Calmodulin antagonists decrease the binding of epidermal growth factor to transformed, but not to normal, human fibroblasts

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Four psychoactive agents which inhibit calmodulin activity were used to study their effect on the binding of epidermal growth factor (EGF) to normal and simian-virus-40-transformed human fibroblasts (WI38). These calmodulin antagonists decreased the binding of ^{125}I -labelled EGF to the transformed, but not to the normal, cell in a dose-dependent manner. The mechanism of this effect appears to be due to a decrease in the apparent affinity of the plasma-membrane EGF receptor for the EGF molecule.

EGF is a well-characterized M_r -6000 mitogenic polypeptide hormone which binds to specific plasma-membrane receptors on its target cells. On binding, the receptors aggregate into clusters at specialized regions on the plasma membrane known as coated pits. These coated pits are internalized as vesicles, which then fuse with lysosomal vesicles, and the EGF-receptor complex is degraded in a process known as receptor-mediated endocytosis (Carpenter & Cohen, 1979; Pasten & Willingham, 1981; Fine *et al.*, 1981).

Calmodulin is a ubiquitous and structurally conserved M_r -17000 regulatory protein, which has four Ca^{2+} -binding sites with high affinity and specificity which activate the molecule to regulate various cellular functions (Klee *et al.*, 1980; Means & Dedman, 1980; Keller *et al.*, 1982). It has been demonstrated to exist in most mammalian tissues (Klee *et al.*, 1980), and we have isolated it from both the normal and simian-virus-40-transformed WI38 cells used in this study (P. V. Bodine & J. J. Tupper, unpublished work). Its activity is inhibited by micromolar concentrations of several psychoactive drugs, which have been shown to bind calmodulin in a Ca^{2+} -dependent manner and to inhibit calmodulin-activated phosphodiesterase (Levin & Weiss, 1979; Weiss & Levin, 1978). These drugs have also been shown to inhibit serum-, EGF- and Ca^{2+} -dependent Na^+ influx in human diploid fibroblasts, presumably by inhibiting calmodulin (Owen & Villerial, 1982). Our present study shows that these same drugs also inhibit in a dose-dependent manner the binding of ^{125}I -labelled EGF to simian-virus-40-transformed

human fibroblasts, but not the binding to normal fibroblasts. The mechanism of this decrease is a lowering of the apparent affinity of the plasma-membrane receptor for EGF.

Experimental

Procedures for cell culture have been previously described (e.g. Tupper & Bodine, 1983). Complete details for the ^{125}I -labelled-EGF binding assay are given in our previous publications (Kegel & Tupper, 1982; Tupper & Bodine, 1983), and the specific methods used in the drug studies are given in the Figure legends.

Results and discussion

Fig. 1 shows the effect of four calmodulin inhibitors on the binding of ^{125}I -labelled EGF to normal and simian-virus-40-transformed WI38 cells. The drugs tested were the antipsychotics trifluoperazine, chlorpromazine and haloperidol, and the anti-depressant imipramine. The drugs used were generously given by Dr. M. L. Villerial, University of Chicago, and were solubilized as described previously (Owen & Villerial, 1982). Vehicle concentration was less than 1.0%, and controls contained vehicle in the absence of drug.

Fig. 1 indicates that all drugs tested inhibited the binding of ^{125}I -labelled EGF to the transformed cell in a dose-dependent manner, and had virtually no effect on the binding of ^{125}I -labelled EGF to the normal cells. The increased EGF binding to the transformed cell as compared with the normal cell observed at 2 ng of EGF/ml has been confirmed

Abbreviation used: EGF, epidermal growth factor.

Fig. 1. Effect of four calmodulin inhibitors on the binding of ^{125}I -labelled EGF to normal and simian-virus-40-transformed WI38 cells

Cells were grown to confluence (approx. 2×10^4 normal cells/cm 2 or 4×10^4 transformed cells/cm 2) in Eagle's basal medium supplemented with 5% (v/v) foetal-calf serum. At the time of the experiment duplicate 35mm-diam. culture plates were replenished with fresh culture media containing the appropriate concentration of one of the four drugs tested. The cultures were then incubated at 37°C for 15min, washed once with iso-osmotic choline chloride (4°C) and placed in binding media at 4°C (Eagle's basal medium plus 0.1% bovine serum albumin) containing 2ng of ^{125}I -labelled EGF (1×10^5 – 2×10^5 c.p.m./ng; New England Nuclear)/ml plus the appropriate concentration of drug. Cultures were incubated at 4°C for 2h (at which time near-maximal binding is achieved; Tupper & Bodine, 1983), washed five times with cold iso-osmotic choline chloride and solubilized in 1M-NaOH. Radioactivity was measured in a gamma counter. The closed symbols refer to the transformed cells as follows: ■, haloperidol; ♦, imipramine; ▲, chlorpromazine; ●, trifluoperazine. The open symbols refer to the normal WI38 cells, with symbol shapes as for the transformed cells. The data are typical of two such experiments. Non-specific binding of the ^{125}I -labelled EGF was less than 10% when assayed with 250-fold excess of unlabelled EGF.

with EGF at saturating concentrations (Tupper & Bodine, 1983). EGF is mitogenic to both cell types (Phillips & Cristofalo, 1980; Kegel & Tupper, 1982), indicating that the absence of an effect in

the normal cell is not due to the absence of functional EGF receptors. Furthermore, those two drugs (trifluoperazine and chlorpromazine) that are considered relatively high-affinity inhibitors of calmodulin activity [I_{50} (dose giving 50% inhibition) less than $50\mu\text{M}$], on the basis of inhibition of calmodulin-dependent phosphodiesterase (Levin & Weiss, 1979; Weiss & Levin, 1978), were more potent inhibitors of ^{125}I -labelled-EGF binding to the transformed cell than the other two drugs (haloperidol and imipramine), which are less potent inhibitors of both calmodulin activity (Levin & Weiss, 1979; Weiss & Levin, 1978) and ^{125}I -labelled-EGF binding.

