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Complex ionic control of [^{3}H]GBR 12783 binding to the dopamine neuronal carrier

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Abstract

At 20°C, [^{3}H]GBR 12783, {1-[2-(diphenylmethoxy)ethyl]4-(3-phenyl-2-([1-^{3}H]propenyl)-piperazine} dissociated from the dopamine neuronal carrier present in rat striatal membranes with a $t_{1/2}$ value of 27 min. At this temperature, KCl, $CaCl_2$ and $MgCl_2$ increased the binding dissociation, revealing that they recognize a binding site which is not mutually exclusive with that of [^{3}H]GBR 12783. The comparison of the ability of KCl to increase the binding dissociation (by 160% at 30 mM KCl) with its potency as a binding inhibitor ($K_i = 2.6 \pm 0.3$ mM) suggests an involvement of two recognition sites for K^+ in binding inhibition, a not mutually exclusive site and another, mutually exclusive, site. Divalent cations mainly inhibited the binding via a mutually exclusive site since 3 mM Ca^{2+} and 10 mM Mg^{2+} increased the binding dissociation by 90% at 20°C whereas their K_i values were 0.049 ± 0.006 and 0.141 ± 0.035 mM, respectively. Involvement of this mutually exclusive site was also supported by the persistence of the binding inhibition elicited by Ca^{2+} and Mg^{2+} at 0°C, a temperature at which they reduced the binding dissociation. At 20°C, 100 mM NaCl did not modify [^{3}H]GBR 12783 binding but it antagonized the binding dissociation elicited by inhibitory cations. Ca^{2+} reduced the off-rate of [^{3}H]GBR 12783 binding at 0°C and in the presence of 100 mM Na^+. Finally, [^{3}H]GBR 12783-binding dissociation was increased by high 'cytosolic' K^+ while 'synaptic' concentrations of Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^- were ineffective. A reduction of $H_2PO_4^-/HCO_3^-$ from 10 to 5 mM and a substitution of 5 mM $H_2PO_4^-/HCO_3^-$ by 5 mM Cl^- increased the binding dissociation, suggesting that an anion-binding site could also regulate the binding

Keywords: Dopamine carrier, neuronal; Binding, in vitro; Ion dependence; K^+ gradient

1. Introduction

A major functional property of the neuronal carriers for neurotransmitters is their dependence upon Na^+, K^+ and/or Cl^- transmembrane gradients which constitute driving forces for these active transport systems. Ionic gradients are likely to be involved in both the regulation of the neurotransmitter binding to the carrier and its preferential inward transport. Several studies dealing with the relationships between the neuronal uptake of dopamine and the binding to its carrier have been developed, using incubation media devoid of inhibitory cations and buffered with bicarbonate/phosphate (Bonnet et al., 1988; Zimanyi et al., 1989; Reith and Coffey, 1993) which buffer physiological fluids (Alberts et al., 1989; Darnell et al., 1990). These studies reveal that the dopamine transport generally depends on the properties of the binding to the carrier. Low Na^+ concentrations, in the 10–50 mM range, allow a maximal level of both dopamine uptake (Zimanyi et al., 1989) and binding to the carrier of pure uptake inhibitors, such as [^{3}H]GBR 12783 {1-[2-(diphenylmethoxy)ethyl]4-(3-phenyl-2-([1-^{3}H]propenyl)-piperazine}, [^{3}H]mazindol, [^{3}H]2 β-carbomethoxy-3β-(4-fluorophenyl)tropane (CFT or WIN 35428) and [^{3}H]cocaine (Bonnet et al., 1988; Zimanyi et al., 1989; Reith and Coffey, 1993; Saadouni et al., 1994). Cations, such as K^+, Ca^{2+} and Mg^{2+}, are not suitable as substitutes for Na^+ since they block the binding to the carrier and the specific uptake of dopamine. In addition, their inhibitory potency on binding or uptake is reduced or suppressed by an increase in Na^+ concentration (Bonnet et al., 1988; Reith and Coffey, 1993; Amejdki-Chab et al., 1992). Thus, all these results support that the ionic sensivity of the [^{3}H]GBR 12783 binding could be representative of that of dopamine binding to the neuronal carrier. On the other hand, direct binding of dopamine to

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the carrier cannot be characterized, probably on account of a too fast dissociation rate.

Previous work has led to the conclusion that Na^+, K^+, Ca^{2+} and transition metal ions could recognize a 'cation' site which is included in the binding domain of the tritiated inhibitors on the dopamine neuronal carrier (Bonnet et al., 1988; Bonnet et al., 1994; Reith and Coffey, 1993). Apart from these studies, little attention has been paid to the characterization of ion-recognition site(s) which could regulate this binding. Hence, the present study was aimed to investigate these sites. For this purpose, we have studied the effects of Na^+, K^+, Ca^{2+}, Mg^{2+} on the kinetic-rate constants of the specific binding of [^{3}H]GBR 12783 to the dopamine carrier, with a special attention to cation concentrations present in or around nerve cells. Some experiments were devoted to the effects of Cl^- which was used as paired anion. Dissociations were achieved by 'infinite' dilution of carrier–[^{3}H]GBR 12783 mixtures brought to binding equilibrium. They were generally performed at 20°C but some of them were performed at 0°C, a temperature which decreases the ability of the carrier to modify its configuration and which results in an inhibition of the transport (Holz and Coyle, 1974; Bonnet et al., 1990).

