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Interactions of cations and anions with the binding of uptake blockers to the dopamine transporter

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Abstract

Uptake blockers and substrates are likely to recognise a common binding domain on the dopamine neuronal transporter (DAT). Among cations that form ionic gradients at the level of the cellular plasma membrane, Na^+ is the only one that can stimulate their binding. The binding stimulation appears over Na^+ concentrations ranging from 0 to 10–60 mM; at higher Na^+ concentrations, binding reaches a plateau or decreases, according to the uptake blocker that is studied. The majority of the other cations, including K^+, Ca^{2+}, Mg^{2+} and $Tris^+$, inhibit the binding of uptake blockers. Several metals impair binding to the DAT and/or the dopamine transport, but, under specific conditions, some of them, and chiefly Zn^{2+}, stimulate binding. The complex relationships between cations, uptake blockers and the DAT suggest that cations recognise at least three different sites: the first one, site 1, is for cation-induced binding inhibition; the second one, site 2, is for Na^+-induced binding stimulation; and the third one, site 3, is for Zn^{2+}-induced binding stimulation. Modelling of the interactions between Na^+, K^+ and radioligands allows a better understanding of the effects of cations at sites 1 and 2, and of uptake blockers at site 1. Some anions also facilitate the binding of uptake blockers to the DAT, as far as they are associated with Na^+. The dependence of the binding of dopamine on ions could be involved in its preferential inward transport and used by uptake blockers for their own binding to the DAT.

Keywords: DAT (dopamine transporter); Cation; Anion; Binding; Uptake blocker

1. Introduction

It is generally an accepted view that the dopamine neuronal transporter (DAT) is deeply involved in the reinforcing properties of several drugs of abuse including cocaine. Mutations in the DAT gene could be associated with generalised anxiety, attention-deficit hyperactive disorder, social phobia and Tourette's disorder. Consequently, it is important to improve our knowledge of the DAT.

As for other neuronal carriers of the same group, the functioning of DAT is dependent upon transmembrane gradients of Na^+, K^+ and/or Cl^-. These ion gradients constitute a driving force for the active transport of dopamine; they are also involved in its binding to the DAT (Chen and Reith, this issue), and in the binding of agents such as cocaine which act as transport (uptake) blockers. Contrary to the different substrates, which are in competition for binding to–and transport by the DAT, uptake blockers only bind to the transporter, but this is sufficient to inhibit the DAT from functioning. In this article, we consider the control of the binding of uptake blockers to the DAT by ions.

It should be mentioned that the majority of the studies referred to were performed using rat DAT (rDAT) present in striatal membrane preparations. At the moment, the features of rDAT seem rather similar to those of the human DAT (hDAT) which has been studied less.

2. Uptake blockers and substrates are likely to recognise a common binding domain on the DAT

Several studies have been undertaken with the aim of characterising the relationships between the binding sites of uptake blockers and those of substrates on the DAT; their results strongly argue for the existence of different binding sites, each specific for one of these agents, but overlapping at the level of a common domain.

Various approaches have provided evidence for this. First, the binding of an uptake blocker or a substrate generally inhibited, in a competitive manner, the binding of another of

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these agents. This inhibition was complete, monophasic, and with a Hill number close to unity, consistent with the fact that these agents recognise mutually exclusive binding sites (i.e. binding sites of these agents are partly common or very close one to another site, so that the binding of one of them totally excludes the binding of another agent at the same site). Several studies have reported results consistent with this (see for example, Calligaro and Eldefrawi, 1988; Zimanyi et al., 1989; Bonnet et al., 1990; Reith and Selmeci, 1992; Billaud et al., 1994; Saadouni et al., 1994), including binding studies performed in the presence of three different competing agents (Reith et al., 1992; Chen et al., 1997b; Corera et al., 2001). Nevertheless, some noncompetitive interactions between uptake blockers (Eshleman et al., 1993) or between dopamine and an uptake blocker (Maurice et al., 1991) have been also reported.

Secondly, the dissociation of the binding of various radiolabelled uptake blockers was not modified by another uptake blocker or a substrate (Andersen, 1987; Zimanyi et al., 1989; Reith and Selmeci, 1992; Corera, personal communication).

Thirdly, several comparisons have shown that different labelled uptake blockers display the same sensitivity to alkylating agents (Reith and Selmeci, 1992; Saadouni et al., 1994; Refahi-Lyamani et al., 1995). Furthermore, uptake blockers and substrates afforded protection against this alkylation (Saadouni et al., 1994; Héron et al., 1994; Reith et al., 1996; Xu et al., 1997), but substrates were less potent than uptake blockers, even when their rather low affinity for the DAT was taken into account (Reith et al., 1996; Xu et al., 1997).

Fourthly, the thermodynamic features of substrate binding were different from those of uptake blockers (Bonnet et al., 1990; Billaud et al., 1994), showing that these drugs constitute two different groups with regard to the DAT. A likely explanation is that, unlike substrates, uptake blockers slowly interact with the DAT, forming two sequential complexes with the DAT (Héron et al., 1994; Do-Régo et al., 1999).

Fifthly, experiments involving photoaffinity ligand incorporation (Vaughan, 1995; Vaughan et al., 1999, 2001) and site-directed mutagenesis provided data indicating that substrates (dopamine) and uptake blockers recognise both specific and shared amino acids. Thus, in tryptophan mutants, there was either parallel variations (W555A: Lin et al., 2000b) or differentiated alterations of the affinity of dopamine and WIN-35,428 (2 α-carbomethoxy-3 α-(4-fluorophenyl)tropane) for the DAT (W84A; W162A for example). Similar results have been found for other mutants (Kitayama et al., 1992; Itokawa et al., 2000; Lin et al., 2000a; Chen et al., 2001; Lin and Uhl, 2002).

Thus, the current opinion is that uptake blockers and substrates likely bind to a central cavity of the DAT, in the medium part of the transmembrane domains, each of them recognising a specific site, but both of them sharing a common binding domain.

3. Among cations that form ionic gradients at the level of the cellular plasma membrane, Na^+ is the only one that can stimulate the binding of uptake blockers to the DAT. The binding stimulation appears over Na^+ concentrations ranging from 0 to 10–60 mM; at higher Na^+ concentrations, binding reaches a plateau or decreases, according to the uptake blocker that is studied

3.1. The Na^+-dependence of the binding of uptake blockers in the presence of Na^+ as the only cation…

As far as it can be estimated, in the absence of Na^+ the affinity of the uptake blocker for the DAT remains very low (Li et al., 2002). The stimulation phase of uptake blocker binding depends on the anion associated with Na^+ (Reith and Coffey, 1993; Corera et al., 2000). This point will be developed further in this article. For higher Na^+ concentrations, from 30 to 300 mM, cocaine binding decreased markedly and in a concentration-dependent manner (Calligaro and Eldefrawi, 1988; Saadouni et al., 1994), and that of cocaine congeners such as WIN-35,428 and RTI-121 (3β-(4-iodophenyl) tropan-2β-carboxylic acid isopropyl ester) was usually moderately impaired under these conditions (Reith and Coffey, 1993; Chen et al., 1997b; Reith et al., 1998). In contrast, the binding of GBR compounds was quite unaffected by medium-to-high Na^+ concentrations (Bonnet et al., 1988; Milner and Jarvis, 1992) whereas that of mazindol decreased slightly around 100 mM Na^+ and then increased at higher concentrations (Zimanyi et al., 1989; Corera et al., 2000).

A comparison of the limited experimental data available suggests that the Na^+ dependency of binding is not very sensitive to the temperature (Bonnet et al., 1988; Wu et al., 1997) or to the DAT origin: rather similar results were found with rDAT inserted in neuronal plasma membranes and hDAT expressed in cell lines (Reith and Coffey, 1993; Chen et al., 2002; but see also Wu et al., 1997).

The biphasic variation of binding with increasing Na^+ concentrations is due to changes in the K_d. As shown in Table 1, this has been demonstrated or reported for GBR compounds (see also Milner and Jarvis, 1992), cocaine and its derivatives, RTI-121 and WIN-35,428 (see also Wu et al., 1997), and for mazindol. Calculation of K_i values starting from IC_{50} values reported by Benmansour et al. (1987), using K_d values obtained under similar experimental conditions (Bonnet et al., 1988), demonstrated that an increase in Na^+ concentration from 10 to 130 mM decreased the affinity of benztropine, nomifensine, methylphenidate and pyrovalerone (not shown). A high Na^+ concentration was reported once to decrease the density of binding sites (B_{max}), without changing K_d (Chen et al., 1997b).

