Diethylstilbestrol Alters the Morphology and Calcium Levels of Growth Cones of PC12 Cells *In Vitro*

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Abstract: J. JANEVSKI, V. CHOH, H. STOPPER, D. SCHIFFMANN AND U. DE BONI. Diethylstilbestrol Alters the Morphology and Calcium Levels of Growth Cones of PC12 Cells *In Vitro*. *Neurotoxicology* **14**(4): 505-512, 1993. *Diethylstilbestrol (DES) is a synthetic estrogen with carcinogenic properties. DES is known to alter cytoskeletal components, including the organization of actin stress fibres in C6 rat glioma cells. In a test of the hypothesis that DES disrupts actin filaments of growth cones in neuron-like cells, DES-induced changes in filopodial lengths were quantified in rat pheochromocytoma (PC12) cells in vitro. DES significantly altered growth cone morphology, with collapse of growth cone filopodia and neurite retraction invariably occurring at a concentration of 10 μM. At 5 μM DES, transient reductions in total filopodial lengths occurred. At DES concentrations of 0.1 nM and 1 nM, reductions in total filopodial lengths occurred in a fraction of growth cones. Evidence exists which shows that growth cone activity and morphology are intimately linked to levels of intracellular, free calcium and that DES increases such levels. Measurements of free intracellular calcium levels by fluorescence microscopy, at times concurrent with the DES-induced reduction in total filopodial lengths, showed that calcium levels were indeed significantly increased by 10 μM DES. Labelling of filamentous actin (f-actin) with FITC-phalloidin showed that the f-actin distribution in growth cones exposed to DES could not be differentiated from the distribution found in spontaneously retracting growth cones. Together with evidence which showed that growth cone motility was not affected, the results are taken to indicate that DES, rather than acting directly on the cytoskeleton, exerts its effects indirectly, by a calcium-induced destabilization of actin filaments in the growth cone.* © *1993 Intox Press, Inc.*

Key Words: Diethylstilbestrol, Rat Pheochromocytoma Cells, Growth Cone, Cytoskeleton, Calcium

INTRODUCTION

The stilbene-type, synthetic estrogen diethylstilbestrol (DES) is considered a non-mutagenic carcinogen. DES decreases the number of spindle and cytoplasmic microtubules (Tucker and Barrett, 1986), disruptions of cytoskeletal components which occur without gene mutation (Barrett *et al.*, 1981). DES also induces aneuploidy (Tsutsui *et al.*, 1983), dislocation of chromatin elements (Schiffmann *et al.*, 1990; Schiffmann and De Boni, 1991) and neoplastic transformation (Barrett *et al.*, 1981). In C6 rat glioma cells, DES induces an astrocyte-like morphology through disruption of stress fibre (actin) organization (Schiffmann *et al.*, 1987), accompanied by a rise in free intracellular calcium (Tas *et al.*, 1992).

Neuronal growth cones are highly motile structures, rich in actin and sensitive to changes in free intracellular calcium concentrations (Lankford and Letourneau, 1989). Rat pheochromocytoma (PC12) cells, when exposed to nerve growth factor (NGF), change from chromaffin-like cells to excitable (Dichter *et al.*, 1977), sympathetic, cholinergic neuron-like cells, complete with growth cones (Greene and Tischler, 1976; Tischler and Greene, 1978). In the present work, PC12 cells were used as a model to test the hypothesis that DES may exert one of its cytotoxic effects by altering the distribution of actin in growth cones and by changing free intracellular calcium concentrations.

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MATERIALS AND METHODS

Cell Culture

PC12 cells were grown at 37°C on glass coverslips forming the bottom of Bionique chambers (Bionique Laboratories, Saranac Lake, New York), essentially of a Rose chamber design. The coverslips were coated with rat tail collagen (Sigma, type VII, 0.3 ml, 0.05 g/ml, 1:1000 acetic acid), and air dried. Culture medium consisted of 85% (v/v) Minimal Essential Medium with Hank's salts and L-glutamine (MEM), 10% (v/v) heat-inactivated horse serum, 5% (v/v) heat-inactivated fetal bovine serum, 50 units/ml of penicillin G, 50 µg/ml streptomycin sulphate, all from GIBCO, and contained 100 ng/ml of 7S nerve growth factor (NGF) (Collaborative Research Inc.). Media containing DES were prepared by adding appropriate aliquots of a stock solution in dimethyl sulfoxide (DMSO), maintaining a final DMSO concentration at or below 0.05% (v/v) in all cases. The use of media with Hank's salts (low bi-carbonate) in lieu of Earle's salts permitted cultures to remain on the heated (37°C) microscope stage for extended periods of time without the use of exogenous carbon dioxide.

