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IONS REQUIRED FOR THE ELECTROGENIC TRANSPORT OF GABA BY HORIZONTAL CELLS OF THE CATFISH RETINA

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SUMMARY

1. Solitary horizontal cells were isolated from catfish retinas. Membrane currents activated by extracellular and intracellular GABA were characterized during a whole-cell voltage clamp.

2. Extracellular GABA activated two currents: a $GABA_A$ current, and an 'influx' current mediated by a GABA transporter. The influx current was studied after the $GABA_A$ current was blocked with 0·5 mM picrotoxin. The influx current required extracellular Na^+ and Cl^-. Extracellular Na^+ could not be replaced by another alkali metal cation.

3. The influx current also depended upon the identity of ions in the intracellular solution. Either an intracellular alkali metal cation or Cl^- was required to produce an influx current.

4. The influx current was inward at −75 mV and decreased as the membrane was depolarized towards +20 mV. When the membrane was depolarized beyond +25 mV, the polarity of the current depended upon the ion composition of the intracellular solution and could be inward, zero or outward.

5. The introduction of GABA into a cell during the course of an experiment produced an outward current. This 'efflux' current was small at −75 mV and increased with depolarization. The efflux current required intracellular Na^+ and Cl^-. Intracellular Na^+ could not be replaced by another alkali metal cation.

6. The efflux current also depended upon the identity of ions in the extracellular solution. An extracellular alkali metal cation was required to produce an efflux current. Removing extracellular Cl^- did not affect the efflux current.

7. The outward movement of GABA produced a local accumulation in extracellular GABA concentration that could be detected by the activation of the $GABA_A$ current. GABA efflux only occurred during conditions that produced an efflux current. Electroneutral efflux did not occur.

8. In the absence of GABA, extracellular alkali metal cations produced a 'leakage' current. The leakage current was inward at −75 mV and decreased as the membrane was depolarized towards +20 mV. When the membrane was depolarized beyond +25 mV, the polarity of the leakage current depended, like the GABA influx current, upon the ion composition of the intracellular solution and could be inward,

zero or outward. The addition of GABA to the intracellular solution produced an efflux current and suppressed the leakage current.

9. We conclude that the transporter mediates electrogenic influx, efflux and leakage. Each mode of operation depends upon ions on both sides of the membrane. Influx and efflux are not symmetrical.

INTRODUCTION

Electrogenic transport of synaptic transmitters appears to offer an alternative to Ca^{2+}-dependent exocytosis as a basic mechanism for voltage-controlled release of transmitter from presynaptic terminals. Perhaps the clearest evidence for transporter-mediated release has come from experiments on cells of the distal retina. Horizontal cells appear to use transporters at vesicle-poor junctions where they release GABA onto adjacent photoreceptors (Schwartz, 1982, 1987; Yazulla & Kleinschmidt, 1983; Ayoub & Lam, 1984). Although evidence is accumulating that transporters release transmitters, the mechanism of action of these 'simple' machines is still relatively unknown.

Coding sequences for a family of Na^+- and Cl^--dependent transporters have recently been identified (see references in Liu, Mandiyan, Nelson & Nelson, 1992). Members of this family transport GABA, glycine, taurine, proline, catecholamines or indoleamines. Sequence homology and the common requirement for extracellular Na^+ and Cl^- indicate that family members share mechanisms for the movement of ions and substrates across membranes.

GABA transport has been studied by measuring isotopic GABA fluxes in tissue slices (Iversen & Neal, 1968), in synaptic vesicles (Martin, 1973), and in liposomes that incorporate a reconstituted transporter protein (Keynan & Kanner, 1988). Na^+-dependent GABA transport has been considered to be a simple reversible system (see for example Blaustein & King, 1976; Pastuszko, Wilson & Ericinska, 1982). However, this conclusion is weakened by at least two experimental problems. Usually, it has been difficult to control separately the solution on each side of a membrane. In addition, membrane voltage has not been directly measured. Direct observation and control of membrane voltage seems essential for the analysis of an electrogenic process. We have studied currents generated by GABA transport with a whole-cell voltage clamp to answer the following questions: (1) What ions are required for transport? (2) Is efflux the reverse of influx? (3) Is transport always electrogenic? (4) Can ions move through the transporter in the absence of GABA? Electrogenic influx of GABA has been observed previously in skate retinal horizontal cells (Malchow & Ripps, 1990) and crayfish stretch receptor neurones (Kaila, Rydqvist, Pasternak & Voipio, 1992). We have now observed a current produced by efflux and also a leakage mode that occurs in the absence of GABA. The results demonstrate that transport is asymmetric. Either *trans* ions have different allosteric effects on each side of the membrane or efflux is not the reverse of influx.

A preliminary report of our findings has been presented at the 22nd annual meeting of The Society for Neuroscience (Cammack & Schwartz, 1992).

METHODS

Horizontal cells were isolated from catfish, *Ictalurus punctatus*, retinas. Catfish were killed by severing the cervical spinal cord and pithing the brain. The procedure for dissociating the retina and maintaining solitary cells was as described previously (DeVries & Schwartz, 1989). The membrane potential of a solitary horizontal cell was controlled by a voltage clamp maintained through a patch pipette in the whole-cell configuration (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The resistance of a fire-polished pipette was 3–4 MΩ. Series resistance usually increased during a whole-cell recording and was typically 5–25 MΩ at the end of an experiment. The patch pipette solution contained (mM): XCl, 100; $CaCl_2$, 1; EGTA, 10; Hepes, 10; adjusted to pH 7·2. X^+ could be Li^+, Na^+, Cs^+, K^+, choline, arginine or *N*-methylglucamine as indicated in the text and figure legends. The extracellular solution contained (mM): XCl, 124; $CaCl_2$, 5·0; $MgCl_2$, 5·0; glucose, 16; Hepes, 2·0; tetrodotoxin, 0·001; adjusted to pH 7·6. When X^+ was K^+, 6 mM Ba^{2+} was added to the extracellular solution to suppress a voltage-activated K^+ current. A change in the predominant anion involved replacing $CaCl_2$ and $MgCl_2$ with their gluconate salts and the remaining Cl^- with an equimolar concentration of either gluconate or D-aspartate as indicated in the text and figure legends. Solutions containing picrotoxin were prepared daily. Usually 15 mg of solid was added to 50 ml of extracellular solution and bath-sonicated for approximately 20 min. GABA (20 mM) was added to the intracellular solution without correcting for the change in osmolarity. The extracellular volume of the bath was approximately 0·2 ml. Cells were usually superfused continuously at 0·25 ml/min. Superfusion was stopped for experiments that detected efflux as illustrated in Fig. 8. The extracellular solution bathing a cell could be altered by changing the superfusing solution or by ejecting solution from a nearby 'puffer' pipette (tip diameter approximately 2 μm). The bath was connected to a Ag–AgCl ground by an agar salt bridge. Changes in junction potential produced by an alteration in extracellular or intracellular solutions were measured as described by Neher (1992) and the data corrected. Current records were low-pass filtered (8-pole Bessel filter with −3 dB at 400 Hz), digitized at 1000 Hz by a 12-bit analog-to-digital converter, and analysed by a computer. Current–voltage curves were measured when the voltage was held at a hyperpolarized potential and then ramped to a depolarized potential at 0·3 V/s. Signal-to-noise ratio was increased by averaging five to ten traces. In most experiments, current–voltage curves were determined before and after a solution change and the difference calculated. Often the procedure could be repeated several times in one cell and we verified that all of the difference currents superimposed. The difference currents observed when the voltage was ramped either from −70 to +70 mV or from +70 to −70 mV superimposed. Therefore, the current–voltage relations are steady-state curves.

