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European Journal of Pharmacology 428 (2001) 1–7

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Potentiation of $GABA_A$ receptor agonists by GABA uptake inhibitors in the rat ventral midbrain

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Received 22 March 2001; received in revised form 6 June 2001; accepted 13 July 2001

Abstract

Whole-cell patch recordings were made from dopamine-containing neurons in the ventral tegmental area (VTA) and substantia nigra zona compacta (SNC). Isoguvacine evoked an outward current (at −60 mV) in a concentration-dependent manner with an EC_{50} of 62 ± 8 μM. The γ-aminobutyric acid (GABA) uptake inhibitor 1-(2(((diphenylmethylene)imino)oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridine-carboxylic acid hydrochloride (NO 711) (3 μM) shifted the isoguvacine concentration–response curve to the left, with a new EC_{50} of 22 ± 4 μM. L-Arginine (3 mM) also shifted the isoguvacine concentration–response curve to the left, with a new EC_{50} of 29 ± 5 μM. L-Arginine (3 mM) increased the currents evoked by GABA (100 μM) and muscimol (1 μM) by 208% and 261%, respectively. The GABA uptake inhibitor 4,5,6,7,-tetrahydroisoxazolo[4,5-*c*]-pyridin-3-ol hydrobromide (THPO) (300 μM) not only mimicked but also occluded the ability of L-arginine (3 mM) to potentiate currents evoked by isoguvacine. Equimolar replacement of Na^+ with choline increased GABA-evoked currents, suggesting that a low Na^+ concentration has an inhibitory effect on GABA transport. Low Na^+ concentration (25 mM) inhibited isoguvacine currents but still occluded the potentiating effects of L-arginine. We conclude that GABA uptake inhibitors potentiate the actions of the $GABA_A$ receptor agonists, isoguvacine and muscimol, probably because they are effective substrates for GABA transporters in the ventral midbrain.

Keywords: Dopamine neuron; γ-Aminobutyric acid (GABA) transporter; Isoguvacine; L-Arginine; Muscimol; NO 711

1. Introduction

In a recent study, our laboratory reported that the amino acid L-arginine potentiates γ-aminobutyric acid (GABA)-mediated synaptic transmission via inhibiting GABA uptake in the ventral midbrain (Shen et al., 1997). We found that L-arginine has advantages over other inhibitors of GABA transporters because it is very water-soluble and its effect quickly reverses during washout from in vitro preparations. However, we were puzzled by a preliminary finding that showed L-arginine also potentiated currents evoked by the GABA analogs isoguvacine and muscimol. These compounds are rigid structural analogs of GABA which were reported to have low affinity for GABA transporters (Korn and Dingledine, 1986; Johnston et al., 1978; Krogsgaard-Larsen and Johnston, 1978). Therefore, the present study was undertaken to explore the effects of well-established blockers of GABA uptake on currents evoked by isoguvacine and muscimol in dopamine neurons in the ventral midbrain slice. Our findings show that uptake inhibitors such as 1-(2(((diphenylmethylene)imino)oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridine-carboxylic acid hydrochloride (NO 711) and 4,5,6,7,-tetrahydroisoxazolo[4,5-*c*]-pyridin-3-ol hydrobromide (THPO) significantly increase the potency of these $GABA_A$ receptor agonists, which suggests that these GABA ligands are indeed effective substrates for GABA transporters.

2. Materials and methods

2.1. Tissue preparation

Sprague–Dawley rats (120–300 g; Bantin and Kingman, WA, USA) were anaesthetized with halothane and

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)01218-3

killed by severing major thoracic vessels in accordance with institutional guidelines. The brain was rapidly removed and horizontal slices (300 μm) containing the midbrain were prepared as described previously (Wu et al., 1995; Shen and Johnson, 1997). Briefly, slices were cut in cold physiological saline with a vibratome and were placed on a supporting net in a recording chamber (volume: 500 μl). The slices were immersed and perfused with a flowing (1.5 ml/min) saline solution which contained (in mM): NaCl, 126; KCl, 2.5; $CaCl_2$, 2.4; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 19; and glucose, 11. This solution was saturated with 95% O_2 and 5% CO_2, and had a pH of 7.35 at 35–37 °C. Using a dissection microscope for visual guidance, the ventral tegmental area (VTA) was identified as the region lateral to the fasciculus retroflexus and medial to the medial terminal nucleus of the accessory optic tract, and the substantia nigra zona compacta (SNC) was identified as a crescent-shaped semilucent region rostral and caudal to the medial terminal nucleus.

