Differential effect of pH on sodium binding by the various GABA transporters expressed in *Xenopus* oocytes

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Abstract **Mouse GABA transporters belong to the family of Na^+- and Cl^--dependent neurotransmitter transporters. The four GABA transporters exhibit unique presteady-state currents when expressed in *Xenopus* oocytes. The properties of the presteady-state currents correspond to their different affinities to Na^+. In the presence of 20 μM GABA and at pH 7.5, the half-maximal uptake activity was 47, 120, 25 and 35 mM Na^+ for GAT1, GAT2, GAT3 and GAT4, respectively. The appearance of presteady-state currents at positive or negative imposed potentials was in correlation with the affinity to Na^+. Changing the external pH differentially affected the GABA uptake and the presteady-state activities of the various GABA transporters. It is suggested that protons compete with Na^+ on its binding site; however, the proton binding is not productive and is unable to drive GABA uptake.** © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Neurotransmitter; γ-Aminobutyric acid; Transport; Transporter; Proton; Sodium

1. Introduction

Sodium-driven transport of organic solutes is quite common in plasma membrane of vertebrate cells. On the other hand, plant fungi and most bacteria primarily utilize the protonmotive force for energizing similar uptake systems [1,2]. Although there is a clear distinction among the transporters that utilize the specific cations, the transition from proton-driven to sodium-driven transport requires very little genetic alteration [3–5]. The family of Na^+/Cl^- neurotransmitter transporters utilizes electrochemical gradients of sodium for driving the uptake of neurotransmitters [6–8]. In most of the family members, two Na^+, one Cl^- and one substrate molecules are co-transported across the membrane, resulting in a net positive charge movement in the same direction. However, the above stoichiometry may vary from one transporter to the other; the affinity to Na^+ and the effect of Cl^- are specific to each transporter and some even require potassium for their transport activity [7,9–11]. It was demonstrated that the prototype of the GABA transporters (GAT1) operates by a co-transport of two Na^+, one Cl^- and one GABA moieties [6]. However, this notion was challenged and it was suggested that the GABA transporter operates asymmetrically and with variable stoichiometry [12].

GAT1, GAT2, GAT3 and GAT4 are four pharmacologically distinct GABA transporters that have been identified in mouse brain and their cDNAs have been cloned and sequenced [14–16]. The uptake mechanism and the electrophysiological properties of GAT-1, the GAT1 homologue from rat, and BGT-1, the canine homologue of GAT2, were previously studied in detail, in cRNA-injected *Xenopus laevis* oocytes and transfected mammalian cells [7,13,17–21]. Considerable differences were found concerning the ion co-transport stoichiometry, steady-state, presteady-state and leak currents. The effect of proton concentrations on the substrate uptake was studied in some of the neurotransmitter transporters [22–26].

It was shown that GABA uptake by GAT1 and BGT-1 is differentially affected by the external pH [20]. In this communication, we examined the effect of pH modulation on GABA uptake and electrophysiology of the four mouse GABA transporters.

2. Materials and methods

2.1. Cloning procedures

cDNAs encoding the mouse GABA transporters GAT1, GAT2, GAT3 and GAT4 were cloned as previously described [14–16]. The cDNAs were subcloned by PCR into a pGEM-HJ plasmid [27]. This expression vector contains 5′ and 3′ untranslated regions from the *Xenopus* β-globin gene. The four plasmids were linearized by *Not*I and the cRNAs were synthesized in vitro (Ambion mMessage mMachine; Austin, TX, USA) using T7 RNA polymerase.

2.2. Expression of mouse GABA transporters in Xenopus oocytes

X. laevis oocytes were handled as described previously [27]. Stage V–VI oocytes were obtained from *X. laevis* and collagenase-treated. Oocytes were injected on the following day with the cRNA of the GAT1, GAT2, GAT3 and GAT4. Injected oocytes were incubated at 18°C in a solution containing 100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 2.5 mM sodium pyruvate, 5 mM HEPES (pH 7.6) and 50 mg/ml gentamicin.

2.3. [^{3}H]GABA uptake in Xenopus oocytes

Uptake experiments were performed 3–5 days after injection. The solution for the uptake experiments contained 100 mM NaCl, 10 mM HEPES, 2 mM Mes, 2 mM KCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The solution was adjusted to the indicated pH using NaOH, Tris base or H_2SO_4. Prior to the uptake experiment, oocytes ($n = 10$) were washed twice in 0.5 ml of the uptake solution. They were then incubated in 0.5 ml of the uptake solution, containing about 0.1 μCi [^{3}H]GABA (Amersham Pharmacia Biotech, UK) and 20 μM cold GABA, for

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30 min at room temperature (25°C). After incubation, oocytes were washed five times with 0.5 ml of the uptake solution. Individual oocytes were solubilized in 100 μl 1% SDS and the radioactivity was determined by scintillation counting.

2.4. Electrophysiological experiments

The two-microelectrode voltage clamp technique was used for the recording of whole-cell-mediated currents as previously described [27]. All experiments are representative of at least five independent repetitions.