The solution within a cell could be changed during the course of an experiment. The method required two patch pipettes. The first pipette established a whole-cell voltage clamp in the usual manner. In addition, a second pipette was sealed onto the cell and operated in the 'current-clamp' mode. The two pipettes contained different intracellular solutions. The membrane occluding the tip of the second pipette was not ruptured immediately. During the experiment, the membrane potential was ramped and the current–voltage relation determined. The second pipette responded to each voltage ramp with a small potential whose amplitude and time course were determined by the resistance of the intact patch occluding its tip. At the appropriate moment, the membrane occluding the tip was ruptured and the solution in the second pipette diffused through the open orifice and into the cell. Rupture of the membrane occluding the tip was indicated by observing the full-sized voltage ramp imposed by the voltage clamp.

Nomenclature. GABA produces a current when added to either the extracellular or intracellular solution. '*Cis*' will refer to the solution on the side of the membrane to which GABA was added. '*Trans*' will refer to the solution on the opposite side of the membrane. The current induced by intracellular GABA is an 'efflux' current. The efflux current was always outward. The current induced by extracellular GABA is an 'influx' current. The influx current was always inward at hyperpolarized potentials, but could be outward at depolarized potentials. None the less, we will maintain our definition even though we have no assurance that GABA moved inward when depolarization produced an outward current.

Abbreviations. Ethyleneglycol-tetraacetic acid, EGTA; γ-aminobutyric acid, GABA; 4-(2-hydroxyethyl)piperazine-1-ethane-sulphonic acid, Hepes; *N*-methylglucamine, NMG; choline, Ch.

RESULTS

Influx

Our preliminary experiments demonstrate that GABA had two electrical effects on solitary horizontal cells. GABA activated a $GABA_A$ current and was also a substrate for an electrogenic transporter. The two currents can be pharmacologically dissected as illustrated in Fig. 1. Membrane voltage was maintained at −70 mV with a whole-cell voltage clamp and the current was sampled at 2 s intervals (Fig. 1*A*). The cell was continuously superfused with the extracellular salt solution. A puff of GABA (200 μM) produced an inward current. Next, picrotoxin (0·5 mM), an inhibitor of the $GABA_A$ current (see Bormann, 1988), was added to the extracellular solution. Subsequently, a puff of GABA with picrotoxin produced a smaller response. The entire procedure was reversible and, as illustrated in the figure, could be repeated.

Current–voltage relations provide additional information. During the interval between each point in Fig. 1*A*, the voltage was ramped from −70 to +70 mV. Current–voltage relations were recorded while the cell was superfused with the extracellular salt solution (trace 1) and during the application of GABA (trace 2). The difference, calculated by subtracting trace 1 from trace 2, is a current induced by extracellular GABA (trace 3). Current–voltage relations were also measured while the cell was superfused with picrotoxin (trace 4) and during the application of GABA and picrotoxin (trace 5). The difference, calculated by subtracting trace 4 from trace 5, is a picrotoxin-insensitive component (trace 6). For comparison, the picrotoxin-sensitive component, calculated by subtracting trace 6 from trace 3, is superimposed in Fig. 1*E* as trace 7.

The picrotoxin-sensitive component appears to be a $GABA_A$ current. Its current–voltage curve (trace 7) reversed near the Cl^- equilibrium potential (apparent reversal potential of −4 mV in five cells, compared with a Cl^- Nernst potential of −8 mV). Moreover, a similar current was produced by muscimol (100 μM, five cells), an agonist for $GABA_A$ channels; however, baclofen (200 μM), a $GABA_B$ agonist, did not produce a current (three cells). The $GABA_A$ current was conveniently inhibited when 0·5 mM picrotoxin was added to the extracellular solutions (except, as noted, the experiments illustrated in Fig. 8).

The picrotoxin-insensitive component had the pharmacology and, as described in the next section, dependence on extracellular Na^+ and Cl^- expected for a current produced by a GABA transporter. It was inhibited by nipecotic acid, a pharmacological agent that interferes with GABA transport (Krogsgaard-Larsen & Johnson, 1975). The current elicited by 0·1 mM GABA was reduced 67 ± 6% (four cells) after the addition of 0·1 mM nipecotic acid. However, nipecotic acid was not a simple antagonist. Higher concentrations (greater than 0·2 mM) produced a current similar to the GABA influx current (namely, the current–voltage relation was similar to Fig. 1*E*, trace 6). The current amplitude produced by 0·5 mM nipecotic acid was approximately half the maximum amplitude produced by GABA. Thus, nipecotic acid behaved as an antagonist at moderate concentrations and as an agonist at high concentrations, a situation which might indicate that nipecotic acid binds the transporter but translocates much more slowly than GABA.