2.2. Electrophysiological recordings

Tightly sealed whole-cell recordings were made with pipettes containing (in mM): potassium gluconate, 130; $MgCl_2$, 2; $CaCl_2$, 1; EGTA, 11; HEPES, 10; ATP, 1.5; and GTP, 0.3. The pH of the internal solution was adjusted to 7.3–7.4 with KOH. Membrane potentials were first observed under current clamp after rupturing the membrane patch. Then, membrane currents were recorded under voltage clamp (holding potential at −60 mV) and amplified with an Axopatch-1B amplifier. Data were acquired and analyzed using pCLAMP software, a Digidata analogue/digital interface (Axon Instruments, Foster City, CA, USA), and an IBM-compatible personal computer. Holding currents were recorded continuously using a MacLab analogue/digital interface, Chart software (AD Instruments, Castle Hill, Australia), and a Macintosh IIVX computer. Series resistance was electronically compensated 50–80% to 10–30 MΩ. Membrane potentials have been corrected for the liquid junction potential (10 mV).

2.3. Current–voltage studies

Current–voltage plots were obtained by measuring currents during hyperpolarizing voltage steps (10–60 mV, duration: 400 ms) from a holding potential of −60 mV. Currents were measured immediately after capacitive transients to minimize the influence of I_h on current–voltage plots. Current–voltage plots show "subtracted" currents, in which currents recorded during control conditions have been subtracted from those currents recorded during the experimental treatment. Therefore, subtracted currents represent "net" currents produced by an experimental condition or treatment.

2.4. Cell identification

Neurons in SNC that were likely to contain dopamine were identified as described previously (Johnson and North, 1992). Dopamine neurons fired broad action potentials spontaneously at 1–5 Hz under current clamp, and exhibited relatively large (> 200 pA) hyperpolarization-activated time-dependent inward current (I_h) in response to a hyperpolarizing voltage step to −120 mV under the voltage clamp. Because responses to GABA receptor agonists and uptake blockers in VTA and SNC were similar, data from these two regions were pooled.

2.5. Drugs

Stock solutions of drugs were diluted at 1:300–1:1000 to the desired concentration in the superfusate immediately prior to their use. A stock solution of dopamine was kept on ice to retard oxidation. Approximately, 30 s was required for the drug solution to enter the recording chamber. This delay was due to passage of the perfusate through a heat exchanger. Bicuculline methiodide, GABA, dopamine hydrochloride, L-arginine, choline chloride, glycine, scopolamine, and β-alanine were obtained from Sigma, St. Louis, MO. Isoguvacine, 4,5,6,7,-tetrahydroisoxazolo[4,5-*c*]-pyridin-3-ol hydrobromide (THPO), muscimol, and 1-(2(((diphenylmethylene)imino)oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridine-carboxylic acid hydrochloride (NO 711) were obtained from Research Biochemicals, Natick, MA.

2.6. Data analysis

Numerical data in the text and error bars in figures are expressed as mean ± S.E.M. In current–voltage plots, a reversal potential was determined by linear regression for each cell, and the mean was calculated by averaging the results from all cells. Two-way analysis of variance (ANOVA) with repeated measures was used to test for significant differences between current and voltage curves (SigmaStat, Jandel Scientific, San Rafael, CA). Significant differences between data points were evaluated using the pairwise multiple comparison method of Student–Newman–Keuls. Two-tailed *t*-tests were used to test for significant differences between holding currents. Differences were considered statistically significant at $p < 0.05$. In evaluating concentration-dependent drug effects, an EC_{50} was calculated for each cell using the KaleidaGraph curve-fitting program (Synergy Software, Reading, PA) on a Macintosh computer. First, data were fitted to the Hill–Langmuir equation: $y = ax/(x + b)$, where y is the magnitude of the effect, a is the maximum effect (E_{max}), x is the concentration of the drug, and b is EC_{50}. Then, amplitudes of currents at each concentration of isogu-

