Temperature effects on the presteady-state and transport-associated currents of GABA cotransporter rGAT1

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Abstract The effects of temperature on the γ-aminobutyric acid (GABA) uptake and on the presteady-state and transport-associated currents of the GABA cotransporter, rat γ-aminobutyric acid transporter 1 (rGAT1), have been studied using heterologous oocyte expression and voltage-clamp. Increasing temperature from 15 to 30°C increased GABA uptake, diminished the maximal value of the relaxation time constant of the presteady-state currents and increased the amplitude of the current associated with the transport of GABA. The curve of the presteady-state charge versus voltage was shifted toward negative potentials by increasing the temperature, while the maximal amount of charge (Q_{max}) remained constant; the τ versus V curve was also negatively shifted by increasing temperatures. Analysis of the outward (α) and inward (β) rate constants as functions of temperature showed that they are affected differently, with a $Q_{10} = 3.4$ for α and $Q_{10} = 1.5$ for β. The different temperature coefficients of the rate constants account for the observed shifts. These observations are consistent with a charge moving mechanism based on a conformational change of the protein; the weaker temperature sensitivity of the inward rate constant suggests a rate-limiting diffusional component on this process.

Key words: Neurotransmitter cotransport; γ-Aminobutyric acid transporter 1; Temperature

1. Introduction

The possibility of heterologous expression in *Xenopus* oocytes has greatly expanded our knowledge of the properties of several cotransporters. However, in the majority of the cases, the experiments have been performed at room temperature [1–3], a situation that, while necessary to keep the amphibian oocytes in optimal condition, is certainly not physiological for the mammalian transporters often used. Studying the effects of temperature on the kinetics and amplitude of the transport characteristics is also a useful means to obtain information on the nature of the process, since the temperature coefficient Q_{10} is related to the complexity of the conformational changes involved. Temperature effects with Q_{10} greater than 3 have been observed for organic substrate uptake in the *Drosophila* serotonin (5HT) transporter [4] and in the glutamate transporter EAAT1 [5], while different results have been reported for the transport-associated currents [4–6]. To date, few results have been published regarding the actions of temperature on the presteady-state currents: in the rabbit Na^+/glucose cotransporter (SGLT1) temperature has a complex, voltage-dependent, effect on the relaxation time constant of this kind of current [7]. The relatively slow relaxation kinetics of the presteady-state currents of the rat γ-aminobutyric acid transporter 1 (rGAT1) appear suitable for this kind of investigation, whose results we report in the following.

2. Materials and methods

2.1. Oocyte expression and electrophysiology

The experimental procedure has been described in detail elsewhere [8]. Briefly, after linearisation with *Not*I, cRNA was in vitro synthesised in the presence of Cap Analog and 200 U of T7 RNA polymerase. Oocytes were tested 72 h after injection of 12.5 ng cRNA. All enzymes were supplied by Promega Italia, Milan, Italy. The cDNA encoding the rat GAT1 cotransporter cloned into the pAMV-PA vector was kindly provided by C. Labarca.

A two-microelectrode voltage-clamp was used (Warner Instruments, Hamden, CT, USA, or Geneclamp, Axon Instruments, Union City, CA, USA). The holding potential (V_h) was kept at −40 mV and voltage pulses to −120, −80, 0 and +40 mV, 0.5 or 1 s in duration, were applied to the oocyte. The presteady-state currents were isolated by subtraction of traces in the presence of SKF89976A (Tocris, http://www.tocris.com); the resulting records were further corrected for any residual leakage, fitted with single exponentials and integrated to obtain time constant and charge data using pClamp 8.0 (Axon Instruments).

2.2. Temperature control and solutions

The external solution was composed as follows (in mM): NaCl, 98; $CaCl_2$, 1.8; $MgCl_2$, 1; HEPES free acid, 5, at pH 7.6; γ-aminobutyric acid (GABA) was added at 100 μM to induce transport-associated currents. Temperature was measured with a small thermistor placed in the bath very close to the oocyte. The temperature was set through a feedback-controlled device (TC 344A, Warner Instruments) that heated a precooled (∼10°C) solution. We found that below 17.5°C the transport-associated current was very small and the presteady-state currents were very slow; at the other end, oocytes became electrically unstable when the temperature approached about 35°C, preventing reliable analysis of the results. Therefore our study was limited to the range 17.5–30°C.

2.3. [^{3}H]GABA uptake

The uptake of 500 μM [^{3}H]GABA (Amersham-Pharmacia Biotech) was measured 3 days after injection. Groups of 8–10 oocytes, from the same batch, were incubated in 100 μl of external control solution at different temperatures for 10 min, dissolved in 200 μl of 10% sodium dodecyl sulphate and radioactivity counted by liquid scintillation.