3. Results and discussion

3.1. Mouse GABA transporters exhibit different presteady-state and steady-state currents

It was demonstrated that, as in several other transporters, sodium binding and release by GAT1, GAT3 and BGT-1 (the canine homologue of GAT2) resulted in the appearance of presteady-state currents [17,18,21,27]. In this work we recorded the presteady-state currents of the four mouse GABA transporters expressed in *Xenopus* oocytes. As shown in Fig. 1A, the transporters exhibit presteady-state currents in the presence of 100 mM NaCl. However, there is a dramatic difference in the appearance of the presteady-state currents in the various GABA transporters. While GAT1 exhibited symmetric presteady-state currents, in both negative and positive imposed potential, GAT2, GAT3 and GAT4 exhibited asymmetric presteady-state currents. In oocytes expressing GAT2, the presteady-state currents were recorded exclusively at negative imposed potentials, whereas oocytes expressing GAT3 and GAT4 gave presteady-state currents exclusively at posi-

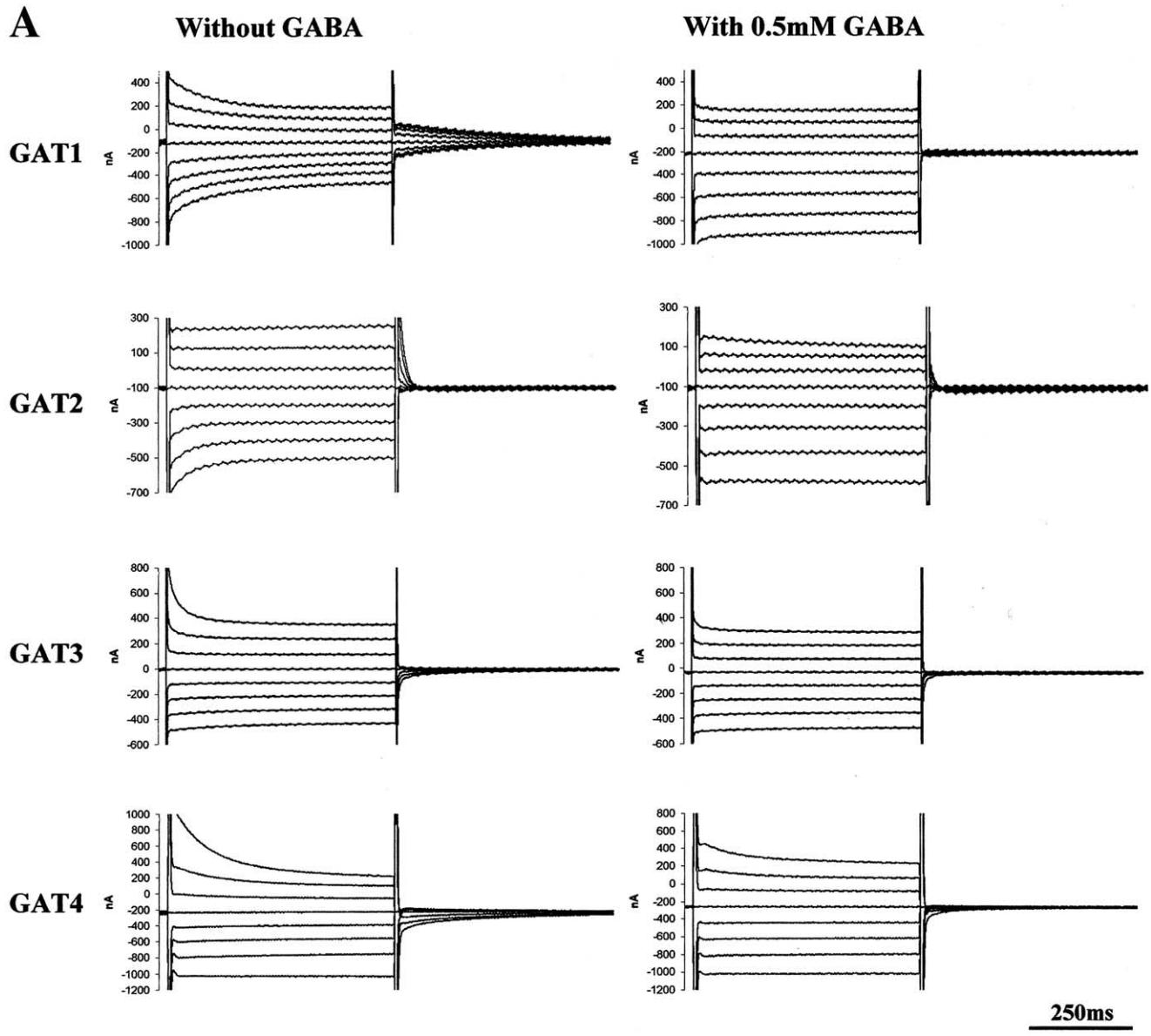

Fig. 1. The presteady-state, the GABA-induced steady-state currents and the *I–V* relationship of the four mouse GABA transporters. A: The presteady-state currents of the four mouse GABA transporters GAT1, GAT2, GAT3 and GAT4 were recorded as described in Section 2. In the presence of 0.5 mM GABA, the presteady-state currents of all GABA transporters disappeared and a GABA-evoked steady-state inward current was recorded. B: At steady-state, the difference between the current traces in the absence and in the presence of GABA yielded the net GABA-evoked, inward current, as the *I–V* relationship of the four mouse GABA transporters.

