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SHORT COMMUNICATION

2,2′,3,3′,4,4′-Hexahydroxy-1,1′-biphenyl-6,6′-dimethanol Dimethyl Ether (HBDDE)-Induced Neuronal Apoptosis Independent of Classical Protein Kinase C α or γ Inhibition

*Anjili Mathur and Mary Lou Vallano**

Department of Pharmacology, Upstate Medical University, Syracuse, NY 13210, U.S.A.

ABSTRACT. Protein kinase C (PKC) isozymes constitute a family of at least 12 structurally related serine–threonine kinases that are differentially regulated and localized, and are presumed to mediate distinct intracellular functions. To explore their roles in intact cells, investigators are developing cell-permeable, isoform-selective inhibitors. 2,2′,3,3′,4,4′-Hexahydroxy-1,1′-biphenyl-6,6′-dimethanol dimethyl ether (HBDDE) is reported to be a selective inhibitor of PKC α and γ with IC_{50} values of 43 and 50 μM, respectively, using an *in vitro* assay. However, data examining the potency and selectivity of HBDDE in intact cells are lacking. Employing rodent cerebellar granule neurons as a model system, we investigated the effects of HBDDE using cell survival as a functional end-point. HBDDE induced an apoptotic form of cell death that was dependent upon protein synthesis and included activation of a terminal executioner of apoptosis, caspase 3. The concentration of HBDDE required for half-maximal cell death was less than 10 μM (~5-fold less than the reported IC_{50} values for PKC α and γ *in vitro*). Furthermore, HBDDE induced apoptosis even after phorbol-ester-mediated down-regulation of PKC α and γ, indicating that this effect is independent of these isoforms. Consistent with this, 2-[1-(3-dimethylaminopropyl) indol-3-yl]-3-(indol-3-yl)-maleimide (GF 109203X), a general inhibitor of all classical and some novel PKCs, did not interfere with survival. Thus, HBDDE should not be used as an isoform-selective inhibitor of PKC α or γ in intact cells. Nevertheless, identification of its target in granule neurons will provide valuable information about survival pathways. BIOCHEM PHARMACOL **60**;6:809–815, 2000. © 2000 Elsevier Science Inc.

KEYWORDS. PKC; cerebellar granule neurons; apoptosis; survival; GF 109203X; HBDDE

PKC† isozymes constitute a family of structurally related serine–threonine lipid-dependent kinases that are encoded by separate genes and ubiquitously expressed [1–3]. They have been implicated in a variety of intracellular processes including exocytosis [4], differentiation [5], growth [6], and survival [7–11]. Based on their homologies and profiles of activation by specific co-factors, they have been classified into three major groups. The classical or conventional PKCs (cPKCs), α, β_I, β_{II}, and γ, require DAG, PS, and calcium for activation. The calcium-insensitive novel PKCs (nPKCs), δ, ϵ, η, and θ, require DAG and PS, whereas the atypical PKCs (aPKCs), ζ and λ/ι, require neither calcium nor DAG, but depend upon PS for activation. Unlike the cPKCs and nPKCs, the aPKCs are insensitive to phorbol ester-mediated activation and subsequent down-regulation [12]. The discovery of multiple PKC isozymes within the same cell, each with unique temporal and spatial patterns of expression, suggests that distinct PKC isozymes mediate distinct intracellular processes [13–15].

To understand the different functions of PKC isoforms in intact cells, investigators are actively developing cell-permeable, isoform-selective inhibitors. Typically, inhibitors are characterized initially using several different purified PKCs, as well as other protein kinases, in an *in vitro* reconstitution assay system. For example, 2-[1-(3-dimethylaminopropyl) indol-3-yl]-3-(indol-3-yl)-maleimide (GF 109203X; also referred to as bisindolylmaleimide I) demonstrates high potency and selectivity *in vitro* for all classical isoforms of PKC (IC_{50} ~ 0.02 μM), when compared with other serine–threonine kinases, tyrosine kinases, and other PKC isoforms [16]. It is structurally similar to staurosporine, a potent but less selective PKC inhibitor, and acts in a competitive manner at the ATP-binding site in the catalytic domain. Moreover, early studies showed that GF 109203X selectively inhibits PKC in two intact systems, platelets and Swiss 3T3 fibroblasts, where its

