Calmodulin antagonists competitively inhibit dexamethasone binding to the glucocorticoid receptor

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1. INTRODUCTION

Steroid hormones exert their glucocorticoid activity by binding reversibly to a receptor protein in the cytoplasm of target cells [1,2]. We have shown [3] that Ca^{2+} inhibit the rate of association of dexamethasone, a semi-synthetic glucocorticoid, with the glucocorticoid receptor. The half-maximum effect was seen at 0.3 μM free Ca^{2+}, a concentration within the range (0.1–1 μM) of fluctuations of free intracellular Ca^{2+} that occur in response to hormonal stimulation in several cell types [4]. Many 'second messenger' functions of calcium (e.g., control of protein phosphorylation) are exerted through calmodulin, a ubiquitous thermostable protein that serves as a multifunctional intracellular calcium receptor [5–8]. Thus, the effect of calcium on glucocorticoid-receptor binding might be mediated by calmodulin, the more so as thermostable factor(s) and phosphorylation processes presumably control glucocorticoid receptor activity [9]. Such a mechanism has been proposed for the acetylcholine receptor [10]. Thus, we determined whether calmodulin antagonists influence dexamethasone binding to the glucocorticoid receptor. The antagonists belonged to the class of antipsychotic phenothiazines, i.e., trifluoperazine (TFP) [11,12], membrane-active compounds, i.e., propranolol and SKF 525A [13], microtubule inhibitors, i.e., vinblastine [14], and new calmodulin inhibitors, i.e., R 24571 [15].

We show here that the calcium effect is not prevented by such antagonists of calmodulin. However, some of these and related drugs (SKF 550 and SKF 625A) competitively inhibit the binding of dexamethasone to its receptor. TFP, the most potent inhibitor, prevents induction of tyrosine aminotransferase by dexamethasone. Thus, calmodulin inhibitors may act as glucocorticoid antagonists, not via calmodulin inhibition but through a direct interaction with the glucocorticoid receptor.

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2. MATERIALS AND METHODS

Frozen pellets of HTC cells grown and harvested as in [16] were disrupted (tight-fitting Dounce homogeniser, 70 strokes) in buffer ($3–5 \times 10^7$ cells/ml) containing 20 mM *N*-[tris-(hydroxymethyl)-methyl]-glycine (Tricine, Calbiochem, San Diego CA) and 10 mM Na_2MoO_4, pH 7.4 at 22°C. Particles were removed by centrifugation at 15 000 rev./min for 20 min (Sorvall SS-34 rotor) and then at 49 000 rev./min for 60 min (Spinco 50Ti rotor). The final supernatant (cytosol) was frozen at – 80°C. Cytosol aliquots (0.1 ml) were incubated in triplicate at 0°C in the homogenisation buffer (final vol. 0.4 ml), in the presence of [^{3}H]dexamethasone, without or with one of the drugs listed below. At the end of the incubation period, 0.2 ml aqueous charcoal suspension (50 mg/ml) containing dextran (5 mg/ml) was added. Incubations were agitated for 10 s and centrifuged to remove free steroid. Radioactivity in 0.25 ml supernatant was counted in picofluor (Packard, Warrenville IL) using a Berthold (Wildbad) scintillation counter (efficiency 58%) to determine macromolecule-bound steroid. Receptor binding was calculated by subtracting

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Table 1

Effect of calmodulin antagonists and related compounds on dexamethasone binding to the glucocorticoid receptor

Drug	Effect on dexamethasone binding		Effect on calmodulin	
	Inhibition of initial rate (%)[a]	Equilibrium K_i, (μM)	App. K_i[b] (μM)	Ref.
Trifluoperazine	75	5	6	[13]
SKF 550	57	7	n.d.	–
SKF 625A	23	19	n.d.	–
Vinblastine	73	29	36	[14]
Propranolol	55	45	180	[13]
SKF 525A	53	88	130	[13]
R 24571	3	$>10^4$	0.005	[22]

[a] At the drug concentrations given in fig. 1A

[b] Apparent equilibrium binding constant [22] or concentration of drug that produces 50% inhibition of calmodulin-mediated stimulation of brain phosphodiesterase [13] or erythrocyte Ca^{2+}-transport ATPase [14]

n.d. = not described

The initial rate of dexamethasone association with the receptor was determined by measuring binding after 30 min incubation. The equilibrium inhibition constants are means from at least 2 expt performed as in fig. 1A. The effects of the drugs on calmodulin are taken from the references cited

non-specific binding determined in parallel incubations containing 0.2 mM $HgCl_2$. Binding assays involving SKF 525A were performed by hydroxylapatite precipitation of the receptor [3] instead of charcoal adsorption of free steroid because this compound bound to charcoal.

