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RAPID COMMUNICATION

Characterization of Two Pituitary GH_3 Cell Sublines Partially Resistant to Apoptosis Induction by Okadaic Acid

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ABSTRACT. Pituitary GH_3 cells die by apoptosis when treated with okadaic acid, a specific inhibitor of ser/thr phosphatases. Incubations starting at concentrations of 5 and 12.5 nM followed by stepwise rises resulted in two populations (the S_1 and S_2 sublines) that proliferated at initially lethal 30 nM. Cells were partially resistant to higher concentrations of okadaic acid and its derivative methyl okadaate. Toxicity of the structurally distinct inhibitors cantharidic acid and calyculin A was differently affected in the two resistant lines. The enhanced expression of the P-glycoprotein was one mechanism of resistance in S_1 and S_2. Resistance was reversed completely (S_1) or partially (S_2) by the addition of verapamil. In addition, phosphatase activity, presumably PP2A, was increased in S_2. Therefore, pharmacokinetic and pharmacodynamic mechanisms can protect pituitary GH_3 cells from apoptotic cell death by okadaic acid. BIOCHEM PHARMACOL **54**;9:967–971, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Ser/thr phosphatases; okadaic acid; GH_3 cells; multidrug resistance (MDR) phenotype

Kinases and phosphatases regulate the phosphorylation state of serine and threonine residues. Ser/thr phosphatases (PP's#) may be separated into at least seven different subtypes: the most abundant are PP1 and PP2A [1]. Additional subtypes are PP2B and PP2C [1], PP3 [2], PP4 [3] and PPT or PP5 [4, 5]. Specific inhibitors of PP's such as okadaic acid [6] and calyculin A [7] are useful tools for studying the participation of these enzymes in cellular responses. The fatty acid polyether okadaic acid displays a higher affinity for PP2A (IC_{50}: 0.5–2 nM) than for PP1 (IC_{50}: 15–50 nM) [8]. In cultured cells tumor promotion [9], stimulated transcription of various genes, e.g. c-jun and c-fos [10], apoptosis induction [11, 12] and inhibition of cell transformation [13] have been reported to be due to an increased phosphorylation state of cellular and nuclear proteins following inhibition of ser/thr phosphatases.

Rat pituitary tumor GH_3 cells [14] are still partially differentiated, since they synthesize prolactin and growth hormone with responsiveness to the physiologic hypothalamic regulator thyrotropin releasing hormone. Tissue-specific cellular functions in GH_3 cells are prominently regulated by phosphorylation and dephosphorylation. When studying the effects of okadaic acid, we observed apoptotic cell death at nanomolar concentrations (30–90 nM) after 24 hr. We selected okadaic acid-resistant GH_3 sublines by increasing the concentration of okadaic acid in the culture medium stepwise. Two isolated sublines that proliferate at the initially lethal concentration of 30 nM were studied for the mechanisms involved in resistance.

MATERIALS AND METHODS

PP1 and PP2A polyclonal antibodies were from UBI (Hamburg, Germany) and P-glycoprotein polyclonal antibody from Dianova (Hamburg, Germany). Okadaic acid and calyculin A were purchased from RBI (Cologne, Germany) and methyl okadaate from Calbiochem (Bad Soden, Germany).

GH_3 rat pituitary cells were maintained in monolayer in Dulbecco's minimal Eagle's medium supplemented with 7% fetal calf serum, streptomycin, penicillin and 0–30 nM okadaic acid at 37° in 5% CO_2. Resistant cells were isolated by increasing the okadaic acid concentration in the culture medium stepwise. Following the treatment with the investigated drugs, cells ($1.5*10^6$) were fixed with 70% ethanol for at least 4 hr at −20° and processed as described in [15]. After isolation of DNA with phosphate-citric acid extraction buffer and subsequent RNase A and proteinase K treatment, the analysis of fragmentation was performed on a 1.5% agarose gel at 10 V/cm for *ca.* 60 min. DNA stained with ethidiumbromide was visualized by UV transillumination at 312 nm.