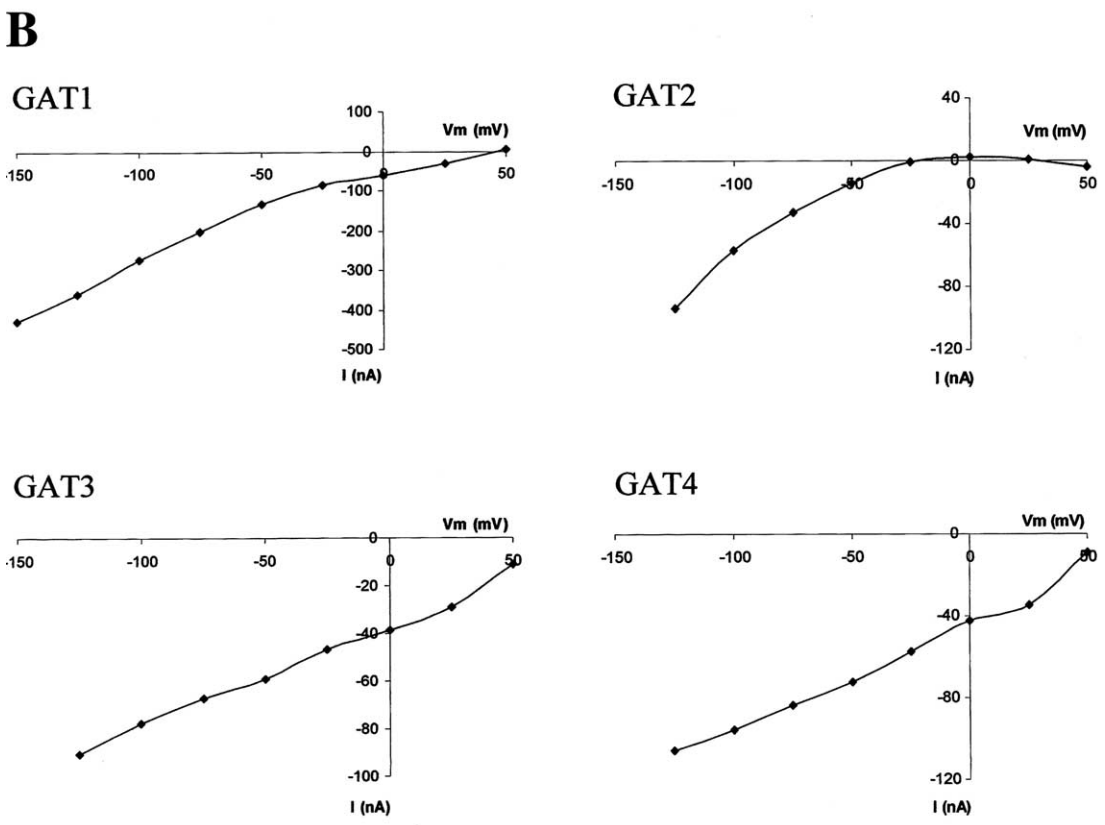

Fig. 1 (*Continued*).

tive imposed potentials (Fig. 1A). Addition of 0.5 mM GABA to the bath caused an inward current in all oocytes expressing the four GABA transporters (Fig. 1A). The GABA-evoked currents were examined as a function of the membrane voltage (V_m) in the voltage range of +50 mV to −125 mV (Fig. 1B). The current–voltage (I–V) relationships of the four GABA transporters were also characteristic for each one of them. While GAT1 and GAT2 showed very small GABA-induced currents at neutral and positive potentials, GAT3 and GAT4 exhibited measurable currents at those potentials. GAT1, GAT3 and GAT4 exhibited a fairly linear relation between −125 mV and +50 mV, suggesting that the rate-limiting step for the GABA transport cycle is voltage-dependent [13,27]. However, in all the four GABA transporters, a deviation from this relation could be observed. The GABA-induced current of GAT2 was rectified at negative potentials. The deviation from linearity was more pronounced when the external Na^+ concentration was reduced (Fig. 2B). The effect of voltage on prolonged GABA uptake is also in variance with the fast kinetic data. While very small fast currents were generated by addition of GABA at neutral and positive potentials (Figs. 1B and 2B), substantial GABA uptake was measured at those potentials when it was allowed to proceed for 5 min (Fig. 2C).

3.2. *The four GABA transporters have different affinities to sodium*

The affinity to sodium of the four mouse GABA transporters was determined by uptake experiments, with oocytes injected with cRNA encoding the various GABA transporters. With all the transporters, increased sodium concentration gave sigmoid saturation curves with a Hill coefficient of about 2. The calculated half-maximal activities for Na^+ were 25 mM for GAT3, 36 mM for GAT4, 47 mM for GAT1 and 120 mM for GAT2. Thus, the presteady-state currents behavior of the four transporters corresponds to the affinity of the transporters to sodium ions. External Na^+ concentration of 100 mM, which is below the K_m of GAT2, yielded presteady-state currents in GAT2-injected oocytes, exclusively at negative imposed potentials. The same Na^+ concentration, which is substantially above the K_m of GAT3 and GAT4, resulted in presteady-state currents exclusively at positive imposed potentials, in oocytes injected with GAT3 or GAT4 (Fig. 1A). With GAT1, in which the Na^+ concentration was slightly above the K_m, the presteady-state currents appeared at all imposed potentials. Changing the external Na^+ concentrations, in GAT1 expressing oocytes, modulated the appearance of presteady-state currents. At high Na^+ concentrations the appearance of the presteady-state currents favored the positive imposed potentials, whereas at low Na^+ concentrations the presteady-state currents were more apparent at negative imposed potentials. Similarly, with the other GABA transporters, adjustment of the external sodium concentrations to slightly above their respective K_m rendered the presteady-state currents to be symmetrical, such as GAT1 at 100 mM NaCl (not shown). External sodium concentrations below the K_m induced presteady-state currents only at negative imposed potentials in all the four GABA transporters. At sufficiently high sodium