* Corresponding author: Dr. M. L. Vallano, Department of Pharmacology, Upstate Medical University, Syracuse, NY 13210. Tel. (315) 464-7969; FAX (315) 464-8014; E-mail: Vallanom@mail.upstate.edu

† *Abbreviations*: PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; HRP, horseradish peroxidase; DIV, days *in vitro*; DAG, diacylglycerol; PS, phosphatidylserine; DTT, dithiothreitol; NMDA, *N*-methyl-D-aspartate; PI-3 kinase, phosphatidylinositol-3 kinase; and PMSF, phenylmethylsulfonyl fluoride.

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functional effects are relatively well characterized. As expected, the effective concentration for half-maximal inhibition of PKC in cultured cells is ~5–10 times higher than its IC_{50} *in vitro* [16]. Subsequent studies have documented both the potency and the selectivity of GF 109203X for cPKCs in several other cell types, where IC_{50} values of 0.1 to 1 μM have been observed [17–20]. Thus, judicious use of these types of agents makes it possible to define the distinct roles of the three classes of PKCs in cell function.

To further distinguish between the roles of individual members of the cPKC group, even more selective inhibitors have become commercially available. 2,2′,3,3′,4,4′-Hexahydroxy-1,1′-biphenyl-6,6′-dimethanol dimethyl ether (HBDDE) is a derivative of ellagic acid that is reported to act as an isoform-selective inhibitor of PKC α and γ with IC_{50} values of 43 and 50 μM, respectively, using an *in vitro* assay [21]. However, in contrast to GF 109203X, data documenting the potency and the selectivity of HBDDE in intact cells are lacking. In the present report, we demonstrate that HBDDE induces neuronal apoptosis under experimental conditions that rule out effects on cPKC α or γ. Thus, it cannot be used as an isoform-selective inhibitor of cPKC α or γ in intact cells. However, identification of its authentic intracellular target will provide important information for investigators in the field of apoptosis.

MATERIALS AND METHODS

Materials

Sprague–Dawley rats were purchased from Taconic Farms. PKC α and PKC γ antibodies were purchased from Transduction Laboratories. PKC ζ antibody (polyclonal; cross-reactive with PKC λ) was obtained from Santa Cruz Biotechnology, Inc. and will be referred to as PKC ζ/λ in the present study. HRP-conjugated anti-goat, anti-rabbit, and anti-mouse secondary antibodies were purchased from the Sigma Chemical Co. Caspase 3 activity was measured using a FluorAce™ Apopain Assay kit from Bio-Rad. The protein kinase inhibitors GF 109203X (bisindolylmaleimide I HCl) and HBDDE were purchased from Calbiochem. All other reagents were tissue culture or molecular biology grade and were obtained from commercial sources.

Cell Culture

Primary cultures of cerebellar granule cells were prepared as previously reported by our laboratory [22]. Briefly, cerebella from 7- to 8-day-old Sprague–Dawley rats were minced, trypsinized, and triturated to dissociate cells. The cells were seeded onto plastic multiwell or 10-cm Corning dishes coated with 10 μg/mL of poly-L-lysine at a density of ~1.25×10^6 cells/mL, and used at ~2 DIV. Cerebellar granule neurons in culture differentiate and develop a mature phenotype when maintained in depolarizing conditions (25 mM KCl- or NMDA-containing medium); otherwise they undergo a massive apoptotic cell death (~70% of total) beginning ~3 DIV [23]. Since our experiments were performed prior to the development of dependence on elevated KCl or NMDA for enhanced survival, the standard growth medium consisted of basal Eagle's medium with Earle's Salts, 10% fetal bovine serum, 2 mM L-glutamine, 0.11 mg/mL of gentamicin sulfate, and 5 mM KCl. However, in some cases the medium was supplemented with elevated KCl or NMDA for purposes of comparison. The growth of non-neuronal cells was limited by the addition of 10 μM cytosine arabinofuranoside to the cultures 20–24 hr after plating. As indicated, different concentrations of HBDDE or GF 109203X were added to cultures between 5 hr and 2 DIV, followed by assessment of cell viability. For PKC down-regulation studies, 500 nM PMA was added to the culture medium at 1 DIV, vehicle or drugs were added ~12 hr later, and cells were assayed at 2 DIV.

