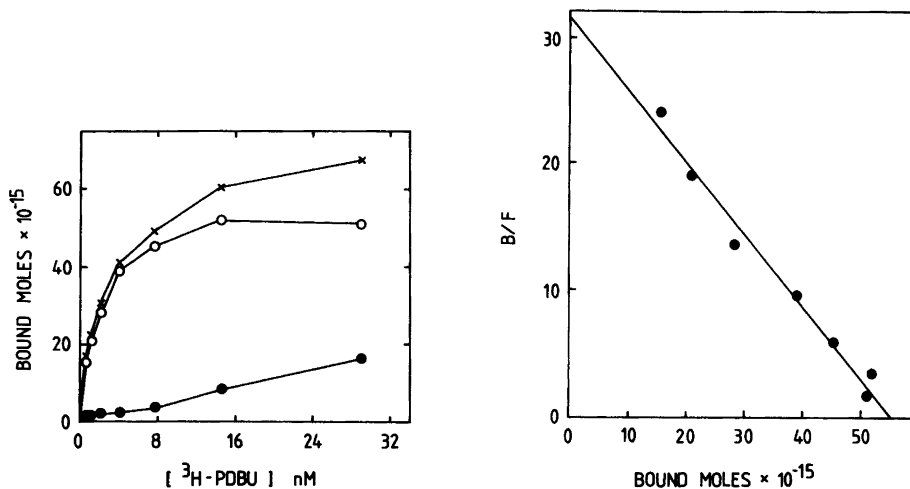


Fig. 5. Total binding (A) and Scatchard analysis of the specific binding (B) of [³H]-PDBU to the protein kinase C fraction obtained from DEAE Sepharose CL-6B. The binding experiments were performed as described in legend to Fig. 4 and under "Experimental". The results from one out of three similar experiments are shown. The correlation coefficient was 0.988 and $K_d=1.75$ nM. (○), specific binding; ●, nonspecific binding; ×, total binding).

Binding of PDBU to the adipocyte protein kinase C. When the DEAE Sepharose CL-6B fractions were assayed for their capability to specifically bind [³H]-labelled PDBU, it was registered that the binding component cochromatographed with the protein kinase C activity (Fig. 4). Scatchard analysis of this binding (Fig. 5) revealed that the protein had an apparent K_d for PDBU of 1.8 nM. Based upon the distribution of binding capacity at 13 nM PDBU between particulate and soluble fraction of 28:72 (not shown), this corresponds to 1.6 pmol of binding sites per ml PCV, to 2.5×10^{11} binding sites per mg of protein (4 mg protein per ml PCV²²) or 63 000 sites per cell (1 ml PCV corresponds to 1.6×10^7 cells when isolated from rats with about 140 g of weight²²). By the assumption that protein kinase C and the PDBU binder are identical, one could calculate the overall content of protein kinase C in the adipocyte to 32 ng/mg protein.

DISCUSSION

The results presented in this report demonstrate the presence of protein kinase C in isolated rat adipocytes and the existence of a receptor for PDBU in these cells. Kikkawa *et al.*²⁴ have recently reported that homogeneous preparations of protein kinase C isolated from rat brain bind PDBU with the same affinity as the intact cell surface receptor and that the dissociation constant for PDBU is identical with the activation constant for protein kinase C. In accordance with these findings, it was not possible to separate protein kinase C activity from the PDBU binding fraction using fractionation of adipocyte supernatant on DEAE-Sepharose CL-6 B (Fig. 4).

The number of PDBU binding sites calculated per adipocyte (6×10^4) is comparable to that found in, *e.g.*, thymoma cells²⁵ but smaller than found in other tissues such as brain, spleen, ovaries and lung.²⁶ In contrast to the situation in brain²⁷ most of the protein kinase C

activity was found in the soluble fraction. The distribution of protein kinase C in the adipocyte in this way resembles the situation in e.g. liver and skeletal muscle.²⁷ Regarding the membrane bound enzyme, the distribution of the specific activity corresponds rather well with the distribution of specific activity of 5'-nucleotidase, a marker for plasma membranes. The activity found in the mitochondrial fraction can possibly be explained by plasma membrane and microsomal contamination.

The activity of protein kinase C was approximately equal to that of protein kinase A, *i.e.* about 50 pmol of [³H] incorporated into histone/min ml PCV. The amount of protein kinase A activity found is lower than described earlier.²⁹ This might be due to nonoptimal assay conditions, to the use of histone instead of protamine as substrate and also to the use of isolated adipocytes in this study as compared to epididymal fat pads in the previous report.²⁹

No activation of adipocyte protein kinase C was seen after addition of PS in the absence of calcium and, likewise, activation by Ca²⁺ required the presence of PS. Addition of diolein or TPA to the enzyme fraction apparently decreased the requirement of calcium for activity. In this respects the adipocyte enzyme have similar properties as protein kinase C isolated from brain⁵ or spleen.⁶ In addition, also the concentrations of calcium and phospholipid required for half-maximal activation are similar. The small effects (10–20 %) observed with diolein and TPA might be due to the impurity of the enzyme preparation (*c.f.* Ref. 6, Fig. 2).

The finding of protein kinase C in adipocytes have prompted us to evaluate a role of this enzyme in hormonal signalling and regulation of adipocyte metabolism. Our results, that are to be published, and those presented in a recent paper by Roach and Goldman,²⁸ indicating an important role of protein kinase C in the regulation of glycogen synthase, suggest that protein kinase C might play a central role in hormone action.

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