Assay of DES on Growth Cone Morphology

Upon exposure to NGF (usually 7 to 12 days) PC12 cells exhibited a neuron-like morphology, including the extension of neurites with growth cones. At this time, the medium was replaced with the same medium but with sera omitted and the chambers were transferred to an inverted Nikon Diaphot microscope equipped with an incubator stage, maintained at 37°C. Serum-free media were employed to avoid binding of DES to serum proteins. Subsequent to a 2 hr adaptation to serum-free conditions, time-lapse photographic recordings (every 6 min) of up to 6 growth cones per culture were initiated, applying the following sequence of conditions: (i) untreated (36 min); (ii) DMSO vehicle (36 min); and (iii) with DES at concentrations ranging from 10 µM to 0.1 nM (36 - 72 min). Thus, for each growth cone, the pre-treatment period served as its own control.

Growth cones were visualized using a 100X oil-immersion, phase contrast objective and photographed on Kodak 2415 Technical Pan film. Photographic negatives of growth cones were projected onto paper and the filopodia traced as linear extensions from the margins of the growth cone body, showing both filopodial length and their point of origin. These tracings were digitized using a Hipad Digitizer (Houston Instruments), connected to a microcomputer equipped with an "in house" program. The total filopodial length for each growth cone was determined as the product of the mean filopodial length and the total filopodial number per growth cone. For each condition, the mean total filopodial length at each specified time point was derived by pooling data from several growth cones in several cultures. Values derived post-treatment, for each growth cone, are expressed as fractions of its mean total untreated filopodial length, the latter obtained by pooling data derived from the time period preceding exposure to DES.

Intracellular Calcium Measurements

Free intracellular calcium levels in growth cones were assessed using Fluo-3 AM ester (Molecular Probes Inc.), loaded in the dark in serum-free medium (4 µM, 22°C, 1 hr). Fluo-3 AM was diluted from a stock solution in anhydrous DMSO in the presence of 1 µl/ml of Pluronic

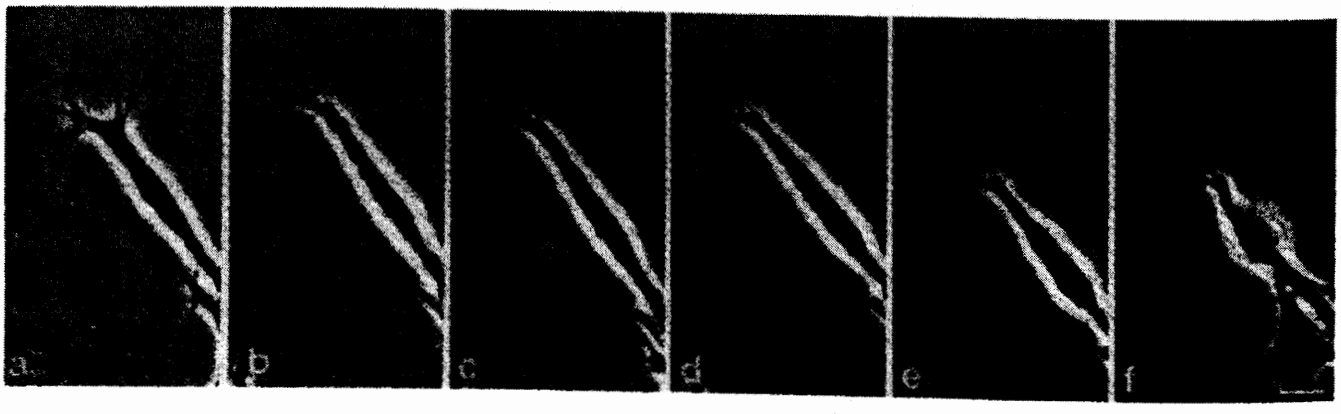

FIG. 1. Time lapse micrographs (6 min) of a PC12 growth cone exposed to 10 µM DES. Note progressive reduction in number and lengths of filopodia (a-d) and subsequent neurite retraction (e-f). Bar = 5 µm.