concentrations, all the transporters induced presteady-state currents only at positive imposed potentials. For example, GAT1 that has a K_m to Na^+ of 47 mM exhibited symmetric presteady-state currents at 100 mM NaCl. When adjusting the external Na^+ concentration to 25 mM, GAT1 exhibited presteady-state currents only at negative imposed potentials, similar to GAT2 at 100 mM NaCl. When the external Na^+ concentration was increased to 200 mM, GAT1 exhibited presteady-state currents only at positive imposed potentials, similar to GAT3 and GAT4 at 100 mM NaCl (Fig. 2A). With GAT2 it was impossible to reach sufficiently high Na^+ concentrations to obtain presteady-state currents exclusively at positive imposed potentials. At an external Na^+ concentration of 100 mM, the GABA-induced *I–V* relationship of GAT1 was fairly linear between +50 and −125 mV. Changing the external Na^+ concentration affected the *I–V* relationship at neutral and positive potentials. At 200 mM Na^+, the *I–V* relationship was linear and decrease of the Na^+ concentration resulted in a non-linear relationship with increased GABA-induced currents at neutral and positive potentials (Fig. 2B). These results are somewhat different than the effect of voltage on the [^{3}H]GABA uptake activity. While small GABA-induced currents were recorded at positive potentials, a substantial GABA uptake was measured in those potentials after 5 min incubation with [^{3}H]GABA (Fig. 2C).

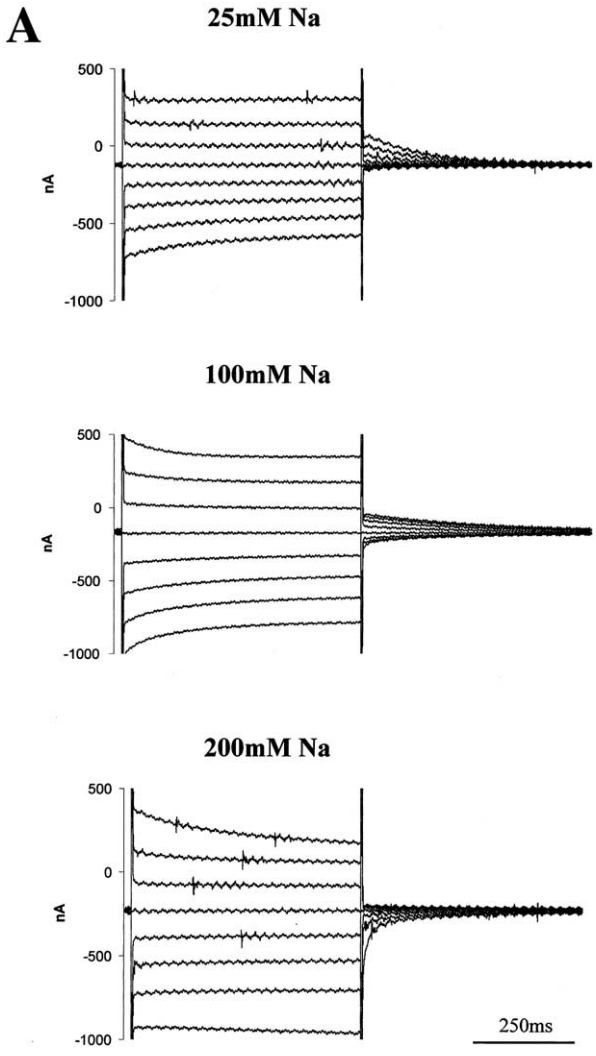

Fig. 2. Sodium concentration affects the appearance of the presteady-state currents of mouse GAT1. A: The effect of different Na^+ concentrations on the presteady-state currents on mouse GAT1 was recorded in the presence of 25 mM NaCl (with 75 mM choline chloride), 100 mM NaCl and 200 mM NaCl. B: The effect of different Na^+ concentrations on the *I–V* relationship of GAT1 was determined by calculating the difference between the current traces in the absence and in the presence of 0.5 mM GABA in different external Na^+ concentrations. C: GABA uptake, by GAT1 expressing oocyte, was measured for 5 min at different potentials under constant voltage clamp in the presence of 100 mM NaCl, as described in Section 2. The data for each bar represent mean ± S.E.M. of five oocytes and the uptake activity is expressed as pmol/h/oocyte.

Fig. 3 depicts a schematic representation of the effect of sodium occupancy on the appearance of presteady-state currents. This scheme is in line with the recent proposal of Binda et al. [31] for rGAT1 and our results for the other GABA transporters support their conclusion that the presteady-state currents may result from exposure and occlusion of positive charges on the surface of the transporters.

3.3. Extracellular pH affects the presteady-state currents

The presteady-state currents of the four GABA transporters were affected by extracellular pH. A potential jump from −25 mV to +50 or −125 in GAT1 expressing oocytes resulted in similar, symmetric presteady-state currents, both at pH 5.5 and pH 7.5. In contrast, at pH 8.5 the presteady-state currents were present mainly at positive imposed potentials (Fig. 4A). Elevating the Na^+ concentration to 200 mM also resulted in a similar phenomenon where the presteady-state currents were apparent only at positive imposed potentials (Figs. 2A and 4). The presteady-state currents of GAT2 and GAT4 observed at pH 7.5 were eliminated at pH 5.5. The presteady-state currents of GAT2, that at pH 7.5 are apparent only at negative imposed potentials, appeared to be more symmetric at pH 8.5, and presteady-state currents at positive imposed potentials could be detected. At pH 8.5, the presteady-state currents of GAT3 and GAT4 also increased at positive imposed potentials (Fig. 4C,D). The changes in the appearance of the presteady-state currents at low pH were similar to the effect of lowering the external sodium concentrations, suggesting a proton competition on the sodium-binding site.

