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SHORT COMMUNICATION

Inhibition of Calcium-Dependent Regulator-Stimulated Phosphodiesterase Activity by Neuroleptic Drugs Is Unrelated to Their Clinical Efficacy

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SUMMARY

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It has previously been suggested that some of the pharmacological and therapeutic effects of certain neuroleptic drugs depend upon their ability to inhibit calcium-dependent regulator-(CDR)-stimulated phosphodiesterase activity. To further test this hypothesis, clinically active and inactive isomers of a number of neuroleptics were examined as CDR inhibitors. Both active and inactive isomers of all the drugs investigated were equally inhibitory in this assay. The IC_{50} values for CDR inhibition obtained for these and other neuroleptics correlated well with their octanol:water partition coefficients. It is concluded, therefore, that the inhibitory effects of these drugs on the CDR protein are unrelated to their clinical efficacy and may be due to nonstereospecific hydrophobic interactions with the CDR protein.

INTRODUCTION

The calcium-dependent regulator protein (CDR) is a low molecular weight, acidic protein which, when complexed with calcium, activates a variety of enzymes. Originally purified as a calcium-binding protein of unknown function (1), CDR was subsequently shown to be an activator of cyclic AMP phosphodiesterase (2, 3) and adenylate cyclase (4, 5) in a variety of tissues and, in addition, to enhance erythrocyte Ca^{++}-Mg^{++}-dependent ATPase (6). More recently, this ubiquitous protein has been found to be an activator of myosin light chain kinase from smooth muscle and phosphorylase kinase from skeletal muscle (7, 8). Thus, there is increasing evidence to suggest that CDR may be an important regulatory protein, with a possible role as a link between intracellular Ca^{++}-availability and cyclic nucleotide metabolism. If this is so, compounds which can specifically inhibit the CDR-stimulation of these enzyme systems may prove to be clinically important or be valuable probes for investigating the physiological role of CDR.

It was previously reported by Levin and Weiss (9) that a variety of structurally unrelated neuroleptic agents, including phenothiazines and butyrophenones, could inhibit CDR-dependent activation of cyclic

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AMP phosphodiesterase. These workers subsequently showed that purified CDR contained 2 high affinity (K_D = 1 μM) and 24 low affinity (K_D = 5 mM) binding sites per molecule for the phenothiazine drug (^{3}H)-trifluoperazine (10). They suggested that this binding might mediate the inhibitory action of trifluoperazine on CDR-stimulated phosphodiesterase activity and possibly on CDR-sensitive adenylate cyclase (11) and further proposed that these effects could explain some of the diverse pharmacological and therapeutic actions of antipsychotic drugs.

In the present study, the clinically active and inactive isomers of three antipsychotics, butaclamol, flupenthixol and chlorprothixene, as well as six other neuroleptics for which isomers do not exist, have been examined as inhibitors of CDR-stimulated phosphodiesterase activity. It was reasoned that if inhibition of CDR-stimulated phosphodiesterase activity is related to the clinical efficacy of these drugs, then only the pharmacologically active isomer of these compounds should be inhibitory. Our results indicate that both isomeric forms of butaclamol, flupenthixol and chlorprothixene are equally inhibitory in this assay, and consequently, the clinical effects of these agents cannot be mediated through the CDR protein. To further understand the nature of this non-stereospecific inhibition of CDR-stimulated phosphodiesterase activity, the octanol:water partition coefficients of these drugs were determined as an estimate of their lipid solubility. It was found that the octanol:water partition coefficients for these drugs correlated very well with the concentration necessary to cause half-maximal inhibition of CDR-stimulated phosphodiesterase activity. Therefore, it is possible that these neuroleptic drugs inhibit CDR-stimulated phosphodiesterase activity through nonstereospecific hydrophobic interactions with CDR.

