J. Pharm. Pharmac., 1977, 29, 664–669 Received June 13, 1977

A model for the selective mode of action of the irreversible monoamine oxidase inhibitors clorgyline and deprenyl, based on studies of their ability to activate a Ca^{2+}—Mg^{2+} ATPase in defined lipid environments

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Clorgyline and deprenyl activated the pure Ca^{2+}-Mg^{2+} ATPase from rabbit muscle sarcoplasmic reticulum when it was in a defined lipid environment of either dimyristoyl lecithin or dipalmitoyl lecithin, apparently by fluidizing the phospholipid that surrounds the protein (the phospholipid annulus). Activation was only significant at temperatures when the annulus placed rigid constraints upon the protein. There was a two order of magnitude difference in the concentration at which the drugs achieved their effect which is thought to be related to their abilities to perturb the phospholipid annulus since it could not be attributed simply to the small differences in their partition coefficients. Clorgyline, which was more potent than deprenyl at effecting fluidization of the phospholipid annulus of the Ca^{2+}-Mg^{2+} ATPase in the defined lipid environments, activated species 'B' monoamine oxidase at concentrations at which it began to inhibit the species 'A' enzyme. Deprenyl did not activate the species 'A' enzyme at any concentration tested. It is suggested that, in tissues where there are multiple forms of mitochondrial monoamine oxidase, the selective action of clorgyline and deprenyl arises from a modification of the single inhibitor binding site on a protein species to yield species 'A' enzyme and species 'B' enzyme. One form of this modification to give the species 'A' monoamine oxidase would be a masking of the inhibitor binding site by lipid such that it could be readily penetrated by clorgyline but less readily so by deprenyl. The other form of the modification would yield the species 'B' enzyme, which like the 'chaotrope-treated' enzyme and presumably 'nascent' monoamine oxidase, would expose the inhibitor binding site to the aqueous environment in which deprenyl would be the more efficacious inhibitor.

Clorgyline (*N*-methyl-*N*-propargyl-3-(2,4-dichlorophenoxypropylamine)) and deprenyl (phenylisopropylmethylpropinylamine) are both irreversible inhibitors of mitochondrial monoamine oxidase (Johnston, 1968; Knoll & Magyar, 1972). In tissues where there are two forms of mitochondrial monoamine oxidase activity, each with distinct substrate specificities, clorgyline preferentially inhibits the species 'A' activity and deprenyl the species 'B' activity (Neff & Goridis, 1972; Houslay & Tipton, 1974; Tipton, Houslay & Mantle, 1976). This selective inhibition of the two enzyme species is characterized by a separation in the effect of inhibition by some two orders of magnitude in drug concentration.

While a solubilized enzyme preparation can be obtained which still demonstrates these effects (Houslay & Tipton, 1973a), removal of lipid material from the enzyme yields a preparation which has no such substrate-related differences in sensitivity to the two drugs (Houslay & Tipton, 1973a; Tipton, Houslay & Garrett, 1973; Tipton & others, 1976), rather it is relatively insensitive to clorgyline and highly sensitive to deprenyl, thus resembling species 'B' activity; however its substrate specificity appears to encompass both 'A' and 'B' enzyme species. Also, solubilization has been shown to change the conformation of the protein (Houslay & Tipton, 1975).

Thus it would seem not unreasonable to assume that the lipid environment of the two enzyme species is at least in part responsible for the selective effects of the two drugs.

These compounds are now shown to cause substantial activation of a pure Ca^{2+}–Mg^{2+} ATPase protein in a defined lipid environment. This activation is considered to be achieved by an increase in the fluidity of the phospholipid surrounding the protein (the phospholipid annulus). The concentration at which the drugs exerted their effect differed

markedly. This difference is discussed in terms of the drugs mode of action as selective inhibitors of mitochondrial monoamine oxidase.

MATERIALS AND METHODS

An homogeneous Ca^{2+}–Mg^{2+} ATPase protein from rabbit skeletal muscle sarcoplasmic reticulum was isolated as described by Warren, Toon & others (1974b). The Ca^{2+}–Mg^{2+} ATPase was found to have 35 molecules of endogenous lipid per molecule of protein (Warren, Toon & others, 1974a, c). The endogenous lipid molecules were then replaced by dioleoyl lecithin (DOL), and titrated with either dipalmitoyl lecithin (DPL) or dimyristoyl lecithin (DML) to yield complexes where the lipid composition was 95% DML or DPL, + 4% DOL + 1% sarcoplasmic reticulum lipid (Warren & others, 1974b).