2. Materials and methods

2.1. Preparation of membrane suspensions

Male Sprague–Dawley rats (150–300 g) were purchased from Charles River (Saint Aubin lès Elbeuf, France). All procedures necessary to prepare membrane suspensions were done at 0–2°C.

Animals were killed by decapitation and the striata were dissected out and homogenized with 10 up-and-down strokes of a Teflon-glass homogenizer (800 rpm) in 10 vols. (w/v) of ice-cold 0.32 M sucrose. The nuclear material was removed by centrifugation at $1000 \times g$ for 10 min and the supernatant was stored. The P_1 pellet was resuspended in an equal volume of sucrose and recentrifuged. The two supernatants were combined and the mixture was centrifuged at $17\,500 \times g$ for 30 min. The resultant pellet P_2 was resuspended in 10 vols. of 10 mM Na^+ medium by sonication for 5 s (microprobe diameter 3 mm; Sonics and Materials, Danbury, CT) and centrifuged ($50\,000 \times g$, 10 min). The 10 mM Na^+ medium contained (mM concentration): NaH_2PO_4 0.3, $NaHCO_3$ 9.7; pH 7.5 ± 0.1. The final pellet was resuspended in the same medium by sonication. The protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.2. Binding experiments

The binding was routinely started by the addition of 400-μl aliquots of [^{3}H]GBR 12783 (0.8 nM final concentration) to silicone-coated tubes containing 1 ml of membrane preparation (50–100 μg protein) and drugs or their vehicles in a total volume of 3.6 ml (10 mM Na^+ incubation medium). The incubation period (1 h at 25°C, 4 h at 0°C) was stopped by rapid vacuum filtration through GF/B filters previously soaked for at least 1 h in 0.5% polyethyleneimine. Each tube was rinsed once; the filters were washed 3 × with 5 ml of ice-cold 10 mM Na^+ medium and the radioactivity counted by liquid scintillation spectrometry (BetamaticV, Kontron, Trappes, France) in 5 ml Optiphase Hisafe II with a 30–35% counting efficiency. The specific binding was calculated by subtracting the nonspecific binding defined in the presence of 30 μM mazindol from the total binding.

2.3. Dissociation experiments

Binding equilibrium was generally achieved by incubation in a silicone-coated beaker of striatal membranes (3 mg protein) and 0.8 nM [^{3}H]GBR 12783 in a total volume of 120 ml of either 10 mM Na^+ medium or 100 mM Na^+ medium containing (mM concentration): NaH_2PO_4 0.3, $NaHCO_3$ 9.7, NaCl 90; pH 7.5 ± 0.1. At the end of the incubation period (4 h at 0°C, 90 min at 20°C), 1-ml aliquots were diluted in 50 ml of dissociation medium containing the chloride form of the tested ions. Preliminary experiments showed that the addition of 10 mM Tris–HCl to a 10 mM Na^+ medium enhanced the dissociation-rate constant of the [^{3}H]GBR 12783 binding from 24 to 40. 10^{-3} min^{-1} at 20°C, demonstrating that Tris–HCl cannot be considered as a convenient buffer for studies of the binding to the dopamine neuronal carrier.

Procedures for stopping the dissociation and determination of filter radioactivity were similar to those already described for binding experiments. Zero time dissociations were made by dilution and immediate filtration. The constancy of the binding was checked and demonstrated by repeating this procedure at regular intervals during the dissociation period.

In a separate set of experiments, binding equilibrium was achieved in a 12 mM Na^+ medium containing 0.3 mM NaH_2PO_4 and 11.7 mM $NaHCO_3$ (pH 7.5 ± 0.1). Dissociation was then performed in this medium containing, when necessary, the chloride form of cations found in the cytosol (150 mM K^+) or in the synaptic cleft (128 mM Na^+, 4 mM K^+, 1 mM Mg^{2+} and 1 mM Ca^{2+}: Alberts et al., 1989; Darnell et al., 1990).

2.4. Association experiments

Rat striatal membranes (100 μg protein) were incubated for 40–90 min with 0.25 nM [^{3}H]GBR 12783 at 20°C in either the 10 mM Na^+ medium or in the same medium containing 90 mM NaCl, 3 mM KCl or 0.1 mM $CaCl_2$. The incubation was stopped as described above.