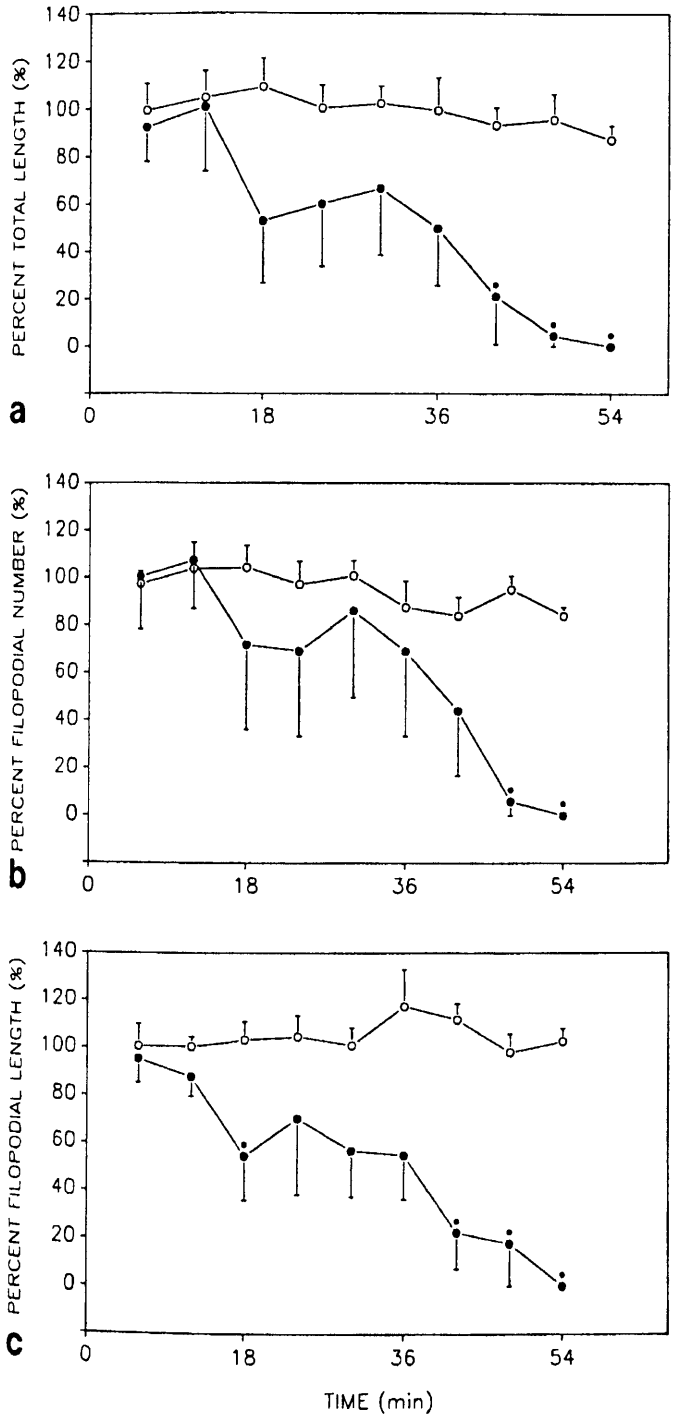

FIG. 2. Plots (± S.E.M.) of responses to 10 μM DES as a function of time. Numbers are expressed as fractions of values derived from a 36 min time period preceding exposure to DES. Open circles; responses to 0.05% (v/v) DMSO vehicle only (9 growth cones, 3 cultures). Filled circles; responses to 10 μM DES (4 growth cones). (a) Mean total filopodial length, (b) Mean filopodial number, (c) Mean filopodial length. ANOVA revealed differences ($p < 0.05$) between DMSO controls and DES-treated cells at the points identified by dots.

F-127 acid (Molecular Probes Inc.). The final concentration of DMSO in the loading dye did not exceed 0.15% (v/v). The loading solution was then removed and replaced with pre-warmed, serum-free medium and the chambers were transferred to the microscope, as above. Following recovery from loading for 1 hr, a baseline fluorescence intensity was recorded (see below) and recordings continued, at 18 min intervals, thereafter. After the first 18 min interval, the cells were exposed to either 0.05% DMSO (control) or to 10 μM DES by quantitative exchange of medium.

