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THE ROLE OF PROTEIN KINASE C AND THE PHOSPHATIDYLINOSITOL CYCLE IN MULTIDRUG RESISTANCE IN HUMAN OVARIAN CANCER CELLS

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Abstract—The present study aimed to investigate the role of protein kinase C (PKC), the phosphatidylinositol pathway (PI) and cytosolic calcium in multidrug resistance (MDR) in human ovarian carcinoma cells. Binding of the phorbol ester 13,14-dibutyrate (PDBu) was 3-fold higher in resistant A2780^{AD} versus sensitive A2780 cells indicating increased PKC activity. However, when inositol phosphate production (IP) was measured in quiescent cells similar total IP release was seen in both lines suggesting no difference in the basal turnover of PI. Non-specific stimulation of the PI pathway was achieved with the calcium ionophore A23187 which increased IP production in a time- and dose-dependent fashion in both cell lines but was significantly less effective in A2780^{AD}. The PI pathway was investigated further using the agonists aluminium fluoride, serum and bombesin but these agents failed to elicit a response. The effect of a wide range of Adriamycin concentrations on the PI cycle and cell growth was also studied. Intracellular calcium was measured with the fluorescent dye fura-2-pentaacetoxymethylester (Fura-2). A23187 produced a rise in cytosolic calcium in A2780 and A2780^{AD} but from a level 3-fold lower in the unstimulated resistant cell line. The dose responsiveness of this effect was greater but irreversible in A2780^{AD} cells. Collectively these results imply that alterations in PI turnover appear not to be responsible for the differences in PDBu binding and calcium handling observed between A2780 and A2780^{AD} and suggests only a minor role for the PI cycle in the maintenance of MDR in human ovarian cancer cell lines.

The treatment of a variety of human cancers is often hindered by the development of drug resistance. Although several differences exist between sensitive and resistant cells in laboratory models the extent to which they are inter-related and their clinical relevance is unclear. Perhaps two of the most common features of MDR are altered drug transport and the appearance of P-170, a large molecular weight membrane glycoprotein [1–4]. Homology of P-170 with bacterial transport proteins [5] and its ability to bind drugs [6] suggests that it actively transports drugs from the cell rendering it drug insensitive. The activity of P-170 appears to be regulated by phosphorylation [3, 7].

Recent studies on MDR have focused on the possible role of second messengers and the enzymes associated with these pathways, particularly protein kinase C (PKC). PKC occupies a central position in signal transduction mechanisms of mitogenic responses to a variety of growth factors and hormones. Activation of PKC results in the phosphorylation of a variety of proteins of which P-170 is a possible candidate [8]. Increased PKC activity has been reported in resistant breast [9], ovarian [10], lung [11], leukemic [12] and fibrosarcoma [13] cancer cells. In resistant breast cells this increased activity was associated with an increased basal and stimulated production of inositol phosphates [14]. Inositol phosphates (IP) along with diacylglycerol (DAG) are products of the phosphatidylinositol (PI) second messenger cascade and their production can result in activation of PKC. The aim of the present study was therefore to investigate the possible involvement of PI turnover and PKC in a human ovarian model of MDR.

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MATERIALS AND METHODS

Chemicals and reagents. Dowex-1-chloride (2% crosslinkage, 100–200 mesh), bombesin and A23187, were obtained from the Sigma Chemical Co. (Poole, U.K.). Fura-2-pentaacetoxymethylester (Fura-2) was obtained from Calbiochem (San Diego, CA, U.S.A.). Adriamycin® (doxorubicin hydrochloride) was obtained from Farmitalia Carlo Erba Ltd (St Albans, U.K.), vinblastine sulphate from Eli Lilly (Basingstoke, U.K.) and Actinomycin-D from Merck Sharp and Dohme Ltd (Hoddesdon, U.K.). All other chemicals were of the highest grade commercially available. Tissue culture reagents and media were obtained from Gibco (Paisley, U.K.). *myo*-2-[^{3}H]Inositol with PTE-271 (1 mCi/mL) was obtained from Amersham International plc (Amersham, U.K.) while [^{3}H]phorbol 13,14-dibutyrate (PDBu) (10.2 Ci/mmol) was obtained from the New England Nuclear Division of Dupont Europe (Stevenage, U.K.). The P-170 antibody, JSB-1, was the generous gift of Dr H. J. Broxterman, Dept. of

Medical Oncology, Free University Hospital, Amsterdam, The Netherlands.