2.5. Calculations

The data obtained in association experiments were plotted according to the second-order rate equation described by Weiland and Molinoff (1981):

$$\ln[LRe(LT - LR \times LRe/RT)][LT(LRe - LR)] = k_{+1}t[(LT \times RT/LRe) - LRe]$$

in which LT is the total concentration of ligand, RT is the total concentration of binding sites determined from the B_{max}. LRe is the concentration of ligand–receptor complex at equilibrium and LR the concentration of the ligand–receptor complex at time t.

The data obtained in dissociation experiments were plotted according to the first-order equation $\ln(LR/LRe) = k_{-1}t$. Binding values obtained in a 4-h dissociation experiment performed at 20°C (Fig. 1) were plotted using a derivative-free nonlinear regression which gave estimate and S.D. value for each parameter (BMDP Statistical Software, Cork, Ireland).

Saturation curves were constructed as a function of free ligand concentrations, as reported by Seeman et al. (1984). IC_{50} values (cation concentrations which inhibit 50% of the control binding) were calculated by nonlinear regression analysis of the specific binding versus the log of the cation concentration (Ligand, Biosoft, Cambridge, UK). K_i values were calculated as described by Cheng and Prusoff (1973) for competitive inhibitors. The significance of change in individual parameters was tested with a Student's t test or a Dunnett's t test when different experimental conditions were compared with a common control group.

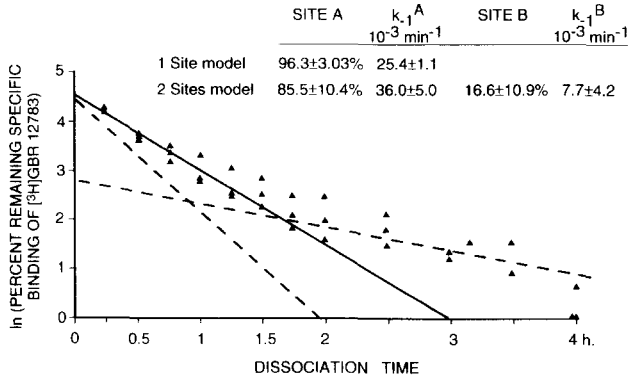

Fig. 1. Analysis of dissociation of [^{3}H]GBR 12783 binding from rat striatal membranes at 20°C. Binding of [^{3}H]GBR 12783 (0.8 nM final concentration) to striatal membranes was brought to equilibrium by incubation in a 10 mM Na^+ medium (1.5 h, 20°C); dissociations were initiated by a 50-fold dilution in same medium kept at 20°C. Binding values were plotted using a derivative-free nonlinear regression (BMDP Statistical Software) which gave estimate and S.D. for each parameter. Presented are individual binding values (from 3 sets of experiments) and regressions obtained using a one-site model (solid line) and a two-site model (broken lines).

2.6. Drugs

[^{3}H]GBR 12783 (29.2–31 Ci/mmol; > 98% purity) was prepared by a reduction of the amide precursor with sodium boro[^{3}H]hydride by Amersham (Les Ulis, France). 10-mM solutions of mazindol (Sandoz, Rueil-Malmaison, France) were prepared with 0.1 M HCl. Subsequent dilutions and solutions of other agents were performed in the incubation medium. Other drugs were from commercial sources.

3. Results

In a first experiment, the dissociation of [^{3}H]GBR 12783 was determined in a 10 mM Na^+ medium, for 4 h at 20°C (Fig. 1). Both a two-site model and a one-site model of regression fitted well with the binding data ($r = 0.99$ and 0.98, respectively). However, the two-site model gave a second population of sites (site B, Fig. 1) which was not statistically different from zero since this value was contained in the 95% confidence limits of the population (16.6% [−4.8 to 38%]). The use of the one-site model indicated that [^{3}H]GBR 12783 slowly dissociated from the carrier with a $t_{1/2}$ value of 27 min at 20°C (Fig. 1). In further experiments, dissociations performed at 20°C were routinely stopped after 90–120 min.

3.1. Kinetic constants of the specific binding of [^{3}H]GBR 12783 at 20°C

The addition of 90 or 290 mM NaCl to a 10 mM Na^+ medium did not modify the dissociation-rate constant of

Table 1
Effect of NaCl, KCl, $CaCl_2$ and $MgCl_2$ on dissociation-rate constant of [^{3}H]GBR 12783-specific binding at 20°C

Experimental conditions	Dissociation-rate constants ($10^{-3} \cdot min^{-1}$) at 20°C
Set 1	
10 mM Na^+ medium	21 ± 2
+ 30 mM KCl	55 ± 2.6 [a]
+ 3 mM $CaCl_2$	39 ± 1.1 [a]
+ 10 mM $MgCl_2$	40 ± 1.3 [a]
+ 90 mM NaCl	21 ± 2
Set 2	
10 mM Na^+ medium	24.3 ± 2.2
+ 290 mM NaCl	28.7 ± 3.5

Binding of [^{3}H]GBR 12783 (0.8 nM final concentration) was brought to equilibrium by incubation of rat striatal membranes in a 10 mM Na^+ medium (1.5 h, 20°C). Dissociations studied at 20°C, during a 120-min period, were initiated by a 50-fold dilution with tested media containing chloride form of different cations. Mean ± S.E.M. values of 3–6 experiments performed in duplicate. [a] Significantly different from control values with $P < 0.01$ (Dunnett's t test).

