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INHIBITION OF PROTEIN KINASE C FROM POLYMORPHONUCLEAR NEUTROPHILS BY LONG CHAIN ACYL COENZYME A AND COUNTERACTION BY Mg-ATP

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SUMMARY. The Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C) from bovine polymorphonuclear neutrophils was inhibited by micromolar amounts of long chain acyl-CoAs. The extent of inhibition at a given concentration of the acyl-CoAs depended on the length of the chain. A chain length of at least 12C was required for inhibition. Inhibition of protein kinase C activity was counteracted specifically by Mg-ATP.

Protein kinase C (PKC) has a widespread occurrence in various tissues of most animals. Besides the fact that polymorphonuclear neutrophils contain a high concentration of PKC (1) and are a convenient source for the purification and the study of this enzyme, many neutrophil functions, including the production of the superoxide anion, appear to be controlled, at least partially, by the activation of PKC (for review, see 2). A number of natural activators, including diacylglycerol, phosphatidyl serine and Ca^{2+} (3), and also long chain fatty acids in the cis-form (4), have been shown to be essential for full expression of the activity of PKC. Recently, attention has been drawn to the potential role that could be played by inhibitory molecules that are naturally present in cells, like polyamines (5), retinal (6), palmitylcarnitine (7), calmodulin (8), gangliosides (9), phosphatidyl serine (under appropriate conditions of incubation) (10), sphingosin (11), and a natural inhibitor of protein nature (12-14). Long chain acyl-CoAs have been reported to either slightly inhibit (7) or to strongly stimulate (15) PKC from brain. Because of our current interest in the role of PKC in neutrophil functions and the central role played by lipid metabolism in the control of the

Abbreviations: PKC, protein kinase C ; Mops, 3-[N-morpholino]propane sulfonic acid ; EDTA, ethylene diamine tetraacetate ; EGTA, ethyleneglycol-bis(β-amino-ethyl ether)tetraacetate ; PMSF, phenylmethyl sulfonyl fluoride ; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone ; TLCK, N-p-tosyl-L-lysine chloromethyl ketone.

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respiration burst of neutrophils (2), we have explored in more detail the conditions of modulation of neutrophil PKC activity by long chain acyl-CoAs. In this paper, we report that long chain acyl-CoAs are efficient inhibitors of PKC at low concentrations, provided they are preincubated with PKC prior to the assay and that this inhibition is counteracted by Mg-ATP, provided Mg-ATP is added together with long chain acyl-CoAs.

EXPERIMENTAL PROCEDURES

Materials. Histone type III S, phosphatidyl serine, diacylglycerol, bovine serum albumin (fraction V), Mops, EDTA, EGTA, mercaptoethanol, PMSF, TPCK, leupeptin, soybean trypsin inhibitor, deoxycholate, Triton X-100 and Tween 20 were purchased from Sigma Co. Acyl-CoAs and CoA-SH were obtained from P.L. Biochemicals, ATP from Boehringer and [γ-^{32}P]ATP from Amersham. Laurylamidodimethylpropylaminoxide was kindly provided by Dr. G. Brandolin.

Biological preparations. The crude cytosolic fraction used as a source of PKC was obtained from bovine neutrophils prepared as described in (16). All the operations were carried out at 0-4°C. Neutrophils were suspended at the concentration of 10^8 cells/ml in a medium consisting of 0.25M sucrose, 10 mM Mops, 2mM EDTA, 2mM EGTA, 50mM mercaptoethanol, 1mM PMSF, TPCK 1µg/ml, TLCK 1 µg/ml, soybean trypsin inhibitor 1µg/ml and leupeptin 10µg/ml. The suspension was subjected to four successive sonications of 15s separated by 30s intervals at 2-6°C, using a Branson sonifier at 20W output. The homogenate was centrifuged at 100 000g for 1h. The supernatant (cytosolic fraction) was either used directly or further processed for partial purification of PKC activity, following the first two steps of the procedure recently described for the purification of the mice brain PKC (17), namely chromatography on DE52 cellulose followed by fractionation with a Mono Q column, using a Pharmacia FPLC system. At this stage, the purification factor was between 70 and 100, with respect to the crude cytosolic fraction. One unit of activity was defined as the amount of enzyme which incorporated 1 nanomol of [γ-^{32}P]ATP into histone III S per min at 37°C.