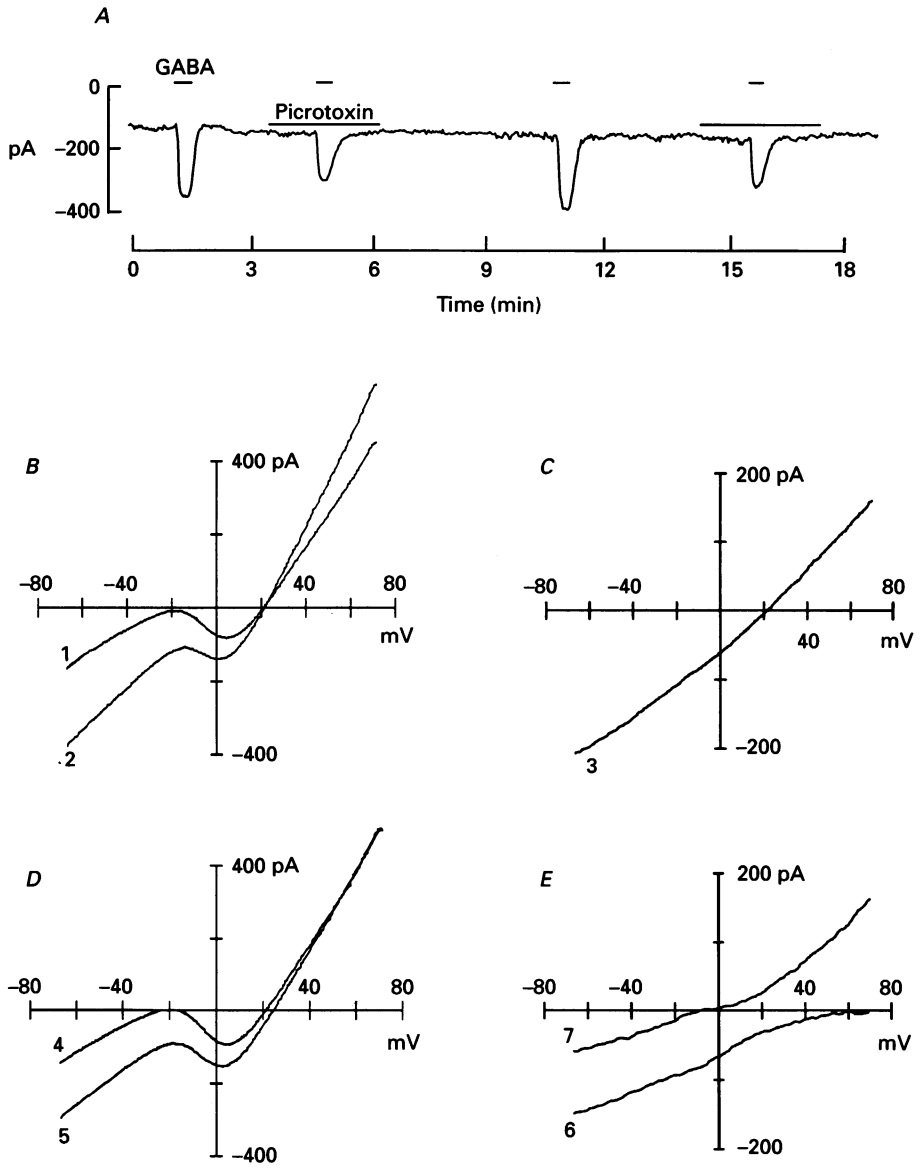

Fig. 1. Extracellular GABA activated two currents: a $GABA_A$ current and ion movement produced by an electrogenic GABA transporter. The two currents could be separated with picrotoxin. *A*, continuous record of membrane current observed at −70 mV. The cell was superfused first with the extracellular salt solution. The timing bars indicate the addition of GABA (0·2 mM) and picrotoxin (0·5 mM). *B*, current–voltage relations measured while the cell was superfused with the extracellular salt solution (trace 1) and during the application of GABA (trace 2). *C*, difference current (trace 3) calculated by subtracting trace 1 from trace 2. *D*, current–voltage relations measured while the cell was superfused with picrotoxin (trace 4) and during the addition of GABA (trace 5). *E*, difference current (trace 6) calculated by subtracting trace 4 from 5. For comparison, the picrotoxin-sensitive current (trace 7) was calculated by subtracting trace 6 from trace 3. Intracellular solutions contained 100 mM KCl.

Cis ions required for influx

Molecular cloning has identified three GABA transporters (Gaustella *et al.* 1990; Clark, Deutch, Gallipoli & Amara, 1992; Borden, Smith, Hartig, Branchek & Weinshank, 1992). All require extracellular Na^+ for the uptake of GABA, but differ in the extent of their dependence on extracellular Cl^-. The GABA influx current behaved most like the transporter cloned and expressed by Gaustella *et al.* (1990). An influx current was only observed when the extracellular medium contained both Na^+ and Cl^-.

The experiment illustrated in Fig. 2*A* demonstrates that the influx current required Na^+ in the extracellular solution. The cell was superfused with a Na^+-free solution (NaCl replaced with choline chloride) and a puff of GABA was delivered. Current–voltage relations were recorded in each solution and the difference calculated (trace 1). In the absence of extracellular Na^+, no current was produced. Next, the cell was superfused with a Na^+-rich solution and the procedure repeated. Now, GABA induced an influx current (trace 2). Similar experiments were completed with other extracellular cations. An influx current did not occur when extracellular Na^+ was replaced by choline (four cells), Li^+ (two cells), K^+ (two cells) or Cs^+ (two cells).

Cl^- was also required (Fig. 2*B*). In the absence of extracellular Cl^- (NaCl replaced with sodium gluconate), a puff of GABA produced no current change (three cells). When the same cell was superfused with a Cl^--rich solution, GABA produced an influx current (trace 4). Thus, the influx current required extracellular Na^+ and Cl^-. In the absence of either ion, an influx current did not occur.

Trans ions required for influx

Relatively little is known about the intracellular ions required for transport. A simple assumption is that intracellular ions are not required. An alternative view is that the system is symmetrical and the same ions act on both sides of the membrane. Neither assumption is correct. The following experiments explored separately the possible roles of small cations and anions. The experiment illustrated in Fig. 3*A* demonstrates the importance of intracellular cations. At the beginning of the experiment, membrane voltage was controlled by a pipette whose intracellular solution lacked small cations and anions. These were replaced with arginine (a cation) and D-aspartate (an anion). A second pipette contained potassium D-aspartate and was sealed to the membrane surface; however, the membrane occluding its tip was left intact. After several minutes the ions in the cell exchanged with the arginine aspartate in the first pipette. At this time, a puff of GABA did not produce a current (trace 1), Next, the membrane occluding the tip of the second pipette was ruptured and potassium D-aspartate diffused into the cell. Subsequently, a puff of GABA produced an inward current (trace 2). The introduction of potassium D-aspartate restored GABA's ability to produce an influx current (three cells). Additional experiments used a single patch pipette and demonstrated that similar current–voltage relations were obtained when Cl^- was absent and the predominant intracellular cation was Li^+ (three cells), Na^+ (five cells), K^+ (seven cells) or Cs^+ (four cells). Thus, an influx current could be observed when the intracellular solution contained an alkali metal cation but lacked Cl^-.

The experiment illustrated in Fig. 3*B* demonstrates the importance of intracellular Cl^-. Again, the experiment began with the membrane voltage controlled by a pipette that contained arginine aspartate. At this time, a puff of GABA did not produce a current (trace 3). Subsequently, the membrane occluding the tip of the second

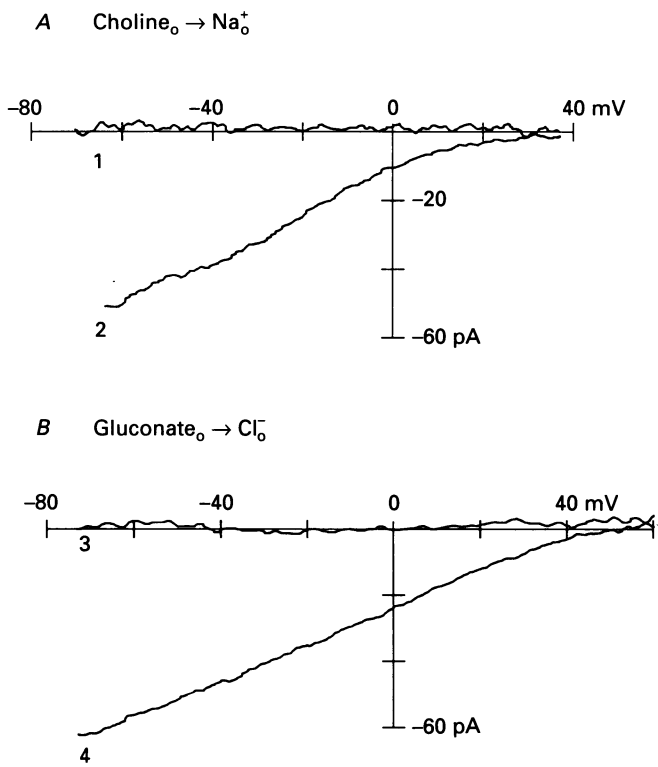

Fig. 2. The influx current required extracellular Na^+ and Cl^-. *A*, the GABA influx current is Na^+ dependent. A cell was superfused first with a Na^+-free solution (NaCl replaced with choline chloride). A puff of GABA (200 μM) produced trace 1. Next, the cell was superfused with a Na^+-rich solution. Now, a puff of GABA produced trace 2. *B*, the GABA influx current is Cl^- dependent. A cell was superfused first with a Cl^--free solution (NaCl replaced with sodium gluconate). A puff of GABA (200 μM) produced trace 3. Next, the cell was superfused with a Cl^--rich solution. Now, a puff of GABA produced trace 4. Extracellular solutions contained 0·5 mM picrotoxin. Intracellular solutions contained 100 mM KCl.

pipette was ruptured and arginine chloride diffused into the cell. Afterwards, a puff of GABA produced an inward current (trace 4). The same result was obtained with another cell. Additional experiments used a single patch pipette and demonstrated that an influx current was absent (six cells) when the pipette lacked both Cl^- and an alkali metal cation, but was present (nine cells) when the pipette contained Cl^- (but still lacked an alkali metal cation). Thus, an influx current could be observed when the intracellular solution contained Cl^- but lacked an alkali metal cation.