Cell Survival Assay

Cell viability was examined using an MTT assay in which MTT is converted to a blue formazan product by the mitochondria of viable cells as previously described [24]. In previous studies, we verified the accuracy of this assay by comparison with a fluorescein diacetate/propidium iodide cell staining assay. MTT (5 mg/mL) was added directly to the culture medium at a final concentration of 0.5 mg/mL, and the cells were reincubated at 37° for 10 min. The solution then was aspirated and replaced with an equal volume of DMSO. The absorbances of samples were quantified using a Hitachi U-2000 spectrophotometer at a wavelength of 540 nm. The color yield was directly proportional to the number of viable cells.

Apoptosis Assay

Fluorometric detection of apoptosis was performed using the FluorAce™ Apopain Assay kit and following the manufacturer's directions with minor modifications. Granule cells were harvested in apopain lysis buffer [10 mM HEPES (pH 7.4), 2 mM EDTA, 0.1% Triton X-100, 5 mM DTT, 1 mM PMSF, 10 μg/mL of pepstatin A, 10 μg/mL of aprotinin, 20 μg/mL of leupeptin]. Measurements of apopain (caspase 3) activity were made using a Perkin Elmer LS50B Luminescence Spectrometer with an excitation wavelength of 395 nm and an emission wavelength of 525 nm. Apoptosis also was assessed using morphological criteria, such as membrane blebbing and shrunken size under phase-contrast microscopy.

Western Immunoblotting

Whole homogenate protein samples were collected and sonicated in homogenization buffer [50 mM Tris (pH 7.4), 2 mM EGTA, 2 mM EDTA, 5 mM β-mercaptoethanol, 0.3 mM PMSF]. Protein content was equalized using a Bradford assay and diluted using homogenization buffer and 1/2 vol. of SDS stop solution [2% SDS, 1.8% β-mercaptoethanol,

10% glycerol, 62.5 mM Tris (pH 6.8)]. Samples were boiled for 2 min before resolving on SDS–PAGE, transferred to nitrocellulose for 2–3 hr at 200–300 mA, and immunoblotted using different PKC antibodies. Immunoreactivity was visualized with chemiluminescent substrate (LumiGlo, Kirkegaard & Perry Laboratories) for 1 min and exposed to x-ray film. The following primary antibodies were used overnight at 4° in blocking solution (5% powdered milk in PBS): PKC α (1:1000), PKC ζ/λ (1:200), and PKC γ (1:5000). The secondary antibodies for PKC α, PKC ζ/λ, and PKC γ, respectively, were HRP-conjugated anti-mouse (1:500), anti-goat (1:4000), and anti-rabbit (1:1000), and were dissolved in blocking solution and used at room temperature for 1–2 hr. Washes were performed using PBS.