3.2. … and in a $Tris^+$-buffered medium

The use of Tris-buffered media gave rather different results. In this case, it is necessary to significantly raise the

Table 1
Representative data concerning the modifications of the parameters of uptake blocker binding evoked by stimulating and inhibiting concentrations of Na^+

Radioligands	Experimental conditions	Binding parameters		References
		Stimulation	Reduction	
[^{3}H]cocaine	rDAT/10 mM Na_2HPO_4 + NaCl	nd	↗ K_d↔B_{max}	Calligaro and Eldefrawi, 1988
	rDAT/10 mM Na_2HPO_4 + NaCl	nd	↗ K_d↔B_{max}	Eshleman et al., 1993
	rDAT/10 mM $NaHCO_3$/NaH_2PO_4 + NaCl	nd	↗ K_d↔B_{max}	Saadouni et al., 1994
[^{3}H]WIN-35,428	rDAT/Na^+-phosphates	↘ K_d↔B_{max}	↗ K_d↔B_{max}	Reith and Coffey, 1993
	hDAT HEK-293/10 mM Na^+-phosphates + NaCl	↘ K_d↔B_{max}	↗ K_d↔B_{max}	Li and Reith, 1999
	hDAT HEK-293/$Tris^+$ + NaBr or $NaNO_3$	↘ K_d↔B_{max}	↗ K_d↔B_{max}	Li et al., 2002
[^{3}H]RTI-121	rDAT/10 mM Na^+-phosphates + NaCl	↘ K_d↔B_{max}	↗ K_d↔B_{max}	Chen et al., 1997c
	rDAT/10 mM Na^+-phosphates + NaCl	nd	↔ K_d ↘ B_{max}	Chen et al., 1997b
[^{3}H]GBR 12783	rDAT/up to 10 mM $NaHCO_3$/$H_2PO_4^-$ + NaCl	↘ K_d↔B_{max}	↗ K_d↔B_{max}	Bonnet et al., 1988
[^{3}H]mazindol	rDAT/up to 10 mM $NaHCO_3$/$H_2PO_4^-$ + NaCl	↘ K_d↔B_{max}	↗ K_d↔B_{max}	Zimanyi et al., 1989

nd: not determined; 'phosphates' corresponds to mixed NaH_2PO_4/Na_2HPO_4.

Na^+ concentration to stimulate uptake blocker binding. Thus, 100–200 mM Na^+ was needed to obtain a maximum stimulation of the binding of GBR 12935 (1-[2-(diphenylmethoxyl)ethyl]-4-(3-phenyl-2-propyl)piperazine) (Janowsky et al., 1986) and nomifensine (Dubocovich and Zahniser, 1985), and even at 600 mM mazindol binding did not reach its maximum (Javitch et al., 1984; Zimanyi et al., 1989). However, $Tris^+$ does not hide the biphasic shape of the Na^+-dependent binding of cocaine, since it peaked at 30–50 mM Na^+ and decreased at concentrations ≥ 100 mM (Kennedy and Hanbauer, 1983). Similar shapes were reported for methylphenidate and WIN-35,428 binding curves, with peaks at 50–100 mM and 70–150 mM Na^+, respectively (Schweri et al., 1985; Li et al., 2002). A study in which the specific binding of GBR 12935, measured in a Tris-buffered medium containing calcium, was not Na^+-dependent deserves to be replicated (Andersen, 1987). IC_{50} values for $Tris^+$ increase with Na^+ concentrations (Table 2A), consistent with the ability of Na^+ to overcome $Tris^+$ inhibition. $Tris^+$ inhibits both uptake blocker binding and dopamine uptake with a rather similar potency (Table 2A). Assays of binding saturation suggest that it could adversely affects both the affinity and the maximum population of binding sites. $Tris^+$ mainly reduced the affinity of GBR compounds, mazindol and WIN-35,428 for the DAT (Javitch et al., 1984; Janowsky et al., 1986; Bonnet et al., 1988; Zimanyi et al., 1989; Reith and Selmeci, 1992; Li et al., 2002), when it reduced the B_{max} of cocaine binding (Kennedy and Hanbauer, 1983; Calligaro and Eldefrawi, 1988; Eshleman et al., 1993). However, the submicromolar affinity of cocaine for the DAT (Kennedy and Hanbauer, 1983; Eshleman et al., 1993) and its high dissociation rate (Calligaro and Eldefrawi, 1988) suggest that a further $Tris^+$-induced decrease in affinity can result in an apparent loss of binding sites. When compared to other buffers, $Tris^+$ seems

Table 2
Representative data concerning the inhibiting potency of $Tris^+$, K^+, Ca^{2+} and Mg^{2+} on the binding of uptake blockers to the DAT

Inhibiting potency (IC_{50})	Experimental conditions	Radioligands	References
(A) For $Tris^+$			
12 mM	10 mM Na^+ ($NaHCO_3$/NaH_2PO_4)	[^{3}H]GBR 12783	Bonnet et al., 1988
36 mM	20 mM Na^+ (mixed phosphates)	[^{3}H]cocaine	Eshleman et al., 1993
13 mM	Tris/HEPES[a] hDAT/HEK293 cells	[^{3}H]WIN-35,428	Li et al., 2002
71 mM	136 mM Na^+ (Krebs Ringer)	[^{3}H]GBR 12783	Amejdki-Chab et al., 1992b
57 mM	136 mM Na^+ (Krebs Ringer)	dopamine transport	Amejdki-Chab et al., 1992b
(B) For K^+/Ca^{2+}/Mg^{2+}			
5.0/0.098/0.27 mM	10 mM Na^+ ($NaHCO_3$/NaH_2PO_4), 0 °C	[^{3}H]GBR 12783	Héron et al., 1996[b]
2.6/0.049/0.14 mM	at 20 °C	[^{3}H]GBR 12783	Héron et al., 1996[b,c]
4.5/0.04/0.08 mM	10 mM Na^+ ($NaHCO_3$/NaH_2PO_4), 20 °C	[^{3}H]WIN-35,428	Corera et al., 2000
20.6/0.86/3.88 mM	+ 90 mM NaCl	[^{3}H]WIN-35,428	Corera et al., 2000
15.3/0.13/0.55 mM	10 mM Na^+ ($NaHCO_3$/NaH_2PO_4), 0 °C	[^{3}H]GBR 12783	Bonnet et al., 1988
78/4.7/≥ 10 mM	+ 120 mM NaCl	[^{3}H]GBR 12783	Bonnet et al., 1988
109/2.6/16 mM	136 mM Na^+ (Krebs Ringer), 37 °C	[^{3}H]GBR 12783	Amejdki-Chab et al., 1992b[d]

Except when indicated, these studies were performed using DAT from rat striatum.
[a] Increasing concentrations of Tris/HEPES.
[b] K_i.
[c] See also Refahi-Lyamani et al. (1995).
[d] See also Billaud et al. (1993).

rather potent as an inhibitor of GBR 12935 binding in a Na^+ medium. In contrast, MOPS (3-(*N*-morpholino)propanesulfonic acid) and HEPES do not significantly affect this binding (Richfield, 1993).