CDR was purified from bovine brain by the procedure of Dedman *et al.* (12). It migrated as a single band on SDS polyacrylamide gel electrophoresis and, in addition, had the UV spectrum and amino acid composition characteristic of pure CDR. CDR-dependent phosphodiesterase was prepared from bovine brain by the method of Ho *et al.* (13). CDR stimulated the specific activity of the phosphodiesterase preparation threefold under the conditions used in this study; however, a tenfold increase in CDR concentration would enhance the specific activity 4.7-fold. This degree of CDR-dependent activation of an impure preparation of phosphodiesterase is consistent with the activation observed by Dedman *et al.* (14). Protein concentrations were determined with a Coomassie Blue staining kit (Bio-Rad) using gamma-globulin as a standard (15). Drugs were donated by the following companies: Lundbeck & Co. A/S, Copenhagen-Valby, Denmark (cis- and trans-flupenthixol, cis- and trans-chlorprothixene); Janssen Pharmaceutica, Beerse, Belgium (spiroperidol, haloperidol and pimozide); Ayerst Laboratories Ltd., Farnborough, U.K. (+) and (−)-butaclamol); Wander Ltd., Berne, Switzerland (clozapine); Delagrange International, Paris, France (sulpiride). In studies of serotonin and dopamine receptor binding with the drug preparations which were used in this work, (+)-butaclamol was at least one thousand-fold more potent than its (−)-isomer as an inhibitor of binding and cis-flupenthixol was one hundred-fold more active than its trans-isomer.[2] Wherever possible, drugs were dissolved in distilled water immediately prior to the start of the phosphodiesterase assay. Clozapine, spiroperidol and haloperidol had to be dissolved in 5.7 mM ascorbic acid and pimozide in 0.01 M HCl. These drugs were then diluted 10-fold into the assay. Control experiments demonstrated that these solvents had no effect on phosphodiesterase activity. Octanol/aqueous-buffer partition coefficients were determined experimentally by the method of Leo *et al.* (16). The aqueous phase was 0.066 M phosphate buffer (pH 6.0 or 8.0), saturated with n-octanol. The organic phase was buffer-saturated, distilled n-octanol. Initial substance concentrations in the aqueous phase were between 50 and 300 μM. The volume of each phase was chosen so as to give good sensitivity for

[2] Drummond and Levitan, unpublished observations.

UV-spectroscopic determination of the drug concentrations.

Whenever possible, experiments were carried out at pH 8.0, the pH of the *in vitro* experiments. However, due to their very high partition coefficients at pH 8.0, some substances were tested at pH 6.0, where the degree of dissociation is higher. Log P at pH 8.0 was calculated using the equation

$$p' = P\frac{1}{1 + 10^{(pKa-pH)}} \quad \text{(ref. 17)}$$

where p' is the apparent partition coefficient at the particular pH and P is the intrinsic partition coefficient of the undissociated free base. The pKa-values were either taken from the Merck Index or from the data sheets of the drug manufacturers. If no pKa-values could be found in the literature, they were determined by spectrophotometric microtitration (18).

The inhibition of CDR-dependent cAMP phosphodiesterase by (+)- and (−)-butaclamol is shown in Fig. 1. Both stereoisomers were equally inhibitory with IC_{50} values of 15 μM. Similar results were found with cyclic GMP as substrate (data not shown). These data are inconsistent with clinical and pharmacological studies, which indicate that the neuroleptic activity of butaclamol resides entirely in the (+)-isomer (19–22). Neither (+)- nor (−)-butaclamol altered CDR-insensitive phosphodiesterase activity at concentrations below 100 μM. In addition to (+)- and (−)-butaclamol, both isomeric forms of flupenthixol and of chlorprothixene were active as inhibitors of CDR-stimulated phospodiesterase (Table 1). The cis-isomer of flupenthixol is the clinically active form (23) and both it and cis-chlorprothixene are more active than their corresponding trans-isomers in pharmacological tests predictive of neuroleptic activity (24–27). Cis- and trans-chlorprothixene had IC_{50} values of 2.4 and 3.0 μM, respectively, against the CDR-stimulated enzyme and values 10-fold higher against the basal or CDR-insensitive enzyme. This latter finding is in conflict with the data shown by Levin and Weiss (9) who reported that the IC_{50} for chlorprothixene against the basal activity of phosphodiesterase was >2 mM. The other neuroleptic drugs shown in Table 1 had IC_{50} values which were comparable with, though somewhat lower than the IC_{50} values reported by Levin and Weiss (9). Since excess amounts of CDR were not used in our assay conditions, it could be expected that lower IC_{50} values were obtained. Filburn *et al.* (28) have shown that the magnitude of neuroleptic inhibition of CDR-stimulated phosphodiesterase activity depends not only on inhibitor concentration but also on CDR concentration. Therefore, since lower CDR concentrations