Cell lines. A2780, a human ovarian cancer cell line and A2780^{AD}, its multidrug resistant counterpart, were kindly provided by Drs T. C. Hamilton and R. F. Ozols, Medicine Branch, Division of Cancer Treatment, NCI, Bethesda, NY, U.S.A. A2780^{AD} had previously been derived by the stepwise exposure of A2780 to increasing concentrations of Adriamycin.

Tissue culture conditions. A2780 and A2780^{AD} cell lines were maintained and passaged in RPMI 1640 containing 10% (v/v) heat inactivated foetal calf serum (HIFCS), 0.25 μg/mL insulin, 0.3 mg/mL glutamine, 100 I.U./mL penicillin and 100 μg/mL streptomycin. Media for A2780^{AD} cells was supplemented with Adriamycin (2 μM final concentration). Prior to any experiment Adriamycin was removed for at least 1 week, cells were then used for no more than 3 weeks during which time levels of P-170 remained high. Cells were incubated at 37° in a humidified atmosphere of 5% (v/v) CO_2.

Extraction of inositol phosphates. Cellular inositol phosphates were extracted and separated essentially according to the method of Downes and Michell [15]. Cells (5×10^6) were plated overnight in 60-mm plastic tissue culture grade petri dishes. Media was then removed and monolayers washed three times with phosphate buffered saline (PBS) and replaced with RPMI containing 0.5% HIFCS. *myo*-[^{3}H]-Inositol (10 μCi) was then added to each dish and left at 37° until equilibrium between endogenous and labeled inositol phosphates had occurred. Before each experiment media was again removed and monolayers washed twice with PBS and replaced with RPMI supplemented with calcium chloride. Monolayers were preincubated with 20 mM lithium chloride for 15 min and at 37°. Incubations were terminated by placing the dishes on ice, removing media and adding 3 mL of ice-cold perchloric acid (3%). After 30 min the lysate was removed and neutralized with 1 M KOH containing 50 mM HEPES and 5 mM EDTA. Cells were also scraped from the plates, pelleted and kept for protein analysis. Neutralized samples were then spun at 2000 rpm for 5 min at 4°. The supernatant was removed and inositol phosphates separated as described below.

Cell pellets were analysed for protein content according to the method of Bradford [16].

Separation of inositol phosphates. Samples were applied to Dowex-1 (100–200 mesh; formate form) anion exchange columns. Free inositol; glycero-inositolphosphates and total inositol phosphates were sequentially eluted with; 10 mL distilled H_2O; 10 mL 5 mM sodium tetraborate/60 mM ammonium formate and 10 mL 0.8 M ammonium formate/0.1 M formic acid, respectively. Aliquots (2 mL) from each fraction were added to 10 mL of Optiphase MP liquid scintillant and radioactivity counted in a Packard Tricarb liquid scintillation counter (model 1900CA, Canberra Packard, Pangborne, U.K.).

Intracellular calcium measurements. Intracellular calcium was measured in scraped cells with the fluorescent dye Fura-2 according to the method of Mendoza *et al.* [17]. Cells were grown on 60-mm plastic tissue culture grade petri dishes. Cells ($2–4 \times 10^6$) were used for each determination. Cell monolayers were washed twice with serum free media, 2 mL of media was added followed by Fura-2 (1 μM final concentration) and incubated at 37° for 10 min. Dishes were then washed twice with electrolyte solution and using a cell scraper cells were scraped into 1 mL of electrolyte solution which was then transferred to a cuvette. Fluorescence was measured in a Perkin–Elmer LS-5 luminescence spectrometer with an excitation wavelength of 336 nm and an emission wavelength of 510 nm. The suspension was stirred continuously and maintained at 37°. Intracellular calcium ($[Ca^{2+}]_i$) was calculated using the formula of Tsien *et al.* [18]:

$$[Ca^{2+}]_i = \frac{220\,(F - F_{min})}{(F_{max} - F)}$$

where F is the fluorescence at the unknown $[Ca^{2+}]_i$; F_{max} is the fluorescence after the Fura-2 is released by the addition of Triton-X (1%) and F_{min} is the fluorescence after the Ca^{2+} in the solution is chelated with EDTA (0.5 M, pH 7.2).

