3-Aminobenzamide inhibition of protein kinase C at a cellular level

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Abstract 3-Aminobenzamide, a known inhibitor of poly-(ADP-ribose)-polymerase has been found in the cell line U-937 to inhibit protein kinase C at the same concentration as poly-(ADP-ribose)-polymerase. 3-Aminobenzamide was not able, however, to inhibit the isolated enzyme. An indirect mechanism of protein kinase C inhibition is proposed.

Key words: 3-Aminobenzamide; Apoptosis; Protein kinase C inhibition; Poly-(ADP-ribose)-polymerase

1. Introduction

Apoptosis is a highly regulated process of cell death. Cells undergoing apoptosis show characteristic morphological changes including plasma and nuclear membrane blebbing as well as chromatin condensation and fragmentation. These morphological changes are usually accompanied by biochemical changes such as the elevation of cytoplasmic Ca^{2+} and internucleosomal DNA fragmentation [1]. These changes distinguish apoptosis from necrotic cell death. In recent years, the role of poly-(ADP-ribosyl)ation in the cell death process has been discussed. Poly-(ADP-ribose)-polymerase (PARP) is a nuclear enzyme that utilizes NAD^+ as substrate to add poly-(ADP-ribose) chains to several chromatin proteins, and that can be activated as result of apoptosis-induced DNA fragmentation [2]. Several inhibitors of PARP have been described, which are used as tools in investigating the role that the enzyme plays in cell death. Among them, 3-aminobenzamide (3-AB) is the most frequently employed since it is supposedly non-toxic and highly specific [3]. Many reports have shown that 3-AB is capable, in a variety of experimental systems, of preventing cell death and apoptosis [4–6].

Conversely, activators of protein kinase C (PKC) have been shown to stimulate apoptosis [7–9], whereas agents that down-regulate or inhibit this kinase can abrogate apoptosis in several model systems [10–12]. Most of the evidence supporting a role for PKC in apoptosis has been obtained from studies with phorbol esters, a class of tumor promoters that bind to the diacylglycerol-binding site on the enzyme and promote its activation. The involvement of PKC in the apoptotic process is also confirmed by the fact that H_2O_2, a reactive oxygen intermediate, can induce apoptosis as well as PKC activation [13–19]. In this work we analyzed the effect of the classical PARP inhibitor 3-AB on PMA- and H_2O_2-induced protein kinase C activity, using both a cellular system and the isolated enzyme.

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The results show that in the cellular system 3-AB inhibits protein kinase C activity in a dose-dependent manner. The protein kinase C inhibition appears to occur via an indirect mechanism since no inhibition by 3-AB takes place with the isolated recombinant enzyme.

2. Materials and methods

2.1. Materials

Purified PKCα was from UBI (New York). The peptide fragment of myelin basic protein (MBP 4–14) was obtained from Bachem Feinchemikalien AG (Dübendorf, Switzerland). [γ-^{32}P]ATP (10 mCi/ml) was from Amersham International. All other chemicals used were of the purest commercially available grade.

2.2. Cell line and cell culture

U-937 cells were cultured in suspension in RPMI 1640 medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) (Sera-Lab, Ltd., UK), at 37°C in T-75 tissue culture flasks (Corning, NY, USA) gassed with an atmosphere of 95% air-5% CO_2. Stock solutions of hydrogen peroxide were freshly prepared in double-distilled water. 3-AB was dissolved directly in the culture medium. PMA was dissolved in DMSO. Because the cytotoxicity of hydrogen peroxide toward cultured cells is dependent on cell density [20], a constant density of 2.5×10^5 cells/ml dish was used at the treatment stage.

2.3. Purified PKC activity

PKCα activity in vitro was performed as follows. After preincubation with 3-AB at 30°C for 20 min, samples were incubated for 10 min at 30°C in 40 μl activation buffer, containing 10 mM MOPS, pH 7.2, 0.5 mM DTT, 100 μM MBP_{4-14}, 0.25 mM ATP, 20 mM $MgCl_2$, 5 μg phosphatidylserine, 5 μg diacylglyceride and 5 μCi [γ-^{32}P]ATP. Reaction was stopped with 20 μl 25% TCA. Fifty μl aliquots were spotted onto 3×3 cm P81 Whatman filter, washed twice with 0.75% phosphoric acid, once with acetone. Radioactivity was counted in a liquid scintillation analyzer.

2.4. PKC activity in permeabilized cells

U-937 cells were subjected to different treatments as indicated in the figure legends. Aliquots of cells were resuspended in a reaction buffer containing 5.2 mM $MgCl_2$, 94 mM KCl, 12.5 mM HEPES pH 7.4, 12.5 mM EGTA, 8.2 mM $CaCl_2$, and assays were started by adding [γ-^{32}P]ATP (9 cpm/pmol, final concentration 250 μM), peptide substrate (final concentration 70 μM), and streptolysin-O (0.3 IU). Samples were incubated at 37°C for 10 min, quenched and analyzed as described previously [21].

