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 promoter 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 promoters 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 promoter 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 promoter 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 (acetoxyethyl 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 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-Amino-Howard 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
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 $2 \times 10^{-4}$ to $2 \times 10^{-3}$ M. At the highest DES concentration ($2 \times 10^{-3}$ 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 $2 \times 10^{-5}$ 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, diacylglycerol (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 Krass et al. (1987) has been used for the acquisition of data (derived from Fig. 1).

<table>
<thead>
<tr>
<th>DES Concentration (μM)</th>
<th>Calcium Levels (nM)</th>
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<tbody>
<tr>
<td></td>
<td>induced</td>
</tr>
<tr>
<td>2</td>
<td>89</td>
</tr>
<tr>
<td>6</td>
<td>137</td>
</tr>
<tr>
<td>20</td>
<td>162</td>
</tr>
</tbody>
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*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 intracellular 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 promoter 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 internal stores is shown (a).

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) ($2 \times 10^{-5}$ 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 ($2 \times 10^{-5}$ M) on identically pretreated cells.
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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REFERENCES