Subsequent experiments demonstrated that the shape of the current–voltage relation was altered when the intracellular solution contained both an alkali metal cation and Cl^-. In Fig. 3*C* the experiment began with the membrane voltage

controlled by a pipette that contained choline chloride. A puff of extracellular GABA produced an inward current (trace 5) that declined as the membrane was depolarized from −80 to approximately +25 mV. When the membrane was depolarized beyond +25 mV, a small, inward current remained nearly constant. Next, the membrane

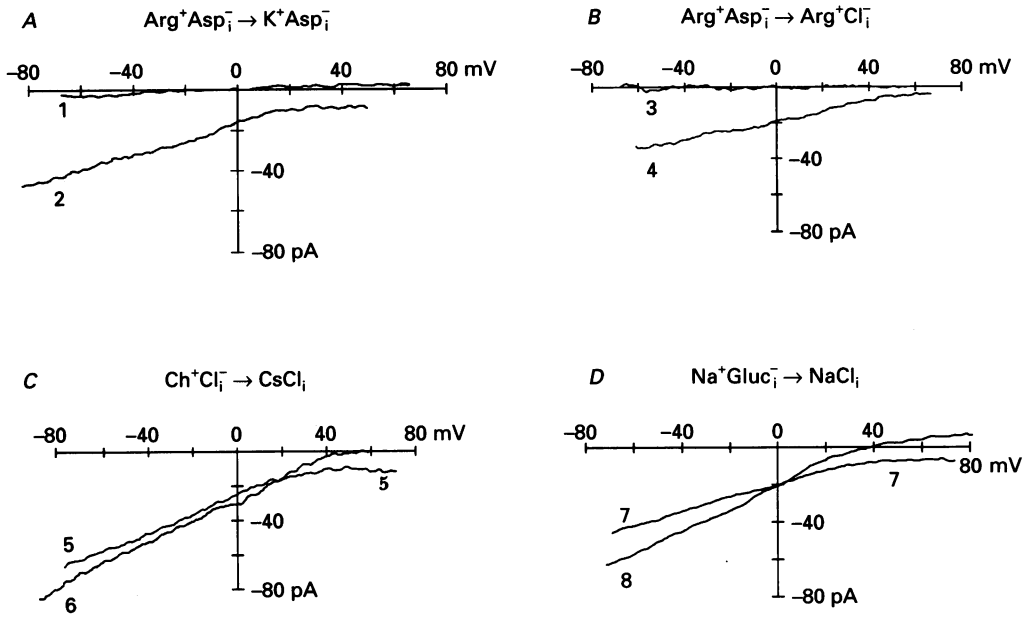

Fig. 3. GABA influx depended upon the identity of ions in the intracellular solution. Each experiment required a second patch pipette to introduce a change in the intracellular solution during the course of an experiment (see Methods). *A*, an intracellular alkali metal cation was sufficient for an influx current. A voltage clamp was maintained with a pipette that contained arginine aspartate. A puff of GABA produced little current (trace 1). The membrane occluding the tip of the second pipette was ruptured and potassium aspartate entered the cell. Subsequently, a puff of GABA produced an influx current (trace 2). *B*, intracellular Cl^- was sufficient for an influx current. A voltage clamp was maintained with a pipette that contained arginine aspartate. A puff of GABA produced little current (trace 3). The membrane occluding the tip of the second pipette was ruptured and arginine chloride entered the cell. Subsequently, a puff of GABA produced an influx current (trace 4). *C*, the shape of the current–voltage curve is altered when an alkali metal cation is introduced into a cell that already contains Cl^-. A voltage clamp was maintained with a pipette that contained choline chloride. A puff of GABA produced trace 5. The membrane occluding the tip of the second pipette was ruptured and CsCl entered the cell. Subsequently, a puff of GABA produced trace 6. *D*, the shape of the current–voltage curve is altered when Cl^- is introduced into a cell that already contains Na^+. A voltage clamp was maintained with a pipette that contained sodium gluconate. A puff of GABA produced trace 7. The membrane occluding the tip of the second pipette was ruptured and NaCl entered the cell. Subsequently, a puff of GABA produced trace 8. Either intracellular Cl^- or an alkali metal cation was sufficient to produce an influx current. The shape of the current–voltage curve was determined by the identities of the intracellular ions (see text). Extracellular solutions contained 0·5 mM picrotoxin.

occluding the tip of the second pipette was ruptured and CsCl diffused into the cell. Subsequently, a puff of GABA produced a current (trace 6) that declined with depolarization until, at approximately +60 mV, the current disappeared (three

cells). No outward current was observed. The shape of the current–voltage curve depended upon the ion composition of the intracellular solution. When the membrane was depolarized beyond +25 mV, the current remained inward if the intracellular solution contained either Cl^- or an alkali metal cation. In contrast, the current approached zero if the intracellular solution contained both Cl^- and an alkali metal cation other than Na^+.

A new feature emerged when the intracellular solution contained both Na^+ and Cl^-. The experiment shown in Fig. 3*D* began with a pipette that contained sodium gluconate. A puff of extracellular GABA produced an inward current (trace 7) that declined as the membrane was depolarized from −80 to +25 mV. As the membrane was further depolarized, a small inward current remained nearly constant. However, after the membrane occluding the tip of the second pipette was ruptured and NaCl diffused into the cell, a puff of GABA produced a current (trace 8) that declined with depolarization until, at approximately +35 mV, the current reversed and became outward. An outward component was only seen when the intracellular solution contained both Na^+ and Cl^-. Thus, Na^+, in the presence of Cl^-, behaved differently from other alkali metal cations.

A difference between Na^+ and other alkali metal cations was also observed in experiments that used a single pipette (Fig. 4). When the intracellular solution contained LiCl (six cells), KCl (nine cells) or CsCl (five cells) an inward current decreased nearly linearly as the membrane was depolarized from −70 to +25 mV and then asymptotically approached zero. In contrast, when the intracellular solution contained NaCl, an inward current decreased nearly linearly as the membrane was depolarized from −70 to +25 mV, reversed near +30 mV, and saturated as an outward current with further depolarization (seven cells). An outward current at depolarized potentials was observed in every experiment in which the intracellular solution contained NaCl.

In the next section we show that intracellular NaCl and GABA are required for an efflux current. We considered the possibility that the outward current seen in Fig. 4*B* occurred after maintaining the membrane voltage at a hyperpolarized potential that allowed the continuous influx of GABA and intracellular accumulation. The following experiments permit us to reject this notion. First, membrane voltage was stepped from −70 to +80 mV (Fig. 4*E*, trace 1). Next, the step was repeated and a puff of GABA (200 μM) was delivered during the depolarization (trace 2). In this experiment, the procedure was repeated five times, and the average difference current is shown in Fig. 4*F*. An outward current developed without a period of prior influx. A similar result was observed in three cells. Thus, the shape of the current–voltage relation of the influx current depended upon the identity of the intracellular ions. In the physiological voltage range (−80 to −20 mV), the current was inward and decreased with depolarization. At depolarized potentials the current could be inward, zero or outward.