For phosphatase assays, cells (1×10^7) were washed

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Abbreviations: PP, ser/thr protein phosphatases; DTT, DL-dithiotreitol; PKA, protein kinase A; BCA, bicinchoninic acid; MTT, thiazolyl blue; PMSF, phenylmethylsulfonyl fluoride; S_1 and S_2, sublines of GH_3 cells cultured on 30 nM okadaic acid; MDR, multidrug resistance.

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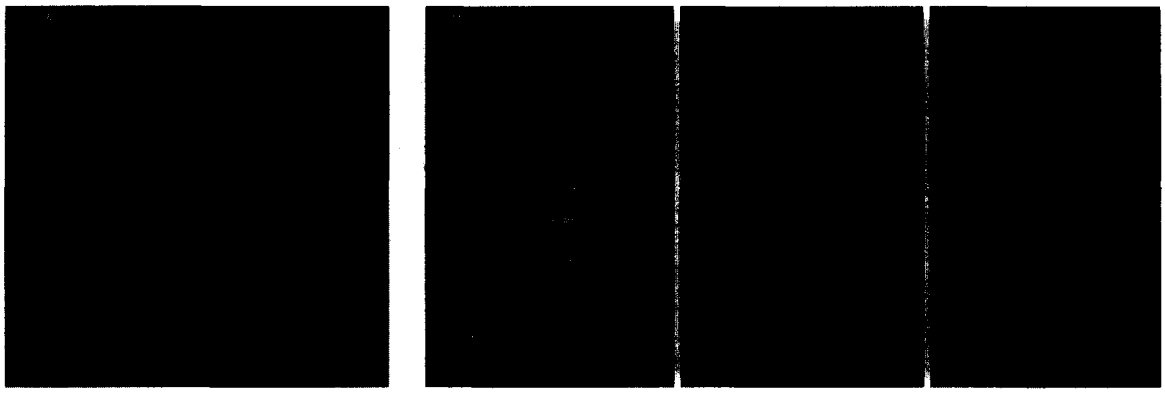

FIG. 1. A. DNA fragmentation observed following a 24 hr treatment of GH_3 cells with 0 (1), 30 nM (2), 60 nM (3), 90 nM (4) and 150 nM (5) okadaic acid and 360 nM methyl okadaate (6). B–D. DNA fragmentation of parental GH_3 (B), resistant lines S_1 (C) and S_2 (D) after 24 hr incubation with no addition (1), 60 nM okadaic acid (2) and 60 nM okadaic acid together with 10 μM verapamil (3).

three times with PBS, resuspended in 50 mM Tris-HCl pH 8, 500 mM NaCl, 7 mM β-mercaptoethanol, 0.1 mM PMSF and sonicated with a Branson Sonifier B 15 (3 min, 50 msec pulses). Phosphatase assays with phosphorylated fluorescent kemptide were performed with the PepTag assay for protein kinase A (PKA) (Promega, Heidelberg, Germany). Dephosphorylation was carried out with 5 μg extract protein, 9 μL buffer (50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 0.1% β-mercaptoethanol) and 0.08 μg phosphorylated kemptide. After 45 min at 30°, samples were boiled for 10 min and electrophoresed on a 0.8% agarose gel (50 mM Tris-HCl pH 8.0) and visualized by UV-light (312 nm).

For Western blot analysis of PP1, PP2A and P-glycoprotein, cells (5×10^6) were washed three times with PBS and scraped off the plate in 0.5 mL 20 mM NaH_2PO_4/Na_2HPO_4 pH 8, 140 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40, 1 mM DTT, 50 μM leupeptin. After 15 min on ice, cells were centrifuged for 15 min at 14000 rpm at 4°. Protein concentration of the supernatant was determined using the BCA assay (Pierce, Heidelberg, Germany). For the detection of P-glycoprotein, membranes were prepared by differential ultracentrifugation [16]. Fifteen μg protein were loaded per lane on a 5% (P-glycoprotein) or 10% (PP1, PP2A) gel. Blots were developed using the ECL system (Amersham, Braunschweig, Germany) following the manufacturer's instructions.

