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Protein Kinase C Inhibitors Arrest the C6 Glioma Cell Cycle at a Mid-G1 Phase Restriction Point: Implications for the Antiproliferative Action of Valproate

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Abstract—The teratogenic mechanism(s) of valproate (VPA) have been suggested to arise through inhibition of proliferation coupled with differentiation at a mid-G1 phase restriction point in the cell cycle. As protein kinase C (PKC) plays a pivotal role in cell proliferation and differentiation, the effect of inhibitors specific for the catalytic and regulatory domains on transit through the G1 phase of the cell cycle was determined. Calphostin C and bisindolylmaleimide GF 109203X produced a dose-dependent decrease in proliferation of C6 glioma with approximate 50% inhibitory concentration values of 10 nM and 1 μM, respectively. Flow cytometric analysis indicated proliferative arrest to be in the G1 phase with the expected concomitant decrease of cells in the G2/M and S phases. Following release from drug-induced proliferative arrest, cells exhibited a synchronous entry into S phase as evidenced by an increase in [^{3}H]thymidine incorporation after approximately 6–8 hr, indicating the restriction point to be in the mid-G1 phase. Using mitotically synchronized cells continuously exposed to valproate (2 mM), PKC activity was found to be significantly reduced in the mid-G1 phase (5.5 hr) but not at an earlier (2.5 hr) time point, implying VPA to exert its effect at a site upstream to the point of proliferative arrest at 5–6 hr into the G1 phase which as yet, remains to be defined.

Abbreviations: ANOVA = analysis of variance; DMEM = Dulbecco's modified Eagle's medium; DMSO = dimethyl sulfoxide; IC_{50} = concentration at which 50% inhibition is achieved; PBS = phosphate buffered saline; PKC = protein kinase C; PLC = inositol phospholipid-specific phospholipase C; VPA = valproic acid.

INTRODUCTION

In vitro studies have demonstrated valproic acid (2-propylpentanoate, VPA) to potently inhibit neural cell proliferation rate at concentrations within twice its therapeutic plasma level (Regan, 1985). This antiproliferative action occurs at a defined restriction point in the G1 phase at which cells assume a differentiated phenotype as judged by altered morphology, gene expression and cell substratum adhesivity (Berezin *et al.*, 1996; Martin *et al.*, 1988; Martin and Regan, 1988 and 1991). This antiproliferative effect becomes apparent in the early G1 phase (Martin and Regan, 1991), during which VPA may perturb signal transduction events leading to the eventual accumulation of cells at the mid-G1 restriction point. In part, this involves suppression of transient glycoprotein sialylation required for passage through this phase of the cell cycle, as is reflected by the reduced affinity of exposed cells for concanavalin A lectin-coated surfaces (Maguire and Regan 1991; unpublished observations).

As VPA sequesters specifically into the neuroepithelium and induces spina bifida during neurulation in both rodent and human populations (Bjerkedal *et al.*, 1982; Dencker *et al.*, 1990; Ehlers *et al.*, 1992; Robert and Guibaud, 1982), this antiproliferative action is likely to result in growth imbalances arising from alterations in differential cell proliferation rates between endodermal and neuroepithelial cell populations. Such differential growth imbalances have been associated with the increased incidence of spina bifida observed in curly tail mutant mouse homozygotes (Copp *et al.*, 1988). The induction of neural tube defects by VPA *in vivo* is reliant on strict structure–activity requirements, which are independent of anticonvulsant potency (Elmazar *et al.*, 1993; Nau *et al.*, 1991). These require the presence of an α-hydrogen atom, a carboxyl function and branching on carbon atom 2 with one chain containing two to four carbon atoms for maximal activity. Similar structural requirements are necessary to concomitantly inhibit

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proliferation and reduce the number of concanavalin A lectin binding sites in C6 glioma (Courage-Maguire *et al.*, 1997).

Recent *in vitro* studies have attributed the antimania qualities of VPA to alterations in signal transduction events (Chen *et al.*, 1994 and 1996). Of particular interest was the observation that chronic exposure of C6 glioma to VPA resulted in a marked decrease in protein kinase C (PKC) activity (Chen *et al.*, 1994). This enzyme is distributed ubiquitously and plays a pivotal role in the transduction of external signals operating by activation of receptor-coupled heterotrimeric G proteins via cross-talk mediated by the products of inositol phospholipid-specific phospholipase C (PLC) and adenylyl cyclase (Liu and Simon, 1996). Activation of PLC generates diacylglycerol and inositol-1,4,5-trisphosphate, leading to the activation of PKC and the mobilization of intracellular calcium (Berridge, 1993). This pathway appears to play a crucial role in neurulation as inositol deficiency increases susceptibility to neural tube defects in genetically predisposed mouse mutants (Cockroft *et al.*, 1992). As PKC plays a pivotal role in cell proliferation and differentiation (Nishizuka, 1992), the effect of inhibitors specific for the catalytic and regulatory domains on transit through the G1 phase of the cell cycle was determined.

