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INTERACTION OF SEROTONIN- AND DOPAMINE-RELATED NEUROTOXINS WITH "SEROTONIN BINDING PROTEINS" IN BOVINE FRONTAL CORTEX

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Abstract—Binding of [^{3}H]serotonin and [^{3}H]dopamine to serotonin-binding proteins (SBP) from soluble extracts of bovine frontal cortex is increased by Fe^{2+}. This group recently attributed this effect of Fe^{2+} to its ability to enhance the oxidation of [^{3}H]serotonin and [^{3}H]dopamine in the presence of dissolved molecular oxygen, and to the ability of the formed oxidation products to bind covalently to cysteine residues of SBP. In this study it is shown that the binding of both ligands is potently inhibited by dopamine as well as by several catecholamine-and serotonin-related neurotoxins: adrenochrome, 5,6-dihydroxytryptamine, 5,7-dihydroxytryptamine, 6-hydroxydopamine and 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline. In contrast, serotonin can only potently inhibit part (36%) of the [^{3}H]dopamine binding, while 1,2,3,4-tetrahydroisoquinoline is only a weak competitor for both ligands. Potent inhibition by the toxins is associated with the presence of electrophilic centres at the aromatic ring, either of the products themselves (adrenochrome) or of their oxidation products (all other competitors). These findings suggest that "SBP" represent an important target for the Fe^{2+}-mediated binding of [^{3}H]-serotonin, [^{3}H]dopamine and related neurotoxins.

Key words: serotonin; dopamine; neurotoxins; serotonin binding proteins; covalent binding; iron

Soluble SBP§ were initially supposed to be involved in the storage of serotonin in serotoninergic neurons [1, 2]. Subsequently, their physiologic role has been extended to include other "housekeeping" functions, such as the protection of serotonin from monoamine oxidase and transport of serotonin [3]. The interaction between serotonin and SBP is dependent on the presence of Fe^{2+} and, based on the occurrence of essential sulphydryl groups at or near the binding site, Tamir and Liu [4] proposed that Fe^{2+} should first bind to sulphydryl groups on SBP and that then up to four serotonin molecules should bind to the trapped iron by coordination bonds.

Recently, SBP have also been demonstrated to bind dopamine and other catecholamines and to be present in tissues, such as retina and adrenal medulla, which are known to contain high levels of catecholamines but only minute amounts of endogenous serotonin [5–7]. Initially, these findings led us to assume that SBP might also be implicated in the housekeeping of catecholamines [5]. Moreover, the elegant "coordination bond" model of Tamir and Liu [4] appeared also to be applicable for dopamine, since its binding was also strongly increased by Fe^{2+} [5]. However, the irreversible nature of the monoamine–SBP association and the inhibitory effect of antioxidants [8] did not comply with this model. To integrate these new findings, the authors proposed an alternative model, wherein the monoamine–SBP interaction should involve: (1) oxidation of the monoamine; and (2) its covalent binding to SBP [8]. In this "covalent bond" model, the role of Fe^{2+} is to promote the oxidation of serotonin and catecholamines instead of directly participating in the binding process. Under aerobic conditions, Fe^{2+} ions are indeed able to generate oxygen free radicals (i.e. superoxide and hydroxyl radicals) [9, 10] which act as potent oxidants for both catecholamines and serotonin [11–13]. The oxidation of catecholamines has been investigated extensively [14, 15] and a number of oxidation products of serotonin have also been identified without ambiguity [16]. Several of these oxidation products (*o*-quinones for catecholamines, quinoneimines and tryptamine-4,5-dione for serotonin) are recognized to bind covalently to external nucleophiles such as sulphydryl groups from proteins [17].

It is no longer possible to reconcile the alleged functional roles of SBP [3, 5] with the binding of oxidation products to these proteins instead of the neurotransmitters themselves and, especially, with the covalent nature of this binding. Indeed, the formation of covalent bonds between oxidation products and proteins has been recognized to represent a potential mechanism by which catecholamine- and serotonin-derived neurotoxins, such as 6-hydroxydopamine and 5,6-dihydroxytryptamine, provoke nerve cell degeneration

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§ Abbreviations: SBP, serotonin-binding proteins; 5,6,-DHT, 5,6-dihydroxytryptamine; 5,7-DHT, 5,7-dihydroxytryptamine; TIQ, 1,2,3,4-tetrahydroisoquinoline.