Efflux

The voltage dependence for GABA efflux has been measured (Schwartz, 1987). A GABA-sensitive neurone was pressed against a horizontal cell and used as a sensitive detector of changes in extracellular GABA concentration. Although the appearance of GABA in the extracellular space was detected, the transport current produced by

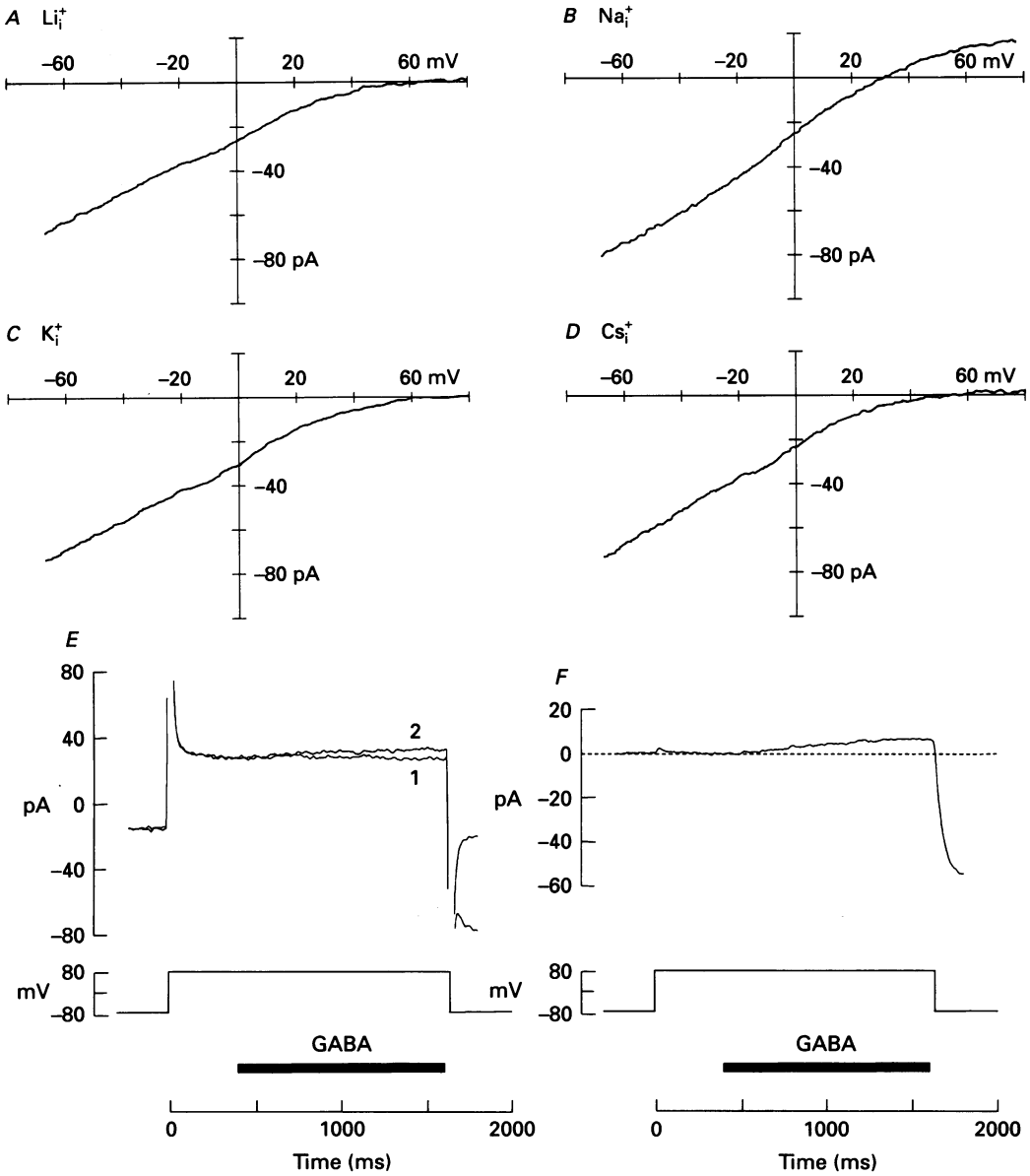

Fig. 4. Intracellular Na^+ had a unique effect on the shape of the influx current–voltage relation. A voltage clamp was maintained by a pipette that contained LiCl (*A*), NaCl (*B*), KCl (*C*) or CsCl (*D*). Influx currents were produced by a puff of GABA (200 μM). Only cells with intracellular NaCl produced an outward current when depolarized beyond +30 mV. An outward current was also seen during a depolarizing step. *E*, the patch pipette contained NaCl. Membrane current recorded when the voltage was stepped from −70 to +80 mV (trace 1). Next, the step was repeated and a puff of GABA (200 μM) was delivered during the time indicated by the horizontal bar (trace 2). *F*, the procedure in *E* was repeated five times and the average difference current, a current induced by GABA, is shown. Extracellular solutions contained 0·5 mM picrotoxin.

electrogenic efflux was not observed. We now describe two procedures that reveal an efflux current (Fig. 5). The first procedure was to measure the current generated when GABA was introduced into a cell during the course of an experiment. At the beginning of the experiment, two patch pipettes were sealed to a cell. One pipette contained a NaCl intracellular solution; the second pipette contained a similar solution with an added 20 mM GABA. A whole-cell voltage clamp was established with the first pipette. After a short control period, the membrane occluding the tip

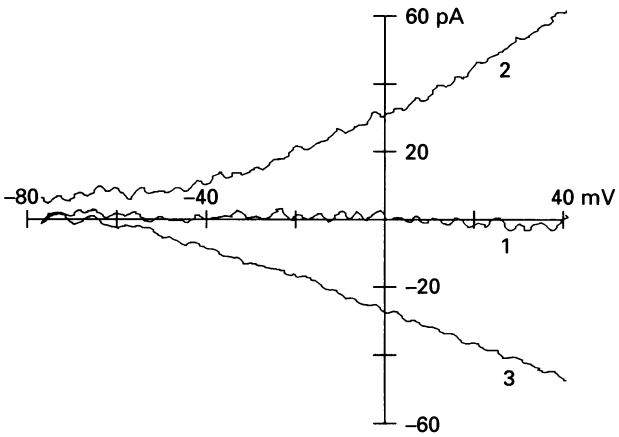

Fig. 5. Two methods for measuring an efflux current. A voltage clamp was established through a pipette that contained NaCl and lacked GABA. A second patch pipette, containing an intracellular solution with 20 mM GABA, was sealed to the cell membrane. A puff of nipecotic acid (100 μM) produced little current change (trace 1). Next, the membrane occluding the tip of the second pipette was ruptured and GABA diffused into the cell. Afterwards, the introduction of GABA produced an outward current (trace 2). Finally, a puff of nipecotic acid produced an inward current (trace 3). Extracellular solutions contained 0·5 mM picrotoxin.

of the pipette containing GABA was ruptured and GABA diffused into the cell. Because two pipettes now exchanged their contents with the cell interior, the concentration of GABA inside the cell must have been less than 20 mM. If each pipette contributed equally, the average intracellular concentration would have been 10 mM. An intracellular concentration of 10 mM is physiologically reasonable for catfish cone horizontal cells (see Lam, 1972). The introduction of GABA produced an outward current that was small at -75 mV and increased with depolarization (trace 2). A similar result was observed in four cells.

