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Differences in the inhibitory effect of Cd^{2+}, Mn^{2+} and Al^{3+} on the uptake of dopamine by synaptosomes from forebrain and from striatum of the rat

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Trace metals influence brain function and behaviour although the underlying mechanism is at present poorly understood [1]. Our interest in their actions have led us to investigate the effects of Al^{3+}, Cd^{2+} and Mn^{2+} *in vitro* on synaptosomal function. These metals appear to exert selective effects since we have found that Al^{3+} and Cd^{2+} were more effective than Mn^{2+} in inhibiting synaptosomal high affinity choline uptake, while only Cd^{2+} was a potent inhibitor of the synaptosomal sodium–potassium activated adenosine triphosphatase [2]. These metals were also found to inhibit synaptosomal uptake of γ-aminobutyric acid at different concentrations for each of the metal while not particularly affecting uptake of either 2-deoxyglucose or phenylalanine [3]. We now report that each of these metals also inhibit dopamine uptake in a different manner.

Materials and methods

Purified synaptosomes were prepared from the forebrain and striatum of adult male rats using sucrose–Ficoll gradients as described previously [2, 4]. Dopamine uptake was determined as previously described [2, 4] based on the method of Nicklas, Puszkin and Berl [5] using [^{3}H]dopamine hydrochloride (sp. act. 5.0 Ci/mmole, final concentration 0.75 μM). Neither the addition of 1 mM ascorbate nor of nialamide as a monoamine oxidase inhibitor [4] made any difference to the uptake rates. Uptake, after initial experiments on the time-course, was restricted to 4 min to minimise effects of the metals, if any, on storage processes.

Results

Forebrain synaptosomes. The effects of Al^{3+}, Cd^{2+} and Mn^{2+} on dopamine uptake were examined in the presence or absence of 1 mM Ca^{2+} to determine whether Ca^{2+} antagonised these effects (for discussion, see [6]). The uptake of dopamine by forebrain synaptosomes increased with time. The presence of each of the metal ions (including Ca^{2+}) limited the increase in uptake to periods of 6 min or less, depending on the metal. Except in the case of Al^{3+}, the incubation of Ca^{2+} with each of the other metal ions resulted in a further decrease of this period of increased uptake. The presence of Ca^{2+}, at 1 mM, resulted in a slight inhibition of the rate of dopamine uptake from 8.7 ± 0.9 (10) to 7.6 ± 1.1 (14) pmole/min/mg protein (mean ± S.D., with number of experiments in parentheses; $P < 0.02$).

The effects of different concentrations of the metals were then determined over a 4 min period which was selected since the observed rates at this convenient period closely approximated initial rates. The rates have been shown not to be altered by the addition of nialamide strongly suggesting that intraterminal events did not influence uptake during this short period [4].

Cd^{2+} inhibited the uptake of dopamine in a dose-dependent fashion at concentrations higher than 10^{-5} M (Fig. 1A). The inclusion of Ca^{2+} did not influence the inhibitory effects of Cd^{2+}. The concentration at which 50% inhibition of uptake occurred (IC_{50}) was similar (4×10^{-4} M) in the absence or presence of Ca^{2+}. Mn^{2+} (Fig. 1B) inhibited the uptake of dopamine in a dose-dependent manner at concentrations higher than 10^{-3} M. The inclusion of Ca^{2+} did not influence this inhibition at these concentrations, IC_{50} being around 2×10^{-3} M in both cases. However, at 10^{-4} M Mn^{2+}, the inclusion of Ca^{2+} stimulated dopamine uptake slightly. Depending on its concentration, Al^{3+} either stimulated of inhibited dopamine uptake (Fig. 1C). This dual action was seen as an apparent increase of the baseline of the dose–response curve (from 100 to 128%). Paradoxically, Ca^{2+} enhanced the inhibitory effects of Al^{3+} at concentrations lower than 10^{-3} M and decreased the IC_{50} from 4 to 2.4×10^{-4} M (Fig. 1C).

