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2-NITROIMIPRAMINE: A SELECTIVE IRREVERSIBLE INHIBITOR OF [^{3}H] SEROTONIN UPTAKE AND [^{3}H] IMIPRAMINE BINDING IN PLATELETS.

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Summary:
The effect(s) of a new imipramine analogue, 2-nitroimipramine, on high affinity [^{3}H] imipramine binding and [^{3}H] serotonin uptake in human platelets were studied. 2-Nitroimipramine was found not only to be a very potent inhibitor of [^{3}H] imipramine binding and [^{3}H] serotonin uptake but was found to irreversibly inhibit binding and uptake simultaneously. This finding supports previous observations from our laboratory and others that high affinity imipramine binding labels serotonin uptake or transport sites. 2-Nitroimipramine should prove an important tool for subsequent studies of the molecular mechanism(s) involved in the transport of serotonin and the binding of imipramine to platelet and brain membranes.

Introduction

High affinity binding sites for [^{3}H] imipramine have been recently demonstrated in rat brain (1), human platelet (2,3) and human brain (4). Recent studies from our laboratory (3, and manuscript in preparation) strongly suggest that these binding sites are involved in the well known inhibition of serotonin uptake induced by tricyclic antidepressants (5,6). 2-Nitroimipramine is one of several new nitro derivatives of imipramine which have been recently synthesized in our laboratory (manuscript in preparation). In the present study we report that in contrast to the parent antidepressant, imipramine, the inhibition of platelet [^{3}H] serotonin uptake and [^{3}H] imipramine binding by 2-nitroimipramine is irreversible in nature. These data strongly support a functional relationship between the high affinity imipramine binding site and the serotonin transport site in

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platelets. Furthermore, our data suggest that 2-nitroimipramine may be a valuable tool for future studies on the purification and characterization of the protein(s) involved in serotonin uptake.

MATERIALS AND METHODS

Preparation of platelets. Blood was collected from medication-free volunteers by venipuncture and gently mixed on ice with an anticoagulant solution containing 16 mM citrate buffer and 1 mM EDTA. Platelets were isolated at 0-4°C using previously described methods (7).

[^{3}H] Serotonin Uptake. Platelets were resuspended in the following buffer: 116 mM NaCl, 4 mM KCl, and 1.8 mM KH_2PO_4, 1.1 mM $MgSO_4$, 25 mM Tris.HCl, 10.9 mM citrate and 5.9 mM dextrose at a pH of 7.4. 425 µl of the platelets suspension (1×10^8 - 1×10^9 platelets per ml) was preincubated at 37°C for 10 min. with 50 µl of buffer or drug. At the end of the preincubation, 25 µl of [^{3}H] serotonin was added (Spec. Act. 28.9 Ci/mmole, NEN, Boston, MA). After 2.0 min. the reaction was stopped by rapidly cooling the tubes on ice. Nonspecific uptake of [^{3}H] serotonin was measured by incubating the tubes at 0-4°C. Platelets were pelleted by centrifugation at 1800 g for 15 min. at 4°C. The supernatant was aspirated and 550 µl of 0.4 N perchloric acid was added to the pellet. A 500 µl aliquot of the perchloric acid extract was counted using Aquasol (NEN, Boston, MA) in a Beckman LS 9000 Liquid Scintillation Counter.

[^{3}H] Imipramine Binding. Platelets were resuspended in the following buffer: 120 mM NaCl, 5 mM KCl and 50 mM Tris.HCl at a pH of 7.4. The incubation mixture contained 180 µl of platelet suspension (~ 1 mg protein/ml), 35 µl of buffer or drug, and 35 µl of [^{3}H] imipramine (Spec. Act. 29.8 Ci/mmole, NEN, Boston, MA). Following incubation at 0-4°C for 60 min., 100 µl of the incubate was quickly diluted in 5 ml ice-cold buffer and filtered under vacuum through Whatman GF/F filters. Filters were washed three times with 5 ml ice-cold buffer and counted in 10 ml Aquasol in a liquid scintillation counter. Specific binding was defined as total binding minus the nonspecific binding. The latter was measured in the presence of 10 µM desipramine.