Ca^{2+}-stimulated ATPase activity was followed as described by Warren & others (1974a). Clorgyline and deprenyl were added to the assay cuvette. At the concentrations of drugs used, the effects observed were not due to any interaction with the coupling enzymes or any non-specific action (there was no evidence of non-linearity in the timecourse of the assays).

Esr spectra were recorded on a Varian spectrometer with the precautions of Hesketh, Smith & others (1976). The lipids were labelled with 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) at a molar ratio of 1:100. The TEMPO parameter f (defined as the fraction of TEMPO bound to DML, 1 mol % of the total lipid in the present study) was determined from the high-field line of the first derivative of the paramagnetic spectrum which was split into two components reflecting the fraction of the probe in the polar (P) and hydrocarbon (H) compartments. f was thus determined from the amplitude of the P and H components as $f = H/(H + P)$ (Shimshick & McConnell, 1973).

Rat liver mitochondrial outer membranes were prepared by the method of Sottocasa, Kuylenstierna & others (1967), as described by Houslay & Tipton (1973b) but using potassium phosphate buffers instead of tris, which inhibited the two enzymes selectively (Fowler, Callingham & Houslay, 1977). Beef heart mitochondria were prepared as described by Ramsey & Tubbs (1975).

Benzylamine oxidation was followed spectrophotometrically (Houslay & Tipton, 1973b) and β-phenethylamine and 5-hydroxytryptamine oxidation were followed using a radioassay as described by Callingham & Laverty (1973). All monoamine oxidase assays were performed at 1 mM substrate concentration at 30°, and results were linear over the times used.

Partition coefficients of the drugs were measured between hexane and 100 mM potassium phosphate buffer, pH 7·2, after up to 40 min shaking to achieve equilibration. The partitioning was followed optically with the aid of linear calibration curves. Partition coefficients, expressed as the ratio of (drug in hexane phase)/(drug in aqueous phase), were essentially independent of drug concentration between 500 $\mu g\ ml^{-1}$ and 5 $mg\ ml^{-1}$.

[^{3}H]-5-HT was from the Radiochemical Centre, Amersham and [2-^{14}C]phenethylamine from NEN, Dreieichenhain, Germany. Clorgyline (M and B 9302) was a gift from May and Baker Ltd, Dagenham, Essex, and deprenyl was a gift from Dr T. J. Mantle, Department of Biochemistry, University of Cambridge. All other chemicals were of A.R. grade from either BDH or Fisons.

RESULTS

Clorgyline and deprenyl both effected a reversible 8 to 10 fold increase in the Ca^{2+}–Mg^{2+} ATPase activity, when the ATPase protein was in a complex with either DML or DPL. However this degree of activation required very different concentrations of the two drugs. Dose response curves (Fig. 1) clearly demonstrate that, irrespective of the lipid-protein complex (DML-ATPase or DPL-ATPase), clorgyline begins to activate and causes maximal activation of the enzyme at concentrations some two orders of magnitude lower than deprenyl. The Ca^{2+}–Mg^{2+} ATPase activity of sarcoplasmic reticulum, or of the DOL-ATPase complex was relatively unaffected by clorgyline (10^{-4} M) or deprenyl (10^{-2} M) at either of the temperatures used for assay, less than 10% activation being noted.

To investigate whether the drugs had an effect on the DML-bilayer, the partitioning of the spin label TEMPO into a DML bilayer was studied. Fig. 2 demonstrates the highly cooperative phase transition at 23° undergone by pure DML, however, the addition of clorgyline or deprenyl at concentrations giving an 8-fold stimulation of Ca^{2+}–Mg^{2+} ATPase activity of the DML-ATPase complex caused an increase in the fluidity of the lipid bilayer and depressed the lipid-phase transition by about 3°. Indeed, an increase in bilayer fluidity, as indicated by an increase in the TEMPO parameter f, was noticeable at the same concentrations as the onset of activation of the DML-ATPase.