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Abbreviations: rGAT1, rat γ-aminobutyric acid transporter 1; GABA, γ-aminobutyric acid

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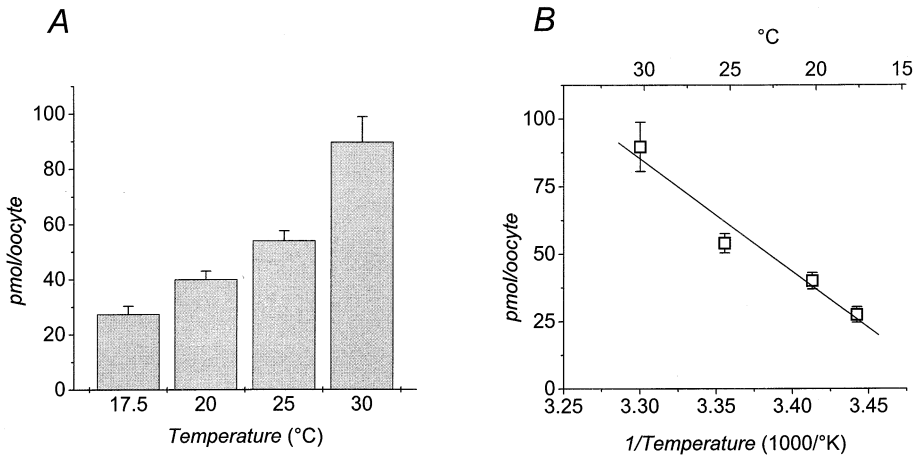

Fig. 1. Temperature effect on the uptake of radiolabelled GABA. A: Data represent differences between injected and non-injected oocytes; incubation time was 10 min at each temperature ($n = 10$ for each group; bars are S.E.M.). B: Arrhenius plot from the data of A; the linear fit indicates a Q_{10} of 2.2. Oocytes from the same batch. Another experiment on a second batch gave similar results.

Temperature control was performed through the use of a thermal cycler model PCR-Sprint (Hybaid).

3. Results

3.1. Effects of temperature on GABA transport

As expected, higher temperatures increased the rate of neurotransmitter uptake: Fig. 1A shows the amount of [^{3}H]GABA accumulated inside oocytes kept at different temperatures between 17.5 and 30°C. These values were converted to an Arrhenius plot in Fig. 1B, from which a Q_{10} value of 2.2 can be estimated.