RESULTS

The potency of 2-nitroimipramine as an inhibitor of [^{3}H] serotonin uptake and [^{3}H] imipramine binding in human platelets was compared with imipramine and other classical tricyclic antidepressants. The uptake of [^{3}H] serotonin into intact platelets was carried out in protein-free physiological medium to avoid the extensive binding of these drugs to proteins and to use comparable conditions for both the uptake and binding studies. The results expressed as IC_{50} values (concentration required to inhibit 50% of either the uptake or binding of radioligand) are shown in Table 1. 2-Nitroimipramine was found to be very potent inhibitor of both

TABLE I

Inhibition of [^{3}H] serotonin uptake and [^{3}H] imipramine binding by tricyclic antidepressants in human platelets

Compound	Inhibition of [^{3}H] Serotonin Uptake IC_{50} (nM)	Inhibition of [^{3}H] Imipramine Binding IC_{50} (nM)
Chlorimipramine	3	7
2-Nitroimipramine	3.5	4
Imipramine	18	7
Amitriptyline	23	7
2-OH imipramine	40	15
Desipramine	160	120

IC_{50} values are the means of three different experiments each done in triplicate with S.E.M. less than 20%.

the uptake of serotonin and binding of [^{3}H] imipramine and is comparable to imipramine and chlorimipramine which are the most potent inhibitors of both uptake and binding respectively.

To ascertain whether the inhibitory effects of 2-nitroimipramine on [^{3}H] imipramine binding and [^{3}H] serotonin uptake were reversible, like the parent compounds, 2-nitroimipramine and imipramine were incubated at low concentration (20 nM) with platelet membranes. After 60 min. of incubation at 0°C the membranes were centrifuged at 1800 g for 15 min. and the supernatant containing the free 2-nitroimipramine or imipramine was removed. The membranes were washed four times by repeated resuspension and centrifugation to eliminate any unbound drug. [^{3}H] Imipramine binding to 2-nitroimipramine-incubated washed membranes was markedly reduced even after extensive washing as is shown in Fig. 1A. Scatchard analysis of the binding data revealed a marked decrease in the number of binding sites (B_{max}) with no change in the apparent affinity (K_d). In contrast, incubation of platelet membranes with imipramine (20 nM) under the same conditions resulted in no significant change in [^{3}H] imipramine binding (Fig. 1B).

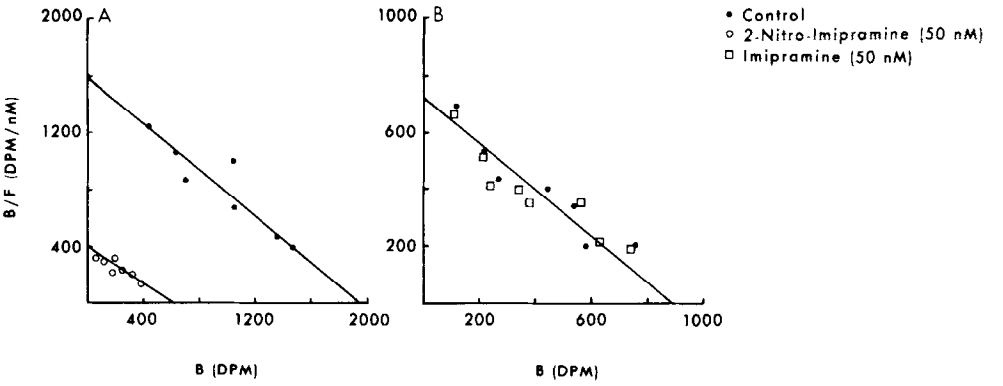

Figure 1 - Platelets were incubated with 20 nM of 2-nitroimipramine or 20 nM of imipramine for 60 min. at 0°C. The binding of [^{3}H] imipramine to the treated membranes was analyzed after removing the free drug by extensive washing of the membranes (see text). Control platelets were incubated with no drug under identical conditions and washed extensively as with treated membranes. B = specific binding of [^{3}H] imipramine, F = concentration of free (unbound) [^{3}H] imipramine.

The uptake of [^{3}H] serotonin into platelets was also measured after incubation with 2-nitroimipramine (150 nM) or imipramine (150 nM) followed by extensive washing of the platelets. The irreversible nature of the interaction of 2-nitroimipramine with the serotonin transport site is demonstrated in Fig. 2. While there was no difference in the kinetics of serotonin uptake after incubation with imipramine followed by washing, there was a marked inhibition of serotonin uptake after incubation with 2-nitroimipramine. Lineweaver Burk analysis of the uptake data using multiple substrate concentrations revealed a significant inhibition of maximal uptake capacity (V_{max}) with no alterations in the apparent rate constant (Km) (data not shown). Comparison of the effects of various concentrations of 2-nitroimipramine on platelet [^{3}H] imipramine binding and [^{3}H] serotonin uptake revealed a linear dose-response relationship (i.e. the greater the inhibition of binding, the greater the inhibition of uptake). However [^{3}H] imipramine binding was inhibited at significantly lower concentrations of 2-nitroimipramine than was [^{3}H] serotonin uptake