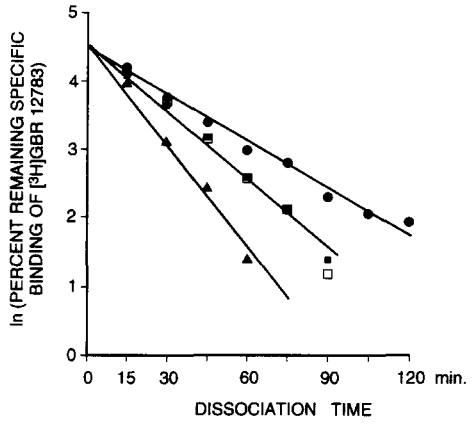

Fig. 2. Effect KCl, $MgCl_2$ and $CaCl_2$ on dissociation of [^{3}H]GBR 12783–carrier complex at 20°C. Binding of [^{3}H]GBR 12783 (0.8 nM final concentration) to striatal membranes was brought to equilibrium by incubation in a 10 mM Na^+ medium (1.5 h, 20°C). Dissociations were initiated by a 50-fold dilution in same control medium (●) containing, when necessary, 3 mM $CaCl_2$ (□), 10 mM $MgCl_2$ (■) or 30 mM KCl (▲). Data are mean ± S.E.M. of 3–6 experiments performed in duplicate. Corresponding dissociation-rate constants are reported in Table 1.

[^{3}H]GBR 12783 binding, revealing that increasing the Na^+ concentration from 10 to 300 mM or the addition of 90 or 290 mM Cl^- did not interfere with dissociation at 20°C (Table 1). The addition of KCl, $CaCl_2$ or $MgCl_2$ to a 10 mM Na^+ medium significantly enhanced the off-rate of [^{3}H]GBR 12783 (Fig. 2; Table 1, Set 1). The off-rate constant was increased by 160% in the presence of 30 mM KCl and by 87–90% in the presence of 3 mM $CaCl_2$ or 10 mM $MgCl_2$.

An equimolar replacement of 90 mM NaCl by *N*-methyl-D-glucamine HCl enhanced the dissociation of [^{3}H]GBR 12783 binding, so that its dissociation-rate constant was increased by 44 ± 9 and 175 ± 32% by 30 and 90 mM *N*-methyl-D-glucamine HCl, respectively ($n = 4$).

The association-rate constant of the [^{3}H]GBR 12783-specific binding was not modified by the addition of 90 mM NaCl or 3 mM KCl to a 10 mM Na^+ medium whereas it was reduced by the addition of 0.1 mM $CaCl_2$ (Fig. 3, Table 2). Calculations of kinetic K_d resulted in similar K_d values (0.15 nM) in both 10 and 100 mM Na^+ media (Table 2, Part A), a result which is substantiated by K_d values obtained in experiments of binding at equilibrium (Table 2, Part B). Furthermore, these latter experiments showed that the affinity of [^{3}H]GBR 12783 for its binding sites was significantly decreased by 0.1 mM $CaCl_2$ whereas it was only slightly affected by 3 mM KCl and 30 μM $MgCl_2$ (Table 2). None of these experimental conditions led to a significant change in the B_{max}.

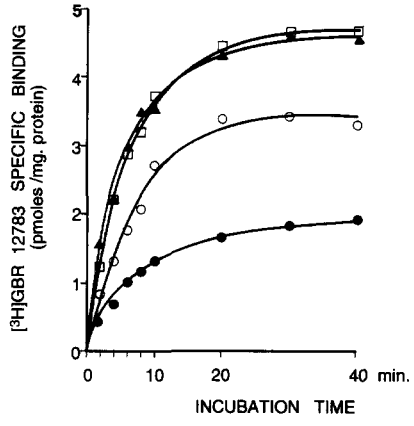

Fig. 3. Effect of NaCl, KCl and $CaCl_2$ on association of [^{3}H]GBR 12783 with striatal membranes at 20°C. Rat striatal membranes (100 μg protein) were incubated with 0.25 nM [^{3}H]GBR 12783 at 20°C in either 10 mM Na^+ medium (▲) or in same medium containing 90 mM NaCl (□), 3 mM KCl (○) or 0.1 mM $CaCl_2$ (●). Experiments dealing with $CaCl_2$ were carried out on a 90 min period. Presented are mean values of 2–4 assays performed in duplicate. Corresponding association-rate constants are reported in Table 2.