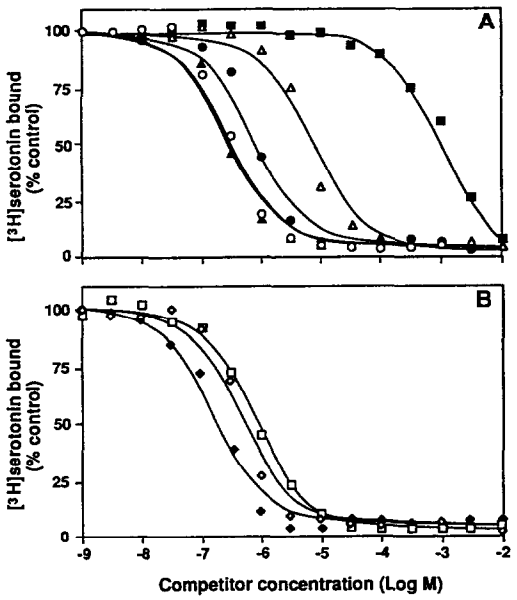

Fig. 1. [^{3}H]serotonin binding to SBP: competition by serotonin, dopamine and related toxins. SBP were incubated for 15 min with 0.1 mM Fe^{2+}, 0.2 μM [^{3}H]-serotonin and increasing concentrations of: *Panel A:* dopamine (○), 6-hydroxydopamine (△), adrenochrome (●), TIQ (■) and 6,7-dihydroxy-TIQ (▲). *Panel B:* serotonin (□), 5,6-DHT (◆), 5,7-DHT (◇): Data are given in % of control (i.e. total binding in the absence of competitor). Values are means of three experiments.

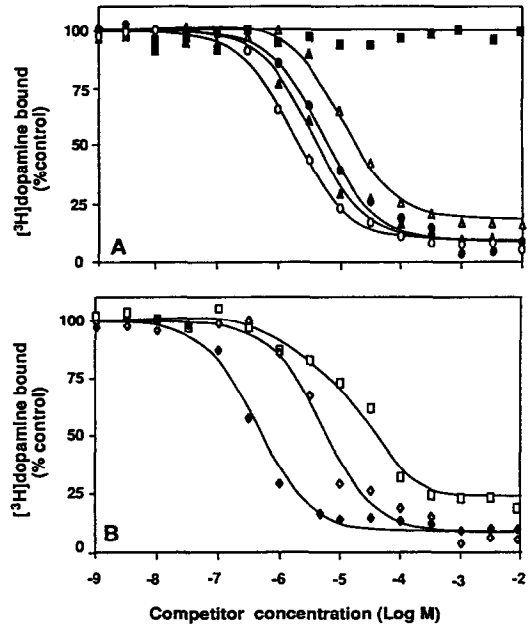

Fig. 2. [^{3}H]dopamine binding to SBP: competition by serotonin, dopamine and related toxins. SBP were incubated for 15 min with 0.1 mM Fe^{2+}, 0.2 μM [^{3}H]-dopamine and increasing concentrations of the compounds listed in the legend of Fig. 1 (same symbols). Data are given in % of control (i.e. total binding in the absence of competitor). Values are means of three experiments.

[18, 19]. This concept is based on the ability of these neurotoxins to undergo fast oxidation and on their pronounced binding to proteins [15, 20, 21].

To obtain further insight into the specificity of monoamine–SBP interactions, the ability of dopamine and serotonin-related neurotoxins to bind to SBP from bovine brain, have been evaluated in this study.

MATERIALS AND METHODS

Materials. 3-Hydroxy [G-^{3}H]tryptamine creatinine sulphate ([^{3}H]serotonin, 8 Ci/mmol) and [7,8-^{3}H]dopamine (49 Ci/mmol) were obtained from Amersham (Amersham, U.K.). 5-Hydroxytryptamine hydrochloride (serotonin), dopamine hydrochloride, 5,6-DHT, 5,7-DHT, 6-hydroxydopamine hydrobromide and adrenochrome were from Sigma (St Louis, MO, U.S.A.); iron sulphate ($FeSO_4$) was from Aldrich Chemie (Belgium). 6,7-dihydroxy-TIQ and TIQ were from Janssen Chimica (Belgium).

Protein preparation. All manipulations were performed at 0–4°. Cerebral frontal cortex samples were dissected from calf brains and homogenized in 0.32 M sucrose/10 mM potassium phosphate buffer (pH 7.5) with an Ultraturax (30 sec) and a motor driven Potter Elvehjem homogeniser (10 strokes, maximum speed), and then centrifuged at 10,000 *g* for 30 min. The supernatant was centrifuged again at 40,000 *g* for 60 min and the remaining supernatant was treated with ammonium sulphate (30% saturation) for 20 min and centrifuged at 15,000 *g* for 15 min. The pellets were resuspended in 20 mM potassium phosphate buffer (pH 7.5) containing 10% glycerol, dialysed against an excess of the same buffer and stored frozen at −20°. Protein concentrations were determined according to Lowry *et al.* [22]; BSA was used as the standard.