4. Like Tris, most other cations, such as K^+, Ca^{2+} and Mg^{2+}, inhibit the binding of uptake blockers

4.1. K^+, Ca^{2+}, Mg^{2+} ...

It is generally accepted that K^+, Ca^{2+} and Mg^{2+} cannot stimulate the binding of uptake blockers and furthermore that they inhibit it. The K^+-induced inhibition is concentration-dependent, complete, and with a Hill number close to unity. Its IC_{50} is in the low millimolar range for 10 mM Na^+, indicating that it is about 50–100 times less inhibitory than Ca^{2+} and Mg^{2+} (Table 2B). These IC_{50} values have been determined essentially against GBR 12783 (1-[2-(diphenylmethoxyl)ethyl]-4-(3-phenyl-2-propenyl)piperazine) and WIN-35,428 binding (Table 2B), but inhibition by a single concentration of K^+ (and Ca^{2+} and Mg^{2+} when indicated) has been reported for GBR 12935 (Janowsky et al., 1986, for K^+, Ca^{2+}, Mg^{2+}), cocaine (Kennedy and Hanbauer, 1983, for K^+, Ca^{2+}, Mg^{2+}; Calligaro and Eldefrawi, 1988), WIN-35,428 (Reith and Coffey, 1993; Wu et al., 1997; Chen et al., 2002), RTI-121 (Chen et al., 1997c), methylphenidate (Schweri et al., 1985, for K^+, Ca^{2+}, Mg^{2+}), BTCP (*N*-[1-(2-benzo(*b*)thiophenyl)-cyclohexyl]piperidine) (Vignon et al., 1988) and mazindol (Javitch et al., 1984; Wu et al., 1997). In a more indirect way, Chen et al. (1997a) demonstrated that Ca^{2+} and Mg^{2+} were inhibitors of the binding of RTI-121 and BTCP.

Only one study failed to demonstrate inhibition of uptake blocker binding by millimolar concentrations of Ca^{2+} and Mg^{2+}, but the authors suggested that this could have been an artefact due to the formation of a calcium phosphate precipitate (Calligaro and Eldefrawi, 1988). Two studies have indicated that the binding inhibition elicited by these cations increases moderately with temperature (Héron et al., 1996), but is not markedly dependent on membrane polarisation (Billaud et al., 1993). Little attention has been paid to these parameters, which are important for understanding the relationships between ions and ligands under physiological conditions (Wu et al., 1997).

Different studies have revealed that Na^+ can overcome the inhibitory potency of K^+, Ca^{2+} and Mg^{2+} (Table 2B). This likely explains why GBR 12935 and WIN-35,428 binding are not impaired by a submillimolar concentration of Ca^{2+} or Mg^{2+} in the presence of a rather high Na^+ concentration (Richfield, 1993; Wu et al., 1997). In other respects, it is worthy to note that the addition of about 100 mM Na^+ reduced by 20–50 times the inhibitory potency of Ca^{2+} and Mg^{2+} (Table 2B; see also Billaud et al., 1993), when that of K^+ was decreased by 4–5 times (Reith and Coffey, 1993; Chen et al., 1997c; Li and Reith, 1999). In a more indirect way, Chen et al. (1997c) also demonstrated that increasing the Na^+ concentration reduced the K^+-elicited inhibition of cocaine and GBR 12935 binding.

K^+ generally decreases the affinity of the uptake blocker. This has been shown for various radioligands including [^{3}H]cocaine (Calligaro and Eldefrawi, 1988), [^{3}H]WIN-35,428 (Wu et al., 1997; Chen et al., 1997c, 2002; Li and Reith, 1999; Corera et al., 2000) and [^{3}H]GBR 12783 (Bonnet et al., 1988). A decrease in the number of RTI-121 binding sites was also reported for a high K^+/Na^+ ratio (30/10 mM; Chen et al., 1997c). This was not observed for lower ratios and the authors stated that some experimental conditions such as ion environment and temperature were likely to produce such an increase in the dissociation rate that RTI-121-DAT complexes disappear during filtration procedures.

Some studies have shown that Ca^{2+} and/or Mg^{2+} decrease the affinity of two tritiated uptake blockers, [^{3}H]GBR 12783 (Bonnet et al., 1988; Héron et al., 1996) and [^{3}H] WIN-35,428 (Corera et al., 2000).

4.2. H^+

Protons also inhibit the binding of uptake blocker to the DAT. A first study performed with Tris-buffered media showed that GBR 12935 binding increased with pH values from 6.5 to 9, the highest tested pH (Andersen, 1987). Two other studies carried out with different buffers and using labelled cocaine derivatives (RTI-121, WIN-35,428) gave similar results, with the exception that binding begun to plateau at pH $\geq$ 8 (Wall et al., 1993; Xu and Reith, 1996). The pH-related increase in binding is due to an increased affinity for the DAT (Andersen, 1987; Xu and Reith, 1996). In an elegant study, Reith et al. demonstrated that the pH dependency of binding was a consequence of a change in DAT protonation and not in ligand charge. So, they compared the effects of changing pH on the ability of three cocaine derivatives to recognise the DAT, namely, WIN-35,428, which is both in a cationic and a neutral form at tested pH values, cocaine methiodide, which is permanently cationic, and benzocaine, which is permanently neutral. Parallel variations in their binding in response to pH change demonstrated that their binding was not dependent on the ligand charge, clearly evidencing the main role of the protonation of the DAT in the binding of uptake blockers. However, one cannot exclude that the dissociation of the last piperazine proton of GBR 12935, which occurs between pH 6 and 8, is not important for the binding of GBR compounds. As previously reported for other inhibitory cations, Na^+ attenuates the effects of protons on WIN-35,428 binding (Chen et al., 2002).

4.3. Li^+, Rb^+, $choline^+$ and others

Several other monovalent cations have been tested for their ability to substitute for Na^+ in studies aimed at

characterising the dependency of binding, or uptake, on Na^+ at a constant anionic concentration. Li^+ is generally described as an inhibitor of the binding of DAT ligands such as GBR compounds (Janowsky et al., 1986; Bonnet et al., 1988; Amejdki-Chab et al., 1992a), cocaine and congeners (Kennedy and Hanbauer, 1983; Calligaro and Eldefrawi, 1988; Reith and Coffey, 1993; Chen et al., 2002), mazindol (Javitch et al., 1984; Zimanyi et al., 1989) and methylphenidate (Schweri et al., 1985).

A similar inhibition of dopamine uptake has been reported (Shank et al., 1987; Amejdki-Chab et al., 1992a; Syringas et al., 2001), but two recent studies suggest that, under some experimental conditions, Li^+ could be neutral rather than inhibitory. So, substitution of 112 mM NaCl by iso-osmotic concentrations of either sucrose or LiCl resulted in a similar decrease in the binding of WIN-35,428 and mazindol to hDAT present in C6 cell membranes. Because chloride ions and sucrose were considered as essentially neutral in the binding process, it follows that the replacement of Na^+ by Li^+ was no more inhibiting than the removal of Na^+ (Wu et al., 1997); Li^+ was as potent as Na^+ in supporting WIN-35,428 binding to hDAT under these experimental conditions. In a second study, substitution of Na^+ by equimolar concentrations of Li^+ produced a reduction in dopamine transport with a Hill number close to unity, in agreement with the dependence of the uptake process on one Na^+ ion and the lack of an inhibiting effect of Li^+ (Chen et al., 1999). It is not established whether the fact that both studies were performed with hDAT expressed by eukaryotic cells (C6 or HEK-293) could explain the discrepancy between these studies and those of the bulk of studies demonstrating Li^+ to be inhibitory, studies performed with rDAT expressed in the plasma membrane from dopaminergic terminals. In fact, expression of a hDAT cDNA in HEK-293 cells allowed the characterisation of a WIN-35,428 binding sensitive to inhibition by Li^+ (Li and Reith, 1999; Chen et al., 2002). Nevertheless, expression of the same hDAT cDNA in different cell lines can produce variations in the dependence of dopamine uptake on Na^+ and Cl^- ions (Syringas et al., 2000, 2001). More work is needed to clarify this point.

Finally, Rb^+, Cs^+, NH_4^+, choline$^+$ and *N*-methyl-glucamine$^+$ have all been reported as agents inhibiting the binding of uptake blockers to the DAT (see Javitch et al., 1984 for Rb^+; Schweri et al., 1985 for NH_4^+; Bonnet et al., 1988 for choline$^+$; Milner and Jarvis, 1992; Amejdki-Chab et al., 1992a for Rb^+, Cs^+, and choline$^+$; Coffey and Reith, 1994; Chen et al., 2002 for *N*-methyl-glucamine$^+$). Choline$^+$, Rb^+ and Cs^+ also impair dopamine uptake in rat synaptosomes (Holz and Coyle, 1974; Shank et al., 1987; Amejdki-Chab et al., 1992a). Choline$^+$ and *N*-methyl-glucamine$^+$ are at least as potent as Li^+ as inhibitors of dopamine transport in LLCPK1 cells expressing rDAT (Gu et al., 1994).