Statistical Analysis

Values stated in the text are means ± SEM and have been analyzed for their statistical significance using an ANOVA, followed by Tukey's test. Values of $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

PKC inhibitors induce apoptosis in a variety of cell types. For example, staurosporine is one of the most potent inhibitors of PKC and is widely used to induce apoptosis, but it is a relatively non-selective inhibitor of serine–threonine kinases [25]. In cerebellar granule neurons, calphostin C, a selective inhibitor of the PKC family of kinases that interferes with DAG binding and, presumably, activation of DAG-sensitive cPKCs and nPKCs, interferes with the survival-promoting effect of brain-derived neurotrophic factor [10]. Alternatively, treatment of granule neurons grown in 5 mM KCl with PKC-activating phorbol esters does not rescue them from naturally occurring death, which led to the conclusion that PKC activation alone was not sufficient to maintain survival in culture [26]. To learn more about the roles of distinct isoforms of PKC in granule cell survival, we used the PKC inhibitor GF 109203X, which is highly effective against all of the cPKCs (IC_{50} values ~0.02 μM *in vitro*) and also effective against the nPKCs δ and ε at a 10-fold higher concentration (IC_{50} values ~0.2 μM *in vitro*). The effects of GF109203X were compared with HBDDE, which is reported to inhibit only cPKC α and cPKC γ [21]. Thus, if cPKC α and/or γ are critical for granule cell survival, then both HBDDE and GF 109203X should be effective inhibitors of cell survival. Alternatively, if cPKC β, nPKC δ, and/or nPKC ε are critical, then GF109203X, but not HBDDE, should be effective. Finally, if none of these PKCs are critical for survival, then neither agent is expected to interfere with survival. To test this, cells were grown in standard 5 mM KCl-containing medium, and vehicle, HBDDE (50 μM), or GF 109203X (0.1 μM) was added 5 hr after initial plating. Cell viability was assessed by the MTT assay at 2 DIV. As shown in Fig. 1A, HBDDE reduced cell viability significantly by ~70%, compared with vehicle-treated or GF 109203X-treated cultures, where no loss of cell viability was observed. Similar inhibitory effects of HBDDE were observed in cultures grown in 25 mM KCl- or NMDA-containing medium (data not shown). In contrast, GF 109203X was ineffective in cells grown in standard medium even when 10- to 100-fold higher concentrations were used over a 6-day treatment period beginning at 2 DIV. (This longer treatment period was employed previously by investigators to maximize death in granule neurons in response to kinase inhibitors [27].) Compared with the untreated control, which was normalized to 100%, survival in response to addition of 1.0 or 10 μM GF 109203X was 99.0 ± 5.6 and 88.0 ± 15.6%, respectively (N = 3; note that the higher concentration of GF 109203X may be sufficient to inhibit protein kinase A, IC_{50} ~ 2 μM *in vitro*). These data suggest that HBDDE inhibits a target other than cPKCs because: (i) GF 109203X, which inhibits all cPKCs and