In viability assays [17], 2×10^4 cells/well were seeded into a 96-well microtiter plate and then incubated for 72 hr with various drugs in serum-free insulin-transferrin-selenite medium followed by the addition of 10 μL MTT solution (5 mg/mL) and incubation for 60 min. After addition of 100 μL 20% SDS in 0.02 N HCl, cells were lysed for 24 hr and MTT conversion measured at 550 and 650 nm using a Molecular Devices UV max microplate reader.

RESULTS

The death of rat pituitary GH_3 cells following the treatment with nanomolar concentrations of okadaic acid was apoptotic since a definite internucleosomal DNA fragmentation was observed. When cells were incubated with okadaic acid for 24 hr at concentrations exceeding 30 nM, the isolated DNA revealed a characteristic 180–200 bp DNA ladder [18] after agarose gel electrophoresis (Fig. 1A). The amount of fragmented DNA increased with the concentration of okadaic acid up to 90 nM. A DNA ladder was also seen with the derivative methyl okadaate (360 nM), which as an inhibitor of phosphatases is at least 5-fold less potent than okadaic acid [8]. No fragmentation was seen with okadaic acid tetraacetate, which does not inhibit ser/thr phosphatases [8]. Long-term incubations revealed that okadaic acid concentrations as low as 15 nM resulted in progressive cell death with treatment periods of a week or longer.

Since cells can develop a resistance to initially lethal concentrations of drugs, we attempted to establish sublines that would survive and finally proliferate at formerly lethal concentrations of okadaic acid. This was achieved by initially culturing cells at okadaic acid concentrations of 5 (S_1) or 12.5 nM (S_2). Surviving cells were incubated in medium with okadaic acid concentrations increased in two or four steps up to 30 nM. After 3 months we obtained two sublines, S_1 and S_2, that in contrast to the parental cells proliferated at an okadaic acid concentration of 30 nM. Both sublines were resistant to even higher concentrations of okadaic acid, since no (S_2) or only a slight (S_1) fragmentation of DNA was observed in the resistant lines when treated with 60 nM okadaic acid for 24 hr (Fig. 1C, D). Interestingly, fragmentation became very prominent when verapamil (10 μM) was included during the treatment. This conversion of resistance suggested that the multidrug resistance (MDR) phenotype [19, 20] might take part in resistance to okadaic acid. To prove this, S_1 and S_2 cells were analysed by Western blotting for the presence of P-glycoprotein. In immunoblots, both resistant sublines revealed a strongly enhanced expression of P-glycoprotein compared to the parental cells (Fig. 2A). Both lines demonstrated an at least 5-fold increase in signal strength, with S_1 cells presenting the strongest signal.