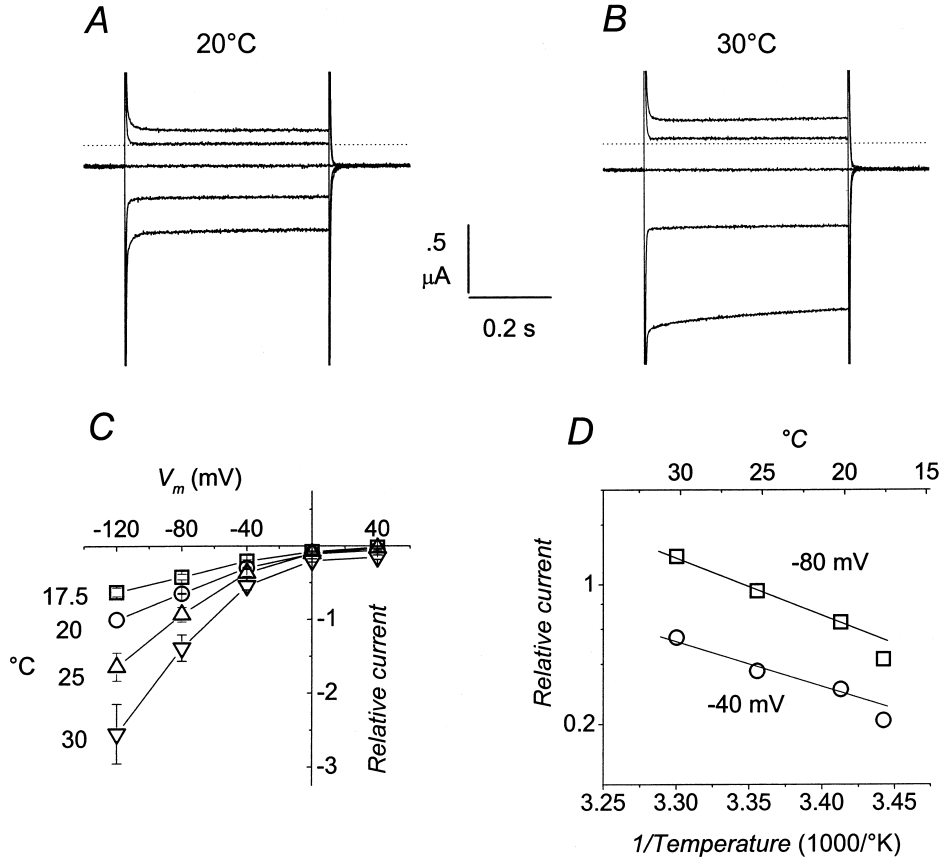

Fig. 2. Effects of temperature on the transport-associated current. A and B: Representative records of the increase induced by temperature in the membrane current of an rGAT1-expressing oocyte in the presence of 100 μM GABA; the dotted lines indicate the zero current level. Voltage pulses to −120, −80, 0 and +40 mV were applied from a holding potential (V_h) = −40 mV. C: *I–V* plots of the transport-associated current at the indicated temperatures; data are means ± S.E.M. from eight oocytes and are normalised to the value at 20°C and −120 mV. D: Arrhenius plot for the current amplitude at the indicated potentials (data from C). Solid lines are linear fits to the three higher temperatures.

3.2. Effects of temperature on the transport-associated current

Fig. 2 illustrates the effect of temperature on the whole-cell current of oocytes expressing rGAT1 and stimulated with 100 μM GABA. In Fig. 2C the current–voltage relationships of the steady-state transport-associated current (obtained as difference between records in the presence and in the absence of GABA) are shown for various temperatures.

The analysis summarised in Fig. 2 indicates, as expected, that temperature increases the amplitude of the transport-associated current; the Arrhenius plot of Fig. 2D shows that the values at the three higher temperatures are well aligned, while the points at 17.5°C are lower. This change of slope below 20°C has been observed in some other instances [9], and attributed to differential action of temperature on different aspects of the process. Since the higher tested temperatures are closer to the operating conditions of the mammalian transporter rGAT1, we have limited the linear fit to the three higher temperatures, obtaining a coefficient (Q_{10}) between 1.8 and 2.2 at the two potentials closest to the physiological range. These values confirm the results of [6] who, in excised oocyte patches and at 0 mV, reported a Q_{10} of 2.2 in the same temperature range. Furthermore, they coincide with the Q_{10} reported above for GABA uptake, reinforcing that charge translocation and substrate transport are based on the same process, as it has been shown for instance, for the 5HT transporter dSERT [4].

3.3. Effects of temperature on the presteady-state current

Fig. 3 shows the effect of temperature on the kinetics of the presteady-state currents: as for the transport-associated current, below 20°C the transients are very slow, and at 15°C fitting and integration of the curves were not reliable. The traces in Fig. 3 show the expected acceleration of the decay phase of the transient currents; relaxations were mono-exponential at all temperatures, consistent with a single apparent two-state system. A negative shift in the amplitude of the transients with increasing temperature may also be noted, which will be better pointed out in the following analysis.

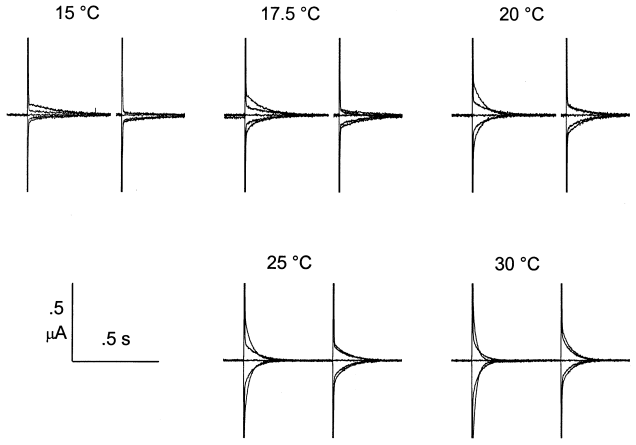

Fig. 3. Temperature effects on the kinetics of rGAT1 presteady-state currents. Voltage pulses to −120, −80, 0 and +40 mV were applied from a holding potential (V_h) = −40 mV. Traces are shown after isolation of the transients from the passive capacitive and leakage currents of the oocyte. Pulses at 15, 17.5 and 20°C were 1 s long, those at 25 and 30°C were 0.5 s long.