The second procedure was to block the efflux current with nipecotic acid. We reasoned that nipecotic acid might bind at an extracellular site and block translocation in both directions. The procedure is also illustrated using the cell of Fig. 5. Before introducing GABA, we performed a control. A puff of nipecotic acid (0·1 mM) was delivered at a time when only the pipette that contained NaCl (and no GABA) communicated with the cell interior. No current change was observed (trace 1). Next, GABA was introduced with the result described above (trace 2). Subsequently, a puff of nipecotic acid produced a current (trace 3) that was zero at -75 mV and increased as an inward current with negative slope as the membrane

was depolarized. The negative slope suggests that nipecotic acid inhibited a standing current. Thus, trace 2 is an outward current produced by intracellular GABA, and trace 3 is the result of nipecotic acid inhibiting a standing efflux current. The two currents are similar. However, there is a small but significant difference that is apparent at hyperpolarized potentials. Trace 2 approaches a finite limit; in contrast, trace 3 approaches zero. We will return to this difference when we describe a leakage mode. Aside from this difference, the two curves are in general agreement and the shapes of their current–voltage curves agree with the voltage dependence previously measured for the movement of GABA across the membrane (Schwartz, 1987).

Cis ions required for efflux

We were interested to know whether efflux, like influx, required both Na^+ and Cl^- in the *cis* solution. The first experiments explored the role of intracellular Cl^-. These experiments used two pipettes. The first pipette contained Na^+ and GABA, but lacked Cl^-. In addition, the cell was superfused with a Cl^--free solution (Cl^- replaced by gluconate) to prevent the non-specific entry of Cl^- from creating a small intracellular concentration. A puff of nipecotic acid (0·1 mM) did not produce a current change (Fig. 6*A*, trace 1). Next, the membrane occluding the tip of the pipette containing a Cl^--rich solution was ruptured and Cl^- diffused into the cell. Subsequently, a puff of nipecotic acid blocked an efflux current (trace 2). A similar result was observed in two cells. Complementary results came from experiments that used a single patch pipette. Nipecotic acid (0·1 mM) had no effect when puffed onto four cells exchanged with an intracellular solution that contained sodium gluconate and GABA, but blocked a standing efflux current when puffed onto seven cells that were exchanged with NaCl and GABA. Thus, efflux required Cl^- in the intracellular solution.

Next, we studied the effect of different intracellular cations. Each experiment required two pipettes. The first pipette contained an alkali metal cation and Cl^-. The second pipette contained a similar solution with added GABA and was used to introduce GABA into the cell during the course of the experiment. An influx current was measured at the beginning of an experiment by applying a puff of GABA. Then, the membrane occluding the second pipette was ruptured. After GABA entered the cell, the efflux current was measured. When, for example, the intracellular cation was K^+ (Fig 6*B*), an influx current was observed (trace 3) but an efflux current (trace 4) did not occur. When Na^+ was the intracellular cation (Fig. 6*C*), influx (trace 6) and efflux (trace 7) currents were both observed (eight cells). An efflux current was not observed when Na^+ was replaced by Li^+ (three cells), K^+ (three cells) or Cs^+ (two cells). Thus, efflux required both Na^+ and Cl^- in the intracellular solution.

It might be argued that influx and efflux currents are mediated by different transporters. Each transporter would move GABA in only one direction across the membrane. The experiments in Fig. 6*B* and *C* were continued to test this notion. First, consider the experiment in which the intracellular cation was K^+ (Fig. 6*B*). At the onset of the experiment, the cell did not contain GABA and a puff of extracellular GABA produced a characteristic influx current (trace 3). After GABA was introduced into the cell, a puff of GABA produced a slightly smaller current (trace 5). The binding of GABA at an intracellular site may impede influx (the difference between traces 3 and 5). Next, consider the case in which the intracellular cation was Na^+

(Fig. 6*C*). Again, a puff of GABA at the onset of the experiment produced a characteristic influx current (trace 6). However, a puff of extracellular GABA applied after GABA was introduced into the cell produced a current with a different current–voltage relation (trace 8). The movement of GABA through the transporter

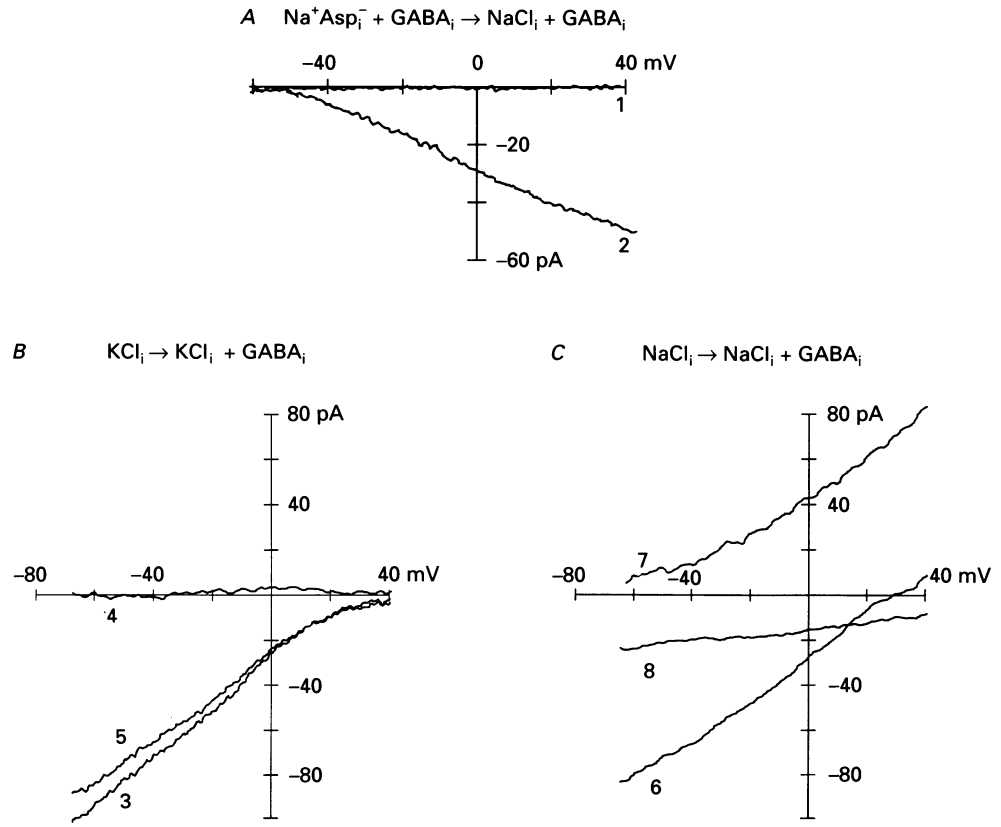

Fig. 6. Efflux required intracellular Na^+ and Cl^-. *A*, a voltage clamp was established with a pipette that contained sodium D-aspartate and 20 mM GABA. The cell was superfused with a Cl^--free solution (Cl^- replaced by gluconate). A puff of nipecotic acid (100 μM) produced little response (trace 1). Next, Cl^- was introduced into the cell from a second patch pipette. Subsequently, a puff of nipecotic acid blocked an efflux current (trace 2). *B*, a voltage clamp was established with a pipette that contained KCl. At the beginning of the experiment, a puff of GABA produced an influx current (trace 3). The introduction of GABA (20 mM) from a second pipette produced little current change (trace 4). Subsequently, a puff of GABA produced a smaller influx current (trace 5). *C*, a voltage clamp was established with a pipette that contained NaCl. At the beginning of the experiment, a puff of GABA produced an influx current (trace 6). The introduction of GABA (20 mM) from a second pipette produced an efflux current (trace 7). Subsequently, a puff of GABA produced trace 8. Extracellular solutions contained 0·5 mM picrotoxin.

during efflux altered the ability of the transporter to simultaneously mediate influx (compare traces 6 and 8). Significant interaction between influx and efflux indicates that both currents are mediated by the same transporter.