Table 2
Effect of NaCl, KCl, $CaCl_2$ and $MgCl_2$ on constants of [^{3}H]GBR 12783-specific binding at 20°C

Part A	Kinetic rate constants of specific binding of [^{3}H]GBR 12783	
Experimental conditions	k_{+1} ($\mu M^{-1} \cdot min^{-1}$)	k_{-1} ($10^{-3} \cdot min^{-1}$)
10 mM Na^+ medium	138 ± 21	21 ± 2
100 mM Na^+ medium	137 ± 10	21 ± 2
10 mM Na^+ medium + 3 mM KCl	122 (128–117)	ND
10 mM Na^+ medium	130 (113–147)	
10 mM Na^+ medium + 0.1 mM $CaCl_2$	76 (69–83)	

Part B	Constants of equilibrium binding of [^{3}H]GBR 12783	
Experimental conditions	K_d (nM)	B_{max} (pmol/mg protein)
10 mM Na^+ medium	0.51 ± 0.08	12.0 ± 0.7
+ 0.1 mM $CaCl_2$	1.52 ± 0.09 [a]	10.8 ± 1.1
+ 30 μM $MgCl_2$	0.73 ± 0.04	12.3 ± 0.7
+ 3 mM KCl	0.78 ± 0.07	11.4 ± 0.5
10 mM Na^+ medium	0.37 ± 0.07	10.7 ± 0.6
+ 90 mM NaCl	0.33 ± 0.07	10.6 ± 1.0

Striatal membranes were incubated at 20°C in 10 mM Na^+ medium. Data are mean ± S.E.M. values of 2–4 experiments in duplicate. Individual values were given when 2 experiments were performed.

Part A: Associations (0.25 nM [^{3}H]GBR 12783 final concentration) were plotted according to equation described by Weiland and Molinoff (1981) in which RT values were determined from respective B_{max} reported in Part B. Dissociations studied at 20°C were initiated by a 50-fold dilution of binding assays (0.8 nM [^{3}H]GBR 12783 final concentration) brought to equilibrium in 10 mM Na^+ medium.

Part B: Saturations were performed in a 10 mM Na^+ medium containing 0.15–4.8 nM [^{3}H]GBR 12783 (1 h, 80 μg protein/assay). Saturation curves were constructed as a function of free ligand concentrations as reported by Seeman et al. (1984). [a] Significantly different from control values with $P < 0.01$ (Dunnett's t test). ND, not determined.

Table 3
Effect of Na^+ and/or its paired anion on dissociation-rate constant of [^{3}H]GBR 12783-specific binding observed at 20°C

Experimental conditions	Dissociation-rate constant ($10^{-3} \cdot min^{-1}$) at 20°C
Set 1	
10 mM $NaHCO_3/NaH_2PO_4$	28.3 ± 1.3
5 mM $NaHCO_3/NaH_2PO_4$	37.0 ± 2.1 [a]
+ 5 mM NaCl	37.0 ± 1.7 [a]
+ 5 mM Na isethionate	34.3 ± 3.0
+ 10 mM sucrose	45.3 ± 7.5
Set 2	
100 mM Na^+ medium	24.5 ± 1.3
+ 30 mM KCl	41.0 ± 3.3 [a]
+ 10 mM $MgCl_2$	36.5 ± 1.6 [a]
Set 3	
12 mM Na^+	20.7 ± 1.7
+ 150 mM KCl	87.0 ± 0.6 [a]
140 mM Na^+	24.0 ± 1.0 [ns]
+ 4 mM KCl + 1 mM $CaCl_2$ + 1 mM $MgCl_2$	26.3 ± 0.3 [b NS]

Binding of [^{3}H]GBR 12783 (0.8 nM final concentration) to striatal membranes was brought to equilibrium by incubation (1.5 h, 20°C) in a 10 mM Na^+ medium (Set 1), 100 mM Na^+ medium (Set 2) and 12 mM Na^+ medium (Set 3). Dissociations were initiated by a 50-fold dilution in tested media kept at 20°C. Data are mean ± S.E.M. values of 3–4 experiments performed in duplicate. Sets 1 and 2: Values were compared with each control group using a Dunnett's t test (values obtained in medium containing sucrose were excluded from comparison after ANOVA): [a] Significantly different from control values with $P < 0.05$. Set 3: Values were compared using Tukey test: Significantly different from control values with [a] $P < 0.001$, [b] $P < 0.05$; [ns] not significant from 12 mM Na^+; [NS] not significantly different from values obtained with 140 mM Na^+ medium.

3.2. Modifications of the [^{3}H]GBR 12783 dissociation observed at 20°C by Na^+ and/or its paired anion

The dissociation-rate constant was significantly increased when the concentration of $NaH_2PO_4/NaHCO_3$ was reduced from 10 to 5 mM; this increase was observed even when a 10 mM Na^+ concentration was maintained by the addition of 5 mM NaCl (Table 3, Set 1). The addition of either 5 mM Na-isethionate or 10 mM sucrose in order to maintain osmolarity seemed to be ineffective in preventing this increase (Table 3, Set 1).