3. Results and discussion

The experiments shown in Fig. 1 were aimed to establish if the protection against apoptosis by 3-AB could be specifically referred to the inhibition of PARP or if protein kinase C, also involved in apoptosis, was affected as well. PKC, measured in U-937 permeabilized cells, has been shown to be sensitive to the concentrations of 3-AB normally used to prevent apoptosis. At 1 mM 3-AB inhibition of PKC activity was approximately 60% when phorbol myrisate acetate was used to activate the kinase. H_2O_2 was also able to stimulate PKC in U-

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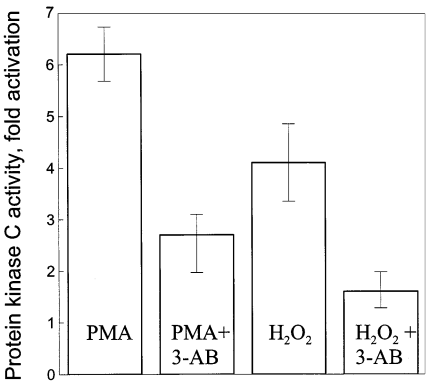

Fig. 1. Protein kinase C activity in permeabilized U-937 cells. U-937 cells have been treated with or without 1 mM 3-AB for 2 h. During the last hour PMA (100 nM) or H_2O_2 (1 mM) were added, as indicated in the picture. The vertical bars represent the ±S.D. of the average of four different experiments.

937 cells, although to a lesser extent relative to the phorbol ester. The inhibition by 3-AB on H_2O_2-stimulated PKC activity was, however, of the same order of magnitude (i.e. 60%).

The following conclusion can be drawn from these experiments. 3-AB is not a specific inhibitor of PARP, being, at the same concentrations, also able to substantially inhibit PKC. Such a lack of specificity should be kept in mind in interpreting results obtained with this tool, especially regarding apoptosis, since programmed cell death is strongly affected by PKC.

A further question has been answered with the experiments of Fig. 2. In the experiment carried out at a cellular level, after 3-AB addition, a dose-dependent inhibition of protein kinase C activation has been seen. Using a recombinant PKCα in vitro no effect of the inhibitor was seen, even at concentrations ten-fold higher than those employed at a cellular level.

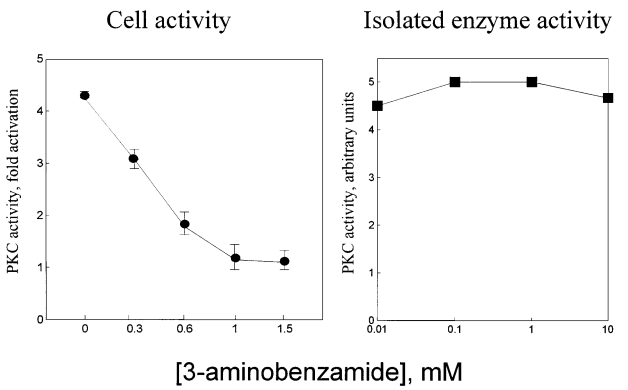

Fig. 2. Effect of 3-AB on protein kinase C activity in permeabilized U-937 cells and with the isolated enzyme. For the measurement in permeabilized cells (left panel), conditions were as described in Fig. 1, and PMA was used as PKC activator. 3-AB concentrations were varied according to the figure. On the right panel the effect of 3-AB on purified, recombinant PKC is shown. Conditions are described in Section 2. The value of the untreated protein kinase C activity was of 4.8 arbitrary units, i.e. not significantly different from those of treated samples. The addition of 3-AB after activation of the enzyme gave results identical to those shown here. The vertical bars represent the ±S.D. of the average of four different experiments.

This result suggests that the inhibition of PKC, seen at a cellular level, should not be due to a direct protein kinase C/3-AB interaction. Rather an indirect mechanism may be postulated, involving changes in the permissive phosphorylation state of the enzyme. The time course of 3-AB incubation with PKC was too short to assume that the compound produced a change in protein synthesis. An analogy can be drawn with the mechanism of α-tocopherol inhibition of protein kinase Cα that has been shown to involve dephosphorylation of the enzyme, possibly by a protein phosphatase 2A [22,23]. The molecular mechanism of PKC inhibition by 3-AB is currently under investigation.

The results presented in this study show that in a cellular system 3-AB inhibits protein kinase C activity in a dose-dependent manner. The protein kinase C inhibition appears to occur via an indirect mechanism since no inhibition by 3-AB takes place with the isolated recombinant enzyme.

Several inhibitors of PARP have been used as tools to show a role of this enzyme in cell death. 3-AB in particular is the most frequently used, since it has been supposed to be non-toxic and highly specific. The data reported here show instead that another enzyme, protein kinase C, is equally inhibited. Since protein kinase C is also involved in apoptosis, caution should be taken in interpreting data on programmed cell death based on the use of 3-AB.

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