Phorbol ester binding. Assay conditions under which the binding of [^{3}H]phorbol-12,13-dibutyrate (PDBu) was measured in A2780 and A2780^{AD} were as described by Gescher and Reed [19]. The concentration of [^{3}H]PDBu was 50 nM (sp. ac. 10.2 Ci/mmol) which was complemented with 50 μM unlabeled PDBu in the case of non-specific binding which was approximately 20% of total binding.

Measurement of cell growth. Cells (5×10^4/well) were plated overnight in plastic 24-well plates. This media was removed, monolayers washed twice with PBS and replaced with media containing 0.5% HIFCS. Twenty-four hours later this media was removed and media containing 0.5% HIFCS and the appropriate concentration of Adriamycin added. After 48 hr cells were trypsinized and counted daily using a Coulter Counter.

RESULTS

Characterization of human ovarian cancer cell lines

In our hands A2780^{AD} exhibited MDR characteristics similar to those previously reported for this cell line [2] i.e. increased resistance to several structurally unrelated drugs, decreased accumulation of Adriamycin and increased expression of P-170 (data not shown).

Inositol phosphate studies

The basal incorporation of [^{3}H]inositol into membrane phospholipids as measured by the release of total IP was studied over a 6 day period (Fig. 1). Production was similar in both cell lines; it initially increased but was maximal after 3 days at which point it plateaued. These profiles suggest no difference in the basal turnover of IPs between the sensitive and resistant cell lines. The calcium ionophore A23187 produced dose related increases in total IP production in A2780 and A2780^{AD}, but was less potent in the latter cell line (Fig. 2). Whereas 5×10^{-7} M A23187 caused a 3.8-fold increase in A2780 it only caused a 1.6-fold increase in A2780^{AD} ($P < 0.05$, Student's paired *t*-test). Bombesin, serum

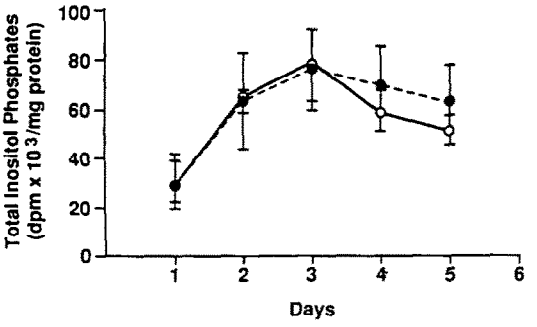

Fig. 1. Total basal inositol phosphate production in unstimulated sensitive A2780 (○) and MDR A2780^{AD} (●) cells over a 6 day period. Each value represents the mean ± SEM of 4 separate experiments each with triplicate determinations.

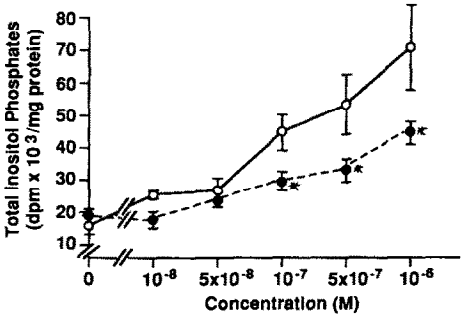

Fig. 2. The effect of varying concentrations of the calcium ionophore A23187 (20 min treatment) on total inositol phosphate accumulation in A2780 (○) and A2780^{AD} (●) cells following 15 min pretreatment with 20 mM lithium chloride. Each value represents the mean ± SD of three experiments each with triplicate determinations. * $P < 0.05$.

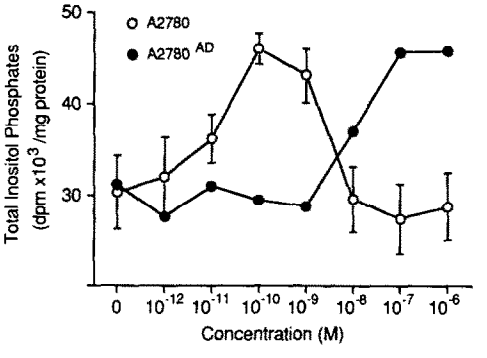

Fig. 3. The effect of varying Adriamycin concentrations (20 min treatment) on total inositol phosphate production following 15 min pretreatment with 20 mM lithium chloride in A2780 cells (○) where each value represents the mean ± SEM of three separate experiments each with triplicate determinations and A2780^{AD} cells (●) where N = 2 with triplicate determinations in each experiment.

and aluminium fluoride had very little or no effect on either A2780 or A2780^{AD} cell lines (data not shown).