The drugs achieve their activation of the DML-and

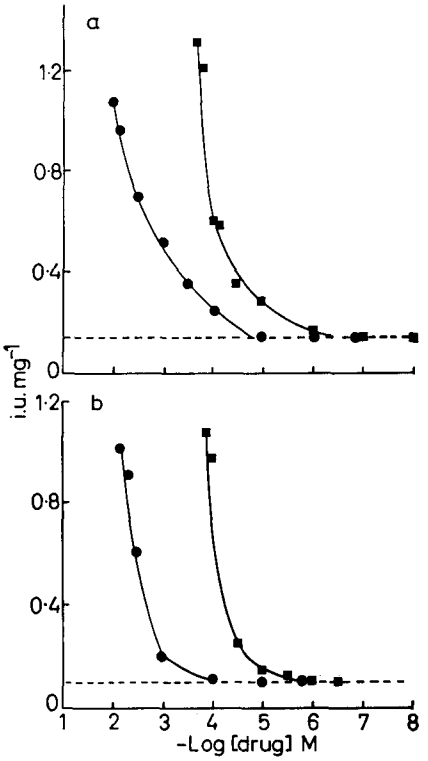

FIG. 1. Activation of lipid-Ca^{2+}-Mg^{2+} ATPase complexes by clorgyline and deprenyl. These results demonstrate the effect of final concentrations of clorgyline and deprenyl in reversibly activating the Ca^{2+}-Mg^{2+} ATPase activity (i.u. mg^{-1}) of a protein in a defined lipid environment at temperatures where the rigidity of the phospholipid annulus normally allows little activity. The effect of clorgyline (■) and deprenyl (●) on (a) DML-ATPase complexes assayed at 26° and (b) on DPL-ATPase complexes assayed at 30°.

DPL-ATPases at concentrations separated by some two orders of magnitude. This effect may have been due to a difference in their partition coefficients, however when these were determined at various temperatures they differed only some 2 to 4 fold (Table 1).

Table 1. *Partition coefficients for clorgyline and deprenyl.* The partition of the drugs between hexane and 100 mM potassium phosphate buffer, pH 7·2 is shown at the various temperatures related to the assays studied.

Drug	λmax (nm)	Partition coefficient °C 20°	25°	30°
Clorgyline	230	83 ± 8 (6)	170 ± 61 (4)	346 ± 95 (6)
Deprenyl	217	45 ± 17 (8)	69 ± 8 (6)	81 ± 32 (16)

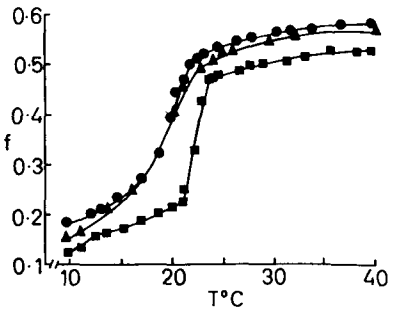

FIG. 2. Effect of clorgyline and deprenyl on the phase transition of DML. The fraction of TEMPO (1 mol % of the total lipid) bound to pure DML (10 mg ml^{-1}) is plotted as a function of temperature. This fraction, the TEMPO parameter f, was determined from the amplitudes of the two components P and H of the high field line of the second derivative of the paramagnetic spectrum as discussed in the Methods section. The TEMPO partition parameter f is plotted against temperature for pure DML (■) DML + 10^{-4}M clorgyline (▲) and DML + 10^{-2}M deprenyl (●).

Lyles & Callingham (1975) have reported that clorgyline at extremely low concentrations could activate the 'B-enzyme' of rat heart mitochondrial monoamine oxidase. This also appears to be true for the 'B-enzyme' activity in mitochondria from rat liver and beef heart. Significant activation of the 'B-enzyme' occurred at concentrations where clorgyline began to inhibit the 'A-enzyme' (Fig. 3). With deprenyl the converse was not observed for the 'A-enzyme' when 'B-enzyme' inactivation began (Fig. 4).

DISCUSSION

The activation of the DML and DPL-ATPase by the drugs was the more striking because at the temperatures chosen for assay the enzyme alone exhibited little ATPase activity. This low activity has been attributed to the constraint imposed on the protein by the relatively immobilized shell of lipids that immediately surround the protein to form the phospholipid annulus, which is in a rigid state at the temperatures of the assay (Warren & others, 1974a; Hesketh & others, 1976). The drugs may have achieved their activation by fluidizing the rigid lipid annulus and relieving the constraint upon the protein in a similar fashion to the local anaesthetic benzyl alcohol, which reduces the rigidity of the annulus and activates the enzyme (Hesketh & others, 1976). This is entirely consistent with the observation that the ATPase activity of sarcoplasmic reticulum or DOL-ATPase is relatively unaffected by clorgyline and deprenyl, for the enzyme in both these preparations (unlike the DML or