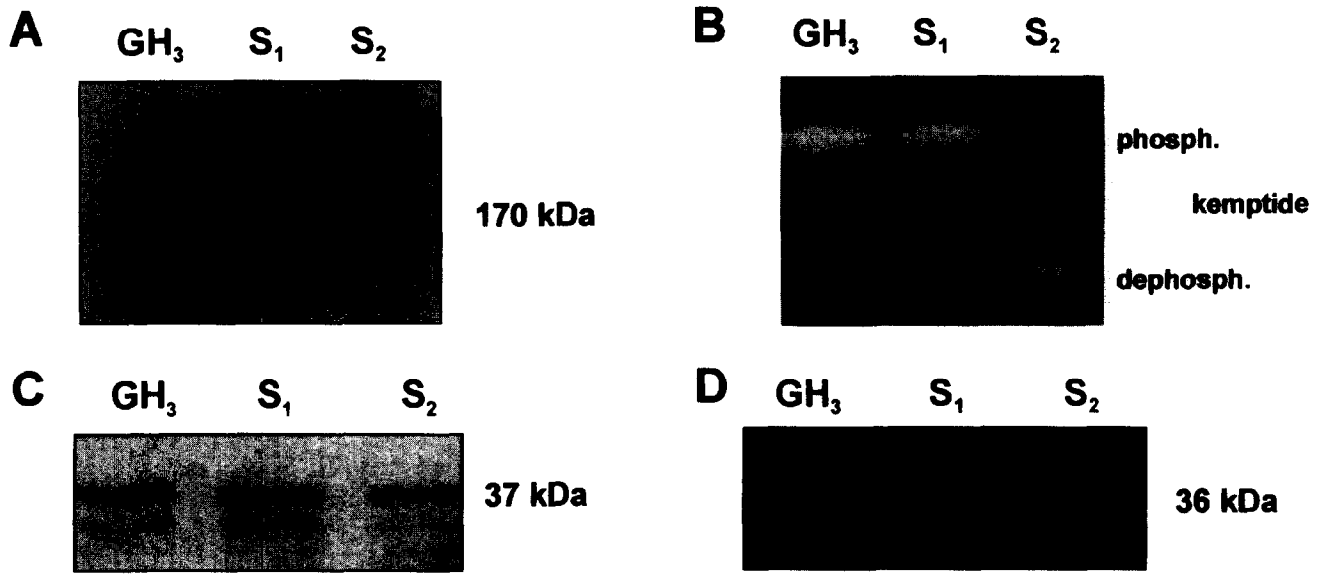

FIG. 2. A. Immunoblot for the 170 kDa P-glycoprotein in parental GH_3 and resistant S_1 and S_2 cells. B. Phosphatase assay with extracts from the 3 lines performed with phosphorylated kemptide as fluorescent substrate. Phosphorylated and dephosphorylated forms were separated by agarose gel electrophoresis and visualized by UV transillumination. C. Immunoblot for PP1 (37 kDa). D. Immunoblot for PP2A (36 kDa). Immunoblots and phosphatase assays are representative of 3 different extracts.

Viability assays were performed to test okadaic acid resistance with respect to substrate specificity and potential cross resistance. GH_3, S_1 and S_2 cells were incubated for 72 hr with okadaic acid (60 nM) ± verapamil (10 μM), methyl okadaate (360 nM), calyculin A (6 nM) and cantharidic acid (10 μM). Cantharidic acid resembles okadaic acid in that sensitivity of PP1 is 10- to 20-fold less than that of PP2A, while calyculin is 10-fold more potent as inhibitor of PP1 [8] as well as inducer of apoptosis in GH_3 cells [21]. After 3 days more than 70% of the parental cells had died at 60 nM okadaic acid, while approximately 60% of S_1 and 90% of the S_2 cells were still alive (Fig. 3A). When verapamil was added together with okadaic acid, more than 80% of parental and S_1 cells died while ca. 35% of S_2 cells survived. After 72 hr with the derivative methyl okadaate (360 nM), less than 20% of parental cells but 90% of S_1 and more than 80% of S_2 survived (Fig. 3B). With the structurally different calyculin A, more than 70% of parental (Fig. 3C) but less than 40% of S_1 and less than 10% of S_2 died. With cantharidic acid (Fig. 3B) only the survival rate of S_2 differed significantly from that of the parental cells (56.4 ± 5.5% and 34.2 ± 2.7%, respectively). In general, with the exception of methyl okadaate, S_2 displayed higher survival rates than S_1 with the tested phosphatase inhibitors.