5. Several metals inhibit the binding of uptake blockers to the DAT and/or dopamine transport; however, under specific conditions, some of them, and chiefly Zn^{2+}, stimulate binding

5.1. About the dual effect exerted by these cations on the binding of uptake blockers

Some studies have indicated that micro- to millimolar concentrations of Co^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+}, and submicromolar concentrations of Cu^{2+} reduce the specific binding of various uptake blockers, including GBR compounds (Bonnet et al., 1994), cocaine and WIN-35,428 (Cao et al., 1989; Bonnet et al., 1994; Wu et al., 1997; Norregaard et al., 1998), mazindol (Wu et al., 1997) and methylphenidate (Schweri, 1994). However, two of these cations, Zn^{2+} and Hg^{2+}, affect the binding of uptake blockers to the DAT in a biphasic manner: they stimulate binding at (sub)micromolar concentrations and inhibit binding at concentrations higher than 5–10 μM (Richfield, 1993; Bonnet et al., 1994; Schweri, 1994; Wu et al., 1997; Norregaard et al., 1998). The behaviour of Co^{2+} and Ni^{2+} seems more complex since at the same concentrations they either markedly stimulate (Richfield, 1993) or dramatically block (Bonnet et al., 1994) the binding of GBR compounds.

A careful examination of the data reveals that the appearance and intensity of the binding inhibition produced by these cations are dependent on the presence of other cations such as Na^+, K^+, Ca^{2+}, Mg^{2+} and Tris$^+$. Thus, Hg^{2+} concentrations which block cocaine binding in 20 mM Na^+-phosphate medium (Cao et al., 1989) stimulate the methylphenidate binding in 100 mM Na^+ Tris$^+$-buffered medium (Schweri, 1994) and WIN-35,428 binding in phosphate-buffered medium containing 127 mM Na^+ (Wu et al., 1997). In the same way, the ability of Cd^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+} or Co^{2+} to impair GBR 12783 binding to rDAT was attenuated by K^+ and Ca^{2+} used at concentrations which reduce the binding of the blocker, and by Na^+ concentrations which protect binding from K^+ or Ca^{2+}-elicited inhibition (Bonnet et al., 1994). Several binding studies suggest that the inhibition of binding and/or uptake produced by Hg^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+} and Co^{2+} could result from their ability to bind to a site containing at least an −SH group (Cao et al., 1989; Bonnet et al., 1994; Schweri, 1994; Norregaard et al., 1998). In contrast, Na^+ did not affect the range of Zn^{2+} concentrations which elicited binding stimulation, suggesting that, in this case, Zn^{2+} binds to another metal-ion recognition site (Bonnet et al., 1994). So, Zn^{2+}, Co^{2+}, Ni^{2+} and Hg^{2+} are likely to recognise two different sites, to produce either a stimulation or an inhibition of binding (Bonnet et al., 1994; Schweri, 1994; Wu et al., 1997; Norregaard et al., 1998). Both sites being occupied by rather similar concentrations of metal ions, a decrease in inhibition elicited by Na^+, for example, reveals or magnifies the stimulation.

5.2. And about Zn^{2+} more specifically

Zn^{2+}-induced biphasic effects are observed for both uptake blocker binding and dopamine uptake: millimolar Zn^{2+} concentrations which stimulate GBR 12783 and WIN-35,428 binding produce a concomitant reduction of dopamine uptake which reaches a plateau representing 30–40% of the control uptake for 10–100 μM Zn^{2+} (Bonnet et al., 1994; Norregaard et al., 1998; Loland et al., 1999). At higher concentrations, Zn^{2+} strongly impairs both processes (Norregaard et al., 1998). The decrease in dopamine uptake results from a decrease in V_{max}, with K_m being unaffected (Bonnet et al., 1994; Norregaard et al., 1998). Effects of inhibiting concentrations of Zn^{2+} on binding parameters have not been described, except for an increase in the dissociation rate of GBR 12783 by 100 μM Zn^{2+} in a 10 mM Na^+ medium (Bonnet et al., 1994). The binding stimulation could be due to an increase in binding affinity. This has been reported for Zn^{2+} (Richfield, 1993; Bonnet et al., 1994), consistent with an enhanced association rate (Bonnet et al., 1994), and for Ni^{2+} and Co^{2+} under experimental conditions allowing binding stimulation to occur (Richfield, 1993). Competition experiments for GBR 12783 and WIN-35,428 binding sites also indicate that stimulating Zn^{2+} concentrations increase the apparent affinity of several other uptake blockers and of low affinity substrates (Bonnet et al., 1994; Loland et al., 2002). In contrast, Zn^{2+} was quite ineffective on (Bonnet et al., 1994), or slightly reduced (Loland et al., 1999), the binding affinity of dopamine and amphetamine derivatives. This agrees with the aforementioned decrease in uptake V_{max} with an unchanged K_m. Nevertheless, two studies suggested that stimulating Zn^{2+} concentrations could be related to an increase in the B_{max} of [^{3}H]WIN-35,428 binding (Wu et al., 1997; Norregaard et al., 1998). This apparent discrepancy could originate from a peculiar ionic environment and/or opposite effects of the Zn^{2+}-induced stimulation and inhibition on the B_{max} parameter. Both studies were performed in a medium containing a high Na^+ concentration, which attenuates the inhibiting component of the Zn^{2+} action on binding. Thus, the increase in B_{max} could result from a strong positive effect of Zn^{2+} on B_{max}, which is not reduced by the negative effect of the inhibition. Under experimental conditions more favourable to expression of this inhibition, the net result of the opposite effects of Zn^{2+} on B_{max} could be null. This reasoning postulates that a decrease in B_{max} is involved in the Zn^{2+}-induced binding inhibition; however, such a decrease has been evidenced for only Cd^{2+} (Richfield, 1993; Bonnet et al., 1994), which is likely to exert its inhibition through the recognition of the same binding site. The discrepancy could also originate from more specific experimental conditions. The stimulating effect of Zn^{2+} could convert a very low-affinity population of binding sites to a high-affinity state, thereby allowing WIN-35,428 binding to occur. Some results concerning the ability of WIN-35,428 and cocaine to compete with GBR 12935 for DAT binding in the presence of stimulating Zn^{2+} concentrations are consistent with this hypothesis (Richfield, 1993). Consistent with this, the Zn^{2+}-induced increase in B_{max} observed for the binding of WIN-35,428 in intact COS 7 cells expressing hDAT could result from an enhancement of the outward facing population of DAT (Norregaard et al., 1998).

6. The complex relationships between cations, uptake blockers and the DAT suggest that cations recognise at least three different sites on the DAT; Zn^{2+} and Na^+ ions allow to establish a simplified identity card for each of them

As already suggested in this article, Na^+, K^+, Ca^{2+} and a large variety of mono- and divalent cations could bind to a first, inhibiting, site (site 1; Fig. 1), which is probably localised in the binding domain of the uptake blockers. The binding stimulation elicited by Na^+ and Zn^{2+} (and its congeners) is likely to result from their association with a second and a third site, which are probably distal from the blocker binding domain (sites 2 and 3: Fig. 1).

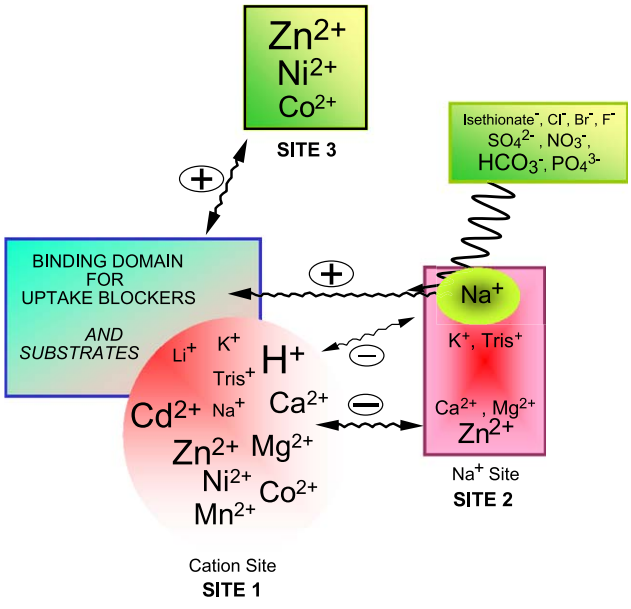

Fig. 1. Schematic representation of the interactions between the binding domain of the uptake blockers and four ion binding sites on the DAT. Squiggly arrows indicate positive (+) and negative (–) allosteric reactions. An arrow starts from the anion binding site and projects on Na^+ at site 2 and on the squiggly arrow corresponding to the positive allosterism between site 2 and site 1 in order to show the permissive role of Na^+ on the stimulating effect of anions. The anion site is drawn in this distal position for convenience. The size of the ions is depicted in relation to their apparent affinity for the different sites. This model is derived from those of Chen et al. (1997c), Corera et al. (2000) and Li et al. (2002).