The effects of Adriamycin on inositol phosphate turnover in A2780 and A2780^{AD} are shown in Fig. 3. Although an identical range of Adriamycin concentrations were tested in both cell lines (10^{-12}–10^{-6} M) only a 20 min exposure to mainly sub-cytotoxic concentrations of Adriamycin were stimulatory in both cell lines (IC_{50} for Adriamycin was 3.5×10^{-9} and 4.1×10^{-7} M in A2780 and A2780^{AD}, respectively).

Intracellular calcium measurements

Compared to A2780 basal levels of $[Ca^{2+}]_i$ were 3-fold lower in the resistant A2780^{AD} cell line (48 vs 150 nM, see Fig. 4). A23187 produced dose related rises in cytosolic calcium in both cell lines (Fig. 4). In the parent cell line these responses (a maximal 3-fold increase at 5×10^{-6} M A23187) were reversible whereas in the resistant cell line these responses (a maximal 5-fold increase at 5×10^{-6} M A23187) were not reversible with time. Bombesin (10^{-10}–10^{-7} M) had no effect on levels of cytosolic calcium in either cell line (data not shown).

Phorbol ester binding

There was approximately a 3-fold increase in PDBu binding in A2780^{AD} in unstimulated cells compared to the parent cell line (0.465 ± 0.075 vs 0.146 ± 0.014 pM/mg protein, respectively, see Fig. 5).

Cell growth studies

At sub-cytotoxic drug concentrations (10^{-11}–10^{-9} M in A2780 and 10^{-10}–10^{-7} M in A2780^{AD}) Adriamycin had no effect on cell growth.

DISCUSSION

The cell membrane and signal transduction are receiving increasing attention as possible new targets in cancer chemotherapy. In this work we have investigated the role of the PI pathway, PKC and cytosolic calcium in MDR with particular emphasis on the PI cycle. Although several studies have reported increased PKC activity in MDR cell lines few have looked at the PI cycle as a possible explanation for this alteration. Such a link has been observed in the human breast cancer MDR cell line MCF-7^{DOX} where a 7-fold elevation in PKC activity was associated with a 3.3-fold increase in the basal rate of hydrolysis of the membrane lipid PIP2 [9, 14]. However, in this present work whilst there was a 3-fold elevation in PKC, there was no evidence of perturbations in the basal activity of the PI cycle. Stimulation of the PI cycle was achieved using the calcium ionophore A23187 which works by activating phospholipase C to hydrolyse PIP2 to inositol-1,4,5-trisphosphate (IP3) and DAG. A23187, as expected, caused a concentration dependent rise in IP, but was significantly less effective in the resistant cell line A2780^{AD}. This may be due to either differences in membrane phospholipid composition or differences in enzyme sensitivity.

Adriamycin has been shown to be cytotoxic

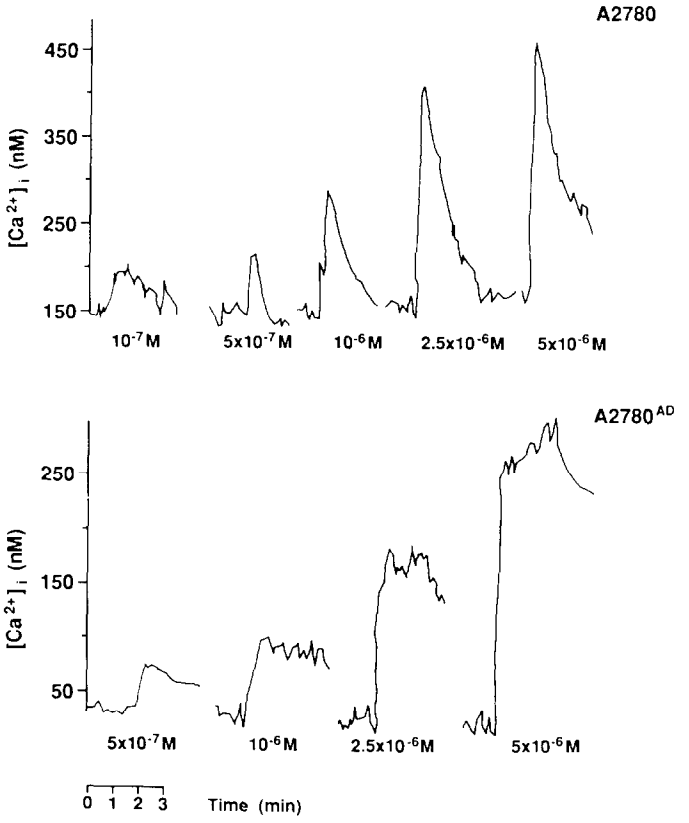

Fig. 4. The effect of varying concentrations of A23187 on cytosolic calcium $[Ca^{2+}]_i$ in A2780 cells (upper traces) and $A2780^{AD}$ cells (lower traces) measured using the fluorescent dye Fura-2 (1 μM final concentration). The fluorescent traces are representative of those obtained in three separate experiments.