Since the S_2 population displayed a higher survival rate

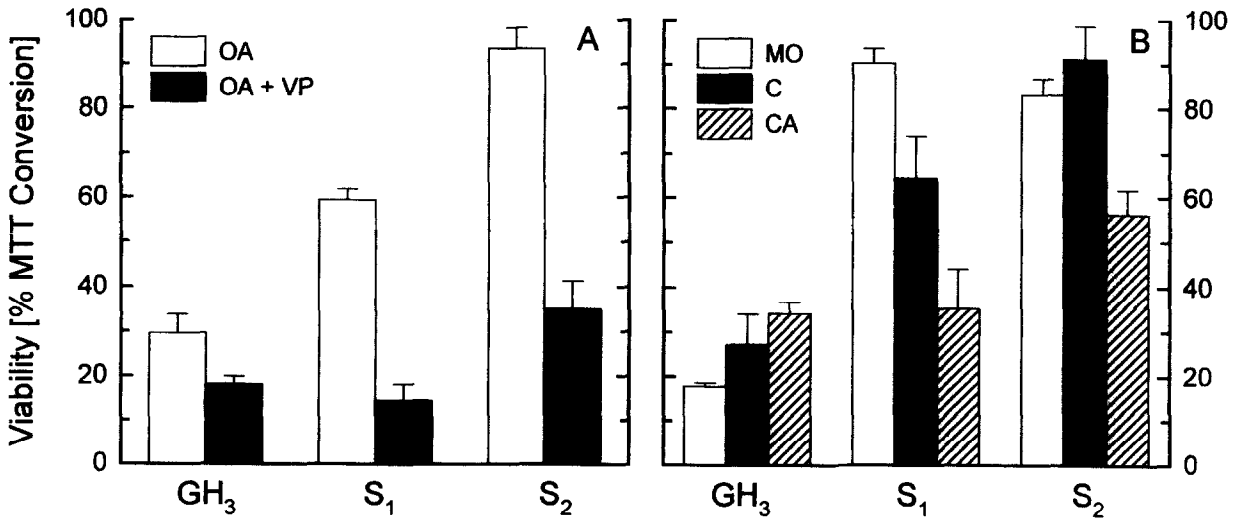

FIG. 3. Viability assays of GH_3 cells and resistant lines S_1 and S_2 treated for 72 hr. A. Incubations with 60 nM okadaic acid (OA) ± 10 μM verapamil (VP). B. Treatment with 360 nM methyl okadaate (MO), 6 nM calyculin A (C) and 10 μM cantharidic acid (CA). Viability was measured by conversion of a methyltetrazolium salt and expressed relative to the respective controls set to 100%. Means ± SEM of 3 separate experiments performed at least in triplicate.

with okadaic acid and the structurally different calyculin A and cantharidic acid, we checked for mechanisms of resistance in addition to the enhanced expression of P-glycoprotein. A potential mechanism in drug resistance is the up-regulation of the cellular targets of drugs. Therefore, we tested for changes in ser/thr phosphatase activity and/or content. When PKA-phosphorylated kemptide was used as a substrate, S_2 cell extracts showed an increased phosphatase activity (Fig. 2B), while extracts of S_1 cells displayed a slightly decreased activity compared to the parental GH_3 cells. Western blot analysis of the individual phosphatase subtypes revealed no change of PP1, a definite increase of PP2A in S_2, and a slight decrease of PP2A in S_1 compared to the parental GH_3 cells (Fig. 2C, D). Densitometric analysis of 3 experiments revealed a mean 1.7-fold increase in phosphatase activity as well as a mean 1.5-fold increase in PP2A signal strength in extracts from S_2 cells. These observations together with the functional differences (Fig. 3) support the hypothesis that especially in subline S_2 additional mechanisms contribute to okadaic acid resistance. Functional and immunological data point to changes in the amount and/or activity of phosphatases, predominantly PP2A.

DISCUSSION

The observation of a DNA fragmentation ladder (Fig. 1A) in GH_3 cells treated with okadaic acid as well as with methyl okadaate together with morphological observations (not shown) characterize cytotoxicity following the prolonged inhibition of ser/thr phosphatases in GH_3 cells as apoptotic cell death. After a 24 hr treatment, this behaviour was observed with concentrations of 30 nM and above. Okadaic acid has been reported to cause apoptosis in different cell lines [11, 12]. Bøe et al. first described apoptosis by okadaic acid according to morphological criteria in GH_3 cells after 3 hours, albeit with the fairly high concentration of 500 nM. We observed apoptotic DNA fragmentation after 24 hours in GH_3 cells treated with 3- to 15-fold lower concentrations of okadaic acid (30–150 nM). Since the derivative methyl okadaate with its lower inhibitory potency also caused DNA fragmentation and apoptotic cell death, albeit at 4- to 6-fold higher concentrations the conclusion that phosphatase inhibition was a trigger for apoptosis was substantiated. This was further supported by observations that other inhibitors such as cantharidic acid albeit at micromolar concentrations, or calyculin A at ca. 8- to 10-fold lower concentrations than okadaic acid also induced apoptosis. Taken together, these data outline that prolonged inhibition of both phosphatases 2A and 1 triggers the signalling cascade for apoptotic cell death in GH_3 cells.