Fluorescence intensities were recorded using a Silicon Intensifier Target camera, connected to a frame grabber (PC Vision, Imaging Technology Inc.) in a microcomputer. Changes in fluorescence intensity, as a function of time, were expressed as the difference in mean pixel intensity between two background areas outside the growth cone, and two areas of equal size over the growth cone (11 X 18 pixels; 2.8 X 4.6 μm). Phase contrast and fluorescent images were photographed from a video monitor. Since different growth cones exhibited different baseline fluorescence intensities, changes in pixel intensities were normalized by expressing them as fractions of the mean, pre-treatment baseline pixel intensity.

RESULTS

Growth Cone Morphology

Exposure to DES at 10 μM resulted in a gradual, progressive reduction in mean total filopodial lengths, invariably followed by neurite retraction (Figs. 1, 2a). At DES concentrations between 5 μM and 0.1 nM,

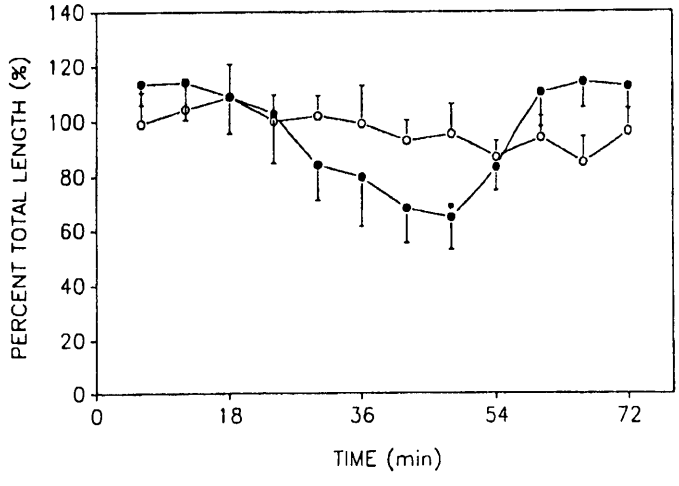

FIG. 3. Plot (± S.E.M.) of the mean total length of filopodia as a function of time. Numbers are expressed as fractions of the mean total filopodial length derived from a 36 min time period preceding exposure to medium containing either 0.05% (v/v) DMSO only (open circles; 9 growth cones, 3 cultures) or 5 μM DES (filled circles; 7 growth cones, 3 cultures). Note the significant transient decrease ($p < 0.05$) at 48 min post-treatment.

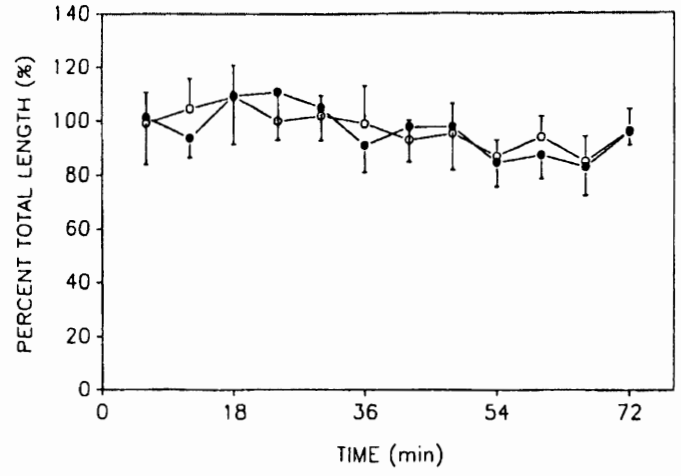

FIG. 4. Plot of mean total length of filopodia as a function of time. Filled circles; responses to exchanges of medium (6 growth cones, 3 cultures). Open circles; responses to exchanges of medium with 0.05% (v/v) DMSO (9 growth cones, 3 cultures). In both cases, numbers (± S.E.M.) are expressed as fractions of the mean filopodial length derived from a 36 min time period preceding exposure. Two-tailed t-tests revealed no differences ($p < 0.05$) in mean total lengths of filopodia throughout time of observation.

