1992

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Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 267, No. 31, Issue of November 5, pp. 22007-22009, 1992

Electrogenic Uptake of γ-Aminobutyric Acid by a Cloned Transporter Expressed in *Xenopus* Oocytes*

(Received for publication, August 20, 1992)

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GAT-1, a γ-aminobutyric acid (GABA) transporter cloned from rat brain, was expressed in *Xenopus* oocytes. Voltage-clamp measurements showed concentration-dependent, inward currents in response to GABA ($K_{0.5}$ 4.7 μM). The transport current required extracellular sodium and chloride ions; the Hill coefficient for chloride was 0.7, and that for sodium was 1.7. Correlation of current and [^{3}H]GABA uptake measurements indicate that flux of one positive charge occurs per molecule of GABA transported. Membrane hyperpolarization from −40 to −100 mV increased the transport current approximately 3-fold. The results indicate that the transport of one molecule of GABA involves the co-transport of two sodium ions and one chloride ion.

Reuptake of neurotransmitter plays a critical role in synaptic transmission. Uptake systems for γ-aminobutyric acid (GABA),[1] the predominant inhibitory transmitter in mammalian brain, have been extensively studied (for review, see Iversen and Kelly (1975) and Kanner and Schuldiner (1987)). Transporter-mediated uptake of [^{3}H]GABA is dependent on external Na^+ (Iversen and Neal, 1968) and also requires Cl^- or certain other monovalent anions (Kanner, 1978). Dependence of GABA uptake on external potassium also has been reported (Martin and Smith, 1972), but this may be an indirect effect due to changes in ion gradients or membrane potential. However, the stoichiometry of GABA transport with respect to other ions has been difficult to determine directly (Radian and Kanner, 1983; Keynan and Kanner, 1988). Evidence that GABA uptake is associated with a net inward movement of positive charge has been obtained from measurements on synaptic vesicles (Blaustein and King, 1976; Radian and Kanner, 1983). More recently, voltage-clamp experiments from horizontal cells of skate retina (Malchow and Ripps, 1990) and from crayfish stretch receptor neurons (Kaila *et al.*, 1992) have provided more direct evidence that GABA uptake is electrogenic; in the stretch receptor, the uptake current contributes quite significantly to the total current induced by GABA.

The recent molecular cloning of cDNAs encoding GABA transporters (Guastella *et al.*, 1990; Nelson *et al.*, 1990; Clark *et al.*, 1992) reveals a high degree of homology with a family of neurotransmitter membrane transport proteins (for review, see Amara and Kuhar (1993)) and allows their functional expression. We have expressed the cloned GABA transporter GAT-1 (Guastella *et al.*, 1990) in *Xenopus* oocytes and used voltage clamp to measure directly the charge movement associated with GABA uptake. We determined its dependence on Na^+, Cl^-, and GABA concentrations, and on membrane potential. In parallel experiments, the number of elementary charges associated with the transport of each GABA molecule was determined by comparing the results of electrical measurements with the uptake of GABA measured using [^{3}H]GABA.

EXPERIMENTAL PROCEDURES

Expression of GABA Transporter in Oocytes—Plasmid pGABAT7 containing a cDNA corresponding to the GAT-1 cDNA clone (Guastella *et al.*, 1990) was generated using polymerase chain reaction techniques as previously described (Blakely *et al.*, 1991). This cDNA was transcribed *in vitro* in a 25-μl reaction (30 min at 37 °C) containing 1 μg of linearized DNA, 500 μM rNTPs, 5 mM $m^7G(5')ppp(5')G$ (Pharmacia LKB Biotechnology Inc.), 10 units of RNAsin (Promega), 10 units of T7 polymerase (Stratagene) in 40 mM Tris, pH 7.5, 50 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, and 30 mM dithiothreitol. Approximately 50 ng of RNA was injected into defolliculated stage V–VI *Xenopus* oocytes.

Uptake Measurements with [^{3}H]GABA—Radiolabeled GABA ([2,3-^{3}H]GABA; 30.7 Ci/mmol; Du Pont-New England Nuclear) uptake measurements were made on individual oocytes in 50-μl assay volumes. Uptake assays were performed at room temperature in recording buffer (see below) containing 100 μM GABA (1 μM [^{3}H]GABA and 99 μM unlabeled GABA). Oocytes were incubated in this uptake buffer for 10–30 min followed by three rapid washes in cold buffer. Uptake of [^{3}H]GABA was linear for up to 60 min under these conditions. Prior to counting, oocytes were lysed in 0.5 ml of 1.0% SDS and then added to 10 ml Scintiverse BD (Fisher) followed by scintillation spectroscopy. Transport rates were calculated (pmol/100 s) from the measured specific activity (cpm/pmol) of the uptake assay buffer.