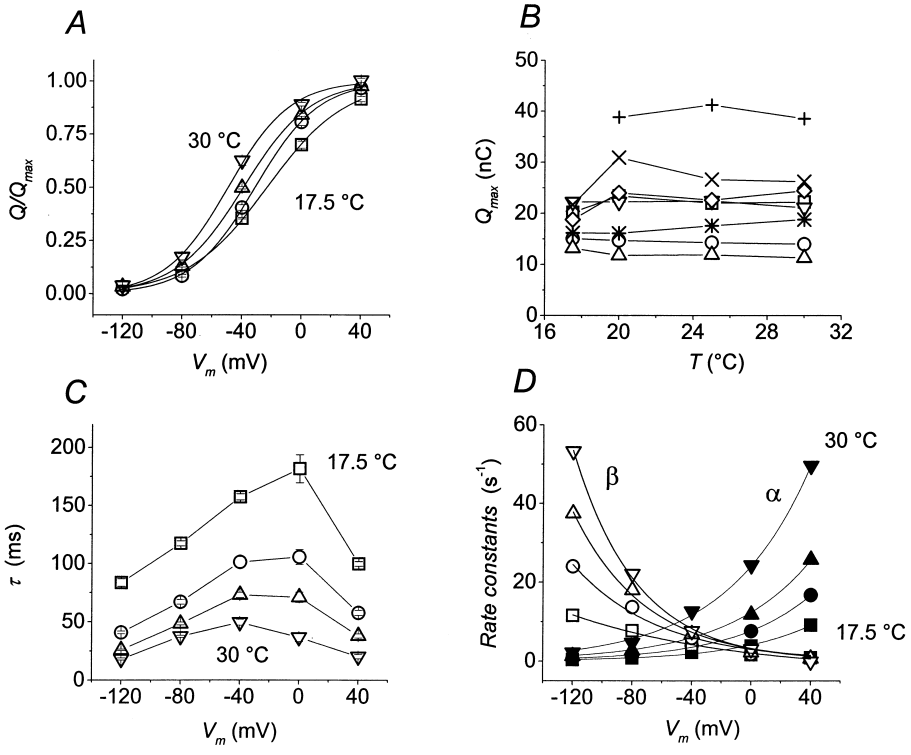

Fig. 4. Effects of temperature on the kinetics parameters of charge movement. A: The sigmoidal Q/V curves, obtained from integration of the transient currents are shifted toward negative voltages by increasing temperatures, while Q_{max} remains constant. B: Data from eight individual oocytes from two batches. C: The relaxation time constant τ showing decrease and also shift with increasing temperatures. D: The unidirectional rate constants α and β are differently increased by temperature. In A, C and D, points are means from the eight oocytes of B and squares represent 17.5°C; circles, 20°C; up triangles, 25°C; down triangles, 30°C. In B, each symbol represents an individual oocyte.

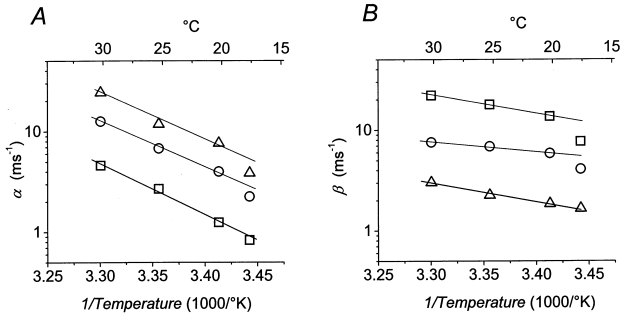

Fig. 5. Arrhenius plots for the unidirectional rate constants. Values of α (A) and β (B) at −80 (squares), −40 (circles) and 0 mV (triangles) are shown as functions of temperature. It can be seen that α is affected more strongly than β and that the values at 17.5°C tend to be lower. The straight lines were fitted to the three higher temperatures.

The transients were analysed as usual [8], by fitting with single exponentials and integrating the traces corrected for leakage and passive oocyte capacity. The results are shown in Fig. 4 and may be summarised as follows: (i) the Q versus V relationship was shifted in the negative direction by higher temperatures (Fig. 4A); (ii) the maximal amount of charge Q_{max} remained constant (Fig. 4B); (iii) the relaxation time constants τ decreased with increasing temperatures (Fig. 4C); (iv) the maximal value of τ also shifted in the negative direction with temperature (Fig. 4C).

While the effect on the amplitude of τ was expected, the shifting effects on Q and τ are rather intriguing. Interestingly, temperature-dependent shifts of the same kind have been reported for the rabbit sodium/glucose cotransporter [7], although their significance was not commented upon.