6.1. A stimulating site for Zn^{2+}: site 3

While knowledge of the site through which Zn^{2+} stimulates the binding of uptake blockers has increased, little is known about its relationships with other binding sites. Considering the ability of Zn^{2+} to produce a binding stimulation at (sub)micromolar concentrations, it had been suggested that it could bind to the ring N-atom of histidine or the carboxylic group of glutamate or aspartate (Bonnet et al., 1994). Mutation analysis of hDAT has demonstrated that histidine 193 (in the second, large, extra-cellular loop), histidine 375, at the top of transmembrane domain 7, and glutamate 396, at the top of transmembrane domain 8, are deeply involved in the formation of a Zn^{2+} binding site responsible for the binding stimulation (Norregaard et al., 1998; Loland et al., 1999; Loland, this issue). Point mutations of each of these amino acids totally remove the Zn^{2+}-induced binding stimulation and the concomitant reduction in dopamine transport, without affecting the binding blockade produced by millimolar concentrations of Zn^{2+} (Norregaard et al., 1998; Loland et al., 1999). This strongly argues for the existence of two distinct binding sites for Zn^{2+} on the DAT. In the same way, the different sensitivity of the stimulation and inhibition produced by Zn^{2+} to cations, and particularly Na^+, and the millimolar affinity of Zn^{2+} for the site at which it produces its binding inhibition are in favour of the existence a second binding site.

According to the hypothesis of Norregaard et al. (1998) and Loland et al. (1999), Zn^{2+} binding at the stimulatory site (site 3) produces a conformational change of the DAT which brings the binding site for uptake blockers in a more favourable configuration for binding. This agrees with results showing that a stimulating concentration of Zn^{2+} increased the association rate of [^{3}H] GBR 12783 binding, without affecting its dissociation (Bonnet et al., 1994). Remembering that the binding of an uptake blocker is likely to involve conformational change(s) of the DAT (Bonnet et al., 1990; Billaud et al., 1994; Ferrer and Javitch, 1998; Do-Régo et al., 1999; Chen et al., 2000; Reith et al., 2001), it follows that Zn^{2+} could either improve DAT recognition by the uptake blocker or favour some conformational transition(s) involved in the consolidating phase of binding (Do-Régo et al., 1999). These results are consistent with the existence of non-mutually exclusive binding sites for Zn^{2+} and uptake blockers at the DAT; they also suggest that Zn^{2+} is devoid of effect on the DAT-uptake blocker complex and/or cannot recognise its stimulating site 3 after the blocker is bound, perhaps as a consequence of the conformational changes generated by the binding of the uptake blocker. Nevertheless, it is very likely that site 3 is rather distal from site 1. The distance between Zn^{2+} and amino acids involved in its binding at site 3 located at the top of transmembrane domains 7 and 8 (0.2 nm: Norregaard et al., 1998; Loland et al., 1999) makes improbable an overlapping with site 1 located in the medium part of transmembrane domains. More investigations are needed to settle these issues.

6.2. A site 1 for the cation-induced inhibition and an allosteric site 2 for the Na^+-induced stimulation (Fig. 1)

Several studies, and particularly studies dealing with binding dissociation and ionic interactions, suggest that, at site 1, the binding of Na^+, K^+ and that of the uptake blocker could be mutually exclusive. First indications for this came from the ability of Na^+ to attenuate the inhibition elicited by several other cations, and from the apparent competition between the binding of four of them and that of [^{3}H]GBR 12783 (Bonnet et al., 1988). This apparent simplicity has been refuted by some major experimental data. The first of them showed that Na^+ was unable to totally overcome the K^+-induced inhibition of WIN-35,428 binding at 0 °C (Reith and Coffey, 1993). More recently, similar data were reported for Na^+ and H^+, and Na^+ and Li^+, respectively (Chen et al., 2002). Later, dissociation studies revealed that K^+, Ca^{2+}, Mg^{2+} and $Tris^+$ accelerated the dissociation rate of the GBR 12783–DAT complex, whereas millimolar concentrations of Na^+ decreased it (Héron et al., 1996; see also Chen et al., 1997c; Corera et al., 1998, 2000). This clearly indicates that these cations could recognise a second, non-mutually exclusive binding site (site 2), distal from the binding domain of the uptake blocker (site 1). However, it is worthy to note that, with the exception of the demonstration that Na^+ can overcome the increase in dissociation rate produced by K^+, Ca^{2+} and Mg^{2+}, there is no more evidence that site 2 actually represents a common, mutually exclusive binding site for all these cations (Héron et al., 1996; Chen et al., 1997c; Corera et al., 2000).

A binding study performed at 37 °C and aiming at modelling the interactions between Na^+, K^+, RTI-121 at the DAT (Chen et al., 1997c) has confirmed that low millimolar Na^+ concentrations were likely to stimulate the binding of the uptake blocker at site 1 through recognition of an allosteric site corresponding to site 2, by positive allosterism. The hypothesis of the existence of allosteric relationships between site 2 and site 1 has been strengthened by more recent data showing that mutations of the DAT can enhance the Na^+-induced stimulation of blocker binding without modifying the Na^+ concentration range which produces this effect (Chen et al., 2002). In this model, Na^+ binds to both site 1 and site 2, and this binding impairs and stimulates uptake blocker binding at site 1, respectively (Fig. 1). At a fixed Na^+ concentration, interactions of Na^+, K^+ and RTI-121 at site 1 are better described as a simple competition for the occupancy of a unique binding domain. Calculations of the apparent affinity of ions for sites 1 and 2, by modelling the experimental data, gave values similar to those observed, i.e. a 15–20 mM affinity for Na^+ at site 2 (in the absence of K^+), and a 5–10 mM affinity for K^+ at site 1 (in the absence of Na^+) (Chen et al., 1997c).

6.3. The consequences of this model

(1) Its binding at site 2 allows Na^+ to overcome the K^+-elicited inhibition by two means, mainly by decreasing the ability of K^+ to bind at site 1, and, for high K^+ concentrations, by competition for recognition at site 2 (in agreement with results of dissociation binding experiments). Thus, Na^+ binding at site 2 stimulates the binding of the uptake blocker at site 1 by positive allosterism, and removes K^+ binding at site 1 by a negative allosteric effect (Fig. 1). Concordant with this hypothesis are the results which suggest that K^+ binding at site 1 impairs Na^+ binding at site 2; low millimolar K^+ concentrations which inhibit the binding of an uptake blocker at site 1 also increase the Na^+ concentration necessary at site 2 to stimulate blocker binding at site 1, and the Na^+ concentration at which this stimulation is maximum (Chen et al., 1997c; Corera et al., 2000). It is noteworthy that negative allosterism also applies for Na^+ binding at site 2 and site 1 (Fig. 1).

(2) The biphasic shape of the curve describing the Na^+-dependence of uptake blocker binding is the result of the marked predominance of the positive allosteric effect resulting from Na^+ binding at site 2, in the initial stimulation phase, and of an equilibrium between this positive effect and a direct inhibition by Na^+ binding at site 1, in the second plateau/reduction phase (Fig. 2). Under these conditions, uptake blockers which are more sensitive to the inhibiting effects of high Na^+ concentrations could be those which are less sensitive to the positive allosteric effect resulting from Na^+ binding at site 2, and/or those for which Na^+ binding at site 1 produces a more intense inhibition of uptake blocker binding (Fig. 2). This proposal implies that the relationships between Na^+ and the uptake blocker at site 1 are not mutually exclusive. This proposal still holds despite the fact that increasing the uptake blocker concentration reduces the Na^+-induced binding inhibition (Corera et al., 2000), which is consistent with competition between Na^+ and the uptake blocker for the recognition of a mutually exclusive binding site (site 1), in line with increased K_d values at high Na^+ concentrations (Table 1).

