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INFLUENCE OF THE CARCINOGENIC OESTROGEN DIETHYLSTILBOESTROL ON THE INTRACELLULAR CALCIUM LEVEL IN C6 RAT GLIOMA CELLS

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Abstract—The synthetic oestrogen diethylstilboestrol (DES) causes a dose-dependent elevation of the cytoplasmic Ca^{2+} concentration in C6 rat glioma cells. This Ca^{2+} rise is caused neither by Ca^{2+} influx nor by release from the Ca^{2+} stores of the endoplasmic reticulum. Therefore it seems likely that DES mobilizes Ca^{2+} from a mitochondrial source. The DES-induced Ca^{2+} signal is remarkably similar to the one induced by the tumour promotor thapsigargin. As this compound causes leakage of calcium from the endoplasmic reticulum it seems possible that DES induces a similar leakage from mitochondrial Ca^{2+} stores. It remains to be established whether the DES-mediated rise in intracellular calcium is causally related to the tumour-promoting properties of this compound.

Introduction

During the last 10 years the molecular mechanisms of tumour promotion have been the subject of intense investigation. A considerable number of tumour promotors appear to be selective activators of the Ca^{2+} phospholipid-sensitive protein kinase C (Nishizuka, 1984; Parker *et al.*, 1984). The physiological activators of this enzyme are diacylglycerols (Berridge, 1981; Michel, 1975), which are produced by the breakdown of membrane lipids in response to extracellular signals. These diacylglycerols have a low affinity for protein kinase C and are rapidly metabolized (Nishizuka, 1986). Phorbol esters mimic diacylglycerols in the activation of protein kinase C (Castagna *et al.*, 1982), but as they are slowly metabolized their presence leads to prolonged activation of the enzyme.

Recent investigations reveal that the tumour promotor okadaic acid inhibits protein phosphatase 1 and 2A (Biajolan and Takai, 1988). It has therefore been suggested that okadaic acid and phorbol esters facilitate tumour formation by increasing the phosphorylation of the same protein(s) (Cohen *et al.*, 1990). The tumour promotor thapsigargin, a naturally occurring sesquiterpene lactone, probably acts by way of a third mechanism: this compound does not activate protein kinase C and does not inhibit phosphatases, but it discharges Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+} ATPase (Thastrup *et al.*, 1990). However, it is not yet known whether the synthetic oestrogen diethylstilboestrol (DES), which also exerts tumour promoting activity, acts by any of the described pathways. We report here that DES induces a discharge of Ca^{2+} from an ionomycin-insensitive intracellular Ca^{2+} store.

Materials and Methods

Chemicals. Fura-2 (acetoxymethyl ester) and ionomycin were purchased from Calbiochem (La Jolla, CA, USA). DES and thrombin were obtained from Sigma Chemical Co. (St Louis, MO, USA) and thapsigargin from Scientific Marketing Association (Barnet, Middlesex, UK).

Cell culture. C6 rat glioma cells were maintained in plastic tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum in a 5% CO_2/95% humidified air atmosphere and were passaged by trypsinization (0.25% trypsin).

Measurement of intracellular Ca^{2+}. Rat glioma C6 cells were washed by centrifugation and resuspended in buffer A (150 mM-NaCl, 5 mM-KCl, 2 mM-$CaCl_2$, 0.4 mM-$MgSO_4$, 25 mM-glucose and 25 mM-HEPES–Tris; pH 7.3). The resuspended cells (10^7 cells/ml) were then mixed with 0.5% volume of 1 mM-fura-2/AM in dimethylsulphoxide. After incubation for 1 hr at 36°C the cells were washed twice and resuspended in buffer A. Assays were carried out at 32°C with a modified 4-8202-Aminco–Bowman spectrofluorimeter (Silver Spring, MD, USA) fitted with a magnetic stirrer and a thermostatted cuvette holder. Intracellular fura-2-fluorescence was measured at two excitation wavelengths (340 and 385 nm) and continuously recorded at 500 nm. Calcium values were calculated from the 340/385 nm ratios as described previously by Kress *et al.* (1987).

Results and Discussion

C6 rat glioma cells harvested from monolayer cultures were brought into suspension and loaded with the intracellular calcium indicator fura-2. After application of DES to these cultures, an increase of the intracellular calcium level was observed as

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Abbreviations: DES = diethylstilboestrol; DMEM = Dulbecco's modified Eagle's medium; IP_3 = inositol 1,4,5 trisphosphate; PIP = phosphatidyl inositol phosphate; PIP_2 = phosphatidyl inositol 2,3-bisphosphate.