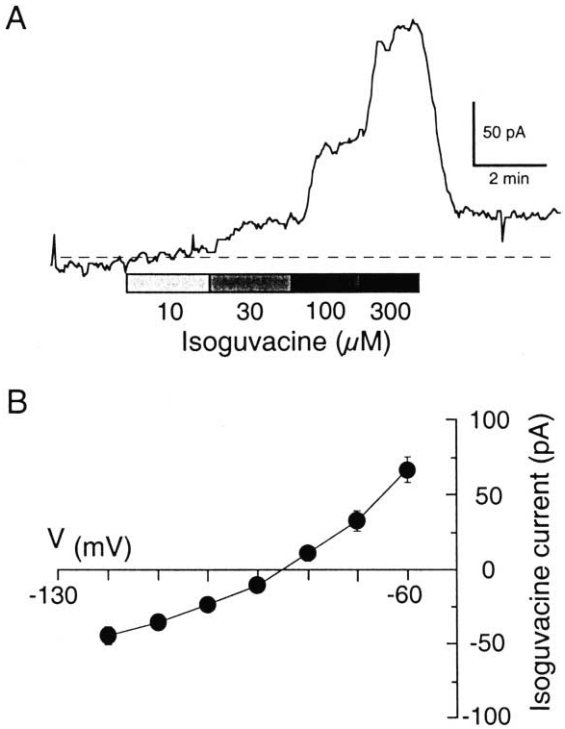

Fig. 1. (A) Isoguvacine evokes outward currents in a dopamine neuron. Concentration of isoguvacine, indicated by the bar shown below the trace, was increased cumulatively. (B) Isoguvacine-evoked currents reverse at the chloride equilibrium potential. This current–voltage relationship was calculated by subtracting the currents obtained in the control from those obtained in the presence of isoguvacine (20 μM) at each test potential for each cell. Each point represents the mean ± S.E.M. of five cells.

vacine were normalized with respect to E_{max} estimated for each cell. Mean EC_{50} and S.E.M. were calculated by averaging the EC_{50} values determined for all cells.

3. Results

3.1. Actions of isoguvacine

The $GABA_A$ receptor agonist isoguvacine evoked an outward current (at −60 mV) which reached its peak within 2 min; recovery was complete within 5–10 min after washout. Repeated superfusion of isoguvacine every 3–5 min gave stable responses for more than 1 h. As seen in Fig. 1A, currents evoked by isoguvacine were concentration-dependent; 10 μM isoguvacine evoked 28 ± 6 pA of outward current (at −60 mV; $n = 8$), whereas 300 μM isoguvacine evoked 235 ± 18 pA of current. Currents evoked by isoguvacine (20 μM) reversed at −82 ± 2 mV ($n = 5$), which agrees with the expected reversal potential for chloride as predicted by the Nernst equation (−83 mV; Fig. 1B). Effects of isoguvacine were completely blocked by bicuculline (30 μM, $n = 5$).

3.2. Potentiation of $GABA_A$ receptor agonists by uptake inhibitors

Although $GABA_A$ receptor agonists such as isoguvacine are usually thought to be poor substrates for GABA transporters (Lodge et al., 1978; Korn and Dingledine, 1986; Krogsgaard-Larsen and Johnston, 1978), Fig. 2A shows that the current evoked by isoguvacine is potentiated by the GABA uptake inhibitor THPO (Bolvig et al., 1999). THPO (300 μM) increased the amplitude of isoguvacine currents by 55 ± 8% (to 109 ± 14 pA, $n = 4$). THPO alone had no effect on holding current. Because we have shown previously that L-arginine inhibits GABA