The addition of 10 mM $MgCl_2$ or 30 mM KCl to a 100 mM Na^+ medium significantly increased the dissociation rate of the [^{3}H]GBR 12783-specific binding (Table 3, Set 2). A comparative study (Fig. 4) revealed that an increase in Na^+ concentration from 10 to 100 mM significantly modified the ability of KCl, $CaCl_2$ or $MgCl_2$ to accelerate the dissociation of [^{3}H]GBR 12783 ($F(2,12) \geq 7.4$, $P < 0.01$; two-way ANOVA) (legend of Fig. 4).

Experiments were undertaken to determine the dissociation rate of [^{3}H]GBR 12783 in media containing cation concentrations found in physiological fluids. The dissociation-rate constant observed in a 'cytosolic' medium containing 150 mM KCl and 12 mM Na^+ ($H_2PO_4^-/HCO_3^-$) was dramatically higher than that observed in a 'synaptic' medium containing 140 mM Na^+, even in the presence of

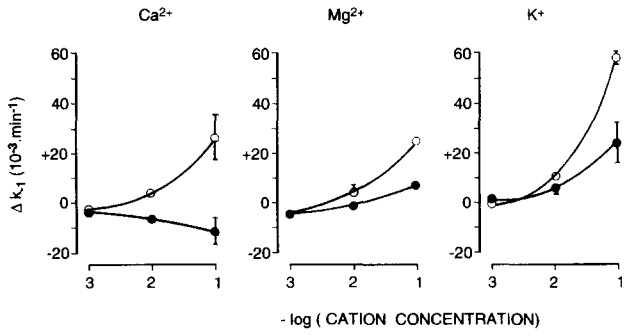

Fig. 4. Effect of NaCl on increase in dissociation-rate constants of [^{3}H]GBR 12783 binding elicited by $CaCl_2$, $MgCl_2$ or KCl. Binding of [^{3}H]GBR 12783 (0.8 nM final concentration) to striatal membranes was brought to equilibrium by incubation in a 10 mM Na^+ medium (1.5 h, 20°C). Dissociations were initiated by a 50-fold dilution in same medium (○) or in a 100 mM Na^+ medium (●). These media contained increasing concentrations of either $CaCl_2$, $MgCl_2$ or KCl. Presented are variations of dissociation-rate constants observed in presence of $CaCl_2$, $MgCl_2$ or KCl relatively to their respective controls (10 and 100 mM Na^+ media). Absolute values were compared using a two-way ANOVA: there were significant interactions between effects of Na^+ and effects of $CaCl_2$ ($F(2,12) = 7.4$, $P < 0.01$), $MgCl_2$ ($F(2,12) = 17.4$, $P < 0.001$) or KCl ($F(2,12) = 11.9$, $P < 0.001$) on [^{3}H]GBR 12783 dissociation-rate constants. Data are mean ± S.E.M. of 3 experiments performed in duplicate.

Table 4
Effect of NaCl, KCl, $CaCl_2$ and $MgCl_2$ on binding of [^{3}H]GBR 12783 observed at 0/20°C and on its dissociation-rate constant at 0°C

Part A Experimental conditions	Dissociation-rate constant ($10^{-3} \cdot min^{-1}$) at 0°C	
10 mM Na^+ medium	5.03 ± 0.03	
+ 30 mM KCl	8.56 ± 0.40 [a]	
+ 3 mM $CaCl_2$	3.81 ± 0.13 [a]	
+ 10 mM $MgCl_2$	4.46 ± 0.36	
+ 90 mM NaCl	7.34 ± 0.49 [a]	
Part B	K_i (mM) at	
Tested ions	0°C	20°C
KCl	5.0 ± 2.3	2.6 ± 0.3
$MgCl_2$	0.266 ± 0.071	0.141 ± 0.035
$CaCl_2$	0.098 ± 0.016 [b]	0.049 ± 0.006

Data are mean ± S.E.M. values of 3 experiments performed in duplicate. Part A: Binding of [^{3}H]GBR 12783 (0.8 nM final concentration) to striatal membranes was brought to equilibrium by incubation in a 10 mM Na^+ medium (4 h, 0°C); dissociations were initiated by a 50-fold dilution in tested media kept at 0°C. Values were compared with 10 mM Na^+ control group using a Dunnett's t test: [a] Significantly different from control values with $P < 0.01$.

Part B: Rat striatal membranes (50–60 μg protein) were incubated with 0.8 nM [^{3}H]GBR 12783 (1 h at 20°C, 4 h at 0°C) in a 10 mM Na^+ medium containing increasing concentrations of KCl, $CaCl_2$ or $MgCl_2$. [b] Significantly different from K_i value obtained at 20°C with $P < 0.05$ (Student's t test). Control binding values were 4.4 ± 0.3 and 3.7 ± 2.2 pmol/mg protein at 0 and 20°C, respectively.

KCl, $CaCl_2$ and $MgCl_2$ at concentrations found in extracellular fluids (Table 3, Set 3).