Trifluoperazine, SKF 550, SKF 525A, and SKF 625A were generous gifts of Smith, Kline and French (Philadelphia PA). Dexamethasone (9α-fluoro-16α-methyl-11β, 17,21-trihydroxy-1,4-pregnadiene-3,20-dione) was a generous gift of Merck, Sharp and Dohme (Rahway NJ). Propranolol was obtained from ICI (Macclesfield), vinblastine from E. Lilly (Indianapolis IN), R 24571 from Janssen Pharmaceutica (Beerse) and [^{3}H]dexamethasone (46 Ci/mmol) from the Radiochemical Centre, Amersham.

3. RESULTS AND DISCUSSION

Rat hepatoma tissue culture (HTC) cells contain steroid receptors that are specific for glucocorticoids and respond to dexamethasone by increased tyrosine aminotransferase activity [1]. HTC cytosol was incubated with [^{3}H]dexamethasone and specific binding was determined after 30 min at 0°C as a measure of the initial rate of association of the steroid with the receptor. Under these conditions, binding is decreased when calcium is added to the incubation and is increased by calcium chelating agents such as EDTA or ethylene glycol bis-(β-amino ethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) [3]. According to our hypothesis, calmodulin antagonists were expected to stimulate the binding by preventing endogenous calcium from exerting its inhibitory effect [3]. However, none of the antagonists tested stimulated the rate of binding; instead, some inhibited binding by up to 75% (table 1).

Calmodulin antagonists are thought to act by binding to a lipophilic region of calmodulin exposed upon interaction of this protein with calcium [5–8, 11,12,17,18]. Perhaps inhibition of dexamethasone binding by these drugs resulted from a perturbation of the hydrophobic [19] steroid-binding site on the glucocorticoid receptor rather than

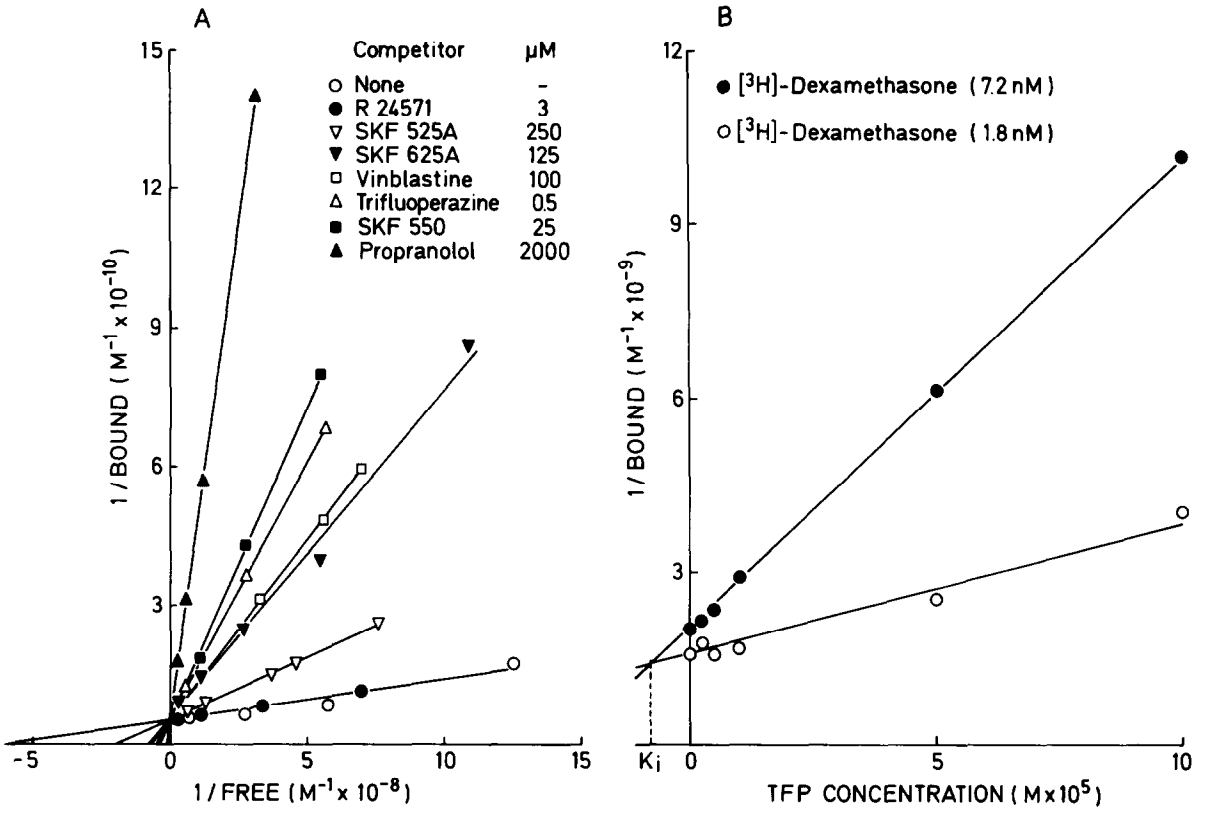

Fig.1. Competitive inhibition of [^{3}H]dexamethasone binding to the glucocorticoid receptor by calmodulin antagonists and related drugs.