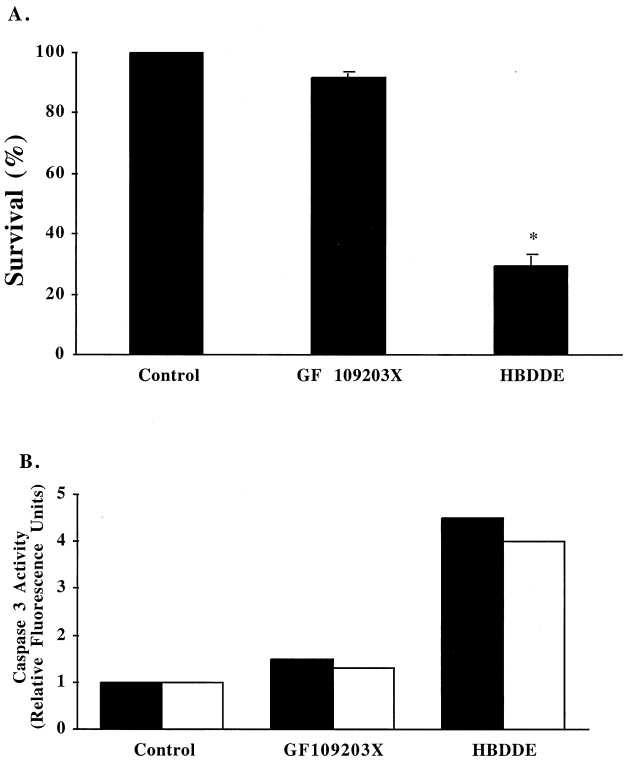

FIG. 1. HBDDE and GF 109203X effects on cell survival and apoptosis. Granule neurons were maintained in standard growth medium containing 5 mM KCl. (A) HBDDE (50 μM) or GF 109203X (0.1 μM) was added to the culture medium 5 hr after initial plating of the cells. Viability was assessed by MTT at 2 DIV. Results are expressed as means ± SEM of percent survival of vehicle-treated control cultures, from at least 3 different cell preparations. Key: (*) significantly different ($P < 0.05$) from untreated cultures. (B) HBDDE (50 μM) or GF 109203X (0.1 μM) was added to the culture medium at 1 DIV, and cell lysates were collected to determine caspase 3 activity 4–5 hr later using a FluorAce™ Apopain Assay kit. Two representative experiments are shown (black bars: experiment 1; white bars: experiment 2). The untreated control culture was normalized to 1 relative fluorescence unit, and was used as a comparison for the drug-treated conditions.

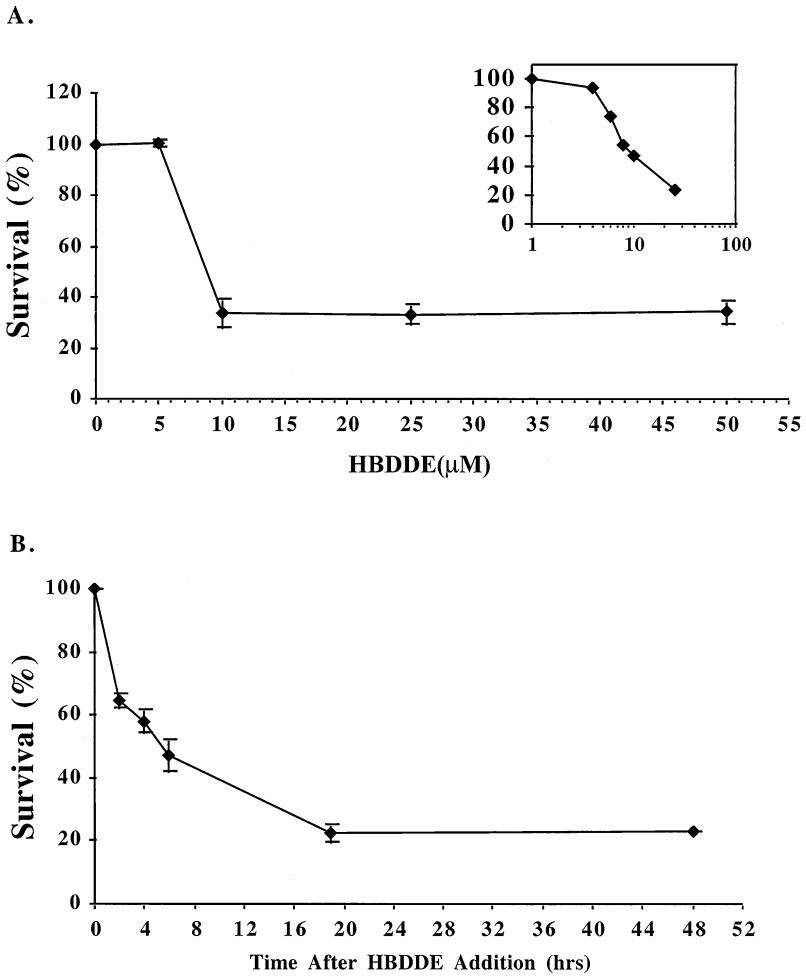

FIG. 2. Concentration and time dependence of HBDDE effects. Granule neurons were maintained in standard growth medium containing 5 mM KCl. (A) Concentration curve. HBDDE (5, 10, 25, and 50 μM) was added 5 hr after plating, and cell viability was assessed by MTT at 2 DIV. Results are expressed as means ± SEM of percent survival of the vehicle-treated control culture from 3 different cell preparations. Inset: more complete concentration curve in a single cell preparation (range: 0–25 μM). (B) Time course. HBDDE (50 μM) was added 5 hr after plating, and cell viability was assessed by MTT at 2, 4, 6, 19, and 48 hr after addition. Data were obtained from 4 different cell preparations, performed in duplicate, and are expressed as means ± SEM of percent survival compared with vehicle-treated control cultures.