3.4. Differential modulation of GABA uptake by extracellular pH

The effect of the extracellular pH on the uptake activity of the four GABA transporters GAT1–4 was examined using [^{3}H]GABA uptake experiments. Extracellular acidification to pH 5.5 slightly decreased the GABA uptake by GAT1 (Fig. 5). On the other hand, external alkalization to pH 8.5 did not affect GAT1 and the activity remained similar to that at pH 7.5. The GABA uptake activity of GAT2-expressing oocytes was dramatically inhibited by extracellular acidification and was reduced to about 20% at pH 5.5. In contrast to the acidification effect, external alkalization to pH 8.5 resulted in a dramatic increase in the uptake activity (Fig. 5). These results are supported by previous observations that demonstrated that the GABA-induced currents, by the canine GAT2 isoform BGT-1 [28], were reversibly decreased by extracellular acidification to pH 5.5 and increased at extracellular alkalization to pH 8.5 [21]. Similar to GAT1, GABA uptake by GAT3 was slightly decreased at pH 5.5 and was quite similar

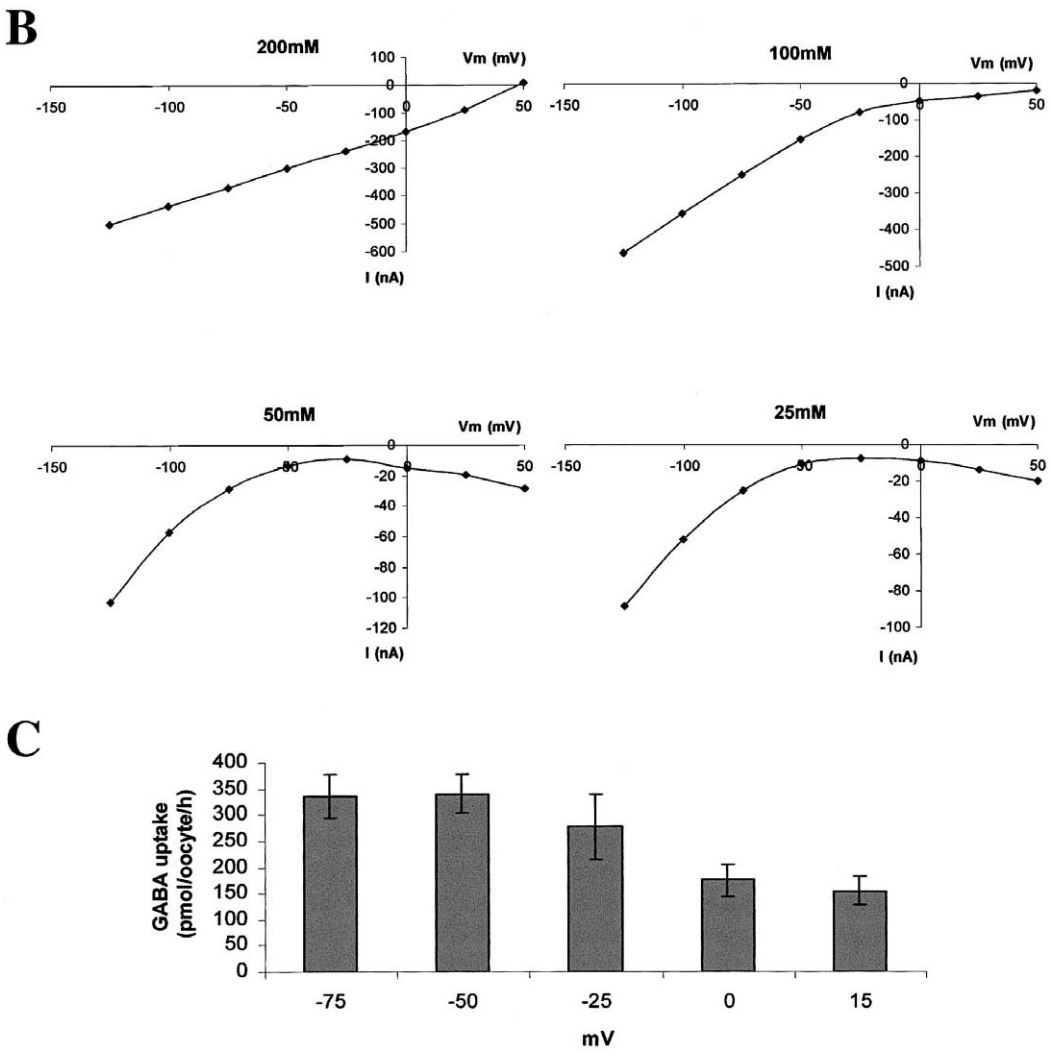

Fig. 2 (*Continued*).

at pH 7.5 and pH 8.5. The effect of extracellular acidification and alkalization on the GABA uptake by GAT4 was similar to the effect on GAT2. At pH 5.5, the uptake activity by GAT4 was decreased and extracellular alkalization resulted in an increase in GABA uptake activity.

The inhibition of GABA uptake activity by extracellular acidification can be explained by competition of the protons with the sodium ions on the sodium-binding site. In order to check this notion, we examined the effect of external pH on GABA uptake activity in the presence of different sodium concentrations. As shown in Fig. 6, when the Na^+ concentration was reduced to 20 mM, a pH decrease from 7.5 to 5.5 resulted in a dramatic decrease in GABA uptake activity by GAT1- and GAT4-injected oocytes. GAT3-injected oocytes that were not sensitive to acidification at 100 mM Na^+ showed a significant inhibition at pH 5.5, when sodium concentration was reduced to 20 mM. GABA uptake by GAT2 was very sensitive to acidic pH and to low sodium concentration. GABA uptake activity by GAT4-injected oocytes was also sensitive to extracellular acidification. When the Na^+ concentration was reduced to 20 mM, the GABA uptake activity was inhibited by decreasing the pH from 7.5 to 5.5. The competition between Na^+ and protons is unlikely to be a result of a single amino acid on the four transporters. GAT3 and GAT4 are highly identical in their amino acid sequences, yet their response to pH is quite different. However, the behavior of the Na^+-binding site in all the four transporters is remarkably similar. This is manifested by the effect of Na^+ affinity on the behavior of the presteady-state currents, and more so by the fact that the pH affected the presteady-state currents in a similar fashion (Fig. 4). At high pH, the presteady-state currents of GAT1 inclined towards positive imposed potentials, as if the Na^+ affinity was increased. Similarly, low pH abolished the presteady-state currents of GAT2 and GAT4. We concluded that protons compete with Na^+ on its binding site but proton binding is not productive and is unable to drive GABA uptake.