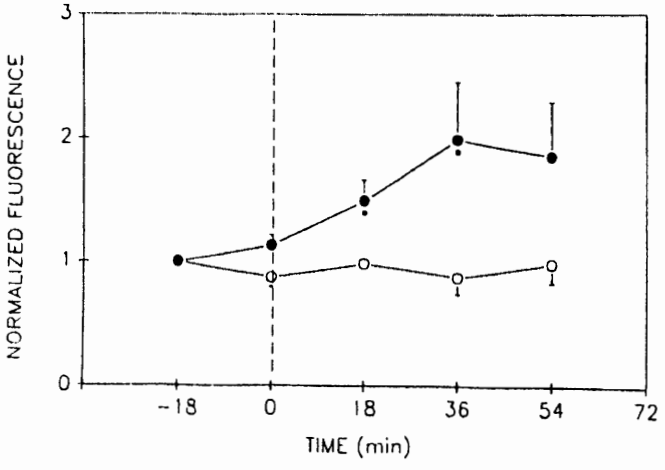

FIG. 5. Plot (± S.E.M.) of mean, normalized fluorescence intensity, indicating increases in intracellular calcium. Open circles; responses to 0.05% (v/v) DMSO vehicle (control; 8 growth cones). Filled circles; responses to 10 μM DES (7 growth cones). Two-tailed t-tests ($p < 0.05$) revealed differences in fluorescence intensities at the post-treatment times identified by dots. Vertical dashed line denotes time of addition of either DMSO or DES.

reductions in mean total filopodial lengths were also observed (Fig. 3). In all cases, reductions in mean total filopodial lengths reflected the effects of DES on both the mean filopodial length and mean filopodial number.

Specifically, upon exposure to 10 μM DES, significant reductions in mean total filopodial lengths were initially observed after 42 min of exposure (Fig. 2a), with mean total filopodial lengths invariably decreasing to zero within 1 hr and both the mean length and the mean number of filopodia showing reductions (Fig. 2b, c).

It was considered that the effects observed might be related to the presence of DMSO vehicle. Although previous studies showed that the use of 0.5% to 1.0% DMSO produced no effects on the cellular morphology of C6 rat glioma cells and of Syrian hamster embryo cells (Tucker and Barrett, 1986; Schiffmann *et al.*, 1987; Schiffmann and De Boni, 1991), the growth cones of PC12 cells, as employed here, retracted following exposure to 0.5% DMSO (data not shown). For this reason, the DMSO concentration was reduced to 0.05%, a concentration at which mean total filopodial lengths were not significantly different ($p < 0.05$) from mean total filopodial lengths in the absence of DMSO (Fig. 4). Moreover, neither growth cone collapse nor neurite retraction was ever observed in any culture exposed to DMSO vehicle alone (13 growth cones, 4 cultures).

In contrast to responses to 10 μM DES, which were characterized by sustained reductions in mean total filopodial lengths (19 growth cones, 5 cultures), at 5 μM DES, mean total filopodial lengths decreased progressively to a minimum and then again increased to values observed prior to treatment (Fig. 3). A direct comparison with vehicle controls revealed a significant reduction in mean filopodial lengths to a minimum, as assessed by a student's t-test ($p < 0.05$). Due to the variability, both in the time of onset of the effect of DES and in the extent of reduction of the mean total filopodial length of individual growth cones, such reductions were not significant ($p < 0.05$) when assessed by ANOVA, in conjunction with tests designed to control for multiple comparisons (Tukey Test; Zar, 1984).

At DES concentrations of 1 nM and 0.1 nM, the responses were restricted to a fraction of the growth cones monitored. In 3 of 4 growth cones treated with 1 nM DES, transient reductions in mean total filopodial lengths were observed, while 2 of 4 growth cones treated with 0.1 nM DES showed such reductions. None of the growth cones exposed to 1 nM and 0.1 nM DES retracted.

To test whether DES also alters growth cone motility, in addition to altering filopodial number and lengths, an index of filopodial orientation was applied (Asymmetry Index for Growth, De Boni and Anderchek, 1986), which represents a dynamic measure of filopodial orientation with respect to the growth cone body. Analyses of the changes in this index, over time, clearly showed that growth cone motility was not altered at times concurrent with reductions in filopodial lengths and numbers (data not shown).