3.3. [^{3}H]GBR 12783 dissociation at 0°C

The dissociation-rate constant calculated for a 10 mM Na^+ medium underwent a 4–5-fold decrease when dissociation (and association) were performed at 0°C instead of 20°C (Table 1, Table 4). At 0°C, the addition of 30 mM KCl or 90 mM NaCl to a 10 mM Na^+ medium significantly increased the [^{3}H]GBR 12783 dissociation-rate constant by 70 and 47%, respectively (Table 4). On the contrary, the addition of 10 mM $MgCl_2$ or 3 mM $CaCl_2$ slightly reduced the dissociation of [^{3}H]GBR 12783; this reduction was significant for $CaCl_2$ (Table 4).

Finally, experiments of equilibrium binding showed that a decrease in temperature from 20 to 0°C increased the K_i values for K^+ and divalent cations on the specific binding of [^{3}H]GBR 12783 by 90–100%; this increase was significant for $CaCl_2$ (Table 4).

4. Discussion

The present data reveal some interesting findings concerning the relationships between ions and the specific binding of [^{3}H]GBR 12783 to the dopamine neuronal carrier: (1) effects of ions on binding are dependent on temperature; (2) ionic dependence of binding is a complex phenomenon which involves various recognition sites for ions; and (3) occupancy of some of these ion-recognition sites decreases the stability of the already established [^{3}H]GBR 12783–carrier complex. Furthermore, some results suggest that K^+ transmembrane gradient could have a role in the inward transport of neurotransmitters.

The results from experiments of dissociation performed at 20°C clearly indicate that the occupancy of recognition site(s) by different cations lowers the stability of the [^{3}H]GBR 12783–carrier complex and hence increases the dissociation of the ligand. Thus, K^+, $Tris^+$, Ca^{2+}, Mg^{2+} and *N*-methyl-D-glucamine$^+$ increased the dissociation-rate constant of the binding of [^{3}H]GBR 12783 when they were added to the medium used for dissociation. These cations have been reported as inhibitors of the binding of [^{3}H]GBR 12783 and other uptake inhibitors (Kennedy and Hanbauer, 1983; Zimanyi et al., 1989; Amejdki-Chab et al., 1992; Reith and Coffey, 1993; Coffey and Reith, 1994). On the other hand, nonspecific effects of Cl^- and/or ionic strength in dissociation experiments carried out at 20°C can be discarded due to the lack of any effect of high NaCl concentrations on dissociation-rate constants and on binding affinity at this temperature (Table 1, Table 2). Furthermore, demonstration of an effect of cations on the [^{3}H]GBR 12783–carrier complex indicates that bindings to cation-recognition site(s) and to the [^{3}H]GBR 12783 site are not mutually exclusive. Finally, Na^+ is also likely to bind to this (these) cation-recognition site(s) since an increase in its concentration reduced the effects of K^+, Ca^{2+} and Mg^{2+} on dissociation (Table 3, Fig. 4).

The involvement of this (these) cation-recognition site(s) in the inhibition of [^{3}H]GBR 12783 binding provoked by Ca^{2+} and Mg^{2+} is partial at best. Thus, at 20°C, Ca^{2+} and Mg^{2+} increased the dissociation-rate constant by only 90% when they were present at concentrations about 100-fold higher than their K_i values (Table 1, Table 4). These results suggest that aforementioned sites for cations are probably involved in binding dissociation resulting from high concentrations of divalent cations but likely not in the competitive inhibition of binding observed in the presence of submillimolar concentrations of Ca^{2+} and Mg^{2+} (Table 2, Table 4). This is also supported by the persistence of the inhibition of binding elicited by Ca^{2+} and Mg^{2+} at 0°C, a temperature at which they improve the stability of the [^{3}H]GBR 12783–carrier complex (Table 4). Consequently, submillimolar concentrations of Ca^{2+} and Mg^{2+} are likely to block the binding through recognition of another binding site. This binding site is mutually exclusive with that of [^{3}H]GBR 12783 since submillimolar concentrations of divalent cations seem to be devoid of any effect on the binding dissociation. Na^+ ions are likely to recognize this site since they decrease the inhibition of binding produced by Ca^{2+} and Mg^{2+} at 0°C (Bonnet et al., 1988). This same site could be the way by which several transition metals, including Zn^{2+}, block the binding of [^{3}H]GBR 12783 (Bonnet et al., 1994). Mutually exclusive binding of Zn^{2+} and [^{3}H]GBR 12783 and reduction of the inhibition of binding produced by transition metals by Na^+ or Ca^{2+} are consistent with this hypothesis (Bonnet et al., 1994). Taken together, all these different cations could block the binding via the recognition of a common site that we could tentatively call 'site for inhibitory cations'.

The ability of Ca^{2+} to decrease the off-rate of [^{3}H]GBR 12783 binding at 0°C or in the presence of a high Na^+ concentration constitutes a somewhat unexpected result (Table 4, Fig. 4). It suggests that Ca^{2+}, and perhaps Mg^{2+} to a lesser extent, could bind to an additional cation site which is virtually not recognized by Na^+. However, this site could be of little interest in physiological conditions since it is recognized only by high millimolar concentrations of Ca^{2+} at 20°C (Fig. 4).