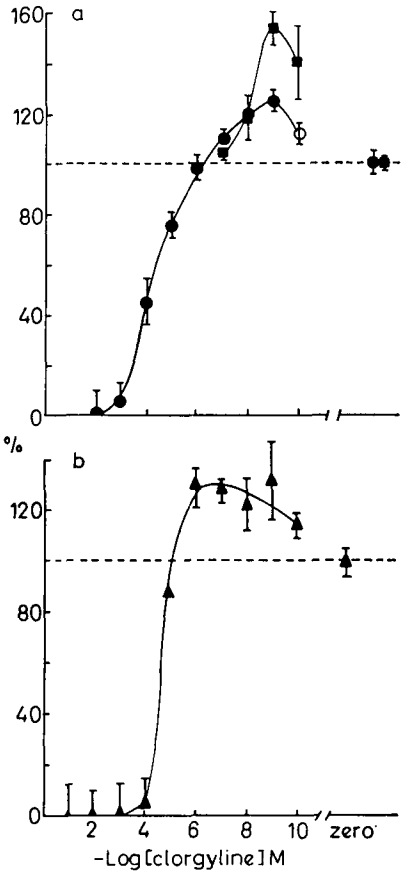

FIG. 3. Effect of clorgyline on species 'B' MAO. (a) Rat liver mitochondrial monoamine oxidase species 'B' activity (%) in the presence of clorgyline was followed using β-phenethylamine (●) or benzylamine (■) as substrate. Both substrates were assayed at 30°; however with β-phenethylamine the enzyme was preincubated with clorgyline for 15 min before assay. With benzylamine a 5 min preincubation was used before initial rate determination. (b) Beef heart mitochondria. β-Phenethylamine was used as substrate. Data are given as s.d. (n = 3).

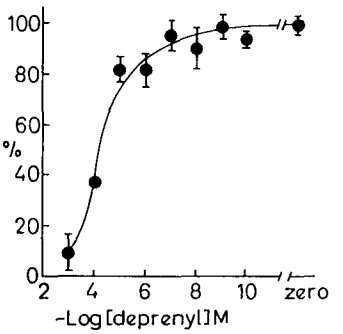

FIG. 4. Effect of deprenyl on species 'A' MAO. Rat liver mitochondrial monoamine oxidase activity (%) was followed using 5-HT as substrate, after a preincubation with deprenyl for 15 min at 20°, with subsequent assay at 30° under identical conditions to those for Fig. 3a. Data are given as s.d. (n = 3).

DPL-ATPase complexes) is in a fluid lipid environment (Lee, Birdsall & others, 1974) and hence exhibits maximal enzyme activity. This suggests that the effect of clorgyline and deprenyl on the DML and DPL-ATPase complexes is at the level of the phospholipid annulus and is not a protein mediated event. Further support for this is the observation that both drugs can fluidize a DML-bilayer, a depression of the transition temperature being detected by the partitioning of the spin label TEMPO, as well as an increase in fluidity as indicated by an increase in the TEMPO parameter f (Fig. 2). The local anaesthetic benzyl alcohol, which activates the DML and DPL-ATPase causes similar shifts (Hesketh & others, 1976).

These results are consistent with previous postulates that it is the rigidity/fluidity of the lipid annulus which determines the Ca^{2+}–Mg^{2+} ATPase activity and that of other proteins that penetrate into the bilayer (see e.g. Hesketh & others, 1976; Houslay, Hesketh & others, 1976; Houslay, Warren & others, 1975). Thus the activity of a Ca^{2+}–Mg^{2+}–ATPase in defined DML/DPL bilayers at temperatures where the rigidity of the annulus lowers the ATPase activity should be a very sensitive means of detecting small molecules which partition into the bilayer to effect a perturbation of it and the lipid annuli surrounding proteins that penetrate the bilayer.

It is apparent that clorgyline at low concentrations is much more effective than deprenyl at perturbing both bilayer fluidity and lipid-protein interactions. This presumably is in part due to structural features of the clorgyline molecule affecting lipid ordering in the bilayer, for there are only small differences in the partition coefficients of clorgyline and deprenyl.

The markedly different concentrations at which these two drugs perturb lipid bilayers may account for their selective mode of action upon mitochondrial monoamine oxidase. It has been proposed that the 'A' and 'B' species of the enzyme found in rat liver, human brain and other tissues are the result of modification of a single protein by lipid (Houslay & Tipton, 1973a) or two different proteins by lipid (Houslay, 1975), this modification by lipid is considered as being responsible for the

selective effects of clorgyline and deprenyl, and also differences in thermostability, electrophoretic behaviour and the substrate specificity of the two species (see Tipton & others, 1976). Both enzymes have an inhibitor binding site for clorgyline or deprenyl where the drugs can presumably interact with the flavin component of the enzyme (see e.g. Chuang, Patek & Hellerman, 1974; Oreland, Kinemuchi & Stigbrand, 1973; Collins & Youdim, 1975).