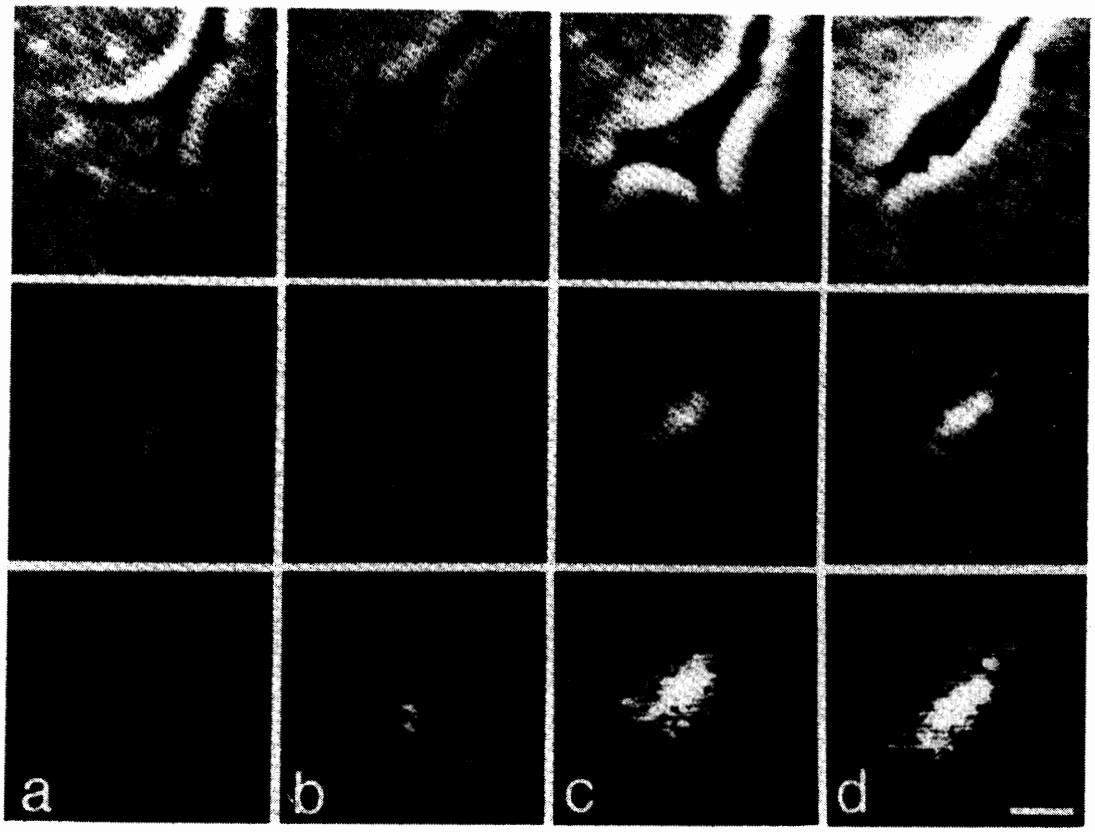

FIG 6. Phase contrast (top row) and corresponding fluorescence micrographs of a representative living growth cone. Note increases in intracellular free calcium as a function of time of exposure to DES (10 μM). Column a) prior to DES addition, columns b) to d) 18, 36 and 54 min after addition of DES, respectively. Middle row shows unprocessed images. Contrast enhanced signals are shown in bottom row. Bar = 5 μm.

Intracellular Calcium

Fluorescence intensity, indicative of the relative free intracellular calcium levels in growth cones, increased progressively upon exposure to 10 μM DES (Figs. 5, 6). In fact, significant changes in mean normalized fluorescence intensities were detectable within 18 min of exposure, compared to controls (Fig. 5). While it was considered that this increase might be related to changes in growth cone morphology (Fig. 6), such as "rounding up", rather than to changes in the absolute level of intracellular calcium, the increase in the integrated intensity clearly exceeds that which could be accounted for by changes in growth cone volume (Fig. 6).

DISCUSSION

The decrease in mean total filopodial lengths concurrent with a rise in free intracellular calcium, as reported here, indicates that DES may exert a cytotoxic effect through mechanisms related to free, intracellular calcium levels. Evidence exists which implicates free calcium levels in the regulation of growth cone behaviour. In fact, it has been shown that a permissive range (Kater *et al.*, 1988) of intracellular calcium levels exists for the elongation of neurites, where both suboptimal and supraoptimal levels are incompatible with neurite extension (Mattson and Kater, 1987; Lankford and Letourneau, 1989, 1991; Kater and Mills, 1991). Neurite outgrowth is proposed to be mediated at least in part by the regulation, via calcium, of actin filament stability. A balance between the assembly and disassembly of the cytoskeleton is postulated to determine the extent of elongation or retraction of neurites (Lankford and Letourneau, 1989, 1991).