Trans ions required for efflux

The efflux current, like the influx current, also depended upon ions on the *trans* side of the membrane. An example is shown in Fig. 7*B*. The cell was continuously superfused with a solution containing NMG-gluconate; the patch pipette contained NaCl and GABA. An intracellular puff of NaCl produced an efflux current (trace 1). Moreover, a puff of sodium gluconate produced a similar current (trace 2). In contrast, a puff of NMG-Cl did not produce an efflux current (trace 3). Other alkali

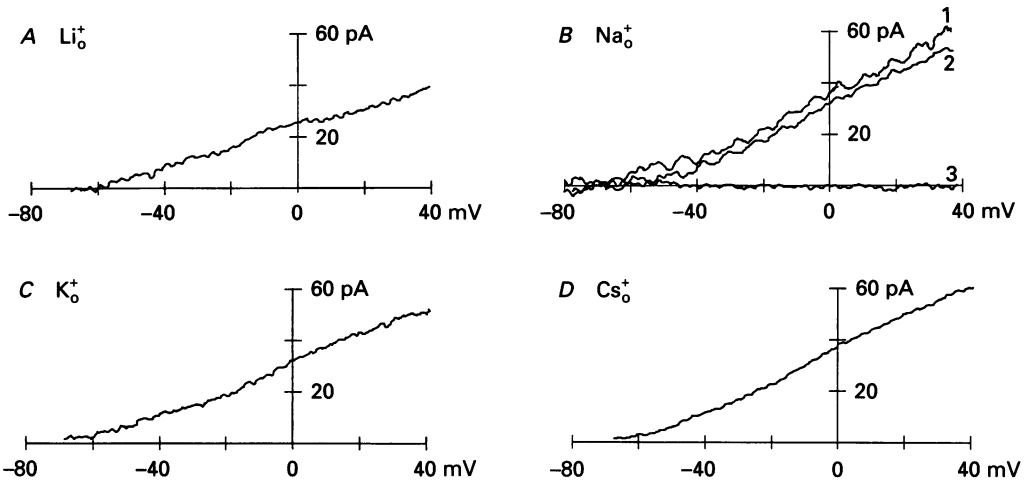

Fig. 7. Efflux required an extracellular alkali metal cation. Cells were continuously superfused with a Na^+-free solution (NaCl was replaced with NMG-Cl). A voltage clamp was established with a patch pipette containing NaCl and 20 mM GABA. A puff of LiCl (*A*), NaCl (*B*, trace 1), KCl (*C*) or CsCl (*D*) produced an efflux current. A puff of sodium gluconate produced a similar current (*B*, trace 2); however, a puff of NMG-Cl did not produce a current change (trace 3). Extracellular solutions contained 0·5 mM picrotoxin.

metal cations could replace extracellular Na^+. Efflux currents were observed when Li^+ (Fig. 7*A*, two cells), Na^+ (Fig. 7*B*, four cells), K^+ (Fig. 7*C*, three cells) or Cs^+ (Fig. 7*D*, three cells) were puffed onto the cell. Consequently, a *trans* alkali metal cation was required; and, unlike the influx current, *trans* Cl^- was not required.

Outward movement of GABA

GABA is an uncharged zwitter ion at physiological pH. The production of a transport current requires the movement of charge with each transport cycle. The presence of a $GABA_A$ current (when picrotoxin was omitted from the extracellular solution) allowed us to determine whether GABA could also translocate without a charge movement. We reasoned that efflux might deliver GABA to the exterior surface of the membrane where it could activate $GABA_A$ receptors before diffusing into the bath (see Akaike, Maruyama, Sikdar & Yasu, 1987). The patch pipette contained NaCl and GABA; the membrane potential was maintained at +80 mV (Fig. 8). These conditions permitted efflux. Activation of $GABA_A$ channels was detected by observing a current change induced by a puff of picrotoxin (trace 1).

Next, transport was blocked by one of two procedures. First, the cell was continuously superfused with nipecotic acid. Now, a puff of picrotoxin did not produce a current change (trace 2). Second, the cell was superfused with a solution that lacked nipecotic acid and held at −80 V, a voltage at which efflux is not expected. Again, a puff of picrotoxin did not produce a current change. Similar results were obtained from three cells. In contrast, picrotoxin did not produce a current change when the intracellular solution contained Li^+ (three cells), K^+ (two

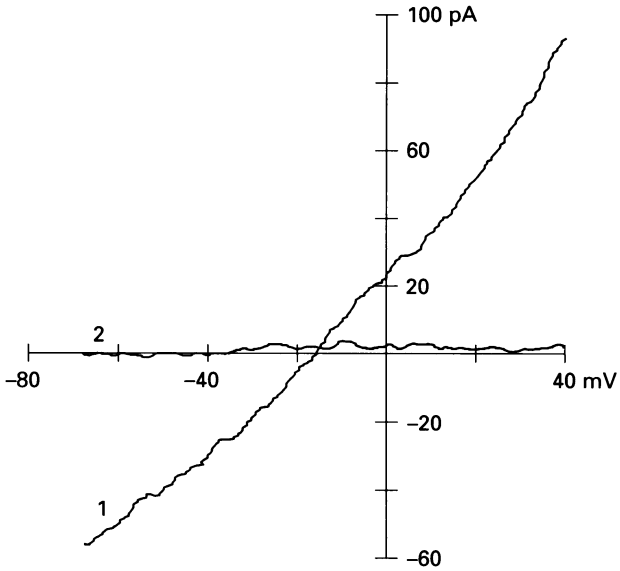

Fig. 8. A method for measuring the outward movement of GABA. The patch pipette contained NaCl and 20 mM GABA. membrane voltage was maintained at +80 mV for several tens of seconds. The cell was superfused with the extracellular salt solution. A puff of picrotoxin (0·5 mM) blocked a $GABA_A$ current (trace 1). Next, the cell was continuously superfused with a solution that contained nipecotic acid (200 μM). Now, a puff of picrotoxin (and nipecotic acid) did not produce a current change (trace 2).

cells) or Cs^+ (three cells). GABA transport only occurred during conditions previously shown to produce an efflux current. The implication is that an electroneutral efflux of GABA does not occur.

Leakage

Influx and efflux currents are produced by the coupled translocation of GABA and ions. The following experiments describe a current that appears to be produced by the uncoupled translocation, or 'leakage', of ions without GABA. We first investigate the identity of extracellular ions required for the leakage current. Next, we determine how intercellular ions affect the shape of the current–voltage curve. Finally, we demonstrate that leakage stops when GABA is introduced into a cell and efflux begins.

A series of experiments demonstrated that only an extracellular alkali metal cation was required for a leakage current (Fig. 9). In each experiment, a voltage clamp was maintained by a patch pipette that contained NMG-gluconate. A cell was superfused with a Na^+- and Cl^--free solution (NaCl replaced by NMG-gluconate). An

extracellular puff of medium containing an alkali metal cation produced a leakage current. A similar current was produced by Li^+ (Fig. 9*A*, three cells), Na^+ (Fig. 9*B*, four cells), K^+ (Fig. 9*C*, two cells) or Cs^+ (Fig. 9*D*, three cells). Thus, Cl^- is not required on either the extracellular or intracellular side of the membrane and any alkali metal cation will produce an inward leakage current.