Binding assay. Samples of protein (0.20 mg/mL) were incubated at 20° in 500 μL of 20 mM potassium phosphate buffer (pH 7.5) with 0.2 μM of [^{3}H]-serotonin or [^{3}H]dopamine, freshly prepared $FeSO_4$ (100 μM) and with other products of interest. $FeSO_4$ was added immediately after the radioligands and competitors. After 15 min incubation, 300 μL of the mixture was applied to a small Sephadex G-50 column (0.7 × 15 cm) equilibrated with phosphate buffer, and eluted with the same buffer. The void volume (1.5 mL) was discarded and the fraction containing labelled protein (1.8 mL) was collected and counted by liquid scintillation counting.

RESULTS

When SBP was incubated with 0.2 μM radioligand and 0.1 mM Fe^{2+} the binding of [^{3}H]serotonin and [^{3}H]dopamine increased with the incubation time to reach a maximum at 15 min. Binding amounted 40

Table 1. IC_{50} values of serotonin, dopamine and related neurotoxins for inhibiting the binding of [^{3}H]serotonin and [^{3}H]dopamine to SBP

Competitor	IC_{50} values (μM) for inhibition of	
	[^{3}H]serotonin binding	[^{3}H]dopamine binding
Dopamine	0.28 ± 0.04	2.3 ± 0.3
6-hydroxydopamine	4.6 ± 1.2	12 ± 2.0
TIQ	1200 ± 350	≫10.000
6,7-dihydroxy-TIQ	0.31 ± 0.01	3.5 ± 0.4
Adrenochrome	0.78 ± 0.10	5.6 ± 1.0
Serotonin	0.59 ± 0.14	1.5 ± 1.0 (36%) 46 ± 1.0 (64%)
5,6-DHT	0.17 ± 0.06	0.40 ± 0.06
5,7-DHT	0.57 ± 0.16	4.0 ± 0.7

Values (means ± SD of three experiments) are derived from the competition binding experiments depicted in Figs 1 and 2

and 57 pmol/mg protein (N = 8). A 15 min incubation time was therefore adopted for the competition binding experiments shown in Figs 1 and 2. Concentrations of competitor at which binding is decreased by half (i.e. IC_{50}) are given in Table 1. The binding of [^{3}H]serotonin and [^{3}H]dopamine to SBP is covalent in nature [8], so that these IC_{50} values do not permit the calculation of the competitor's equilibrium dissociation constants (since they describe reversible interactions).

The data shown in Figs 1 and 2 reveal that the covalent binding of [^{3}H]serotonin and [^{3}H]dopamine to soluble SBP from calf brain is potently inhibited by the related toxins 6-hydroxydopamine, 5,6-DHT, 5,7-DHT and 6,7-dihydroxy-TIQ. In contrast, TIQ (which lacks hydroxyl groups at the aromatic ring) is nearly inactive. The binding of both radioligands is also potently inhibited by adrenochrome, an oxidation product of adrenaline.

It has consistently been shown for SBP from bovine brain, retina and adrenal medulla that the binding of [^{3}H]dopamine is more pronounced than the binding of [^{3}H]serotonin [5–7]. Competition binding experiments in this (Figs 1 and 2) and previous studies [8] are in accordance with this view. They show that unlabelled dopamine can effectively compete with [^{3}H]serotonin for the majority of its binding sites, whereas unlabelled serotonin can only effectively compete with [^{3}H]dopamine for a limited fraction of its sites.

DISCUSSION

The competition binding data presented in Figs 1 and 2 are fully compatible with earlier structure–binding activity relationship studies, wherein it was shown that serotonin analogues bind to SBP when the indole ring contains at least one hydroxyl group and that the presence of an intact catechol moiety is primordial for the binding of catecholamine derivatives [5, 23]. This structural requirement can easily be explained in light of the "covalent bond model"; i.e. monoamines with the appropriate structure are able to undergo rapid oxidation and their oxidation products possess one or more electrophilic centres at the aromatic ring (Figs 3 and 4) so that they can covalently bind to external nucleophiles such as sulphydryl groups from proteins. The inhibitory effect of adrenochrome also complies