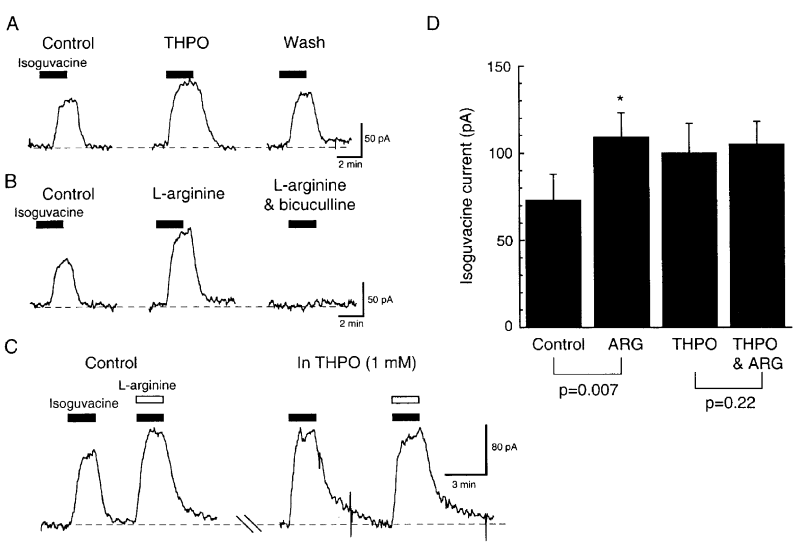

Fig. 2. Inhibitors of GABA uptake potentiate the currents evoked by $GABA_A$ receptor agonists. Currents evoked by isoguvacine (30 μM) were potentiated by 300 μM THPO (A) and by 3 mM L-arginine (B) in the same dopamine neuron. THPO or L-arginine was perfused 5 min prior to the application of isoguvacine. In the presence of L-arginine, bicuculline (30 μM) blocked all the currents evoked by isoguvacine. (C) THPO (1 mM) occludes the ability of L-arginine (3 mM) to potentiate the current evoked by isoguvacine (30 μM). All currents in (C) were recorded in the same neuron. (D) The bar graph shows that the abilities of L-arginine (3 mM) and THPO (1 mM) to potentiate isoguvacine (30 μM)-evoked currents are not additive. Each data bar represents data from the same four dopamine neurons.

uptake in the midbrain slice (Shen et al., 1997), we were interested to know if L-arginine would also potentiate effects of isoguvacine. As shown in Fig. 2B, L-arginine (3 mM) increased the amplitude of currents evoked by 30 μM isoguvacine by 40 ± 10% (100 ± 17 pA in L-arginine compared with the control current of 73 ± 15 pA; $p < 0.01$, $n = 4$). However, as seen in Fig. 2C, L-arginine (3 mM) failed to produce a further increase in currents evoked by isoguvacine in the presence of THPO (105 ± 13 pA; $p = 0.22$, $n = 4$). Because THPO occluded the ability of L-arginine to potentiate isoguvacine-induced currents, this suggests that both THPO and L-arginine act via a common mechanism of action, i.e. inhibition of GABA uptake. The summary graph in Fig. 2D summarizes these experiments with L-arginine, THPO and isoguvacine.

Fig. 3 shows that L-arginine increases the amplitude of currents evoked by GABA (Fig. 3A) and muscimol (Fig. 3B). L-Arginine (3 mM) increased the currents evoked by GABA by 208 ± 41% ($n = 10$), and muscimol (10 μM)-evoked currents were increased by 261 ± 63% ($n = 3$). However, L-arginine failed to enhance the current evoked by either glycine (1 mM, $n = 4$) or β-alanine (1 mM, $n = 3$) (data not shown), presumably because these ligands are not substrates for the subtypes of GABA transporter expressed in the ventral midbrain.

3.3. Concentration-dependent potentiation of isoguvacine by uptake inhibitors

Fig. 4A shows the concentration–response curves for isoguvacine alone and in the presence of 3 μM NO 711 or 3 mM L-arginine. NO 711 is a potent inhibitor of GABA transporters (Bolvig et al., 1999). These data show that both NO 711 and L-arginine shift the concentration–response curve of isoguvacine to the left. Whereas the control EC_{50} value of isoguvacine alone was 62 ± 8 μM ($n = 8$), NO 711 (3 μM) reduced the isoguvacine EC_{50} to 22 ± 4 μM ($n = 8$). Similarly, 3 mM L-arginine reduced the isoguvacine EC_{50} to 29 ± 5 μM ($n = 6$). Reversal potentials for currents evoked by isoguvacine (20 μM) in 1 μM NO 711 (−82 ± 1 mV, $n = 3$) and 10 mM L-arginine (−87 ± 1 mV, $n = 3$) were not significantly different from the reversal potential for currents evoked by isoguvacine alone.

Fig. 4B shows the concentration–response curves for NO 711 and L-arginine on currents evoked by 10 μM isoguvacine. A low concentration of NO 711 (0.3 μM) increased the amplitude of isoguvacine current by 85% (from 26 ± 3 to 48 ± 5 pA, $n = 7$), and 10 μM NO 711 increased the isoguvacine current by 366% (to 121 pA).