Electrophysiology and Data Analysis—Two-microelectrode voltage clamp recordings were made at 20–22 °C with a Dagan TEV-200 clamp amplifier filtered at 20 Hz and digitized using an Axon Instruments TL-1 interface controlled by computer using the pCLAMP Clampex program. Electrodes (0.1–0.5 MΩ) were filled with 3 M KCl. The bath solution contained (in mM): NaCl (96), HEPES-NaOH, pH 7.5 (5), KCl (2), $CaCl_2$ (1.8), $MgCl_2$ (1). In experiments where the concentrations of sodium or chloride were varied, these were substituted by Tris or gluconate, respectively. Oocytes were continuously superfused with buffer, which could be rapidly changed to one containing the indicated concentration of GABA. Current-voltage curves were obtained by voltage ramps of 4 s in duration, generally from −150 mV to +50 mV. Ramps were measured before addition of GABA and again during the peak current following addition of GABA; the currents were then digitally subtracted using the Clampfit program. Current as a function of GABA concentration ([GABA]) was fitted by least squares to $I = I_{max} \times [GABA]^n/(K_{0.5}{}^n + [GABA]^n)$ where I is the current, I_{max} is the maximal current, and $K_{0.5}$ and n are constants. Values of $K_{0.5}$ and n were determined by fitting the results from individual oocytes in which five or more different concentrations were

* This work was supported by a Hutchings award from the Burroughs Wellcome Fund and National Institutes of Health Grants DA07595 (to S. G. A.) and DA03160 (to R. A. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GABA, γ-aminobutyric acid; $K_{0.5}$, apparent dissociation constant; GAT-1, a GABA transporter clone obtained from rat brain.

applied, they are expressed as mean ± S.E. Figures show curve fits to averaged data from all oocytes tested.

RESULTS

GABA evoked inward currents in oocytes expressing GAT-1, but not in water-injected or non-injected oocytes (n = 18). The current did not decline significantly during applications lasting up to several minutes, and its amplitude was dependent on the concentration applied (Fig. 1*A*). Nipecotic acid, which is a widely used inhibitor of GABA uptake (Krogsgaard-Larsen and Johnston, 1975), also caused a dose-dependent inward current in cells expressing GAT-1 (Fig. 1*B*). The $K_{0.5}$ for the GABA-dependent current was 4.7 ± 0.8 μM (n = 5), and the Hill coefficient was 1.12 ± 0.06; for nipecotic acid the values were 19 ± 7 μM and 1.05 ± 0.14 (n = 3). The maximal current for nipecotic acid, determined by fitting data to the logistic function described under "Experimental Procedures," was 86 ± 7% that of the I_{max} for GABA in the same oocytes.

Dependence on Na^+ and Cl^- Concentrations—The current evoked by GABA was abolished when the extracellular Na^+ or Cl^- were replaced by Tris or gluconate, respectively; removal of extracellular potassium was without effect (n = 3). The concentration dependence of sodium and chloride were examined at a fixed concentration of GABA (100 μM) (Fig. 2). Fitting of the chloride concentration-response data to the logistic function gave a Hill coefficient of 0.71 ± 0.03, and $K_{0.5}$ of 19 ± 2 mM (n = 4; Fig. 2*A*). The dependence of the GABA-induced current on the sodium concentration was clearly sigmoidal, with a Hill coefficient of 1.74 ± 0.15 and $K_{0.5}$ of 73 ± 10 mM (n = 6; Fig. 2*B*).

Charge Movement per GABA Molecule—To determine the number of elementary charges translocated with a molecule of GABA, the rate of [^{3}H]GABA uptake was measured in oocytes expressing GAT-1 and compared with the GABA-induced currents in other oocytes from the same batch. Uptake assays were performed in the same buffer used for voltage-clamp recording, with a GABA concentration of 100 μM (see "Experimental Procedures"). In five batches of oocytes, the uptake was (pmol/100 s) 8.3 ± 0.4 (n = 5), 2.9 ± 0.4 (n = 12), 3.1 ± 0.7 (n = 5), 8.3 ± 1.6 (n = 7), and 8.0 ± 0.3 (n = 5). Current measurements were made in oocytes clamped at their resting membrane potential (range −40 to −60 mV), also using 100 μM GABA. In the same five batches of oocytes the currents were (in nA): 9.5 ± 2.0 (n = 6), 3.6 ± 0.9 (n = 14), 3.6 ± 0.7 (n = 9), 11.8 ± 2.1 (n = 8), and 9.8 ± 0.9 (n = 5), respectively. Assuming 1.6 × 10^{-19} coulomb/elementary charge, then 1.29 ± 0.05 (n = 5) net positive charges enter the cell per GABA molecule.