(3) The apparent competition between Na^+ and K^+ at site 1 suggests that the interaction of K^+ with site 2 is negligible. In fact, under identical experimental conditions, K^+, Ca^{2+} and Mg^{2+} were actually more powerful as inhibitors of binding, as a consequence of their binding at site 1, than as promoters of binding dissociation due to their binding at site 2 (Héron et al., 1996; Chen et al., 1997c; Corera et al., 2000). Consistent with a predominant effect of K^+ at site 1 are the recent results which indicate that mutations of the DAT which enhance allosteric interactions between site 2 and site 1 do not affect the K^+-induced inhibition (Chen et al., 2002). In contrast, $Tris^+$, at concentrations producing 50% inhibition of binding, doubles the dissociation rate of the GBR 12783–DAT complex, showing that the allosteric component plays a significant role in its binding inhibition potency (Héron et al., 1996).

(4) The apparent mutually exclusive binding at site 1 should be reconsidered. As illustrated by the ability of Na^+, Zn^{2+} or K^+ to modify the K_d of blocker binding because they bind to an allosteric site (2 or 3), it becomes evident that apparent competitive inhibition of binding at site 1 can result from recognition of a different, not mutually exclusive, binding site. The decreased affinity of a ligand in the presence of a cation, or the ability of a cation to reduce the inhibition elicited by another cation, could result from allosteric interactions between different binding sites. So, the apparent affinity of an uptake blocker for the DAT could be the net result of its affinity for various ion-bound states of

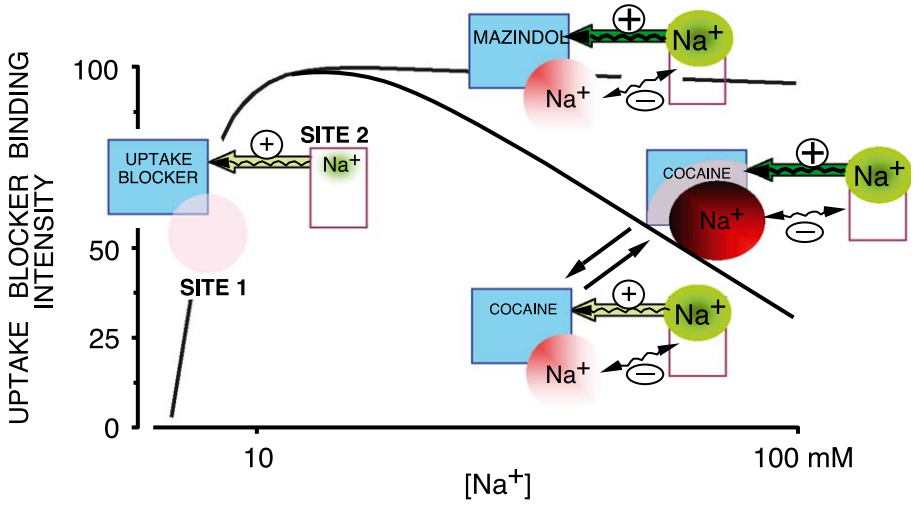

Fig. 2. Schematic representation of the biphasic effect of Na^+ on the binding of mazindol and cocaine to the DAT. The stimulation phase is the consequence of an exclusive or a predominant (as a function of the Na^+ concentration) positive allosteric effect resulting from Na^+ binding at site 2. The second phase results, for mazindol, from an equilibrium between a stronger positive effect and a direct inhibition by Na^+ binding at site 1. For cocaine, the second, decreasing, phase, could result from a low ability of Na^+ bound at site 2 to stimulate cocaine binding and/or a higher potency of Na^+ as binding inhibitor at site 1.

the transporter. Consequently, more work is needed to ascertain the mutually exclusive binding of ions and uptake blocker at site 1.

6.4. Even though this model seems rather satisfactory, it does not match perfectly with some experimental data

(1) If allosteric effects exist between Na^+ binding at site 2 and blocker binding at site 1, the reciprocal of the Na^+-induced positive allosterism between site 2 and site 1 should be a higher Na^+ binding at site 2 following an increase in the binding of the blocker at site 1. A first experiment performed using two very different concentrations of either WIN-35,428 or mazindol (0.2 and 20 nM) failed to demonstrate this (Corera et al., 2000). A second one, carried out using a smaller range of WIN concentrations (4–16 nM), gave more positive results (Chen et al., 2002). However, one cannot exclude that this result was not due to the ability of increasing concentrations of WIN-35,428 to overcome the binding inhibition produced by $Tris^+$ (HEPES), present in the medium, at site 1. As a consequence, the negative allosterism between sites 1 and 2 should be attenuated and Na^+ binding at site 2 favoured.

(2) The demonstration of the negative allosterism for ion binding at sites 1 and 2 is rather problematic. While it was found necessary in a first model dealing with RTI-121 binding (Chen et al., 1997c), it became negligible in a second modelling study concerning Na^+, K^+, and WIN-35,428 interactions with hDAT (Li and Reith, 1999). This later study confirms that Na^+ is likely to stimulate blocker binding at site 1, by a positive allosteric effect resulting from its binding at site 2, but it also suggests that the effects of Na^+ and K^+ at sites 2 and 1 are totally independent. Consequently, the aforementioned modifications of the Na^+ stimulation phase by low K^+ concentrations (Chen et al., 1997c; Corera et al., 2000) could be simply due to a dual effect of ions on blocker binding, stimulation by Na^+ binding at site 2, and inhibition by K^+ binding at site 1. Thus, increasing the K^+ concentration means that the Na^+ concentration must be raised to increase the positive allosteric effect and to overcome the deleterious effect of K^+ at site 1.

It is noteworthy that the negative allosterism which was significant in a study performed at 37 °C (Chen et al., 1997c) became negligible in another study carried out at 21 °C (Li and Reith, 1999). So, it is tempting to suggest that temperature could be an important parameter for revealing, or masking, this negative allosterism. Conformational changes of the DAT and allosteric effects are likely to be improved by an increase in temperature. Consistent with this are the results of earlier mentioned thermodynamics studies, and of a binding dissociation study in which a decrease in temperature, from 20 to 0 °C, attenuated, or even reversed, the effects of K^+, Ca^{2+} and Mg^{2+} on the dissociation of a GBR 12783–DAT complex (Héron et al., 1996). Thus, the inability of Na^+ to totally overcome the K^+-induced binding inhibition which was observed at 0 °C (Reith and Coffey, 1993) could be due to an impairment of allosteric effects, including negative allosterism, at this temperature. This inability of Na^+ was not found again in rather similar assays conducted at 20 °C (Corera et al., 2000). However, this negative allosterism may not be strong enough to remove the binding inhibition produced by Li^+ and H^+, whereas a reinforcement of the allosteric relationships, as a consequence of point mutations of the DAT, improved the ability of Na^+ to reduce this cation-induced binding inhibition (Chen et al., 2002).