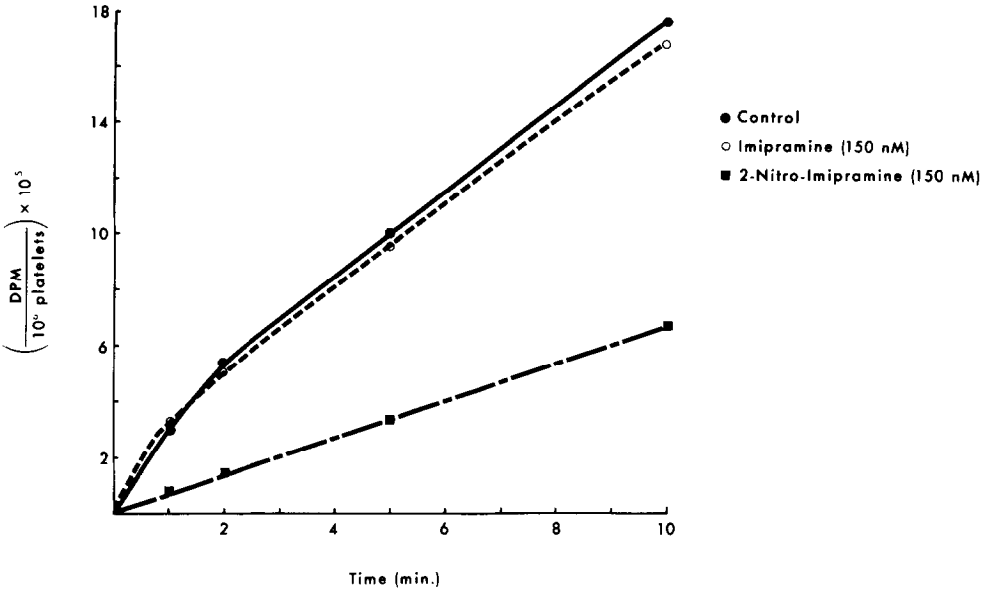

Figure 2 - Platelets were incubated at 0°C for 60 min. with 150 nM 2-nitroimipramine, 150 nM imipramine and no drug for control. After removing the free drug by repeated washings (x4) the uptake of [^{3}H] serotonin was measured as described in the text. A highly significant inhibition of serotonin uptake ($p < 0.001$) was observed only in the 2-nitroimipramine incubated platelets.

(Fig. 1 and 2) suggesting that a more complex stoichiometry exists between the two events.

In a separate series of experiments more extensive washing of the 2-nitroimipramine-incubated membranes (as many as 10 times) did not eliminate the marked inhibition of [^{3}H] imipramine binding or [^{3}H] serotonin uptake (data not shown) indicating that a very slow dissociation of the 2-nitroimipramine was unlikely to account for the apparent irreversible inhibition of both uptake and binding.

DISCUSSION

The high affinity imipramine binding sites that have been recently demonstrated in rat (1) and human brain (4) as well as in human platelets (2,5) appear to be specific for tricyclic antidepressants. Nevertheless, the exact pharmacological and physiological significance of these binding

sites is unclear since attempts to correlate the in vitro potency of various antidepressants in displacing [^{3}H] imipramine binding with their behavioral potency has been unsuccessful (1). We have previously suggested (3) that these binding sites appear to be related to the inhibition of serotonin uptake produced by tricyclic antidepressants and this hypothesis was recently supported by a study showing a good correlation between binding and uptake in rat brain synaptosomes (8). In the present study further evidence for an association between the high affinity imipramine binding site and serotonin uptake was provided by the development of an irreversible ligand for this site. 2-Nitroimipramine was found to be a very potent inhibitor of both [^{3}H] imipramine binding and [^{3}H] serotonin uptake in human platelets. The finding that both [^{3}H] serotonin uptake and [^{3}H] imipramine binding are irreversibly inhibited by 2-nitroimipramine further supports the idea that the two are functionally interrelated. The use of radiolabelled 2-nitroimipramine as an irreversible ligand of the high affinity imipramine binding site may be an important tool for subsequent purification and characterization of the protein(s) involved in both the binding of imipramine and the uptake of serotonin in platelets and brain membranes.

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