Voltage Dependence of GABA-induced Current—The effect of voltage on the rate of GABA transport was investigated by ramping the membrane potential of voltage-clamped oocytes from −150 to +50 mV in the presence and absence of 100 μM GABA (Fig. 3*A*) and subtracting the membrane currents (Fig. 3*B*). Membrane hyperpolarization caused a large increase in the GABA-induced current. At depolarized potentials the

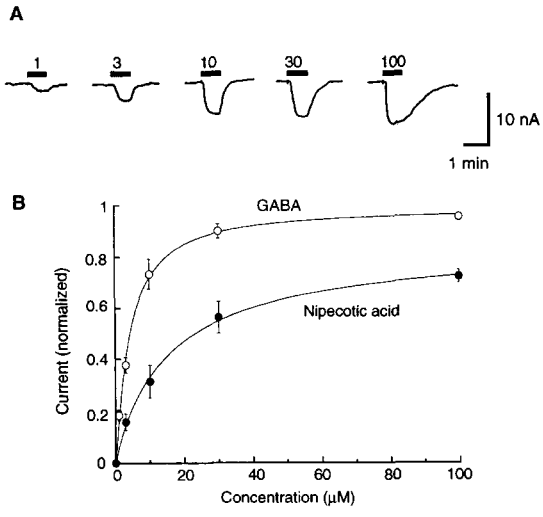

FIG. 1. *A*, inward currents evoked by GABA in an oocyte expressing GAT-1 (μM). Cells were voltage-clamped at −60 mV and GABA (concentration indicated by each trace, μM) was superfused for the period shown by the *bar*. *B*, concentration-response curves for inward currents induced by GABA (*open symbols*, n = 5) and nipecotic acid (*closed symbols*, n = 3). Data were normalized to the maximal current evoked by GABA in the same oocyte.

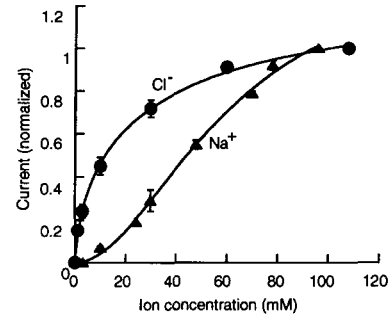

FIG. 2. **Na^+ and Cl^- dependence of GABA uptake current.** Concentration-response curves for Cl^- (*circles*) and Na^+ (*triangles*) in the presence of 100 μM GABA. Inward currents measured in oocytes voltage-clamped at −60 mV were normalized to the response in control buffer (98.5 mM Na^+/104 mM Cl^-). *Points* are mean ± S.E. The *lines* are fitted by minimizing squared errors to $I_{max} \times [ion]^n/(K_{0.5}^n + [ion]^n)$.

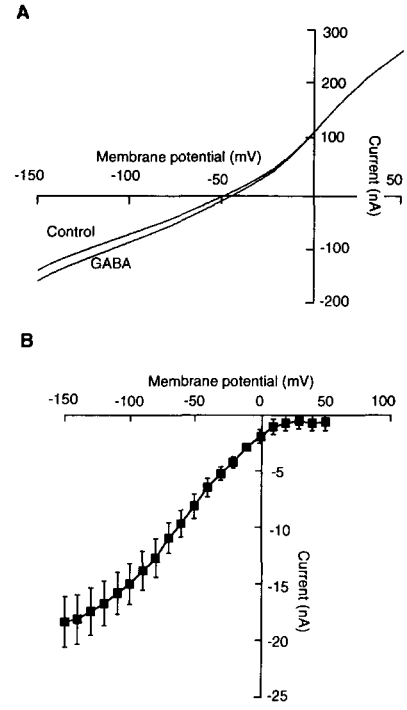

FIG. 3. **Voltage dependence of GABA uptake.** *A*, current-voltage curves in a representative oocyte were measured as described under "Experimental Procedures." *Curves* shown were obtained in the absence (*top*) and in the presence (*bottom*) of 100 μM GABA. *B*, current-voltage plot of the GABA-specific inward current, determined by subtraction of control current from current in presence of 100 μM GABA. *Points* are mean ± S.E. (n = 7).