some nPKCs, including the putative targets of HBDDE, was ineffective at concentrations that inhibit PKCs in a variety of other cell types [16–20]; and (ii) HBDDE induced cell death at much lower concentrations than expected in an intact cell system for inhibition of cPKC α or γ.

We next examined whether the type of cell death induced by HBDDE was apoptotic, since the MTT assay does not provide this information. Members of the caspase family, in particular caspase 3, are cysteine proteases that typically serve as terminal effectors of apoptosis [25]. To determine if the nature of cell death induced by HBDDE is apoptotic and involves activation of caspase 3 (also referred to as apopain), a FluorAce™ Apopain kit was used. Cells were maintained in standard growth medium, vehicle or kinase inhibitors were added at 1 DIV, and cell lysates were collected 4–5 hr later. Figure 1B shows that, in two separate cell preparations, cultures treated with HBDDE (50 μM) exhibited a marked increase in caspase 3 activity compared with the untreated control cultures or cultures incubated with GF 109203X (0.1 μM). When examined under phase-contrast microscopy, cells incubated with HBDDE, but not GF 109203X, showed morphological changes characteristic of apoptosis (e.g., cell shrinkage and membrane blebbing; data not shown). These results are consistent with those obtained using the MTT assay and further indicate that HBDDE induces neuronal apoptosis as well as activation of caspase 3 in cerebellar granule neurons.

As mentioned above, the observation that HBDDE potently reduced cell viability at a concentration similar to its reported *in vitro* IC_{50} was unexpected, and suggested a target other than PKC α or γ. The lack of effect of GF 109203X, at concentrations 5- to 100-fold greater that its *in vitro* IC_{50}, on cell survival is consistent with this theory. To further characterize the effects of HBDDE, a concentration–response curve was constructed. HBDDE concentrations ranging from 5 to 50 μM were added to cells maintained in standard medium 5 hr after initial plating. Cell viability was subsequently assessed at 2 DIV by MTT assay. Figure 2A shows that the IC_{50} for HBDDE-induced cell death lay within the 5–10 μM range, at least an order of magnitude less than expected if HBDDE were acting through inhibition of PKC α or γ.

The time course for HBDDE-induced cell death is shown in Fig. 2B. HBDDE (50 μM) was added 5 hr after plating cells in standard medium, and cell viability was assessed by MTT assay 2, 4, 6, 19, and 48 hr later. After 6 hr, there was an ~50% reduction in cell survival compared with untreated cultures (100%). By 19 hr after addition of the inhibitor, there was an ~80% reduction in viability.