From the present and previous findings it is possible to propose a model to account for the observed selective inhibitory action of clorgyline and deprenyl on mitochondrial monoamine oxidase. In this model the 'A-enzyme' is envisaged as having a single inhibitor binding site for clorgyline and deprenyl that is isolated from the aqueous environment by the lipids of the bilayer, i.e. the inhibitor binding site on the protein is buried in the bilayer membrane. Thus, for the inhibitor to act on the 'A-enzyme' it must first enter the bilayer and then penetrate the immobilized lipids of the annulus to interact at the binding site on the protein.

The 'B-enzyme' would have an identical inhibitor binding site, but this would be freely exposed to the aqueous environment. A similar exposed binding site would presumably be displayed by solubilized enzyme preparations since their protein surfaces, previously buried in the bilayer, are now exposed to the aqueous environment. Thus, proteins solubilized by Triton X100 treatment and then either treated with 'chaotropic agents' or passed through DEAE-cellulose columns to severely deplete them of lipid (and detergent) show aggregation phenomena characteristic of membrane proteins with exposed hydrophobic faces and display the inhibitor sensitivity of the 'B-enzyme', i.e. they are relatively sensitive to deprenyl and insensitive to clorgyline (Houslay & Tipton, 1973a; Tipton & others, 1976). In all systems where an inhibitor binding site is presumably freely exposed to the aqueous environment deprenyl inhibits the enzyme at much lower concentrations than clorgyline (Tipton & others, 1976).

The reverse of this situation, that is the inhibitor binding site of the 'A-enzyme' is masked by lipid can be used to explain clorgyline's action because it can far more easily enter the bilayer (higher partition coefficient) and perturb lipid-protein interactions by fluidizing the bilayer and the lipid annulus (activates DML/DPL-ATPase), and thus it might well be expected to interact with the enzyme at far lower concentrations than deprenyl. Lipid masking this site would affect the dielectric constant of the inhibitor site environment and hence affect the interaction of the inhibitor with the enzyme. Such a model implies that the first order rate constants for inhibition of the 'A' and 'B' enzymes by clorgyline and also by deprenyl would be very different and indeed this does appear to be so (T. J. Mantle, personal communication).

This comparatively simple model offers an explanation of the selective action of the monoamine oxidase inhibitors clorgyline and deprenyl on the enzyme in intact membranes and when solubilized. It also rationalizes the observations that detergent treatment, under conditions that either do not solubilize the enzyme or bring it into solution as a protein-lipid-detergent complex, reduces the sensitivity of the enzyme to clorgyline (Houslay & Tipton, 1973a; Houslay & Tipton, unpublished observations). This treatment presumably affects the lipid interaction at the inhibitor binding site on the 'A-enzyme', tending to make it more hydrophilic in character.

Activation of the 'B-enzyme' by clorgyline at very low concentrations where the onset of inhibition of the 'A-enzyme' begins, may well be attributable to its fluidizing effect on the lipid annulus of the enzyme. This observation strongly supports that clorgyline can be expected to interact with the 'A-enzyme's' inhibitor binding site at low concentrations through its ability to perturb the bilayer much more effectively than deprenyl.

The insertion of a purified, solubilized monoamine oxidase protein into defined lipid bilayers should in the final analysis solve the problems of substrate specificity, inhibitor sensitivity and other phenomena associated with multiplicity. This will pose problems such as lipid specificity, and changes in the conformation of the protein which may become altered by detergent treatment (Houslay & Tipton, 1975). As a prelude to any such study there are advantages in investigating a biochemically and physically well-defined system such as the Ca^{2+}–Mg^{2+} ATPase from rabbit muscle sarcoplasmic reticulum. By elimination of any direct action on the protein by clorgyline or deprenyl, it has been possible to separate the observations that the drugs have an action on monoamine oxidase at the level of the phospholipid annulus.

As clorgyline is a very effective perturbing agent of the lipid bilayer, it is likely to have local anaesthetic properties and also to perturb membrane localized systems in the cns other than monoamine oxidase. Thus caution is required in attributing any *in vivo* biochemical or behavioural

results using 'A-enzyme' selective inhibitors entirely to inhibition of the 'A-enzyme'.

Acknowledgements

My thanks for the excellent technical assistance of Mr R. W. Palmer and to Dr T. R. Hesketh for help with the esr studies. I am grateful to Drs G. B. Warren and J. C. Metcalfe for discussion and to Professor A. A. Eddy for laboratory facilities and encouragement.

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