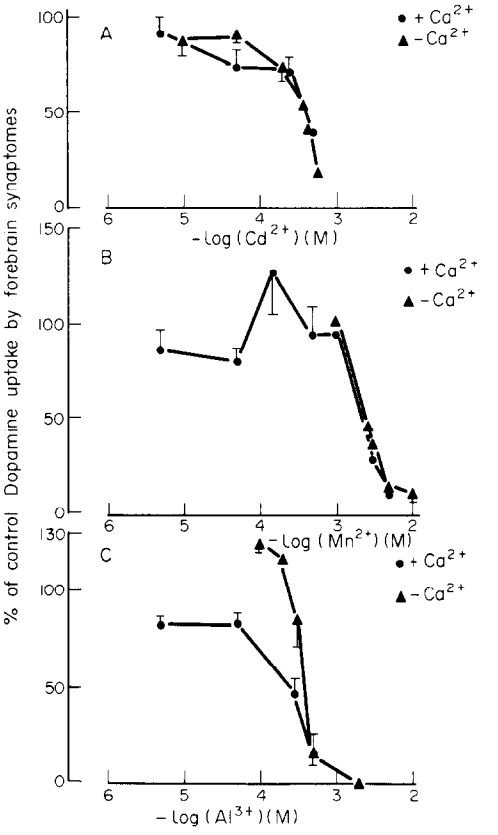

Fig. 1. Effects of Cd^{2+}, Mn^{2+} and Al^{3+} on dopamine uptake by forebrain synaptosomes. Values are means ± S.D. for 3–5 separate experiments; in each experiment determinations were performed in triplicate.

Striatal synaptosomes. To determine whether there were regional variations in the sensitivity of dopamine uptake to these metals, the experiments were repeated this time using synaptosomes isolated from the striatum, a region highly enriched in dopaminergic nerve endings. The rate of uptake of dopamine in the absence or presence of Ca^{2+} was similar (38.1 ± 5 (5) and 36.3 ± 6.4 (5) pmole/min/mg protein, respectively; $P > 0.1$). Cd^{2+} inhibited dopamine

uptake in a dose-dependent fashion at concentrations greater than 10^{-4} M (Fig. 2A). The presence of Ca^{2+} slightly shifted the dose–response curve of Cd^{2+} to higher concentrations, indicating an antagonistic effect. The IC_{50} for Cd^{2+} was increased from 2.5 to 4×10^{-4} M. Mn^{2+} also inhibited dopamine uptake in a dose-related manner at concentrations greater than 10^{-4} M. In this case, the antagonistic influence of Ca^{2+} was clearly seen as a large shift of the dose–response curve to higher concentrations, with the IC_{50} being increased from 1.7 to 6.3×10^{-3} M in the presence of Ca^{2+} (Fig. 2B). Al^{3+} stimulated dopamine uptake at low concentrations, with maximal stimulation at 10^{-4} M and inhibited uptake at higher concentrations (Fig. 2C). Ca^{2+} had an antagonistic effect, since the maximal stimulation was seen at a higher concentration of Al^{3+} (2.5×10^{-4} M; see Fig. 2C). Despite an apparently similar IC_{50} (4×10^{-4} M, Al^{3+}) it was obvious that the dose–response curve in the presence of Ca^{2+} and that obtained in its absence were dissimilar (Fig. 2C).

Discussion

The inhibitory effects of Cd^{2+}, Mn^{2+} and Al^{3+} on dopamine uptake by forebrain synaptosomes were easily distinguishable from one another, with Mn^{2+} being the least effective. Although the IC_{50} values for Cd^{2+} and Al^{3+} were similar, the effects of these two could be distinguished on the basis of antagonism by Ca^{2+} of Al^{3+}, but not Cd^{2+} (Figs. 2A and 2C). These metal ions also affected striatal and forebrain synaptosomes differently. Their inhibitory effect on dopamine uptake by striatal synaptosomes was, in all cases, antagonized by Ca^{2+}, with a definite resultant increase in the IC_{50} values for both Mn^{2+} and Al^{3+}. Furthermore, the antagonistic effect of Ca^{2+} on Al^{3+} was more complex than on the other two metal ions.