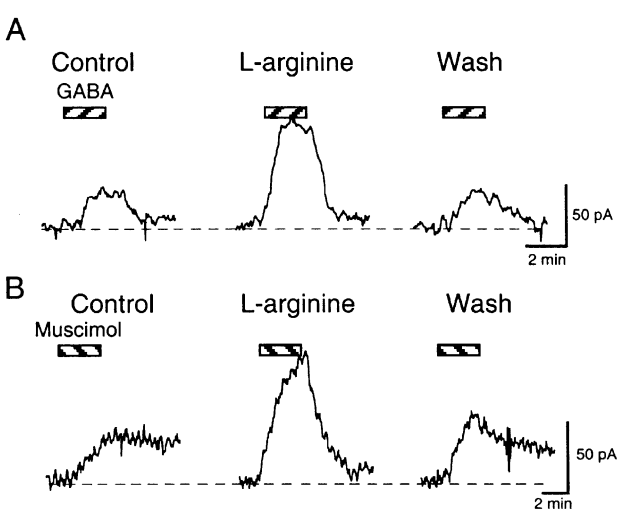

Fig. 3. L-arginine (3 mM) potentiates the currents evoked by 100 μM GABA (A) and 1 μM muscimol (B). L-Arginine was perfused 5 min prior to the application of GABA or muscimol.

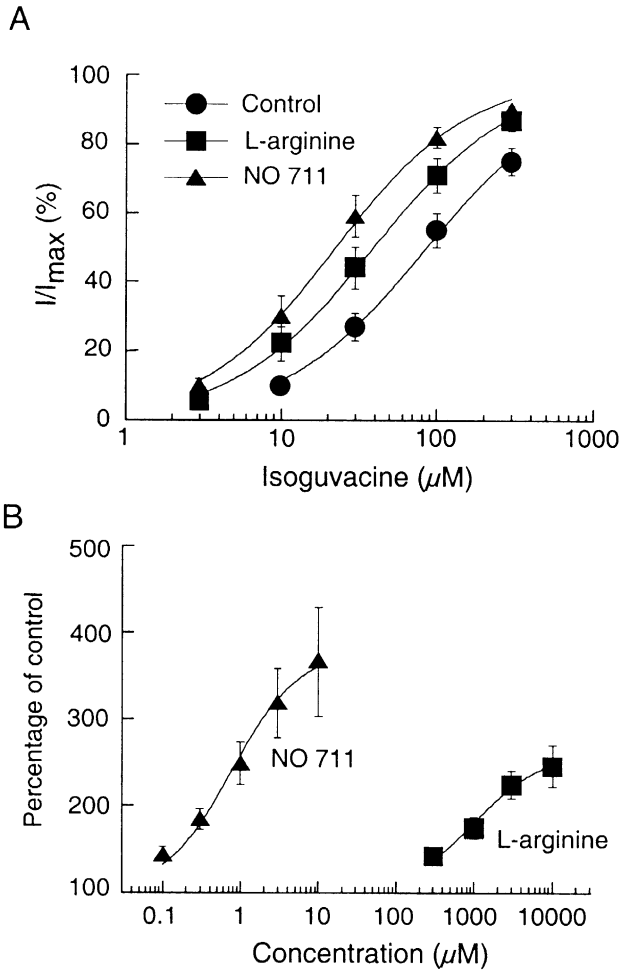

Fig. 4. Uptake inhibitors NO 711 and L-arginine potentiate the currents evoked by isoguvacine. (A) Concentration–response curves for isoguvacine in the absence and presence of L-arginine (3 mM) or NO 711 (3 μM). Amplitudes of currents were normalized to the E_{max} estimated by the Hill–Langmuir equation. Each point is the average of six to eight neurons. Note that both L-arginine and NO 711 shift the isoguvacine concentration–response curve to the left. (B) Percentage increases in peak currents evoked by isoguvacine (10 μM) in the presence of various concentrations of NO 711 or L-arginine. Each point represents the mean ± S.E.M. of six to eight cells.

The EC_{50} of NO 711 for potentiation of isoguvacine currents was about 1 μM. L-Arginine, at a concentration of 1 mM, increased the isoguvacine currents by 69% (from 29 ± 3 to 49 ± 6 pA, $n = 7$), and 3 mM L-arginine increased the isoguvacine currents by 225%. The EC_{50} of L-arginine was about 1 mM.