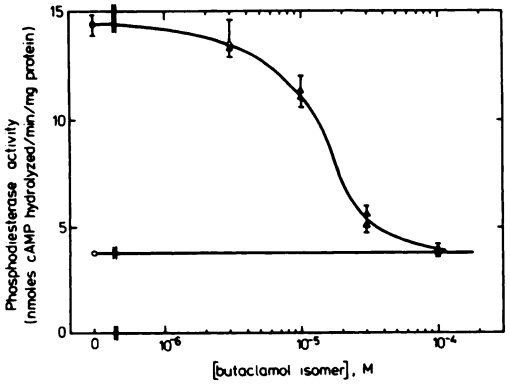

FIG. 1. *Inhibition of CDR-stimulated phosphodiesterase by* (+)- *and* (−)-*butaclamol*

Phosphodiesterase activity was measured by a modification of the Thompson and Appleman procedure (35). All reactions were carried out in 0.4 ml volumes at pH 8.0 containing 5 mM $MgCl_2$, 40 mM Tris, 0.1 mM EGTA, 4.0 mM 2-mercaptoethanol (0.01 mM cAMP, 200,000 cpm [^{3}H] cAMP), 10.2 μg of CDR-depleted phosphodiesterase and drugs as indicated. Tubes containing CDR (100 ng/ml) also contained 0.3 mM $CaCl_2$. Incubations were conducted for 10 minutes at 30° and terminated by boiling for 45 seconds. Conversion of 5′-AMP reaction product to adenosine was accomplished by the addition of 0.1 ml of 5′-nucleotidase (0.5 ng/ml) for an additional 10 minute incubation at 30°. This reaction was terminated by the addition of 1.0 ml of methanol, and adenosine was separated from unhydrolysed cAMP on a 1.0 ml column of BioRad resin, AG 1-X2. All samples were counted by liquid scintillation and corrected for per cent recovery of adenosine which ranged from 85 to 95%. Background values containing no enzyme ranged from 1.0 to 1.5% of total counts added. (△) CDR-stimulated phosphodiesterase in the presence of (+) butaclamol, (▲) CDR-stimulated phosphodiesterase in the presence of (−) butaclamol, (●) CDR-stimulated phosphodiesterase activity in the absence of butaclamol, (○) basal level phosphodiesterase in the absence of CDR and butaclamol. The error bars shown represent the range of experimental values measured in triplicate.

TABLE 1

Effects of neuroleptics on activated and unactivated phosphodiesterase of bovine brain

Phosphodiesterase activity was measured as described in the legend to Fig. 1. The IC_{50} (activated) values were calculated as the concentration necessary to produce 50% inhibition of CDR-stimulated phosphodiesterase. The addition of 0.3 mM $CaCl_2$ to phosphodiesterase in the absence of CDR caused a 10% reduction in activity. The IC_{50} (unactivated) values were estimated as the concentration of drug required to induce a 50% reduction of phosphodiesterase in the absence of calcium and CDR.

Class of drug	Drug	Activated	Unactivated	Average clinical dose	Octanol/aqueous buffer Partition coefficient log *P*
		IC_{50}, μM	IC_{50}, μM	(mg/day)	
Phenothiazine	1) chlorpromazine	6.0	100.0	550.0	4.04[a]
Thioxanthene	2) cis-chlorprothixene	2.4	30.0	315.0	4.6[a]
	3) trans-chlorprothixene	3.0	22.0	—	4.6[a]
	4) cis-flupenthixol	5.0	>100.0	5.5	4.25
	5) trans-flupenthixol	3.3	>100.0	inactive	4.25
Butyrophenone	6) haloperidol	41.0	100.0	8.0	3.36
	7) spiroperidol	17.0	100.0	0.5	3.03
Diphenylbutylamine	8) pimozide	0.7	>100.0	4.5	4.88[a]
Dibenzodiazepine	9) clozapine	19.0	>100.0	320.0	3.32
Benzamide	10) sulpiride	>100.0	>100.0	500.0	−0.50
Benzocycloheptapyridoisoquinoline	11) (+)-butaclamol	15.0	>100.0	40.0	4.35[a]
	12) (−)-butaclamol	15.0	>100.0	inactive	4.35[a]