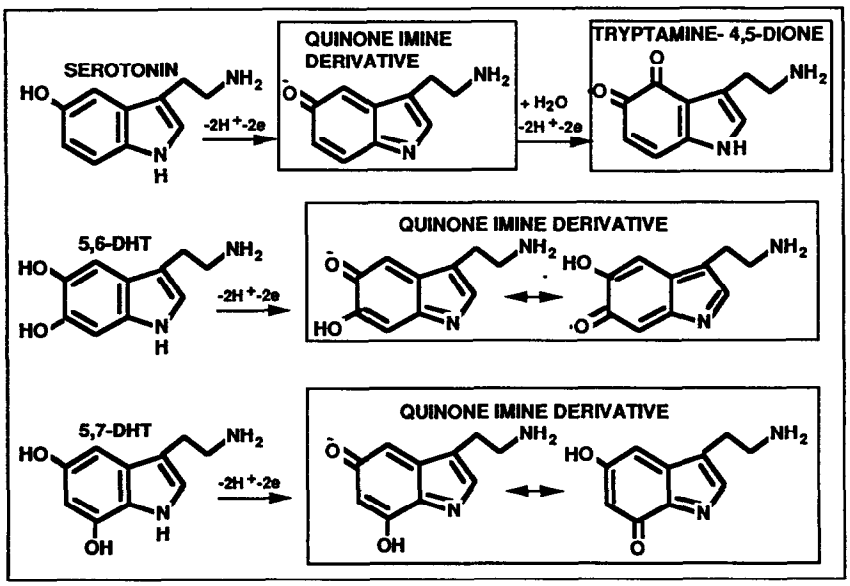

Fig. 3. Serotonin and related toxins: chemical structure of the compounds and their oxidation products. Compounds with presumed sites for nucleophilic attack are presented in boxes. According to Wrona and Dryhurst [16, 17] and Sinhababu and Borchardt [31].

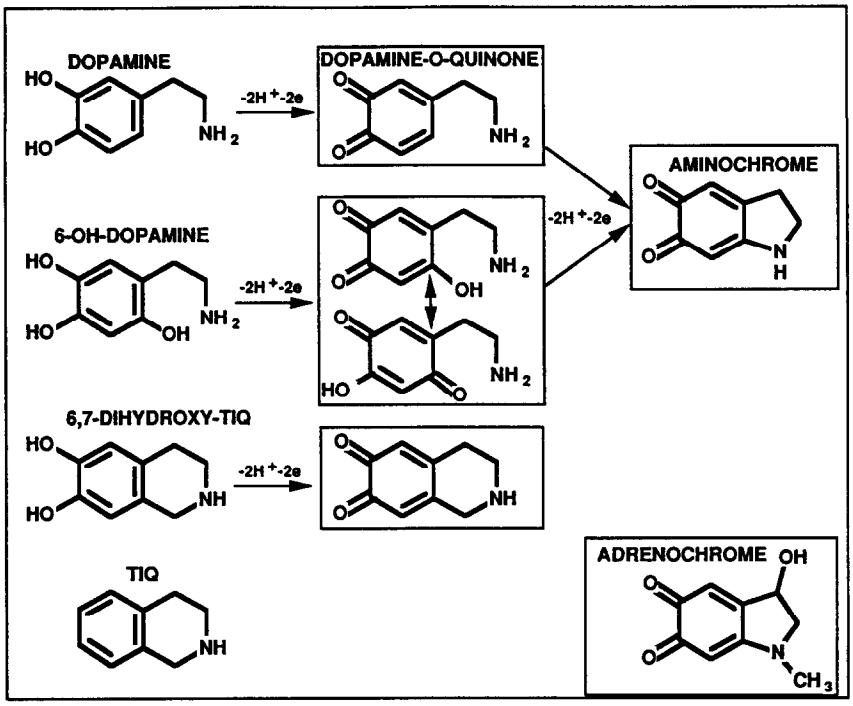

Fig. 4. Dopamine and related toxins: chemical structure of the compounds and their oxidation products. Compounds with presumed sites for nucleophilic attack are presented in boxes. According to Maguire *et al.* [42] and Graham *et al.* [15].

with the "covalent bond" model [8] since this compound possesses electrophilic centres at the aromatic ring (Fig. 4). On the other hand, adrenochrome is unable to form coordination bonds with trapped iron, so that its effect is clearly not compatible with the earlier "coordination bond" model of Tamir and Liu [4].