3.4. Potentiation of isoguvacine is Na^+-dependent

Because the activity of GABA transporters is Na^+-dependent (Kavanaugh et al., 1992), we reasoned that removal of extracellular Na^+ would impair the ability of transport inhibitors to potentiate currents evoked by isoguvacine. As seen in Fig. 5A, reducing the superfusate concentration of Na^+ to 25 mM (by equimolar substitution of choline chloride for Na^+) significantly increased the amplitude of currents evoked by GABA; this finding is consistent with the Na^+-dependence of the GABA transporter. However, the low superfusate concentration of Na^+ occluded the ability of L-arginine to potentiate currents evoked by GABA (right panel, Fig. 4A). Similarly, the low Na^+ concentration in the superfusate also prevented L-arginine from potentiating currents evoked by isoguvacine (Fig. 5B). Thus, isoguvacine (100 μM) plus L-arginine (3 mM) produced an outward current of 61 ± 11 pA in the low Na^+ superfusate; this current was not significantly different from that evoked by isoguvacine alone as recorded in the low Na^+ concentration. Interestingly, the low Na^+ concentration reduced the isoguvacine currents by $30 \pm 7\%$ (91 ± 13 pA in control vs. 63 ± 10 pA in 25 mM Na^+; $p < 0.05$), whereas the low Na^+ superfusate increased the GABA-mediated currents (compare Fig. 5A and B). In all experiments in which choline was substituted for Na^+, scopolamine (1 μM) was added to block possible cholinergic effects of choline. A summary of the data obtained in low extracellular Na^+ concentration is presented in Fig. 5C.

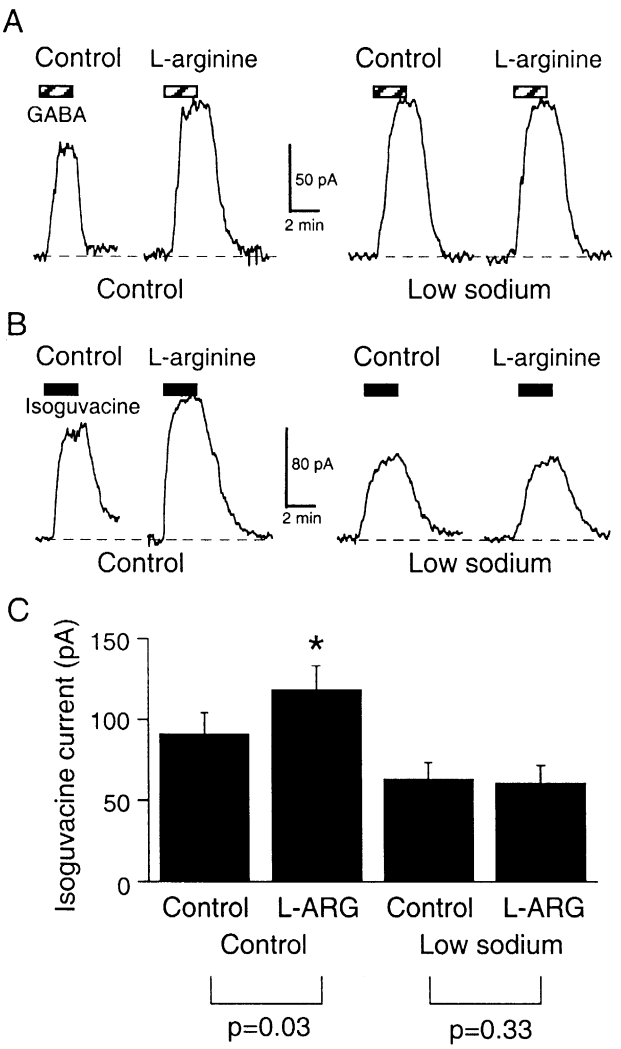

Fig. 5. The superfusate that contains a low extracellular concentration of Na^+ (25 mM) occludes the ability of L-arginine (3 mM) to potentiate currents evoked by 500 μM GABA (A) or 100 μM isoguvacine (B). L-Arginine was perfused for 5 min prior to the application of GABA or isoguvacine. Choline was substituted for Na^+ in the superfusate. Note that currents evoked by GABA (A) were increased by the low Na^+ superfusate, but currents evoked by isoguvacine were not (B). (C) The bar graph showing that L-arginine (3 mM) fails to potentiate currents evoked by isoguvacine (100 μM) in the low Na^+ superfusate. Each data bar represents results from the same four neurons. Asterisk indicates a significant increase in current compared to control ($p < 0.05$).