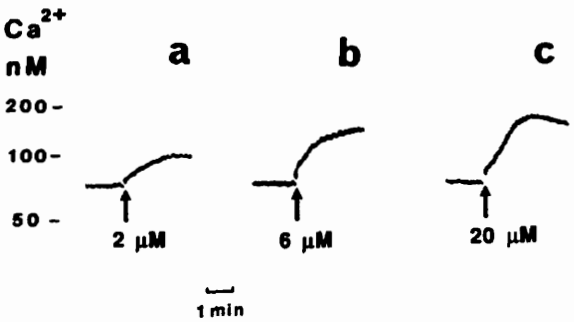

Fig. 1. Fura-2 fluorescence traces of DES-induced Ca^{2+} rise in C6 rat glioma cells. For continuous registration the emitted fluorescence signal (500 nm) was recorded only at the 340 nm excitation wavelength. DES was added at (a) 2×10^{-6} M, (b) 6×10^{-6} M and (c) 2×10^{-5} M. Arrows indicate the time of DES application. The tracings are representative of at least four independent experiments.

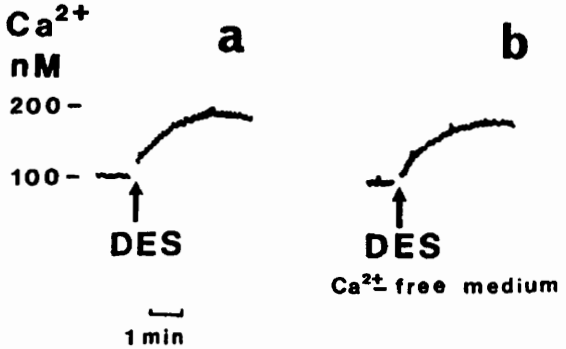

Fig. 2. Effect of Ca^{2+}-free medium on the DES-induced Ca^{2+} rise. DES (2×10^{-5} M) was added in buffer (see Materials and Methods) (a) with and (b) without 2 mM-$CaCl_2$.

determined by fura-2 fluorescence (Fig. 1). The calcium concentration reached a maximum after 2–3 min and declined slowly thereafter. This effect is dose-dependent in the range from 2×10^{-6} to 2×10^{-5} M. At the highest DES concentration (2×10^{-5} M), Ca^{2+} increases from a basal level of approximately 75 nM to 162 nM (Table 1).

We also applied a number of different oestrogens to investigate whether the observed influences on the calcium level correlate with hormonal action. All compounds were assayed at a concentration of 2×10^{-5} M. The steroid oestrogen oestrone did not induce a significant rise in intracellular calcium concentration, but the stilbene oestrogen hexoestrol, and the non-oestrogenic compound Z,Z-dienoestrol, yielded a response similar to the one caused by DES. Stilbene itself induced a comparable but less pronounced signal. It therefore seems unlikely that the observed calcium changes are related to oestrogenicity.

To identify the source of the DES-mediated intracellular calcium rise, we omitted extracellular Ca^{2+}. This removal had only a minor effect on the DES-induced calcium elevation (Fig. 2). Furthermore, DES may cause an increased hydrolysis of the phosphatidyl inositol 2,3-bisphosphate (PIP_2), which produces the second messenger, diacylglcyerol (an activator of protein kinase C) and inositol 1,4,5-trisphosphate (IP_3), which releases calcium from the endoplasmic reticulum. Low concentrations of neomycin (0.05–1 mM) have been reported to bind PIP_2 preferentially to PIP, thus preventing PIP_2 hydrolysis (Lodhi *et al.*, 1979).

Table 1. Effect of DES on the cytoplasmic Ca^{2+} level in C6 rat glioma cells. The method of Kress *et al.* (1987) has been used for the acquisition of data (derived from Fig. 1)

DES concentration (μM)	Calcium levels (nM)		
	Induced	Control*	Increase
2	89	56	33
6	137	77	60
20	162	74	89

*Untreated.
Control: Ca^{2+} levels of cells before application of DES. Induced: maximum Ca^{2+} level after DES application. Increase: DES-mediated maximum Ca^{2+} increase.

Addition of 1 mM-neomycin, which completely inhibited thrombin-induced IP_3 production and Ca^{2+} mobilization from endoplasmic reticulum calcium stores (Carney *et al.*, 1985; Tas and Koschel, 1990) had no effect on the DES-induced calcium elevation (not shown).