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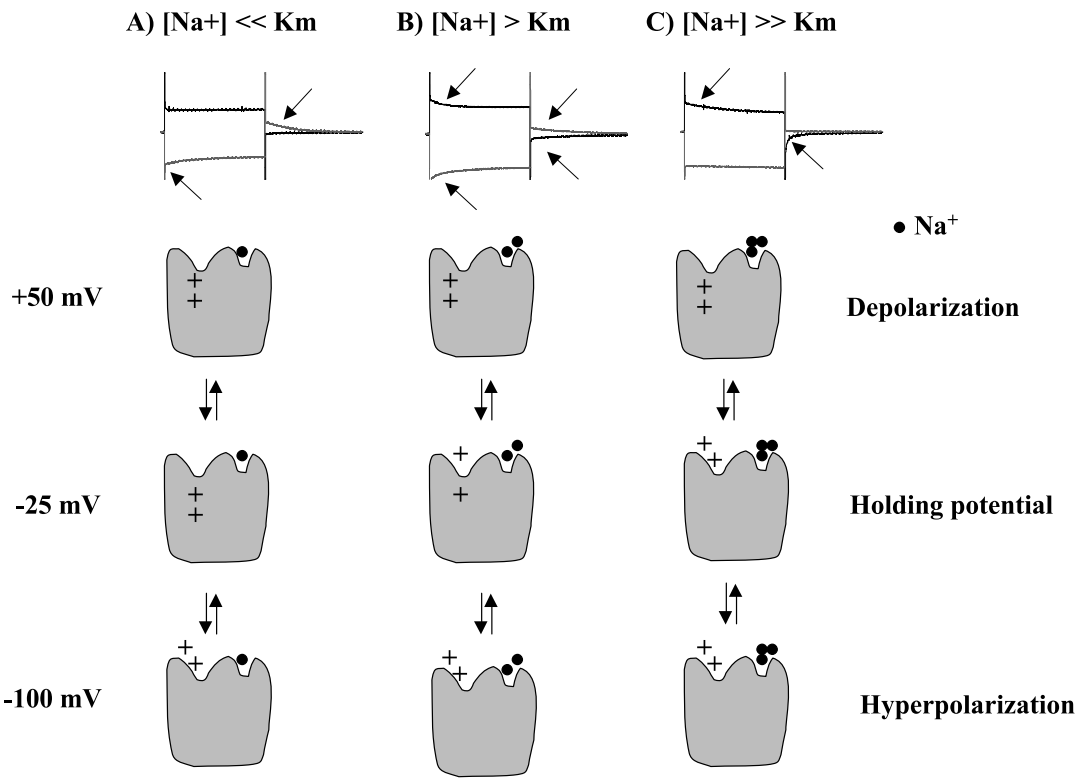

Fig. 3. Schematic representation of the effect of Na^+ on the presteady-state currents generated by GABA transporters. In the upper panel the graph shows recordings at two imposed potentials, positive (+50 mV) and negative (−100 mV).