In general, shifts in the position of the Q/V and τ/V curves reflect asymmetrical changes in the unidirectional rate constants of the process [8]. We have then derived the curves for the outward (α) and inward (β) rate constants of the charge movement from the equations [6]:

$$\alpha = Q/\tau$$

$$\beta = (1-Q)/\tau$$

These relationships are plotted in Fig. 4D for the four temperatures tested: the graphs show that the effect of temperature on α is stronger than on β, giving rise to the negative shift of Q and τ.

The values of α and β have been plotted in Fig. 5 in an Arrhenius plot for three potentials: −80, −40 and 0 mV. As for the transport-associated current (Fig. 2D), the points at the lowest temperature (17.5°C) tend to fall out of the straight line that fits well the other three temperatures. In any case the slope of the lines for α is significantly higher than for β, giving an average Q_{10} of 3.35 for α and of 1.49 for β.

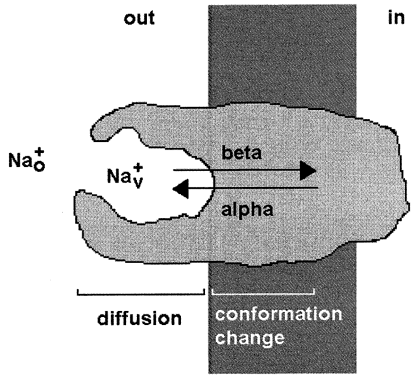

Fig. 6. Schematic model showing the presence of a vestibule whose access is in series with a conformational change giving rise to intramembrane charge movement. The model may account for the asymmetrical effect of temperature on the unidirectional rate constants α and β.

4. Discussion

As stated in Section 1, the interest of investigating temperature effects on the functioning of mammalian cotransporters is two-fold: on one hand to acquire data on the kinetic properties of the system at more physiological temperatures and, on the other, to gain new insights on the molecular processes involved, on the basis of the Q_{10} values.

Regarding the first aspect, we have observed that the temperature coefficients of the GABA uptake and of the transport-associated current are constant between 20 and 30°C, both with a Q_{10} around 2.1; this value is lower than those reported for the *Drosophila* 5HT transporter dSERT [4], and for the human glutamate transporter EAAT1 [5], however, the estimates for dSERT and EAAT1 were done at lower temperatures (10–22°C), a range where the Arrhenius plots become steeper in our experiments, indicating that there is no contrast, and which, in any case, is rather far from physiological temperatures of mammalian transporters.

The second aspect is particularly important since in the recent years different models for the functioning of transporters have been proposed, some of which are based on rather simple diffusional mechanism and do not require, in principle, conformational changes of the protein [10,11]. Determination of the temperature dependence may help in this respect, since Q_{10} values below 1.5 are indicative of diffusional processes [12], while conformational changes are generally characterised by a stronger temperature dependence [13] and therefore by higher Q_{10} values.

We have found that temperature affects to different degrees various parameters of the transport activity of rGAT1. The Q_{10} value of about 2.0 for the amplitude of the transport-associated current confirms a previous report based on isolated macropatches [6]. The uptake experiments give a value of Q_{10} in agreement with that obtained for the transport-associated current, suggesting that the charge flux and the substrate flux are probably dependent on the same thermodynamically coupled mechanism, and indicating that the processes responsible for the translocation of organic substrates should involve a conformational change of the protein. This, in any case, does not exclude electrodiffusional models of transport.

The effect of temperature on the presteady-state currents, in the absence of organic substrate, is more complex since we have seen that the shift induced on the Q/V and τ/V relationships may be interpreted as a differential effect on the unidirectional rate constants. Our analysis shows that the Q_{10} for the inward rate β is lower than that for the outward rate α. While the Q_{10} for α clearly suggests a conformational change, the value for β is at the limit between diffusional processes and conformational modifications [12].

These results appear to be consistent with an hypothesis that we have put forward recently [8]: the existence on the extracellular side of the transporter of a vestibule through which Na^+ (and perhaps Cl^- and the organic substrate) must pass before proceeding to the steps giving rise to the charge movement (Fig. 6).

Following this idea, and taking into account the differential effects of temperature on the unidirectional rate constants, we may envisage a process in which a charge-displacing conformational change may occur after a diffusion-limited entry of Na^+ in the vestibule. In this way the outward rate constant α would be related only to the conformational change, while the inward rate β would depend on both a change in conformation and on the diffusional arrival of sodium ions in the vestibule. In this scheme, the lower value of Q_{10} for β is explained by the weaker effect of temperature on diffusion, representing a bottleneck step in the overall mechanism.

As mentioned in Section 3, a temperature-induced shift of the Q/V and τ/V curves has been observed also in the Na^+/glucose transporter SGLT1 [7], and therefore the proposed mechanism might be a general feature of cotransporters.

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