MATERIALS AND METHODS

Cell culture

The C6 glioma were cultured in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, UK) supplemented with 10% foetal calf serum (Gibco-Biocult, UK), 200 mM glutamine and 100 μg/ml gentamicin (Sigma Chemical Co. Ltd, UK) and were maintained in a humidified atmosphere of 9% CO_2 at 37°C. Cells were passaged using 0.025% trypsin in DMEM. The tissue culture plastics were purchased from Costar (UK). Synchronized populations of C6 glioma were obtained by a mitotic selection procedure (Axelrad and McCulloch, 1957). The loosely adhering and rounded post-mitotic cells were dislodged from the monolayer into the medium by gentle tapping of the culture flask. The cells were gathered by centrifugation and seeded (10^4 cells/cm^2) into multiwell plates using fresh medium.

Antiproliferative effects were assessed by direct haemocytometer counting of trypsinized cells or by use of a nuclei counting procedure (Sanford *et al.*, 1951). Freshly trypsinized cells were seeded (10^4 cells/cm^2) into 25-cm^2 flasks for direct cell counting or into multiwell dishes for nuclei counting. The cells were allowed to recover for 24 hr before being exposed to the relevant drug for 48 hr. Drugs were dissolved in dimethyl sulfoxide (DMSO), the concentration of which never exceeded 1% of the tissue culture medium. Following exposure, nuclei counting was performed in duplicate by replacing the medium with 500 μl crystal violet (0.25, w/v) in citric acid (0.1 M). The cells were incubated with this nuclei releasing and staining solution for 30 min at 37°C. Then gentle trituration with a Gilson micropipette was performed in order to obtain an even suspension of stained nuclei which were counted using a haemocytometer. Values significantly different ($P < 0.05$) from the control were established by analysis of variance (ANOVA) and Dunnett's post-test.

The length of S phase in cells released from a 48-hr drug exposure period was determined by measuring the point of increased ^{3}H-thymidine incorporation during continued culture in full medium under standard conditions of incubation. Briefly, this involved washing the cells with warm DMEM and adding 1 μCi ^{3}H-thymidine (sp. act. 26.5 Ci/mmol; Amersham International, UK) to separate duplicate wells for 1 hr, every hour. Incorporation of ^{3}H-thymidine was determined by solubilizing the cells in 2% sodium dodecyl sulfate and estimating the number of counts in perchloroacetic acid (0.5 M) precipitates which had been solubilized in 2 M NaOH. Values significantly different ($P < 0.05$) from time zero were established by ANOVA and Dunnett's post-test. Previous pulse chase studies with ^{3}H-thymidine have demonstrated populations of cells synchronized by mitotic selection or following exposure to VPA to enter S phase at 11–12 hr and 5–6 hr, respectively, indicating this agent to arrest the cells in the mid-G1 phase of the C6 glioma cell cycle (Martin and Regan, 1991).

Flow cytometric analysis

The distribution of cells within the various phases of the cell cycle was determined by flow cytometric analysis. Cells were seeded at 2×10^4 cells/ml in 25-cm^2 tissue culture flasks and were incubated for 24 hr at 37°C and 9% CO_2 to allow recovery from the trypsinization procedure. The cells were then incubated with the appropriate agent for a further 48 hr, harvested using 0.025% trypsin, pelleted by centrifugation and fixed using a 70% ethanol–30% phosphate buffered saline (PBS) solution for at least 30 min. Subsequently, the cells were pelleted by centrifugation and resuspended in PBS containing RNAase (1 mg/ml; Sigma) and the propidium iodide fluorochrome (400 μg; Sigma) which intercalates with DNA. Following incubation at 37°C for 30 min in the dark, the cells were analysed for fluorescence in a Becton Dickinson FacStar Plus processor using an argon-ion laser tuned to 488 nm. Values significantly different ($P < 0.05$) from the control were determined using Student's *t*-test.