Because the antipsychotic drugs used (trifluoperazine, chlorpromazine and haloperidol) are known to have membrane-stabilizing effects, owing to their hydrophobic nature (Landry *et al.*, 1981), it is possible that the decreased EGF binding to the transformed cell is not due entirely to the inhibition of calmodulin, but conceivably to differences in membrane composition in the two cell types. However, the doses of trifluoperazine and chlorpromazine used have been previously reported not to generate non-specific membrane effects on Na^+ flux in another human fibroblast cell line (Owen & Villeria, 1982). Furthermore, the I_{50} values for inhibition of ^{125}I -labelled-EGF binding by the high-affinity calmodulin inhibitors (trifluoperazine and chlorpromazine) are within the range of the I_{50} values observed for their inhibition of calmodulin-dependent phosphodiesterase (Levin & Weiss, 1979; Weiss & Levin, 1978).

Fig. 2 shows a Scatchard analysis (Scatchard, 1949) of the effect of the most potent of the inhibitors of EGF binding (trifluoperazine) on the binding of ^{125}I -labelled EGF to simian-virus-40-transformed WI38 cells. From the Scatchard plot the approximate number of EGF receptors per cell and the apparent dissociation constants were determined. Under control conditions, the transformed WI38 cell possesses approx. 6×10^4 receptors per cell, with an apparent dissociation constant of $3 \times 10^{-9}\text{M}$. The receptor number is within the range reported for other human fibroblasts (4×10^4 – 10×10^4 receptors per cell; Carpenter & Cohen, 1979). The dissociation constant is 10-fold greater than previously reported for human fibroblasts (Carpenter & Cohen, 1979), but the previous studies were not conducted at 4°C, and thus are not directly comparable. In the presence of $15\mu\text{M}$ -trifluoperazine, the transformed WI38 cell exhibits no significant change in receptor number; however, the apparent dissociation constant for EGF binding of $6 \times 10^{-9}\text{M}$ represents a 2-fold decrease in receptor affinity for EGF. Virtually identical data were obtained in the presence of $30\mu\text{M}$ -trifluoperazine.

Fig. 2. Scatchard analysis of the effect of trifluoperazine on the binding of ^{125}I -labelled EGF to simian-virus-40-transformed WI38 cells

Binding assay was carried out on duplicate cell cultures as described in Fig. 1. Specific radioactivity of the binding media was 1.3×10^3 c.p.m./ng of EGF and trifluoperazine was present at $15 \mu\text{M}$. ●, Control cells; ○, drug-treated cultures. Inset illustrates actual binding as a function of external EGF concentration. Equivalent results were obtained in a separate experiment with $30 \mu\text{M}$ -trifluoperazine. Correlation coefficients, $r = -0.982$ (control) and -0.951 ($15 \mu\text{M}$ -trifluoperazine).

The mechanism by which specific growth factors trigger renewed or continued cellular proliferation is unknown. It has been suggested that growth-factor binding to its receptor is sufficient to initiate the mitogenic signal and that subsequent internalization and degradation serve to limit the cellular response (Aharonov *et al.*, 1978; Maxfield *et al.*, 1979). Others suggest that internalization of mitogens is central to the proliferative signal (Fox & Das, 1979). Within either of these models there are possible functional roles for calmodulin. EGF binding to its receptor induces phosphorylation of several proteins, including the receptor itself (Cohen *et al.*, 1980). In this regard, the phosphorylation of certain membrane proteins has been shown to be Ca^{2+} - and calmodulin-dependent (Schulman & Greengard, 1978). Thus the effect of calmodulin inhibitors on

EGF binding could reflect inhibition of a calmodulin-regulated process (protein phosphorylation?) necessary for formation of a functional EGF-receptor complex.

The observation that all four calmodulin inhibitors affected EGF binding to the transformed cell only is of particular interest, in view of our previous studies. Transformed WI38 cells, as well as numerous virally and chemically transformed cells, have greatly decreased growth requirements for Ca^{2+} as compared with their normal counterparts (Boynton & Whitfield, 1976; Tupper & Zorngniotti, 1977; Hazelton *et al.*, 1979). Specific temporal Ca^{2+} requirements have been identified in the G1 phase of normal WI38 cells, which are lacking in transformed cells (Tupper *et al.*, 1980). Also, G1-phase progression is blocked by calmodulin inhibitors (Hidaka *et al.*, 1981). Since many cellular Ca^{2+} requirements may reflect a requirement for Ca^{2+} -activation of calmodulin, one might expect that the decreased Ca^{2+} requirements of transformed cells could reflect a lesion in a Ca^{2+} -calmodulin-regulated process. This could also be reflected in a decreased sensitivity to calmodulin inhibitors. This is clearly not the case for EGF binding. The transformed WI38 cell has a low extracellular Ca^{2+} requirement for growth but a high sensitivity to calmodulin inhibition of EGF binding. The reverse is true for the normal WI38 cell. Furthermore, neither cell type exhibits a requirement for extracellular Ca^{2+} actually to bind EGF, although Ca^{2+} is required for the subsequent internalization of EGF by the normal, but not by the transformed, cell (Tupper & Bodine, 1983).

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