When incubated for more than a week, GH_3 cells did not survive okadaic acid concentrations even as low as 15 nM. Stepwise increments in the concentrations of okadaic acid in the culture medium resulted in the selection of okadaic acid-resistant cells. Cells originally prone to apoptotic cell death by okadaic acid at concentrations of 30 nM and above can survive and proliferate at this initially lethal concentration and will survive even higher concentrations (60 nM) without displaying intense signs of apoptosis (Fig. 1C, D). In accordance with the viability data (Fig. 2A), S_1 displayed a lower resistance in DNA fragmentation (Fig 1C–2) than S_2 (Fig. 1D–2) when treated with 60 nM okadaic acid alone. S_2 revealed a slight spontaneous DNA fragmentation without treatment (Fig. 1D–1), which was confirmed in flow cytometric analysis where a small apoptotic population was observed in untreated cells (data not shown).

A major cause of the observed resistance seemed to be the increased expression of the P-glycoprotein in both resistant populations. The MDR phenotype reflected the resistance to the structurally similar derivative methyl okadaate fairly well, since S_1 with the highest rate of P-glycoprotein expression also revealed the highest survival rate with this drug. The conclusion that this mechanism contributes to okadaic acid resistance was functionally supported by the reversion of okadaic acid resistance with micromolar concentrations of verapamil. At these concentrations, it is believed that verapamil acts as a resistance modifier independently from its calcium channel blockade, presumably as a competing substrate of the P-glycoprotein [18, 19]. This was supported by the observation that structurally and functionally different drugs that have been reported to reverse MDR, e.g. reserpine, abolished resistance to okadaic acid in S_1 cells (data not shown). On the other hand, structurally different phosphatase inhibitors and the combination of okadaic acid with verapamil were best tolerated by S_2 with its higher activity of phosphatases, presumably PP2A. Taken together, these data suggest that additional mechanisms contribute to the observed resistance to okadaic acid.

In conclusion, GH_3 cells that initially respond to okadaic acid at concentrations of 30 nM with apoptotic cell death, acquire resistance when cultured in medium supplemented with stepwise increased okadaic acid concentrations. Analysis of the resistant sublines (S_1 and S_2) suggests that pharmacokinetic as well as pharmacodynamic mechanisms mediate resistance either alone or in combination. Different culturing conditions resulted in separate sublines proliferating for many months in the presence of 30 nM okadaic acid. Increasing the concentrations in the medium in two steps resulted in subline S_2, which is characterized by an increase in P-glycoprotein as well as an increased ser/thr phosphatase activity and content, especially of PP2A. Raising the okadaic acid concentrations in four steps resulted in resistant cells (S_1) that revealed an at least 5-fold increase in P-glycoprotein but a decreased total phosphatase activity. Therefore, more than one mechanism took part in the resistance to the phosphatase inhibitor okadaic acid in GH_3 cells. Firstly, expression of the P-glycoprotein results in the MDR phenotype, which protects cells presumably by an increased extrusion of the toxic drug. Secondly, changes in the activity of the targets of the

phosphatase inhibitor, predominantly PP2A, seemed to prevent, at least within a limited concentration range, the initiation of the signal cascade which otherwise triggers apoptosis. Further experiments are in progress to identify additional mechanisms of resistance and to obtain cells that might be resistant to okadaic acid as well as other phosphatase inhibitors without the involvement of an increased P-glycoprotein expression.

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