Determination of PKC activity

Enzyme activity was determined separately in both cytosol and particulate fractions using a commercial assay kit (Amersham International, UK) which is based on the catalysed transfer of [γ^{32}P]ATP to the threonine group of a peptide acceptor molecule specific for PKC. Samples were

prepared by washing the adherent cells with Tris–HCl (30 mM), pH 7.5, containing KCl (125 mM), magnesium acetate (5 mM), EGTA (5 mM) and β-mercaptoethanol (45 mM; Sigma), leupeptin (2 μg/ml; Sigma), benzamidine (10 μM; BDH, UK) and aprotinin (2 μg/ml; Sigma) as protease inhibitors. The cells were then scraped into the above buffer, containing 10 mM KCl, and lysed using low intensity sonication. Nuclei and unbroken cells were removed from the cell lysate by centrifugation (1000 rpm × 5 min) through the same buffer containing 25% glycerol and the supernatent was recentrifuged (100,000 *g* × 30 min) to obtain the cytosolic and particulate fractions. The latter fraction was solubilized for 1 hr at 4°C following high intensity sonication in a 50 mM Tris–HCl buffer, pH 7.5, containing EGTA (10 mM), β-mercaptoethanol (7.7 mM), benzamidine (10 μM), phenylmethylsulfonyl fluoride (1 mM; Sigma), leupeptin (2 μg/ml), aprotinin (2 μg/ml) and 0.1% Brij 58 (Sigma), centrifuged (100,000 *g* × 30 min) and the supernatant retained. PKC activity was determined in 25-μl sample aliquots and was linear over a 0.5–17-μg range of protein concentrations, as determined by the method of Bradford (1976). Aliquots of 10 μg were used routinely for activity determinations. Enzyme samples prepared from cells exposed to 1% DMSO served as controls. The activities obtained in the cytosol and particulate fractions were combined to yield total PKC activity and values significantly different ($P < 0.05$) from the control were determined using Student's *t*-test.

RESULTS

The involvement of PKC in the regulation of the C6 glioma proliferation rate was determined using calphostin C (Sigma) and bisindolylmaleimide GF 109203X (Calbiochem, UK), which are specific inhibitors of its regulatory and catalytic domains, respectively (Kobayashi *et al.*, 1989; Toullec *et al.*, 1991). Exposure to either agent resulted in a dose-dependent decrease in cell proliferation rate in the absence of any apparent cytotoxicity (Fig. 1). Calphostin C exerted the most potent effect with an approximate IC_{50} (concentration at which 50% inhibition is achieved) in the region of 10 nM and approximately 95% of the cell population was arrested at the highest concentration evaluated (20 nM). Similar results were obtained with bisindolylmalimide; however, the dose-dependent effect was not as profound as that observed with calphostin C. The IC_{50} value was approximately 1 μM and over 80% of the cells were arrested with a 10-fold higher concentration of the drug.

Flow cytometric analysis of drug-arrested cells demonstrated both calphostin C and GF 109203X to exert their effects in the G1 phase of the cell cycle. Using calphostin C (20 nM) the majority of cells (90 ± 4%) were found to reside in the G1 phase as compared with those of the untreated controls (66 ± 1%). Similarly, GF 109203X (5 mM) arrested 77 ± 1% of the cells in the G1 phase. Furthermore, the expected concomitant decrease of cells in the G2/M and S phases was observed to be similar using both calphostin C (3 ± 1 and 4 ± 2%, respectively) and GF 109203X (9 ± 0.3 and 9 ± 0.3%, respectively).

To locate more precisely the point of proliferative arrest in the G1 phase, cells were released from a 48-hr exposure to either calphostin C (20 nM) or GF 109203X (5 μM) by washing the cells briefly with warm DMEM, replacing full medium and determining the time of entry into the S phase using [^{3}H]thymidine incorporation as an index (Fig. 2). Cells released from a calphostin C-mediated proliferative arrest exhibited a slow increase in [^{3}H]thymidine incorporation after approximately 5–6 hr; however, this did not become significant until 8 hr. This indicated the restriction point to be located at approximately 6–8 hr into the G1 phase, as this has been demonstrated previously to be 11–12 hr in duration (Martin and Regan, 1991). In contrast, release from GF 109203X-induced arrest resulted in a synchronous entry into S phase which was significant at 6 hr following release, suggesting