To unequivocally determine whether HBDDE induces

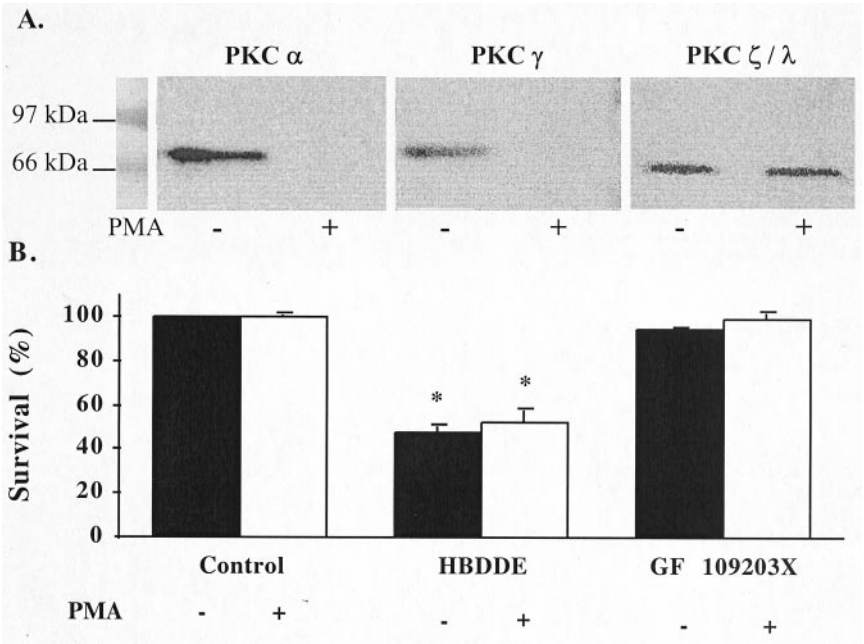

FIG. 3. HBDDE-induced cell death after PKC down-regulation. Cells were maintained in 5 mM KCl-containing medium, and 500 nM PMA (+) was added to half of the plates at 1 DIV. (A) After an overnight incubation, cells were harvested, and protein was resolved on 6% SDS–PAGE, transferred to nitrocellulose, and assayed for immunoreactive PKC α, γ, or ζ/λ by western immunoblotting. The apparent molecular masses of the immunoreactive proteins were estimated by interpolating from a plot of the relative mobilities of the standards against the log of their molecular weights. Masses of ~75, ~76, and ~67 kDa were obtained for PKC α, γ, and ζ/λ, respectively, which are in good agreement with the published values of 76.8, 77.5, and 67.2 kDa [29]. (B) HBDDE (50 μM) or GF 109203X (0.1 μM) was added to the culture medium at 2 DIV, and cell viability was assessed by MTT 6 hr later. Data represent results obtained from 3 different cell preparations, performed in duplicate, and are expressed as means ± SEM of percent survival compared with vehicle-treated cultures. Key: (*) significantly different ($P < 0.05$) from vehicle-treated control cultures.

apoptosis through inhibition of PKC α or γ, PKC down-regulation studies were performed. Classical and some novel, but not atypical, isoforms of PKC are sensitive to down-regulation after prolonged exposure to phorbol esters, such as PMA [28, 29]. Thus, if HBDDE remains effective in inducing apoptosis after down-regulation, then these PKC family members can be ruled out as intracellular targets. To test this, cells grown in 5 mM KCl for 1 DIV were treated overnight with 500 nM PMA. Down-regulation of PKC α and γ, but not PKC ζ/λ, was verified by western immunoblotting (Fig. 3A). After overnight exposure of cells to PMA or vehicle, HBDDE (50 μM), GF 109203X (0.1 μM), or vehicle was added to the culture medium. Six hours later, cell viability was assessed by MTT assay. The addition of 500 nM PMA alone to the cultures did not influence cell viability, compared with control cultures (Fig. 3B). Consistent with the data presented in Fig. 2B, the addition of HBDDE to cultures not pretreated with PMA reduced survival by ~50%, compared with control cultures, whereas GF 109203X had no effect. Importantly, the addition of HBDDE to PMA-treated cultures significantly reduced cell survival by the same amount, ~50%, compared with non-PMA-treated cultures receiving HBDDE. These data demonstrate that HBDDE induces apoptosis by inhibiting a different target enzyme than PKC α or γ.