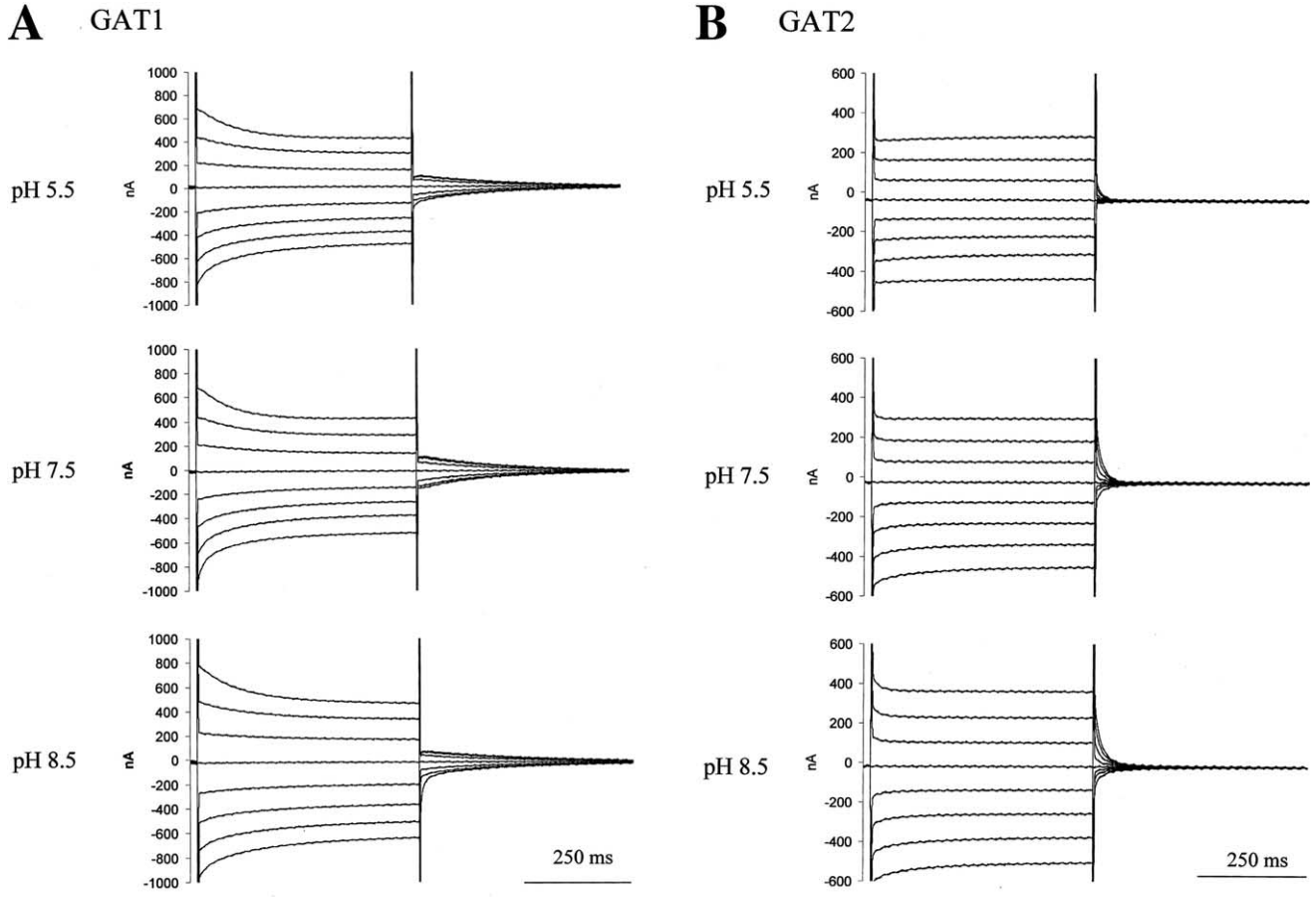

Fig. 4.

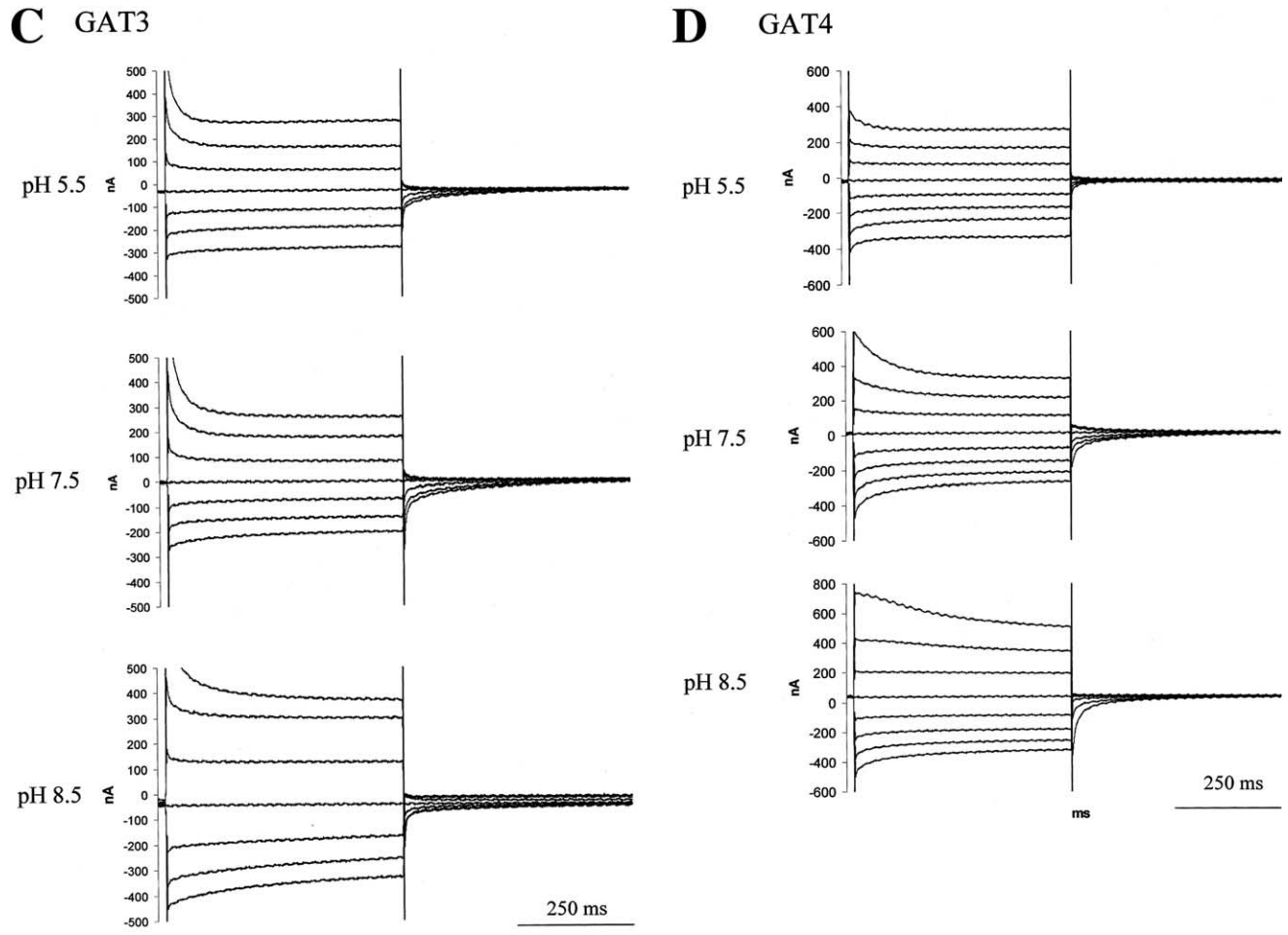

Fig. 4. (*Continued*). The effect of extracellular pH on presteady-state currents of the mouse GABA transporters. The effect of pH 5.5, 7.5 and 8.5 on the presteady-state currents of the GABA transporters, GAT1 (A), GAT2 (B), GAT3 (C) and GAT4 (D) was measured as a response to step changes in the membrane voltage. The membrane was held at −25 mV and stepped to a series of test voltages from +50 mV to −125 mV in steps of 25 mV, at different pHs.