(3) There are some reasons to believe that allosteric effects between ions binding sites could occur. Negative, or positive, allosterism between ion binding sites could explain two series of data. The first one concerns the high sensitivity of divalent cations to Na^+. As previously seen, an increase in the Na^+ concentration from 10 to 100–130 mM produced a 20- to 50-fold decrease in the Ca^{2+}- or Mg^{2+}-induced inhibition, when the K^+-inhibition underwent a 4- to 5-fold decrease (Table 2B). Similar large decreases were observed for Zn^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+} and Cd^{2+} (Bonnet et al., 1994). The ability of some of these cations to recognise site 2 is likely not involved in their inhibiting potency (Bonnet et al., 1994) and, consequently, in the differential effect of Na^+. It is tempting to speculate that all divalent cations possess a stabilising or stimulating effect on blocker binding which becomes increasingly evident when their inhibiting potency is overcome by Na^+. This has been discussed previously for Zn^{2+} and some other metals. The ability of Ca^{2+} to reduce the dissociation rate of a blocker–DAT complex in high Na^+ medium has been shown, but Mg^{2+} failed to produce the same effect (Héron et al., 1996). Ca^{2+} and Mg^{2+} probably do not recognise the stimulating binding site 3 for Zn^{2+} since they slow down the association rate of the blocker whereas Zn^{2+} decreases it (Bonnet et al., 1994; Héron et al., 1996). Another possibility is that the occupancy of site 1 by a divalent cation results, by strong negative allosterism, in a dramatic reduction of Na^+ binding at site 2 so that binding at site 1 of a Na^+ ion, instead of a divalent cation, has less effect on the binding of Na^+ at the stimulating site 2 (Fig. 3A). The second set of data concerns a recent study performed in $Tris^+$ (HEPES)-buffered medium. In this case, addition of 5 mM Na^+ resulted in an increase in the ability of K^+, Li^+ and H^+ to inhibit the binding of an uptake blocker (Chen et al., 2002). A possibility is that the binding of $Tris^+$ at site 2 produces a more intense negative effect on ion binding at site 1 than that induced by Na^+. As a consequence, Na^+ binding at site 2, instead of $Tris^+$ binding, attenuates the negative allosteric effect on ion binding at site 1, increasing K^+ binding at site 1 with the subsequent inhibition of blocker binding (Fig. 3B). So, these results prompt the question whether the negative allosterism between ion binding sites does exist and whether the different cations are equipotent in producing this allosteric effect.

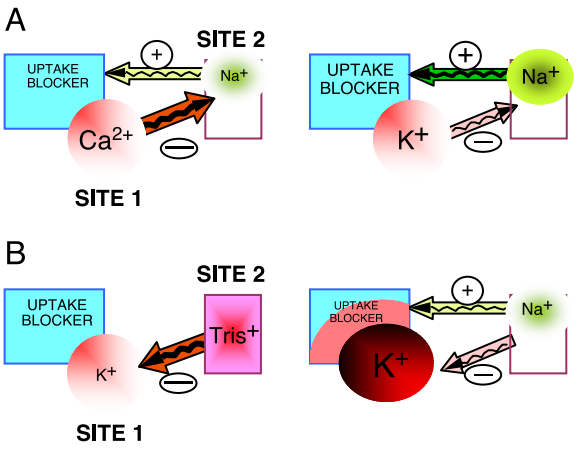

Fig. 3. Schematic representation of two potential cases of differential negative allosterism for ions. (A) For a similar occupancy of site 1, Ca^{2+} produces a more intense negative allosteric effect on Na^+ binding at site 2 (left part) than does K^+ (right part). Consequently, the competition between Na^+ and Ca^{2+} at site 1 should reduce the inhibition more markedly than the competition between Na^+ and K^+ at this site. (B) In medium devoid of Na^+ ions, the negative allosterism resulting from the binding of $Tris^+$ at site 2 markedly attenuates K^+ binding at site 1 (left part), so that the K^+ inhibition is higher when low Na^+ concentrations allow Na^+ to bind at site 2 (right part).

6.5. Contrary to site 3, the localisation of sites 1 and 2 on DAT remains uncertain

As previously stated, binding sites for uptake blockers and substrates probably share a common binding domain at the level of a central cavity of the DAT. However, the localisation of the ion recognition sites that should be present at this level (site 1) and distal from this site (site 2) remains rather obscure because of a virtually complete lack of investigations concerning this point. Some studies have been performed using hDAT/hNET (human norepinephrine transporter) chimeras, in order to characterise parts of the transporter involved in the Na^+ dependence of dopamine transport (Syringas et al., 2000, 2001); their results are not meaningful since the binding of dopamine to the DAT seems to be Na^+ independent (Li et al., 2002). However, it is noticeable that the region called cassette 1, which starts from the amino terminus of the transporter through the two first transmembrane domains, is strongly involved in the higher $Tris^+$-sensitivity of DAT-operated transport as compared to NET-operated transport. Thus, when compared to a substitution by sucrose, a substitution of NaCl by Tris/chloride was significantly more inhibitory when cassette 1 of hDAT was present in the parental transporter and in chimeras, and rather less inhibitory in the presence of cassette 1 of hNET (Syringas et al., 2000). Studies of site-directed mutagenesis and photoaffinity ligand incorporation have shown that some amino acids of the two first transmembrane domains could be important for the binding of dopamine or uptake blockers to the DAT (Kitayama et al., 1992; Vaughan et al., 1999; Lin et al., 2000a,b).

Cysteine residues are likely to be involved in cation binding at site 1. As suggested in various studies, the rank order of potency of Hg^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+} and Co^{2+} as binding inhibitors was rather characteristic of binding to –SH group(s) (Cao et al., 1989; Bonnet et al., 1994; Schweri, 1994; Norregaard et al., 1998). The ability of dithiothreitol to reverse the binding inhibition produced by Zn^{2+} and Cd^{2+} is also consistent with this view (Bonnet et al., 1994). In the same way, Na^+, K^+, Ca^{2+} and Mg^{2+}, used at concentrations active at site 1, either potentiate or reduce the ability of two ionic hydrophilic sulphydryl reagents to inhibit the binding of an uptake blocker (Refahi-Lyamani et al., 1995).

Cysteine residues are also important for the binding of an uptake blocker at site 1: uptake inhibitors and substrates can provide protection against the alkylation of cysteines involved in uptake inhibitor binding (Johnson et al., 1992; Reith and Selmeci, 1992; Richfield, 1993; Saadouni et al., 1994; Xu et al., 1997). More recently, mutation studies have shown that most of the cysteines in the DAT are not directly involved in the binding of WIN-35,428 but that cocaine binding alters the accessibility of cysteines to sulphydryl-specific agents (Ferrer and Javitch, 1998), consistent with the involvement of some cysteines in the conformational changes that the DAT undergoes following the binding of an uptake inhibitor (Bonnet et al., 1990; Ferrer and Javitch, 1998; Do-Régo et al., 1999; Reith et al., 2001) or during the uptake process (Chen et al., 2000).

7. Some anions also facilitate the binding of uptake blockers to the DAT, as far as they are associated with Na^+

The anion associated with Na^+ can significantly affect the intensity of the stimulation of binding (Reith and Coffey, 1993). Thus, the binding of GBR 12783, mazindol, cocaine and WIN-35,428 peaked at 10 mM Na^+ when the cation was associated with HCO_3^-/HPO_4^- (Bonnet et al., 1988; Zimanyi et al., 1989; Saadouni et al., 1994; Corera et al., 2000), whereas higher Na^+ concentrations were needed when the associated anions were mixed phosphates (Reith and Coffey, 1993; Corera et al., 2000). Anions exert their binding stimulation exclusively when they are associated with Na^+; association with a potent anion does not overcome the ineffectiveness of the cation K^+ to stimulate uptake blocker binding (Vignon et al., 1988). Consequently, it seems that Na^+ plays a very specific permissive role in the binding stimulation produced by anions (Fig. 1).

Associated with Na^+, anions increased uptake blocker binding with the following rank order of potency: $HCO_3^- > PO_4^{3-} \geq SO_4^{2-} > NO_3^- \geq Cl^- = Br^- = F^- =$ isethionate$^-$ (Diliberto et al., 1989; Reith and Coffey, 1993; Billaud et al., 1993; Corera et al., 2000; Chen et al., 2002). It is probably the chaotropic effect of I^- which allows it to stimulate the binding of WIN-35,428 (Reith and Coffey,

1993; but see also Corera et al., 2000), methylphenidate (Schweri, 1987) and mazindol (Corera et al., 2000), but it is generally accepted that other halides are ineffective as co-stimulating ion for the binding of uptake blockers. However, Cl^- was described in a single study as stimulating mazindol binding in an experiment in which it was added with Na^+ to a $Tris^+$-buffered medium (Javitch et al., 1984). It is possible that Cl^-, and other anions, could be more stimulatory when they are added to media containing ions unfavourable to blocker binding. This result could also be due to mazindol itself, since its sensitivity to high Na^+/anion concentrations seems rather specific. So, addition of 100 mM Na^+ to a 30 mM $NaHCO_3/NaH_2PO_4$ medium reduced WIN-35,428 binding by 30–40%, irrespective of which anion was associated with Na^+. In contrast, under the same conditions, $HCO_3^-/H_2PO_4^-$ and isethionate$^-$ reduced mazindol binding by more than 50%, when other anions, and particularly Cl^- and Br^-, were less inhibitory (Corera et al., 2000). Thus, at high Na^+ concentrations, the anion dependence of the binding of the uptake blockers could be specific for each of them.