[a] Log *P* calculated from log *P* pH 6.0 and pka.

and lower substrate concentrations were used in this study, a direct comparison with the data of Levin and Weiss is not possible. Table 1 also presents data for the average dose of these drugs used in clinical practice (29, 30). Linear regression analysis gives a very poor correlation coefficient ($r = 0.018$; $p > 0.05$) between these two sets of data, even if the inactive isomers are omitted. In addition, the concentration of these drugs in the plasma of patients receiving neuroleptics is in the nanomolar range and this is several hundred times less than the IC_{50} values for CDR inhibition (31).

The neuroleptic drugs as a class have been shown to influence a vast array of membrane-associated events and enzyme activities (for review see ref. 32). These effects are generally attributed to their high lipid (and hence membrane-) solubility (33). In order to examine the relationship between the inhibition of CDR and lipid solubility, the octanol:water partition coefficients were determined for all of the neuroleptic compounds tested. The octanol:water partition coefficient is considered an index of lipid solubility for a molecule and the partition coefficients of these drugs correlate very well with the IC_{50} values obtained for CDR inhibition. This correlation is expressed in Fig. 2 where the $\log \frac{1}{IC_{50}}$ is plotted against the log of *P*, the partition coefficient. The correlation coefficient is $r = 0.786$ ($p < 0.005$), indicating that the inhibition of CDR is closely related to the lipid solubility of these drugs. It is interesting to note that sulpiride has very low lipid solubility (log $P = -0.50$) and was the only neuroleptic drug tested which had little or no inhibitory effect on CDR. Levin and Weiss (34) have recently shown that the binding of (^{3}H)-trifluoperazine is specific to CDR and not to other calcium binding proteins, although low affinity binding to Troponin C does occur. These data, and the calcium dependence of neuroleptic binding by CDR suggests that an electrostatic interaction may be involved. However, CDR undergoes a conformational change upon binding calcium (11) and it is quite possible that this change exposes a hydrophobic region of CDR which can then bind lipid soluble neuroleptic drugs in a non-stereospecific manner rendering the CDR protein unable to activate phosphodiesterase.

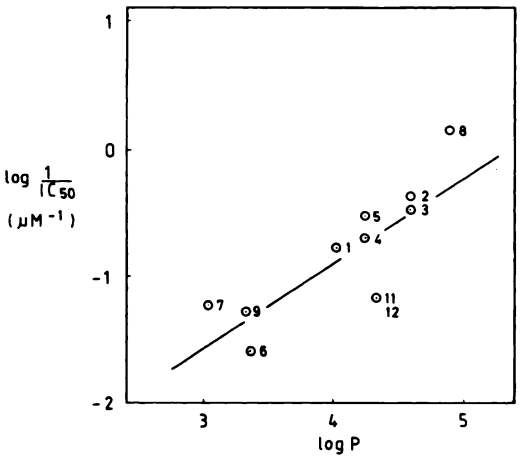

FIG. 2. *Correlation between inhibition of CDR-stimulated phosphodiesterase by neuroleptics and the octanol:water partition coefficients for these drugs*

Ordinate: the inverse of the IC_{50} values (μM) for neuroleptic inhibition of CDR-stimulated phosphodiesterase activity taken from Table 1 (log scale). Abscissa: the logarithm of the partition coefficients for the neuroleptic agents shown in Table 1. *P* refers to the undissociated form of the drug.

In conclusion, the minimum criteria which need to be fulfilled before a neuroleptic effect can be considered at all specific or relevant are: 1) that the drugs should be effective in nanomolar concentrations, and 2) that (+)-butaclamol should be stereospecifically active (31). Neither of these criteria is fulfilled for neuroleptic inhibition of CDR-stimulated phosphodiesterase activity and hence it is concluded that this effect is unrelated to the therapeutic action of these drugs.

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