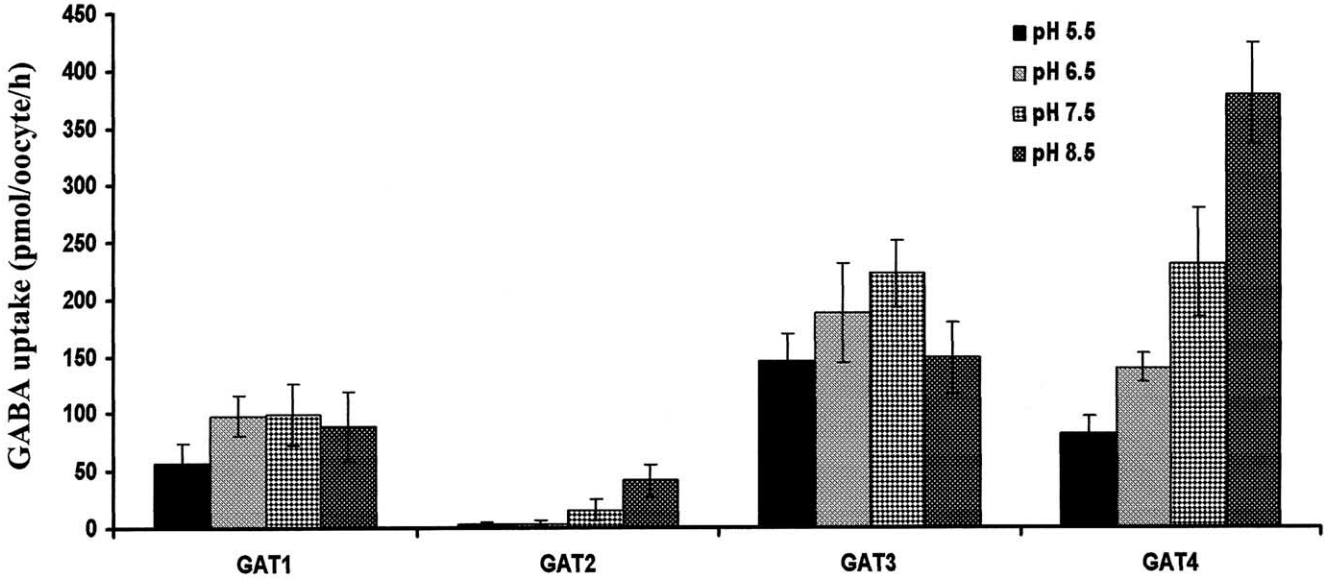

Fig. 5. The effect of extracellular pH on the [^{3}H]GABA uptake into oocyte expressing mouse GABA transporters. GABA uptake experiments were performed as described in Section 2, using 10 injected oocytes for each pH tested. The data for each bar represent mean ± S.E.M. of 10 oocytes expressed as pmol/h/oocyte.

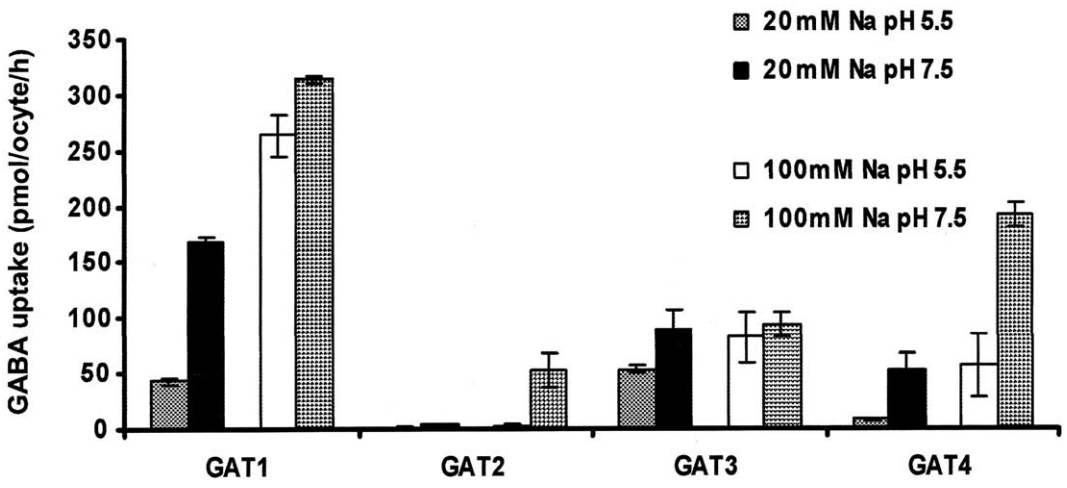

Fig. 6. The effect of external pH and Na^+ concentrations on the [^{3}H]GABA uptake activity in oocytes expressing mouse GABA transporters. GABA uptake experiments were performed as described in Section 2, using 10 injected oocytes, for each pH and Na^+ concentration. The data for each bar represent mean ± S.E.M. of 10 oocytes in pmol/h/oocyte.

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