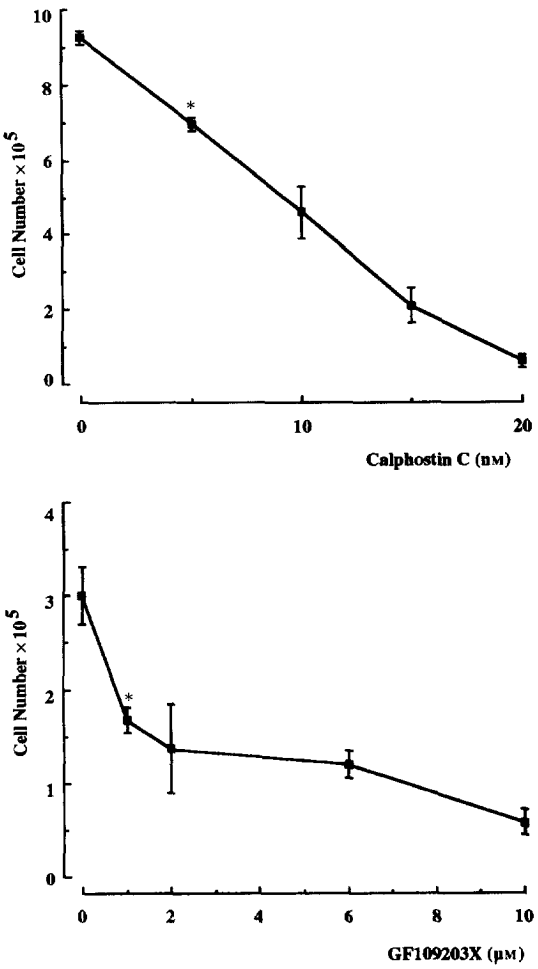

Fig. 1. Dose-dependent effect of PKC inhibitors on the C6 glioma proliferation rate. The values represent the mean ± SEM (n = 3) and the first significant point with respect to the control is indicated with an asterisk.

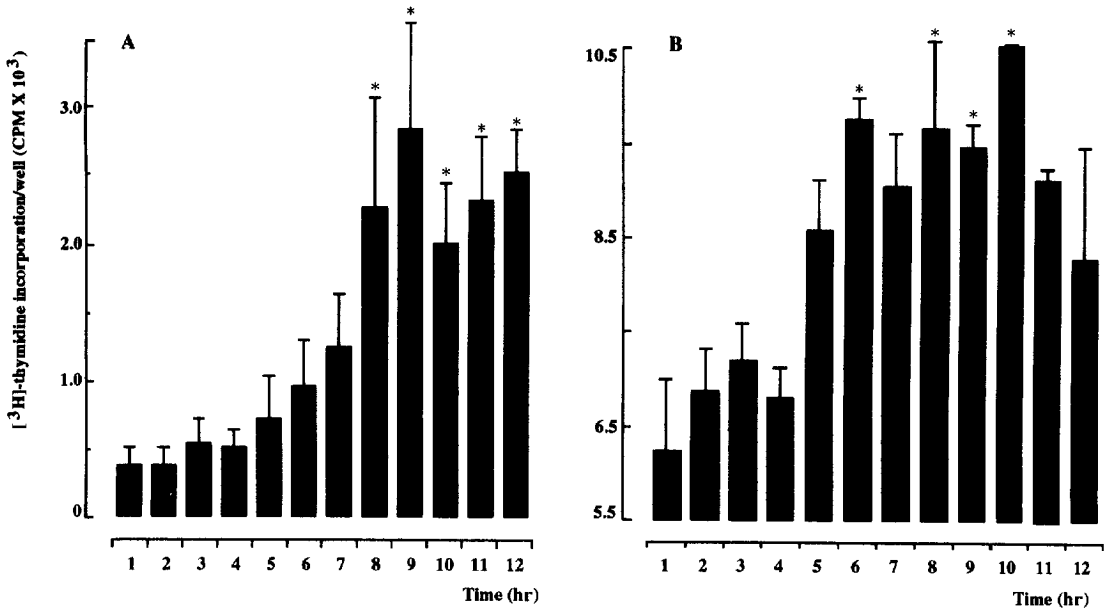

Fig. 2. Time of entry into S phase following release from proliferative arrest arising from PKC inhibition. Incorporation of [^{3}H]thymidine following release from calphostin C and bisindolylmaleimide GF 109203X is illustrated in panels A and B, respectively. The values represent the mean ± SEM (n = 3) and those significantly different from time zero are indicated with an asterisk.

the restriction point to be approximately 6 hr into the G1 phase. The ability of GF 109203X to define the point of proliferative arrest more precisely most likely reflects the relative antiproliferative potencies of the PKC inhibitors employed. Cells arrested with the more potent calphostin C inhibitor may be expected to require a longer recovery time for continued passage through G1. Collectively, both results suggest a regulatory role for PKC at a defined restriction point in the mid-G1 phase of the cell cycle.