Assay of inhibition of PKC activity by acyl-CoA. The assay was performed in two stages ; the first one consisted in preincubation of PKC with acyl-CoA and the second stage in the assay of PKC activity. Either the crude neutrophil cytosolic fraction or the partially purified PKC after suitable dilution was preincubated at 37°C for 5 min in the presence of acyl CoA in 0.07ml of a medium containing 20mM Mops, pH 7.4 and 10mM mercaptoethanol. $MgCl_2$ and ATP or other nucleotides were added as indicated. Incubation was started by the addition of 0.02 ml of the preincubation mixture to 0.08 ml of a medium containing 20 mM Mops pH 7.4, 5 mM $MgCl_2$, 1mM $CaCl_2$, 70µM [γ-^{32}P]ATP 10^6cpm, 50 µg histone III S, phosphatidyl serine 20 µg/ml and diacylglycerol 2 µg/ml. The incubation reaction was allowed to proceed at 37°C. It was terminated by the addition of 0.5 ml of ice-cold 10% trichloroacetic acid and 0.5 ml of a solution of bovine serum albumin at 2 mg/ml. The protein was collected on Millipore HAWP 0.45 µm membrane filters, and washed three times with 1 ml of ice-cold 10 % trichloracetic acid. Radioactivity on the filters was measured by liquid scintillation counting in 10 ml of toluene and Triton X-100 scintillation fluid (18). A blank assay was performed by

adding trichloroacetic acid and bovine serum albumin to the incubation medium prior to the addition of enzyme. Control conditions also included the absence of Ca^{2+} and lipids in the incubation medium. The amount of PKC in the medium was adjusted so that the assay was linear up to 10min. In routine assays, the phosphorylation reaction lasted for 5min, so that the [^{32}P] radioactivity incorporated into histone IIIS was proportional to PKC activity. Protein content was analyzed by the method of Bradford (19) with bovine serum albumin as reference.

RESULTS

1. Factors determining inhibition of PKC activity by palmityl-CoA.

Preincubation of PKC with palmityl-CoA in the absence of ATP was required for inhibition of the enzyme. In fact when directly added to the assay medium in the presence of [γ-^{32}P]ATP, and in accordance with Shoyab (15) palmityl-CoA was not inhibitory, and it even enhanced the PKC activity. The extent of stimulation afforded by 20 µM palmityl-CoA was however limited and routinely less than 30%.

Preincubation times as short as 30 s with palmityl-CoA were sufficient for maximal inhibition of PKC. For convenience, in routine assays, a 5 min-preincubation with palmityl-CoA was used. The dose-effect curves in Figure 1 were obtained with the crude cytosolic fraction from bovine neutrophils and with the partially purified preparation derived from the neutrophil cytosolic fraction (cf.

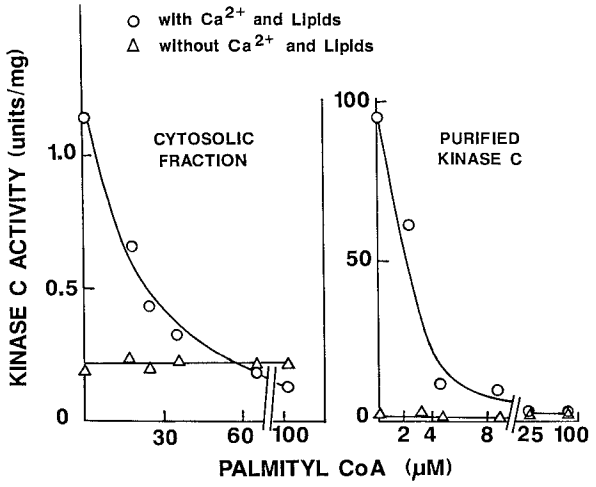

Figure 1. Effect of increasing concentrations of palmityl-CoA on the PKC activity present in the cytosolic fraction from bovine neutrophils and in a partially purified preparation. The experiment was carried out in two steps, a preincubation step in which palmityl-CoA was left to react with PKC and an incubation step which consisted in the transfer of [^{32}P] phosphate from [γ-^{32}P]ATP to histone III S. In the case of the crude cytosolic fraction, a complementary assay was carried out in the absence of added $CaCl_2$ and lipids.

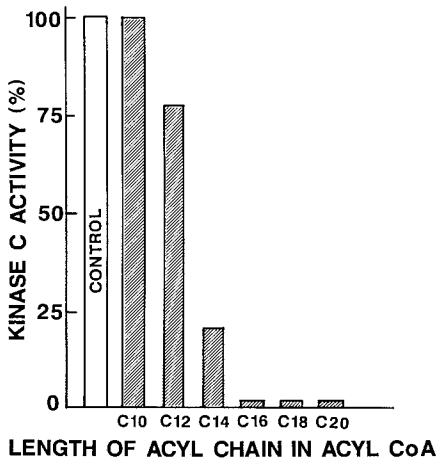

Figure 2. Effect of the acyl chain length on inhibition of PKC by acyl-CoAs. The experimental conditions were the same as those described in the legend of Fig.1. The crude cytosolic fraction from bovine neutrophils was used and acyl-CoAs were added to the preincubation medium at the final concentration of 70µM. The data presented have been corrected for the basal kinase activity observed in the absence of added $CaCl_2$ and lipids.