4. Discussion

Uptake by transporters is an important mechanism for terminating the action of endogenous neurotransmitters. In contrast, the chemical structures of drugs and receptor ligands that are not endogenous to the central nervous system usually contain structural characteristics which make them poor substrates for active uptake by transporters. Nevertheless, our results suggest that the $GABA_A$ receptor agonists, isoguvacine and muscimol, are significant substrates for GABA transporters. In support of this conclusion, we found that: (1) agents that are well-known to inhibit GABA uptake, such as THPO and NO 711, effectively potentiated currents evoked by $GABA_A$ receptor agonists; (2) L-arginine, which we have shown previously to inhibit GABA uptake (Shen et al., 1997), also potentiated $GABA_A$ agonists; and (3) reduced concentration of extracellular Na^+ occluded the ability of uptake blockers to potentiate $GABA_A$ receptor agonists. Because currents evoked by $GABA_A$ receptor agonists were potentiated by NO 711 and THPO—but not those evoked by β-alanine—our findings are most consistent with the uptake by the GABA transporter isoform known as GAT-1 (Clark and Amara, 1994; Borden et al., 1994).

Our results are surprising, considering the low affinities that have been reported for the binding of $GABA_A$ receptor agonists to uptake sites (Krogsgaard-Larsen and Johnston, 1975). For example, muscimol reportedly binds to the

uptake sites in rat cortical synaptosomes with a K_d of about 50 μM (DeLorey and Brown, 1992). Another study found that muscimol is bound to transporters in rat cerebral cortical slices with an affinity in the low millimolar concentration range (Johnston et al., 1978). A lack of a functional interaction of muscimol and/or isoguvacine with GABA transporters has been reported in Skate retinal glial cells (Qian et al., 1993), crayfish stretch receptor neurons (Krause et al., 1981), dorsal root ganglia (Gallagher et al., 1983), and hippocampus (Korn and Dingledine, 1986; Rovira et al., 1984). On the other hand, Brown and Scholfield (1984) reported that inhibition of uptake by nipecotic acid or low extracellular Na^+ concentration potentiated the electrophysiological effects of muscimol and isoguvacine in olfactory cortical neurons. In contrast, others showed that inhibitory effects of muscimol, but not isoguvacine, could be potentiated by nipecotic acid in cat spinal cord (Lodge et al., 1978). It is possible that these conflicting results are due to tissue- and/or species-dependent differences in the expression of GABA transporter isoforms.

It is interesting to note in our finding that a reduced extracellular concentration of Na^+ caused a reduction in the amplitude of the currents evoked by isoguvacine, even though the low Na^+ media occluded the ability of L-arginine to block uptake (Fig. 4). A similar finding was reported by others who found that currents evoked by muscimol were reduced in low Na^+ media (Constanti and Nistri, 1981). One possible explanation is that the reduced agonist current is due to receptor desensitization caused by the accumulation of GABA when uptake was inhibited by reduced Na^+ concentration. However, this mechanism would not be consistent with our results which showed that low Na^+ greatly increased the current evoked by exogenous GABA. Alternatively, isoguvacine may be taken up by the transporter into nerve terminals where it causes release of GABA by a heteroexchange mechanism (White and Snodgrass, 1983). Consequently, when uptake of isoguvacine is blocked by low Na^+, the evoked current is smaller in part because the isoguvacine-induced release of GABA is also reduced. A third possibility is that a low Na^+ concentration interferes with the binding of agonists —but not of GABA—to the $GABA_A$ receptor. It is unclear which is the correct explanation, but our data are consistent with either the second or third possibility.

In summary, we have shown that effects of the $GABA_A$ receptor agonists, isoguvacine and muscimol, are potentiated by inhibitors of GABA uptake. The most plausible explanation is that these GABA receptor agonists are effective substrates for GABA transporters in the ventral midbrain, and their uptake are inhibited by uptake block. When using these agonists in in vitro pharmacological experiments, it may be important for the accurate interpretation of data to keep in mind the possibility that actions of these drugs may be influenced by ongoing levels of active uptake. This may also be important in clinical studies in which the combined use of a GABA receptor agonist (e.g. progabide) and a GABA uptake inhibitor (e.g. tiagabine) may be explored as a new treatment for epilepsy (Fisher and Blum, 1995). Our data would suggest that the combined use of an uptake blocker and a $GABA_A$ receptor agonist could have a synergistic action when used clinically.

Acknowledgements

This work was supported by USPHS Grants MH40416 and NS35437.

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