In accordance with previous data [8], it is shown that serotonin only competes with high affinity for part of the [^{3}H]dopamine sites (Fig. 2), whereas dopamine competes with high affinity for all the [^{3}H]serotonin binding sites (Fig. 1). These findings are explained by assuming that some of the binding sites on SBP could be more accessible to the oxidation products of dopamine than those of serotonin [8]. However, it is clearly shown in the present study that the serotonin-related neurotoxins 5,6-DHT and 5,7-DHT act as potent competitors at all the [^{3}H]dopamine binding sites. It may therefore be concluded that the binding sites for oxidized catecholamines are all accessible to oxidized indoleamines and, hence, that the difference in the binding capacity of [^{3}H]serotonin and [^{3}H]dopamine should be attributed to other factors, such as differences in their rate of oxidation or the electrophilicity of the oxidation products.

The neurotoxic activity of the dopamine-analogue 6-hydroxydopamine and of the serotonin-analogues 5,6-DHT and 5,7-DHT have already been investigated in great detail. These products only display major toxicity in a limited number of neurons and it is generally accepted that this neuroselectivity is related to the presence of specific uptake systems on the target neurons. Moreover, the toxicity of these products is also thought to be inherent to their property to undergo rapid oxidation [15, 20, 21, 24]. To date, three major molecular mechanisms have been advanced to explain the oxidation-related toxic effects of these molecules.

Firstly the auto-oxidation of 6-hydroxydopamine is well known to produce superoxide radicals, hydrogen peroxide and hydroxyl radicals [9, 11]. These reactive oxygen species, and more particularly the hydroxyl radicals, are extremely cytotoxic and they are prone to produce cell damage by, for example, peroxidation of membrane lipids and breaking of DNA [25, 26].

Secondly, initial work by Saner and Thoenen [20] and subsequent studies by others [18, 27–30] have led to the "alkylation theory", according to which the oxidation products of dopamine- and serotonin-related toxins may undergo covalent attachment to proteins. It has been demonstrated by Rotman *et al.* [29] that neuroblastoma cells take up 6-hydroxydopamine and that most of the intracellular ligand is covalently bound to proteins. Hence, such mechanism is also likely to be operative under *in vivo* conditions.

Thirdly, Sinhababu and Borchardt [31] proposed "redox cycling" as a possible mechanism for the neurodegenerative effects of 5,7-DHT. According to this mechanism, the monoamine should constantly cycle between its oxidized and reduced forms, thereby depleting the cell's oxygen as well as certain

reducing agents such as NADPH. Redox cycling of neurotoxins is also likely to occur under *in vivo* conditions since, after intracerebral injection of either 6-hydroxydopamine or its quinone to mice, the presence of both products become readily detectable in the brain [32, 33].

Although the exact mode of toxicity of those neutrotoxins has not been demonstrated, all three mechanisms could act in concert to produce neurodegeneration and their relative importance could vary from one toxin to another. Interestingly, some of the oxidation products of serotonin and catecholamines are cytotoxic as well. A striking example is given by the ability of adrenochrome to produce myocardial damage and functional cardiac impairment [34, 35]. In the same line, marked neurotoxicity is also displayed by tryptamine-4,5-dione, a recently discovered oxidation product of serotonin [13, 32, 36, 37]. Since both products react avidly with sulphydryl groups and since they are not liable to undergo rapid further oxidation, their cytotoxic action could be preferentially related to their ability to modify proteins.

The activity of certain proteins, such as the catechol *O*-methyltransferase enzyme and G proteins, has been reported to be irreversibly decreased after their covalent modification by monoamine oxidation products [38, 39]. Modification of these proteins, and presumably also of other key proteins, could therefore interfere with the normal cell's metabolic functions and, ultimately, provoke the destruction of nerve terminals. The marked inhibitory action of the investigated catecholamine- and serotonin-related neurotoxins on the [^{3}H]serotonin– and [^{3}H]-dopamine–SBP association suggests that these proteins constitute intracellular targets for these toxins. Therefore, it should be interesting to establish the actual nature of these proteins and the implication of chemical modification on their physiological activity. In this context, SBP are usually described to comprise of two peptides with molecular weights of 45 and 56 kDa [5, 40]. However, these proteins can no longer be regarded to possess a monoamine-housekeeping function, so that their contribution to the cell's metabolic functions are completely unknown.

Finally, since Fe^{2+} is capable of enhancing the oxidation of serotonin and dopamine, and since the resulting oxidation products bind covalently to SBP [8] and other proteins [38, 39], it may even be speculated that these monoamines could be cytotoxic by themselves, especially in situations where the concentration of iron is elevated and the concentration of protecting agents, such as glutathione, is low. Such a condition has been reported to occur in the substantia nigra from parkinsonian brains [41]. This group is currently investigating the Fe^{2+}-mediated binding of monoamines to intracellular proteins on cultured cell lines, and the potential cytotoxicity thereof.

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