We have previously reported [2] that Cd^{2+}, at 10^{-5} M, completely abolished synaptosomal Na–K-ATPase activity but had no pronounced effect on choline uptake into synaptosomes. The data presented in this paper clearly establish the lack of correlation between the inhibitory effects of Cd^{2+}, Mn^{2+} and Al^{3+} on synaptosomal dopamine uptake and on synaptosomal Na–K-ATPase activity (see Table 2 of Ref. [2]) and support previous suggestions that the electrochemical potential gradient, maintained by the activity of Na–K-ATPase, cannot be the only driving force responsible for the synaptosomal uptake of amines [2].

Colburn and Maas [7] have suggested that formation of ATP–metal–catecholamine chelate may play an important role in the regulation of uptake and storage of catecholamines in the nerve-endings. However, it seems unlikely that the inhibitory effects of Cd^{2+}, Mn^{2+} and Al^{3+} on synaptosomal dopamine uptake can be entirely attributed to metal–amine chelate formation because no marked difference in uptake occurred in the presence or absence of 1 mM Ca^{2+} although Colburn and Maas [7] have demonstrated that Ca^{2+} chelates with noradrenaline.

We conclude that Cd^{2+}, Mn^{2+} and Al^{3+} inhibit uptake of dopamine into synaptosomes, each in a different manner. The present results together with previous observations that these metals exert selective effects on synaptosomal Na–K-ATPase and choline uptake [2] strongly suggest that these metal ions interact differentially with the membrane. This conclusion is compatible with our observations that the inhibition of dopamine uptake into forebrain synaptosomes by Cd^{2+}[8], Mn^{2+} [9] and Al^{3+} (J. C. K. Lai, unpublished data) show dissimilar kinetic features.

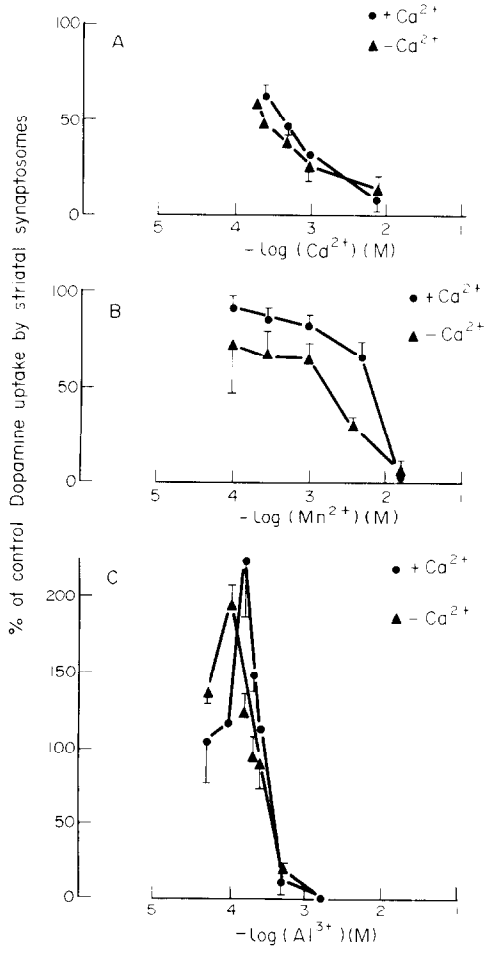

Fig. 2. Effects of Cd^{2+}, Mn^{2+} and Al^{3+} on dopamine uptake by striatal synaptosomes. Values are means ± S.D. and derived from 3 experiments. In each experiment 4 determinations were performed.

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Hydralazine as an inhibitor of lysyl oxidase activity

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Many of the unique properties of connective tissue, such as its high tensile strength and the low solubility of its constitutive structural proteins, are due to the oxidative deamination of the ε-amino group of specific lysine and hydroxylysine residues in collagen or elastin by lysyl oxidase to form the aldehyde precursors of the cross-links in these important structural proteins (Ref. 1 and references therein).