DES causes a dose-dependent elevation in the free intracellular calcium levels in C6 rat glioma cells, likely by a discharge of calcium from intracellular stores and by an inhibition of calcium extrusion from the cell (Tas *et al.*, 1992). While a causal relationship cannot be established based on the present work, the increased levels of free calcium, induced by DES in growth cones of PC12 cells, as described here, may have been induced by an analogous mechanism. Such an effect may have

elevated free calcium levels beyond the permissive range (Kater and Mills, 1991) leading to inhibition of growth cone activity. Alternatively, DES may exert its effects by altering the growth cone response to a variety of stimuli which act as environmental cues during neurite outgrowth. Such cues include electrical activity (Fields *et al.*, 1990) and neurotransmitters (Haydon *et al.*, 1987; McCobb *et al.*, 1988), cues which are themselves considered to act via calcium-associated second messenger pathways.

Actions of DES can be separated into different effects, some of which are not related to its estrogenicity. For example, DES has been shown to act on elements of the respiratory chain (Schulz *et al.*, 1990) and has been shown to exhibit genotoxic properties (Tucker and Barrett, 1986; Ozawa *et al.*, 1989; Schiffmann and De Boni, 1991). Given the observation that neurite and growth cone activity may be significantly altered by agents which affect free calcium levels, together with the observation that DES raises such levels, it may be proposed that at least one component of its cytotoxicity may be expressed by an action on mechanisms which control free calcium levels.

Responses to changes in intracellular calcium are graded, so that larger shifts from optimal levels result in more significant changes in behavioral responses (Kater and Mills, 1991). The observations of transient reductions in lengths and number of filopodia at 5 μM DES, and of their sustained reduction leading to neurite retraction at 10 μM DES, suggest that responses to DES are similarly graded (Figs. 2, 3). Compensatory responses to non-optimal, free intracellular calcium concentrations permit re-establishment of normal growth cone activity (Kater and Mills, 1991). Such responses have been observed in many cell types, where changes in intracellular calcium levels are transient (Fields *et al.*, 1990; Mills and Kater, 1990). The transient reductions in total filopodial lengths, at 5 μM DES, as reported here, may therefore occur as a result of such compensatory responses.

It must be considered that DES may act through mechanisms not associated with changes in free calcium. Growth cone collapse, independent of changing calcium concentrations, has indeed been demonstrated (Ivins *et al.*, 1991). In these cases, morphological changes associated with growth cone collapse were not accompanied by changes in growth cone calcium levels, but rather, were ascribed to regulation by second messengers other than calcium. Alternatively, a direct action of DES on the actin cytoskeleton must be considered. Given that filopodial dynamics may be dependent on a balance between assembly and disassembly of filamentous actin (f-actin) at the periphery of growth cones (Lankford and Letourneau, 1989; Okabe and Hirokawa, 1991), an agent which disrupts, rather than modulates, such assembly and disassembly may be expected to affect filopodial motility. The observation that growth cone motility was not significantly altered at times concurrent with transient reductions in filopodial lengths may be taken to indicate that mechanisms of assembly and disassembly of f-actin were still functional in the presence of DES. Moreover, in line with the hypothesis that DES may act directly on the cytoskeleton, the distribution of f-actin in retracting growth cones exposed to DES would be expected to differ from that of spontaneously retracting growth cones. This hypothesis is not supported, however, by the observation that labelling of f-actin with FITC-phalloidin resulted in no detectable differences in the morphology of the actin network in growth cones exposed to 5 μM and 10 μM DES, compared to growth cones retracting spontaneously (data not shown). This is in keeping with results of work on neurons *in vitro* which had shown that neurite retraction is associated with a calcium-regulated destabilization of peripheral actin filaments, regardless of the type of stimulus inducing the retraction (Lankford and Letourneau, 1989). Such a mechanism is supported by the absence of a DES-specific effect on the distribution of f-actin and of an associated change in motility, as shown by the present work. The changes in free intracellular calcium levels, taken together with the latter observations, suggest that DES does act on growth cones indirectly, possibly by a calcium-induced destabilization of peripheral actin filaments.

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