Experimental Procedures). About ten times more palmityl-CoA was required to inhibit 50% of PKC in the crude cytosolic extract than in the purified preparation ; this is probably due to the fact that palmityl-CoA has a strong propensity to bind to a number of proteins, due to its amphiphilic nature. Half inhibition of the partially purified PKC was achieved with only 2.5µM palmityl-CoA. The inhibitory potency of palmityl-CoA depended on temperature ; twice as much palmityl-CoA was required to inhibit 50% of PKC activity when preincubation was carried out at 25°C compared to 37°C.

No effect of palmityl-CoA was observed when the medium was deprived of Ca^{2+} and lipid. The crude cytosolic fraction exhibited a significant level of enzymatic activity independent of Ca^{2+} and lipid. This could be due either to the presence of protein kinase(s) different from PKC, or to the proteolytic degradation of PKC, generating an enzyme form insensitive to lipid and Ca^{2+}.

2. Specific effect of long chain acyl-CoAs as inhibitors of PKC.

Acyl-CoAs with a fatty acyl chain length of less than 10 C atoms had no effect on PKC activity, even at concentrations as high as 70 µM (Figure 2). For inhibition to become effective, a chain length equal to or higher than 12 C was necessary. Maximal inhibition was observed with chain lengths of 16 to 20 C. Unsaturation did not modify the inhibitory efficiency of long chain acyl-CoAs ; for example, oleyl-CoA was as efficient as stearyl-CoA.

Table I. Counteracting effect of ATP on the inhibition of PKC by palmityl-CoA

Enzyme preparation	conditions of preincubation	PKC activity (Units/mg)
Cytosolic fraction	control	1.2
	70μM palmityl-CoA	0.1
	70μM palmityl-CoA +0.2mM $MgCl_2$	0.2
	70μM palmityl-CoA +0.2mM $MgCl_2$+20μM ATP	0.9
Partially purified PKC	control	85
	4.5μM palmityl-CoA	7
	4.5μM palmityl-CoA +0.5mM $MgCl_2$	15
	4.5μM palmityl-CoA +0.5mM $MgCl_2$+20μM ATP	39
	4.5μM palmityl-CoA +0.5mM $MgCl_2$+20μM ADP (or AMP)	8
	9μM palmityl-CoA +0.5mM $MgCl_2$	4
	9μM palmityl-CoA +0.5mM $MgCl_2$+20μM ATP	14

The experimental conditions are the same as those described in the legend of Figure 1, except that ATP (or the other nucleotides) and $MgCl_2$ were present in the preincubation medium at the indicated concentrations. Corrections were made for the basal activity observed in the absence of $CaCl_2$ and lipids, and for dilution when unlabeled ATP was present in the preincubation medium.

When used at a concentration of 70 μM under the same conditions as palmityl-CoA, palmitic acid and CoA-SH did not exhibit any inhibitory effect, and 100 μM palmityl-carnitine inhibited 60% only of the activity of the partially purified PKC.

3. Specific reversal by Mg-ATP of the palmityl CoA-dependent inhibition of PKC.

In preliminary assays carried out with the cytosolic fraction of bovine neutrophils, it was found that ATP added together with palmityl-CoA could reverse the inhibition of PKC activity caused by palmityl-CoA, and that Mg^{2+} was required for the counteracting effect of ATP. However, Mg^{2+} **per se** binds to palmityl CoA in a reversible reaction to form a precipitable, inactive, Mg-palmityl-CoA complex (20). A compromise between these two effects of Mg^{2+} was found with Mg^{2+} concentrations equal to, or lower than 0.5mM. The experiment reported in Table I illustrates the reversal by Mg-ATP of the

palmityl-CoA dependent inhibition of PKC assayed with the crude cytosolic fraction from bovine neutrophils and the partially purified PKC. In both cases, the small relief of inhibition caused by Mg^{2+} alone was due to the decrease of the effective concentration of palmityl-CoA in the medium by partial insolubilisation, as mentionned above. Interestingly, a marked reversal of inhibition, probably by competition, was observed when micromolar concentrations of ATP were added. ATP could not be replaced by ADP or AMP. Other nucleotides like GTP, UTP and CTP were also ineffective (not shown). At the respective concentrations of added ATP and $MgCl_2$, i.e. 100 times more $MgCl_2$ than ATP, it could be calculated on the basis of the Mg-ATP stability constant (21) that more than 90% of ATP was in the form of Mg-ATP. Omission of Mg^{2+} in the medium prevented reversal of the palmityl CoA-dependent inhibition of PKC by ATP (not shown). There was no reversion of PKC inhibition by ATP when ATP was added after preincubation of PKC with long chain acyl-CoAs. A direct assay to test whether the reversal of inhibition by Mg-ATP was strictly competitive was not feasible because of the experimental conditions of the inhibition of PKC by palmityl-CoA ; in fact, the counteracting effect of ATP on palmityl-CoA was followed by the enzymatic reaction of phosphorylation in which [γ-^{32}P]ATP was used at a fixed concentration.