Kucharz and Dróżdż [2] found an increase of soluble collagen and a decrease of insoluble collagen in the skins of guinea pigs with a collagen-disease-like syndrome produced by long-term hydralazine administration, and they suggested the possibility of inhibition of lysyl oxidase with hydralazine.

In this paper we demonstrate the *in vitro* inhibition of lysyl oxidase activity by hydralazine.

Materials and methods

Hydralazine hydrochloride was purchased from the Tokyo Kasei Kogyo Co. Ltd., Tokyo. [4,5-^{3}H]Lysine (60–80 Ci/mmole) was obtained from the New England Nuclear Corp., Boston, MA, and pyridoxal-phosphate from the Sigma Chemical Co., St. Louis, MO. Other chemicals were reagent grade.

Lysyl oxidase was extracted from 16-day-old chick embryo aortas with 4 M urea and 0.16 M NaCl in 0.1 M potassium phosphate buffer, pH 7.4 [3]. After removing the urea by dialysis against 0.05 M potassium phosphate buffer, pH 7.4, overnight at 4°, the enzyme solution was subjected to NaCl fractionation (0–20%) to precipitate and remove the collagen co-extracted with urea into the enzyme solution. The resultant supernatant fraction was dialyzed against the same buffer and used as the enzyme solution for lysyl oxidase assay. Poor linearity of the enzyme dose curve with crude enzyme preparations was greatly improved by removing the collagen (unpublished data). The specific activity of the enzyme preparation was 997 cpm·(mg protein)$^{-1}$·hr^{-1}.

[4,5-^{3}H]Lysine-labeled collagen substrate was prepared from 16-day-old chick embryo calvaria parietal bones by essentially the same method as has been reported [4].

Lysyl oxidase activity was assayed by measuring the tritium released from ^{3}H-labeled collagen substrate following the conversion of specific lysyl residues in collagen into the corresponding δ-semialdehyde, allysine [4]. The incubation mixture contained: 0.3 ml of tritiated collagen substrate (600,000 cpm), 0.4 ml of 1 M NaCl in 0.1 M $KHPO_4$ buffer (pH 7.4), 0.3 ml of enzyme preparation, and water to 2.0 ml. The tritiated substrate was preincubated at 37° for 60 min to reconstitute the fibrils. After a 3-hr incubation at 37° in a shaking water bath, the reaction was terminated by the addition of 0.2 ml of 50% trichloroacetic acid. Tritiated water formed during the incubation was collected by vacuum distillation and 1.5 ml of the distilled water was counted with 10 ml of Bray's solution.

Results and discussion

Figure 1 shows the inhibition of lysyl oxidase activity by hydralazine at different concentrations. Fifty percent inhibition was attained at a concentration of about 30 μM. A similar degree (at about 20 μM) of inhibition was reported on partially purified prolyl hydroxylase from embryonic chick cartilage [5].

To test the reversibility of the inhibition of lysyl oxidase activity, the enzyme was incubated for 2 hr at 0° or 37° in the presence of 5 mM hydralazine and then dialyzed intensively against 0.16 M NaCl in 0.1 M potassium phosphate buffer, pH 7.4. The inhibition of lysyl oxidase activity by hydralazine was, however, essentially irreversible. Furthermore, the labeled collagen substrate was incubated

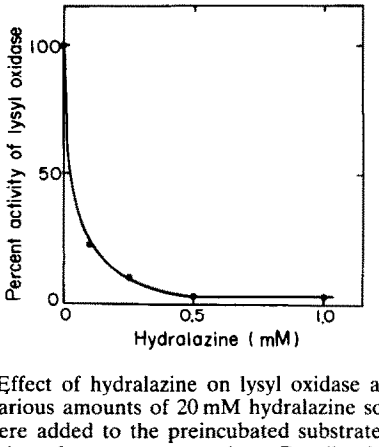

Fig. 1. Effect of hydralazine on lysyl oxidase activity *in vitro*. Various amounts of 20 mM hydralazine solution in water were added to the preincubated substrate prior to the addition of enzyme preparations. Details of the assay procedure are described under Materials and Methods.