To investigate whether DES releases calcium from internal stores by way of an IP_3-independent mechanism, we depleted the calcium stores of the endoplasmic reticulum by application of ionomycin. After two applications of ionomycin, the mitogen thrombin, which mobilizes calcium from the endoplasmic reticulum (Tatakis *et al.*, 1989), no longer induced a calcium signal, thus indicating that the calcium stores were depleted (Fig. 3b). However, DES still induced a calcium rise under these conditions (Fig. 3c), indicating that it mobilizes calcium from a different source.

In view of the similarity of the calcium signals caused by DES and the tumour promotor thapsigargin (Thastrup *et al.*, 1990) a comparison of both substances under similar incubation conditions was performed. Thapsigargin (1 μM) induced a slightly higher calcium rise than 20 μM DES; although the overall shape of the signals was remarkably similar (c.f. Fig. 4a with 4c). However, after depletion of the

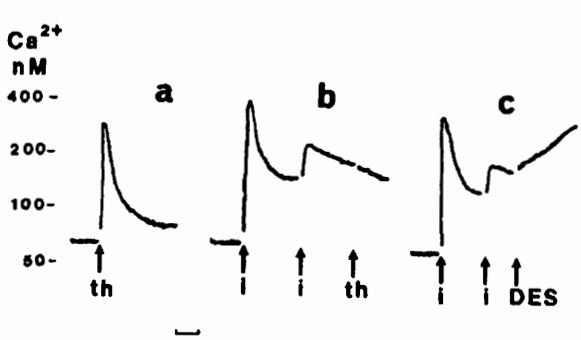

Fig. 3. Effect of thrombin and DES on the cytoplasmic Ca^{2+} concentration after depletion of internal Ca^{2+} stores by ionomycin. The rise in Ca^{2+} concentration induced by thrombin (final concentration 1 unit/ml) is shown in (a). The endoplasmic Ca^{2+} stores were depleted by two additions of ionomycin (i) (2×10^{-5} M). The effect of thrombin (final concentration 1 unit/ml) on cells with ionomycin-depleted Ca^{2+} stores is shown in (b) and (c) shows the effect of DES (2×10^{-5} M) on identically pretreated cells.

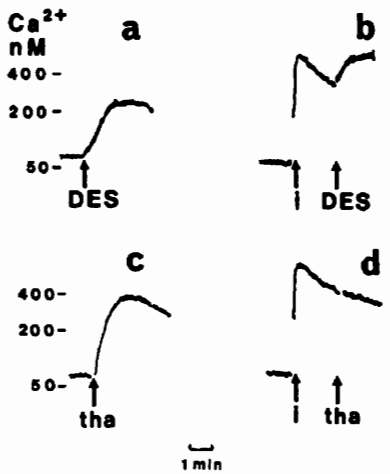

Fig. 4. Influence of thapsigargin (tha) and DES on the cytoplasmic Ca^{2+} concentration in cells with normal or depleted Ca^{2+} stores. Effects of DES (2×10^{-5} M) and thapsigargin (10^{-6} M) are shown for normal cells (a and c) and for cells in which the endoplasmic Ca^{2+} stores were depleted by ionomycin treatment (b and d).

endoplasmic calcium stores with ionomycin, DES still induced a calcium rise, unlike thapsigargin (c.f. Fig. 4b with 4d). We therefore concluded that DES does not mobilize Ca^{2+} from the endoplasmic reticulum. Instead, it seems likely that it releases calcium from mitochondrial stores. This is in agreement with the fact that DES also induces mitochondrial respiration deficiency in *Saccharomyces cerevisiae*, probably by interaction with the mitochondrial membrane (Stopper and Metzler, 1991). Thus, DES may interact in a similar way with mammalian mitochondria.

In contrast to physiological, receptor-mediated calcium signals, the initial rise of the DES- and thapsigargin-induced signals and their subsequent decrease are relatively slow. The shape of both signals may be attributed to an interference with calcium homoeostasis. As thapsigargin causes a leakage of calcium from the endoplasmic reticulum, it seems likely that the DES-induced Ca^{2+} elevation is due to a similar leakage of calcium from mitochondria. Furthermore, investigations to discover whether the DES-mediated rise in intracellular calcium is causally related to the tumour-promoting properties of this compound are necessary.

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