K^+ ions competitively block the specific binding of [^{3}H]GBR 12783. The K^+ – induced decrease in affinity observed at 20°C (Table 2) is consistent with the significant reduction in the affinity reported in a previous study performed at 0°C (Bonnet et al., 1988). K^+ at 30 mM increases the dissociation-rate constants by 70 and 160%, at 0 and 20°C, respectively (Table 1, Table 4). Thus, K^+ ions are likely to lower the stability of the [^{3}H]GBR 12783–carrier complex via a cation-recognition site which is not mutually exclusive with the binding site for the radioligand. This cation-recognition site should be located so close to the binding site for [^{3}H]GBR 12783 that K^+

effects on dissociation partly persist at 0°C, a temperature which decreases the ability of the carrier to modify its configuration. On the other hand, the mild increase in binding dissociation elicited by a K^+ concentration 6-fold higher than its K_i value at 0°C makes it probable that K^+ ions produced also a binding inhibition through a binding site for cations which cannot be recognized when the GBR 12783–carrier complex has been made. It is necessary to further characterize this site but it possibly corresponds to the so-called 'site for inhibitory cations'.

Na^+ ions are remarkable for their ability to antagonize inhibition of binding and [^{3}H]dopamine uptake produced by K^+, Ca^{2+} and Mg^{2+} (Bonnet et al., 1988; Amejdki-Chab et al., 1992; Billaud et al., 1993). Furthermore, the present results demonstrate that Na^+ interacts with cation-recognition site(s) by which K^+, Ca^{2+} and Mg^{2+} increase the dissociation-rate constants of [^{3}H]GBR 12783 binding (Table 3, Fig. 4). It is noteworthy that an increase in Na^+ concentration and a decrease in temperature resulted in some comparable effects: (1) they both reduced the increase in binding dissociation produced by inhibitory cations (Table 4, Fig. 4); (2) they both revealed that Ca^{2+}, and Mg^{2+} to a lesser extent, can exert a stabilizing effect on the [^{3}H]GBR 12783–carrier complex (Table 4, Fig. 4); and (3) they both reduced the ability of Mg^{2+} and Ca^{2+} to block the specific binding of [^{3}H]GBR 12783 (Bonnet et al., 1988; Table 4). These data suggest that a decrease in temperature could improve the Na^+ binding to the carrier and consequently its effects on [^{3}H]GBR 12783 binding. In this respect, the increase in the off-rate of binding produced by 90 mM NaCl at 0°C (Table 4) could be an improvement of the marginal effect of 290 mM Na^+ at 20°C (Table 1). In other respects, the increase in binding dissociation induced by NaCl could be involved in the reduction of binding affinity for [^{3}H]GBR 12783 and other tritiated inhibitors which has already been observed in the presence of high Na^+ concentrations (Bonnet et al., 1988; Zimanyi et al., 1989; Reith and Coffey, 1993; Saadouni et al., 1994). However, one cannot exclude that Cl^- alone could stimulate the dissociation of the [^{3}H]GBR 12783 binding at 0°C, even if Cl^- is considered to produce little or no effect on radioligand binding to the dopamine carrier (Reith and Coffey, 1993).

As far as the effect of anions is concerned, it is noteworthy that a reduction of $H_2PO_4^-/HCO_3^-$ concentration from 10 to 5 mM significantly increased the dissociation rate of [^{3}H]GBR 12783 binding (Table 3). This increase was also observed when 5 mM NaCl was used as substitute for 5 mM $NaH_2PO_4/NaHCO_3$. These results are consistent with previous data showing that $H_2PO_4^-$ and HCO_3^- could increase the affinity of uptake inhibitors in the 0–30 mM range concentration (Reith and Coffey, 1993). Furthermore, they suggest that a not mutually exclusive site for anion could be involved in this effect.

According to the model developed by Zimanyi et al. (1989) (see also Lester et al., 1994) for the transport of dopamine through the neuronal membrane, the neurotransmitter-binding site faces alternatively outwards and inwards during the uptake process. Interestingly, cation concentrations present in extracellular fluids did not modify the dissociation of [^{3}H]GBR 12783 whereas high 'cytosolic' K^+ concentrations dramatically increased the off-rate of the binding (Table 3, Set 3). Thus, if similar K^+ effects on dopamine binding occur, this could underlie a major involvement of the K^+ transmembrane gradient in the preferential inward transport of dopamine.

In conclusion, Ca^{2+} and Mg^{2+} are likely to block the binding of [^{3}H]GBR 12783 to the dopamine neuronal carrier through binding to a 'site for inhibitory cations'. From a physiological point of view, changes in dissociation-rate constants produced by these cations seem to be of little interest. K^+ ions probably recognize two cation sites which are involved in a binding inhibition and a dissociation of the binding which is already established. Na^+ ions reduce binding inhibition and binding dissociation elicited by K^+, Ca^{2+} or Mg^{2+}.

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