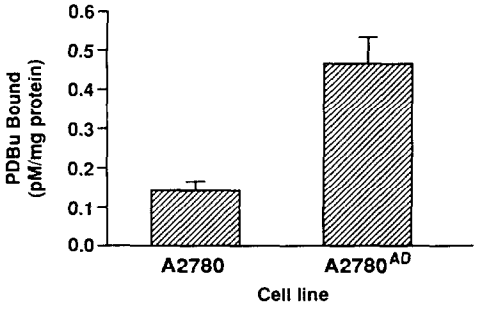

Fig. 5. Binding of the phorbol ester (PDBu) to cell membranes of A2780 and $A2780^{AD}$ cells. Each value represents the mean ± SD of three experiments each done in quadruplicate.

without entering the cell when covalently coupled to agarose beads of a diameter too large to be internalized suggesting a specific interaction with the membrane [20]. At sub-cytotoxic concentrations, Adriamycin has actually been shown to promote cell growth under conditions where there is a lack of growth factors in the medium [21]. Vichi and Tritton [21] have postulated that at sub-cytotoxic drug concentrations Adriamycin acts as a growth factor by stimulating mitogenic signal transduction pathways whereas at higher concentrations it causes cytotoxicity by an overstimulation of the same membrane initiated signals. In the ovarian cell lines studied in this work, sub-cytotoxic Adriamycin concentrations elicited a PI response both in A2780 and $A2780^{AD}$ consistent with a membrane action but they did not alter growth in low serum conditions. The notion of an overstimulation of the PI pathway at higher cytotoxic drug concentrations was not borne out in the parent cell line A2780.

No role for cytosolic free calcium in MDR has recently been reported in Ehrlich ascites cell lines even though the resistant clones had a 3-fold lower level than the parent cell line (50–80 vs 140–180 nM, respectively [22]). These figures are almost identical to the values reported in this work for human ovarian cell lines. The difference in Ehrlich ascites was attributed to the over expression of the calcium binding protein sorcin in the resistant clones [22]. In $A2780^{AD}$ the calcium ionophore A23187 produced an unusual response where the increase in cytosolic calcium failed to return back to its pre-stimulation level. Since A23187 works through the plasma membrane, this effect may be due to the ionophore compromising membrane integrity and/or may be related to changes in membrane phospholipid composition. Changes in phospholipid patterns have

been reported for a variety of MDR cell lines including P388 leukemia [23], rat glioblastoma [24] and Friend leukemia [25].

The level of PKC is closely related to the number of phorbol ester binding sites [26]. The 3-fold increase in phorbol ester binding recorded in $A2780^{AD}$ supports results obtained by Ming Yang *et al.* [27] where PKC activity, measured by the incorporation of ATP into histone 1, was also 3-fold higher in $A2780^{AD}$. Although elevated PKC has now been reported for many different models of MDR, no differences in PKC activity were reported recently in MDR MOLT-3 human acute lymphoblastic leukemia cell lines [28]. This model, however, has the added complication that cells were also made resistant to the lipophilic antifolate trimetrexate.

Increased PKC activity observed in MDR cell lines may be of particular importance in the development of drug resistance because of its possible role in P-170 phosphorylation and activation. PKC isolated from KB-3 cells can phosphorylate P-170. Exposure of these cells to phorbol esters results in increased PKC activity, phosphorylation of P-170 and decreased drug accumulation characteristic of MDR [8]. Also, in the MCF-7 parent cell line, phorbol ester treatment increased PKC activity and induced the MDR phenotype [9].

In summary, our data show that in a human ovarian model of MDR there is an increase in PKC activity and differences in calcium handling but this is not associated with alterations in basal or stimulated turnover of the PI cycle. Mechanisms unrelated to changes in PI must, therefore, be responsible for this increased PKC activity. These nonetheless may be important in the development of MDR in ovarian cancer and once identified might represent new targets for drug intervention.

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