Because of the amphiphilic character of long chain acyl-CoAs, we addressed the question of whether detergents could also inhibit PKC. Indeed Triton X-100, laurylamidodimethylpropylaminoxide and deoxycholate at concentrations of 0.5 to 1% inhibited between 50 and 100% of PKC activity. However, for the same extent of inhibition, concentrations of palmityl-CoA 100 times lower were sufficient. Moreover, the inhibition caused by detergents was not reversed by Mg-ATP, pointing to the specific interactions of long chain acyl-CoAs with the nucleotide binding site of PKC.

DISCUSSION

As shown in this paper, inhibition of PKC by long chain acyl-CoAs depends on the sequence of addition of acyl-CoAs with respect to PKC and the other components of the medium. When added to the assay phosphorylation medium together with PKC and [γ-^{32}P]ATP, long chain acyl-CoAs stimulate the PKC activity ; when preincubated with PKC in the absence of ATP, they are strongly inhibitory. We shall discuss briefly : 1. the physiological relevance of PKC inhibition (or

stimulation) by long chain acyl-CoAs at the cellular level ; 2. the molecular basis of the interactions of long chain acyl-CoAs with PKC.

Long chain acyl-CoAs have been reported to inhibit a number of enzymes at concentrations lower than 50 µM, which correspond to the concentrations found in liver and heart cells (for review, see 22). However, the criticism can be raised that inhibition might be due to the formation of acyl-CoA micelles, which behave as detergents. In recent papers, the critical micellar concentrations (CMC) of long chain acyl-CoAs have been carefully assessed. Various ranges of values for the CMC of palmityl-CoA have been given, 30 to 60 µM (23), 3 to 60 µM (24), and 7 to 250 µM (25). Although there are differences between these CMC values, it is clear that inhibition of purified PKC by concentrations of palmityl-CoA lower than 4µM (Fig. 1) is not likely due to the surfactant properties of micellar palmityl-CoA. A stronger criticism is the propensity of long chain acyl-CoAs to bind to cellular membranes, which results in a marked decrease of their actual concentration in the cytosol fluid. Finally, we have to recall that inhibition of PKC by palmityl-CoA is counteracted by micromolar concentrations of Mg-ATP which may correspond to the actual situation in neutrophils (26). All these considerations make it doubtful that long chain acyl-CoAs exert control over the level of PKC activity in neutrophils.

More interesting is the physico-chemical implication of the inhibitory effect of long chain acyl-CoAs in terms of interactions with PKC. The requirement of preincubation of palmityl-CoA with PKC (in the absence of Mg-ATP) for inhibition of the kinase activity, and the decrease of inhibition when palmityl-CoA is added together with Mg-ATP, suggest that palmityl-CoA interacts with the nucleotide binding site of PKC, probably by its CoA moiety. As inhibition of PKC by preincubation with palmityl-CoA cannot be reversed by Mg-ATP added in a second stage, even at relatively high concentrations, it is suggested that PKC reacts with palmityl-CoA only in its resting state (in the absence of ATP), probably at the nucleotide binding site, thus inhibiting the PKC activity. This behavior recalls that of phosphatidyl serine, which activates PKC when added together with ATP in the phosphorylation medium, but inhibits PKC when preincubated with the enzyme prior to the addition of ATP (10).

The requirement of a chain length higher than 12 C for PKC inhibition points to the importance of the hydrophobic character of the acyl chain. The fact that neither free CoA nor free fatty acids are able to inhibit PKC indicates that the integrity of the long

chain acyl-CoAs is required for inhibition. Palmityl carnitine, which has no nucleotide residue in its structure, exhibits a much lower inhibitory efficiency than palmityl-CoA. It is suggested that the acyl chain moiety of long chain acyl-CoAs interacts with the lipid regulatory domain of PKC (27, 28) in the same manner as activating lipids, like diacylglycerol and phosphatidyl serine (3) whereas the CoA moiety probes the nucleotide binding site. Double occupancy of both the lipid domain and the nucleotide binding site of PKC by long chain acyl-CoAs would result in inhibition of the enzyme, whereas binding of long chain acyl-CoAs to the lipid domain only of PKC would activate the enzyme. On this basis, chemically derivatized palmityl-CoA and other long chain acyl-CoAs, including fluorescent, photoactivable and spin labeled derivatives, could hopefully be used for probing strategic regions in PKC.

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