The discovery that PKC isoforms exhibit distinct tissue distributions, activation profiles, and substrate preferences [13, 14] indicates that they have distinct functions in biological processes. The roles of individual PKC isoforms in mediating cell survival have been studied in a number of systems with inconsistent results. For example, Kelly *et al.* [30] demonstrated that leukemia cells can be rescued from apoptosis through activation of a PKC α-mediated pathway. A separate report has shown both cPKCs and nPKCs to be critical for the survival of leukemia cells [8]. Still other studies have demonstrated that the aPKCs protect leukemia cells [7] as well as PC12 cells [31, 32] from apoptosis. As stated above [10, 26], there is evidence for and against a role for phorbol ester-sensitive PKCs (e.g. cPKCs/nPKCs) in granule cell survival pathways. Clearly, the roles of different isoforms in mediating survival are complex and may be determined in part by the nature of the apoptotic insult, cell type, and stage of maturation.

In the present study, we provide evidence that the putative PKC α and γ inhibitor HBDDE induces neuronal apoptosis independent of an effect on classical and novel PKCs. This hypothesis is based on the following observations: (i) HBDDE was maximally effective in inducing apoptosis in intact neurons at concentrations that were well below its IC_{50} values for PKC α and γ *in vitro*; (ii) HBDDE remained optimally effective in inducing apoptosis after phorbol ester-mediated down-regulation of cPKCs and, presumably, nPKCs; and (iii) in protein extracts from granule cells grown in standard medium, Lin *et al.* [11]

reported that all cPKCs, nPKC ϵ, and aPKC λ/ι are detectable by western immunoblotting using isoform-specific antibodies. The nPKCs δ, θ, and η or aPKC ζ were not detectable. Since nPKC ϵ is a target of GF 109203X (IC_{50} ~0.2 μM *in vitro*), the lack of effect of 10 μM GF109203X in intact cells suggests that nPKC ϵ, the only nPKC in granule neurons, is not a key component of the HBDDE-sensitive survival pathway.

Two other observations should be noted: (i) Down-regulation of cPKCs and nPKCs did not enhance the degree of HBDDE-mediated apoptosis. Rather, the degree of cell death was equivalent in PMA-treated and untreated neurons. This is in contrast to a study in proleukemia cells, where ara-C-mediated apoptosis was augmented by phorbol-ester-mediated down-regulation of PKCs [8]. This result indicates that transmodulation by these PKCs is not operative in the HBDDE-sensitive survival pathway in granule neurons; and (ii) In some cell types, agents that induce apoptosis are dependent on macromolecular synthesis, and their detrimental effects can be attenuated by pretreatment of cells with cycloheximide, a protein synthesis inhibitor [33]. In four separate cell preparations receiving cycloheximide followed by HBDDE, cycloheximide pretreatment completely rescued cells from HBDDE-induced death (unpublished results). This finding indicates that the apoptosis-inducing effect of HBDDE depends upon protein synthesis.

If cPKCs and nPKCs are not primary mediators of granule cell survival in our model, then what kinases are important? The atypical family of PKC isoenzymes (PKC ζ and PKC λ/ι) is insensitive to phorbol ester activation and down-regulation [28, 29]. In non-neuronal cells [7, 34, 35] and the neuronal-like PC12 cell line [31, 32], recent evidence points to a major role for aPKCs in survival. In non-neuronal cells, it is well established that aPKCs are activated by PI-3 kinase [36, 37]. Moreover, PI-3 kinase mediates the survival-promoting effect of insulin-like growth factor-1 in granule neurons [33, 38, 39]. Thus, it is possible that HBDDE induces apoptosis in granule neurons through inhibition of its downstream effector aPKC, presumably λ, the form that is expressed in these cells. Alternatively, HBDDE could be exerting its apoptotic effects through inhibition of protein kinase B (Akt), which was originally identified as a PKC-like kinase [40, 41]. Akt is a general mediator of growth factor-dependent survival and is a downstream target of activated PI-3 kinase in sympathetic [42] and cerebellar granule [38] neurons. Thus, HBDDE may directly or indirectly inhibit one or both of these enzymes. Currently, there is no *in vitro* information about the effects of HBDDE on aPKCs or Akt, but further analysis should provide valuable information regarding their respective roles in neuronal survival and apoptosis.

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