(A) Aliquots of HTC cel cytosol were incubated in triplicate at 0°C as in table 1 except that 1 mM EDTA was added to the buffer and the [^{3}H]dexamethasone concentration was varied, without or with fixed concentrations of the drugs as indicated. After 16 h, specific dexamethasone binding was determined by charcoal assay and the results presented as double-reciprocal plots [20].

(B) The experiments were performed as above using fixed concentrations of [^{3}H]dexamethasone and varying concentrations of TFP. The data are plotted according to Dixon [21] and yield K_i 7.7 µM for TFP.

from an effect on calmodulin. If so, the antagonists should inhibit dexamethasone binding even in the absence of free calcium. This was the case since EDTA did not prevent the antagonists from depressing dexamethasone binding to its receptor.

To further investigate this phenomenon, binding experiments were conducted at equilibrium (16 h, 0°C) by using various [^{3}H]dexamethasone concentrations with fixed concentrations of the inhibitors and in the presence of EDTA. Double-reciprocal plots of the data (fig.1A) were compatible with competitive inhibition either at, or close to, the steroid-binding site [20]. This alternative was investigated in experiments designed according to [21] using the most potent inhibitor, TFP. The plots were linear (fig.1B), which is consistent with a purely competitive phenomenon. If this effect were unrelated to calmodulin inhibition then the potency of the drugs as calmodulin antagonistst might not match their potency as inhibitors of dexamethasone binding. A comparison of the respective inhibition constants (table 1), albeit suggestive of a discrepancy, did not give a definite answer. We therefore used R 24571, a novel and more specific calmodulin antagonist [15] that has an affinity for calmodulin 3 orders of magnitude greater than TFP [22]. At a

concentration (50 nM) that saturates calmodulin R 24571 did not influence the rate of dexamethasone binding to its receptor over a wide range (10 nM–10 μM) of free Ca^{2+} (not shown). Furthermore, R 24571 at the highest concentration compatible with solubility (3 μM) did not influence dexamethasone binding whether in absence (table 1) or presence (fig.1A) of EDTA. We also tested other membrane-active compounds, SKF 550 and SKF 625A (potent inhibitors of the extraneuronal catecholamine uptake process [23]). Each of the drugs depressed cell-free dexamethasone binding (table 1), apparently through a competitive inhibition mechanism (fig.1A).

These results, together with the lack of a calcium requirement for inhibition of dexamethasone binding by these drugs, make it unlikely that calmodulin mediates the effect of calcium on the glucocorticoid receptor. We conclude that several calmodulin antagonists, including the potent compound TFP, inhibit glucocorticoid action by competing with the hormone for the steroid-binding site. Consistent with this interpretation TFP inhibited the induction by dexamethasone of tyrosine aminotransferase in HTC cells without affecting uninduced enzyme levels (fig.2).

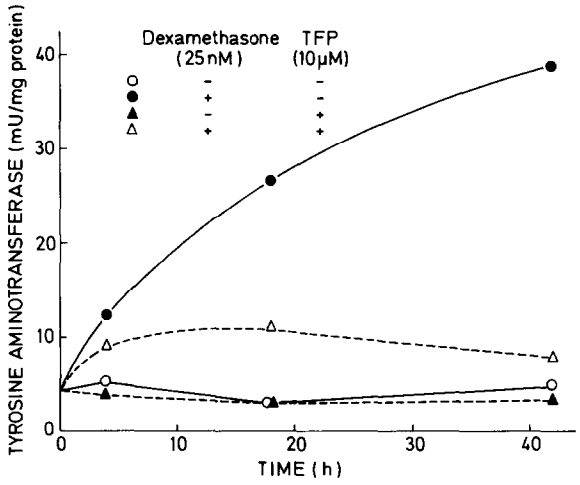

Fig.2. Inhibition of TFP of tyrosine aminotransferase induction by dexamethasone in HTC cells. Duplicate cultures of HTC cells in serum free medium (1) were exposed to 25 nM dexamethasone or to the vehicle, ethanol (0.25%, v/v). Half of each culture also received 10 μM TFP. Incubations were kept at 37°C and aliquots removed at the times indicated for determination of tyrosine aminotransferase activity [1].

Inhibition of biochemical processes in intact cells exposed to TFP has often been taken as evidence for an involvement of calmodulin [24–26]. Our finding that TFP and functionally related drugs interfere with glucocorticoid receptor action draws the attention to an alternate mechanism because glucocorticoid hormones control the activity of a number of enzymes [2]. The antipsychotic effect of TFP is not related to inhibition of calmodulin [27,28], nor is its capability to reduce ATP levels in lymphocytes [29]. The potential antiglucocorticoid activity of TFP adds a new facet to the pharmacologic properties of phenothiazines and could give a clue to some of their side-effects [30]. Likewise, interaction of membrane-active compounds with the intracellular glucocorticoid receptor might account for hitherto unexplained observations such as the inhibition by propranolol of tyrosine aminotransferase induction by dexamethasone in chick embryo liver cells [31].

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