The stimulating effect of the anion was demonstrated to result from an increase in affinity (Reith and Coffey, 1993). In the same way, a decrease in $NaHCO_3/NaH_2PO_4$ from 10 to 5 mM increased the dissociation rate of a DAT–blocker complex. This effect was not overcome by the addition of 5 mM NaCl or Na/isethionate, consistent with the inability of these anions to stimulate the binding of uptake blockers (Héron et al., 1996).

The localisation of the site at which anions can stimulate uptake blocker binding and its relationship with other sites on the DAT remain unknown.

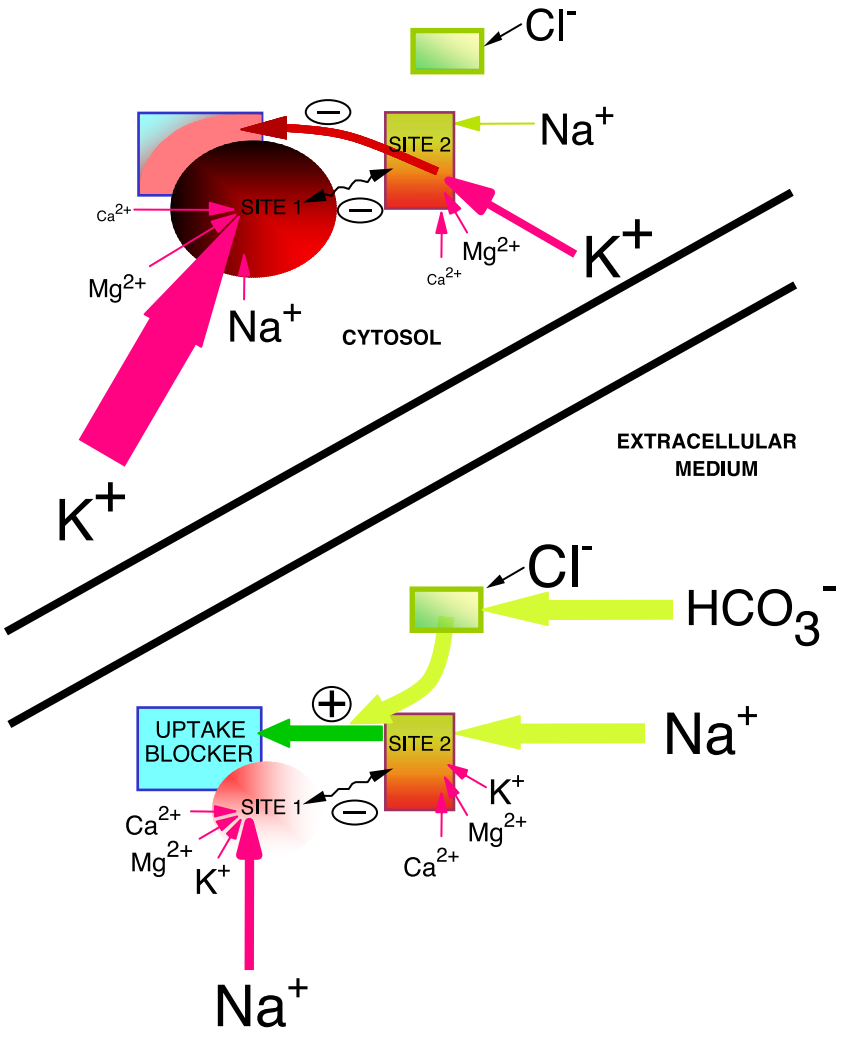

Fig. 4. Schematic representation of interactions of Na^+, K^+, Ca^{2+}, Mg^{2+} and anions with the DAT, in cytosolic and extracellular media. This representation postulates that all ion sites are equally accessible to ions specifically present in each of the compartments. The size of the ions is depicted in relation with their respective concentrations in each compartment. The intracellular concentration of Ca^{2+} is set at a high level (0.1 mM), since blockade of the DAT is particularly important immediately after depolarization-induced release of dopamine. Except for anions, the size of the arrows is proportional to the ratio of ion concentration/apparent affinity for each of the sites (taking into account the Na^+ concentration in each of the compartments). The HCO_3^- concentration in the extracellular medium (30 mM: Baggott, 1992) is considered as maximally stimulating uptake blocker binding in the presence of Na^+.

8. The dependence of the binding of dopamine on ions could be involved in its preferential inwards transport and used by uptake blockers for their own binding to the DAT

Some information has become available on the relationship between Na^+, K^+ and other ions, DAT and dopamine at its binding site. Na^+ does not seem to be strictly necessary for dopamine binding but it protects this binding against the inhibition produced by K^+ (Li and Reith, 2000; Li et al., 2002). Modelling of the interaction between Na^+, K^+, dopamine and the DAT yields a model which displays some similarities with that described for uptake blockers (Li and Reith, 2000; Li et al., 2002; Chen and Reith, this issue). In particular, in both models, K^+ recognises the cation site 1, in the binding common domain, and a second, distal site (site 2), at which it promotes the dissociation of dopamine. In the same way, Na^+ recognises both cation sites. Furthermore, Cl^- ions stimulate substrate binding to the DAT (Amejdki-Chab et al., 1992b; Wall et al., 1993; Gu et al., 1994; Li and Reith, 2000) through a «disinhibiting» effect on the binding of dopamine at site 1.

The dopamine uptake process begins by the binding of dopamine to its site located in the central cavity of the DAT, an extracellular gate being opened: DAT is in an outward facing position. Dopamine binding is rather easy because of favourable ion concentrations, i.e. high Na^+/Cl^- concentrations and a low K^+ concentration (Baggott, 1992). After some conformational changes such as closing of the extracellular gate and opening of the cytosolic gate, the loaded dopamine binding site is exposed to a combination of high K^+/low Na^+ concentrations which favour binding of K^+ at site 2. As a consequence, the dissociation of dopamine and its release in the cytosol occur. The probability that the DAT can load dopamine in this inward facing position is rather low since intracellular ion concentrations (high K^+, Mg^{2+}, low Na^+) are unfavourable for binding. However, a recent study suggests that the Na^+-inward current which is associated with dopamine uptake could be sufficient to allow the binding of dopamine to the inward facing transporter (Khoshbouei et al., 2003).

A possibility is that uptake blockers show opportunistic behaviour (Fig. 4). Their binding to the DAT is highly favoured when the DAT is in an outward facing position since high Na^+ stimulates their binding and removes the inhibiting potency of quite low extracellular concentrations of K^+, Ca^{2+} and Mg^{2+}. Furthermore, moderate extracellular concentrations of HCO_3^- (Baggott, 1992) associated with Na^+ have a stimulating effect on their binding (Fig. 4). In contrast, for the same reasons as for dopamine binding, when in an inward-facing position the DAT does not bind to the uptake blocker, but this situation lasts only a moment because the disappearance of the outward-facing forms of the DAT, which are bound to the uptake blocker, accelerates the reorientation of free inward-facing forms to free outward-facing configurations which are sensitive to the blocker.

So, why is site 1 so sensitive to physiological concentrations of cations, since Na^+, K^+, Ca^{2+} and Mg^{2+} are inhibitory at this site? A possibility is that amino acids of the central binding cavity that bind these cations are dramatically important for the DAT conformational changes involved in the transport process itself. We have previously underlined the involvement of –SH groups both in the transport process and in the sensitivity of site 1 to certain inhibiting cations. Carboxylic groups of acidic amino acids, which probably bind physiological cations, could be also important for dopamine transport. Under these conditions, the driving force for the binding of dopamine to its transporter is Na^+ binding at site 2, which removes the cation-induced inhibition at site 1, by a negative allosteric effect. Na^+ ions could be necessary for dopamine binding only for their removal of the inhibition of binding at site 1. Then, uptake blockers could take advantage of this situation twice because their binding is stimulated by Na^+ binding at site 2. Stimulating, is it not?

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