GABA current approached zero but did not reverse, and at strongly hyperpolarized potentials (less than −100 mV) the current began to saturate (Fig. 3*B*).

DISCUSSION

This study directly demonstrates that uptake of GABA by the cloned transporter GAT-1 is associated with an inward movement of positive charge; correlation of the voltage-clamp current measurements and [^{3}H]GABA transport across the membrane suggests a stoichiometry of approximately one net positive elementary charge per molecule GABA transported. It has previously been demonstrated, for the transporter reconstituted in proteoliposomes, that flux of GABA is coupled to the flux of both Na^+ and Cl^- (Keynan and Kanner, 1988); [^{3}H]GABA uptake mediated by the cloned GAT-1 also requires both Na^+ and Cl^-(Guastella *et al.*, 1990; Keynan *et al.*, 1992). The present work (Fig. 2) shows that the dependence on Cl^- differs markedly from that for Na^+; the Hill coefficient for Cl^- was 0.7, whereas that for Na^+ was 1.7 (Fig. 2). A Hill coefficient greater than 1 for Na^+ has also been observed in studies of radiolabeled GABA uptake (Martin and Smith, 1972; Keynan *et al.*, 1992). Assuming that GABA is transported in the zwitterionic form (which greatly predominates at physiological pH), the data suggest a stoichiometry of Na^+:Cl^-:GABA of 2:1:1 during a cycle of transport. The concentration-response curve observed for Na^+ is consistent with such a stoichiometry, as it indicates cooperative binding of more than one ion to the transporter (Fig. 2). The quantity of positive charge associated with GABA flux was significantly greater than 1 elementary charge (1.29 ± 0.05; $p < 0.05$, paired t test). Possible reasons for this include transport of some fraction of GABA in the cationic form or partial uncoupling of sodium or chloride from GABA transport. In view of the steep voltage dependence of the GABA transport (Fig. 3), it is also possible that the membrane potentials in the two sets of experiments are not the same; in the experiments on uptake of [^{3}H]GABA, the cells would be expected to depolarize several millivolts as a result of the inward current, and this would reduce the influx.

Studies of [^{3}H]GABA flux in synaptosomes or plasma membrane vesicles (Blaustein and King, 1976; Kanner, 1978; Haycock *et al.*, 1978) and GABA-induced currents in retinal cells (Malchow and Ripps, 1990) have suggested that GABA transport is voltage-dependent. The present study found that hyperpolarization strongly increases the inward current induced by GABA. In the range 0 to −80 mV the current increased exponentially with hyperpolarization, approximately 3-fold for 50 mV. At positive potentials, the current approached zero but did not reverse in the voltage range examined. The theoretical equilibrium reversal potential for the transporter current based on a coupling stoichiometry of 2:1:1 for Na^+:Cl^-:GABA is given by $E = RT/F \times (\ln(Na_o/Na_i)^2 + \ln(Cl_o/Cl_i) + \ln(GABA_o/GABA_i))$. Assuming intracellular concentrations of Na^+ and Cl^- of 6 and 33 mM, respectively (Barish, 1983), the GABA transporter reversal potential would be +171 mV when the intracellular and extracellular concentrations of GABA are equal; this is outside the range of potentials experimentally attainable. At hyperpolarized potentials the current-voltage curve tended to saturate. This behavior is similar to that reported for Na^+-dependent transport of glucose (Umbach *et al.*, 1991), but it is different from the glutamate co-transporter in retinal glial cells (Brew and Atwell, 1987) and from facilitated transport of arginine mediated by the cloned y^+ transporter,[2] where currents continue to increase exponentially with hyperpolarization in this voltage range. This saturation may indicate that the rate-limiting step at hyperpolarized potentials is not voltage-dependent (Laeuger, 1991).

It should now be possible to determine systematically the effect of membrane potential on the kinetic constants for Na^+, Cl^-, and GABA, and to formulate a simulation of the transport process. Such a simulation will be helpful for testing kinetic models, and for the interpretation of experiments in which site-directed mutagenesis is used to determine the regions of the transporter molecule that are involved in the binding and translocation of ions and GABA. Similar experiments using analogs of GABA that are also transported, such as nipecotic acid, may lead to a more refined molecular understanding of this transporter.

Acknowledgments—We thank Yanna Wu and Wendy Fairman for expert technical assistance.

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J. Biol. Chem. 1992, 267:22007-22009.