Given that both PKC inhibitors defined a restriction point similar to that at which VPA exerts a coincident antiproliferative action, the influence of this latter agent on enzyme activity was determined during transit through G1 phase. Using mitotically synchronized cells continuously exposed to VPA (2 mM) or vehicle alone, PKC activity was found to become significantly reduced in the mid-G1 phase (5.5 hr) but not at an earlier (2.5 hr) time point (Fig. 3), implying this agent to exert an indirect inhibitory effect on enzyme activity at its site of proliferative arrest. VPA-induced inhibition of PKC activity was observed only in the cytosol and not in the particulate fraction (0.12 ± 0.03 *v.* 0.12 ± 0.02 pmol ^{32}P transferred/μg protein/min in VPA-treated and control cells, respectively). Furthermore, particulate activity only accounted for 4% of total activity which is consistent with that observed by Courage *et al.* (1995) in A549 lung carcinoma cells.

DISCUSSION

PKC plays an important role in the transduction of cellular signals elicited by growth factors which play a pivotal role in the regulation of cell proliferation and differentiation (Nishizuka, 1992). As a consequence, it is not surprising that the specific PKC inhibitors employed in this study potently inhibited the C6 glioma proliferation rate. Calphostin C proved to be a much more effective antiproliferative agent compared with that of GF 109203X, as has been observed for other cell lines (Courage *et al.*, 1995). Flow cytometric analysis

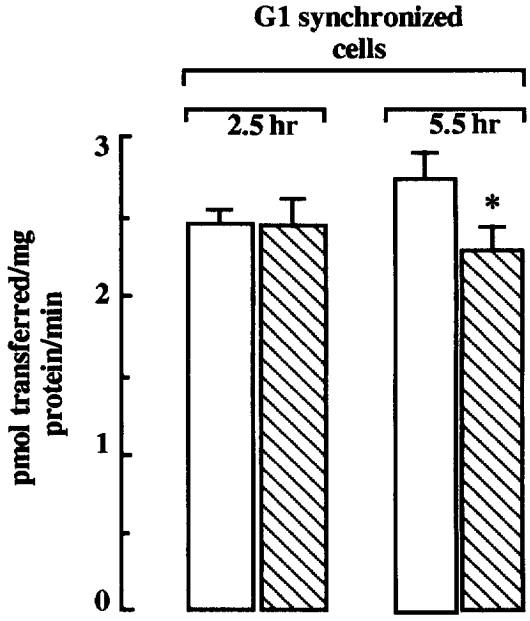

Fig. 3. Influence of VPA on total PKC activity in synchronized cells at increasing times into the G1 phase of the cell cycle. The shaded columns represent activity in the presence of 2 mM valproate. Values are as the mean ± SEM (n = 3) and that significantly different from the control is indicated with an asterisk.

demonstrated both agents to restrict the cell cycle in the G1 phase, since 80–90% of the cells accumulated in this phase with a concomitant decrease in those resident in the G2/M and S phases.

Proliferative arrest was reversible as removal of either inhibitor resulted in a synchronous entry into S phase after approximately 6–8 hr, thereby defining a PKC-dependent restriction point in the mid-G1 phase of the cell cycle. Progression through the G1 phase of the cell cycle is remarkably sensitive to protein synthesis inhibition and requires the presence of specific mitogenic growth factors such as platelet-derived growth factor (Zetterberg and Larsson, 1985). Exposure of Swiss 3T3 fibroblasts to this growth factor results an immediate activation of the PKC δ and ϵ isoforms and a subsequent steady decline in their expression during late G1 (Olivier and Parker, 1994). These findings support the existence of a PKC restriction point in the G1 phase of the cell cycle.

The proliferative restriction point defined by PKC inhibitors is remarkably similar to that observed with VPA (Martin and Regan, 1991). This occurs in the early G1 phase and eventually results in an accumulation of cells at the 5–6-hr time point at which they exhibit a differentiated phenotype (Berezin *et al.*, 1996; Maguire and Regan, 1991; Martin *et al.*, 1988; Martin and Regan, 1988 and 1991). At this point a significant decrease in total PKC activity is observed. This appears to be a consequence of proliferative arrest rather than direct inhibition as, in the presence of valproate, enzyme activity remains unaltered during the early G1 phase. As VPA initiates its antiproliferative effects in the early period (1–4 hr) of the G1 phase (Martin and Regan, 1991), inhibition of PKC in the mid-G1 phase must arise from a perturbation in upstream signal transduction events which may relate to the ability of VPA to attenuate activation of receptor-coupled heterotrimeric G proteins (Chen *et al.*, 1996). Thus, the concensus opinion emerging is that VPA exerts its effect at a site upstream to the point of proliferative arrest at 5–6 hr into the G1 phase which as yet, remains to be defined.

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