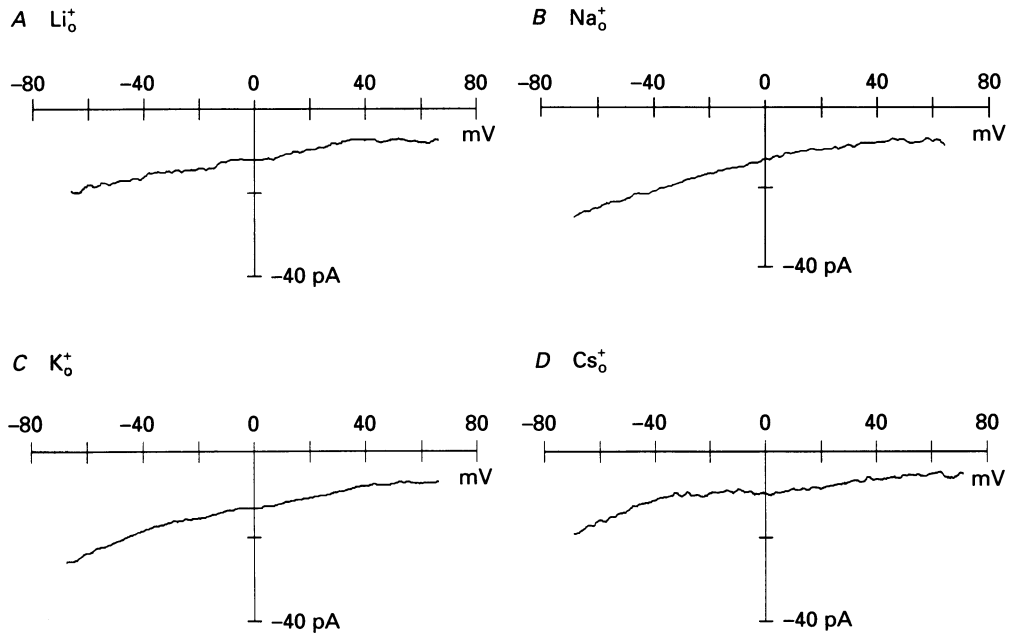

Fig. 9. An alkali metal cation is required for a leakage current. In each experiment, a voltage clamp was maintained by a patch pipette that contained NMG-gluconate. The cell was superfused with a Na^+- and Cl^--free solution (extracellular NaCl replaced by NMG-gluconate). An extracellular puff of medium containing Li^+ (*A*), Na^+ (*B*), K^+ (*C*) or Cs^+ (*D*) produced a leakage current. Extracellular solutions contained 0·5 mM picrotoxin.

The series of experiments illustrated in Fig. 10 demonstrate that intracellular ions affected the shape of the current–voltage curve. Each part of the figure illustrates a result obtained with a different intracellular solution. Each experiment began with a cell continuously superfused with a Na^+-free medium. During the course of the experiment, first Na^+ and then Na^+ with GABA was puffed onto a cell.

When the patch pipette contained choline chloride (Fig. 10*A*), a puff of extracellular Na^+ produced an inward current that declined as the membrane was depolarized from −80 to +20 mV. Thereafter, as the membrane was further depolarized, a small inward current remained nearly constant (trace 1). For comparison, a puff of Na^+ and GABA produced a current (trace 2) that also had an inward component at depolarized potentials (compare with Fig. 3, traces 4 and 5). A similar result was observed in three cells.

When the patch pipette contained sodium D-aspartate (Fig 10*B*), a puff of extracellular Na^+ produced an inward current that declined as the membrane was depolarized from −80 to +20 mV. Thereafter, as the membrane was further

depolarized, a small inward current remained nearly constant (trace 3). For comparison, a puff of Na^+ and GABA produced a current (trace 4) that also had an inward component at depolarized potentials (compare with Fig. 3, trace 7). A similar result was observed when the intracellular solution lacked Cl^- and contained Li^+ (three cells), Na^+ (four cells), K^+ (two cells) or Cs^+ (three cells). In each case, the leakage and influx currents had inward components at depolarized potentials.

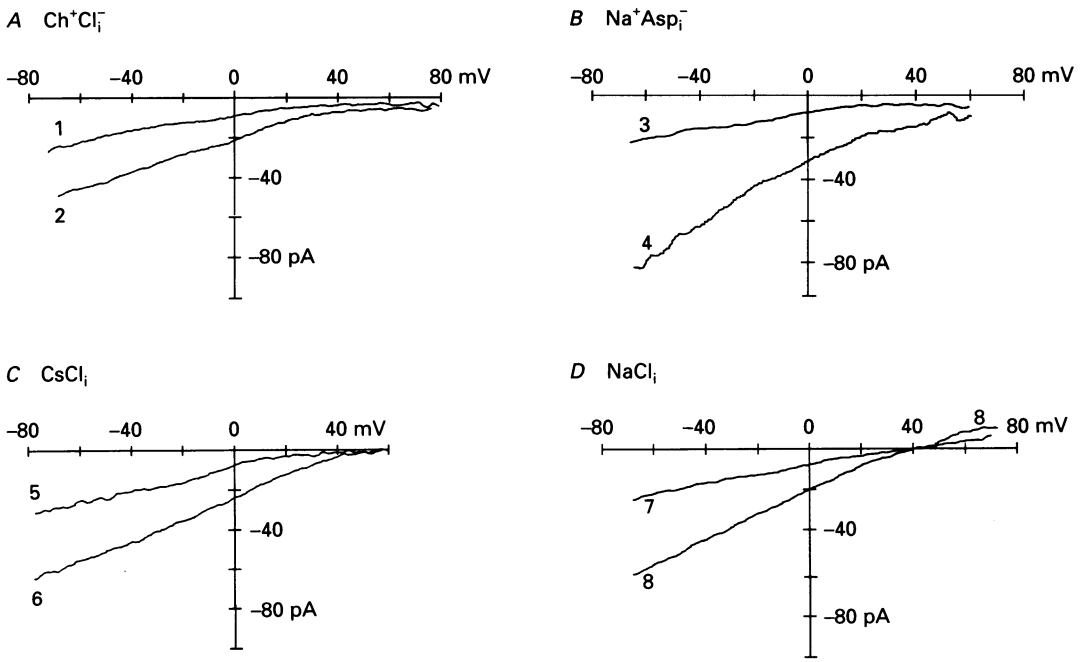

Fig. 10. Intracellular ions affected the shape of the leakage current–voltage relation. In each experiment, the cell was continuously superfused with a Na^+-free solution (NaCl was replaced with choline chloride). *A*, the patch pipette contained choline chloride. A puff of NaCl produced trace 1. A puff of NaCl and GABA (200 μM) produced trace 2. *B*, the patch pipette contained sodium D-aspartate. A puff of NaCl produced trace 3. A puff of NaCl and GABA (200 μM) produced trace 4. *C*, the patch pipette contained CsCl. A puff of NaCl produced trace 5. A puff of NaCl and GABA (200 μM) produced trace 6. *D*, the patch pipette contained NaCl. A puff of NaCl produced a current with inward and outward components (trace 7). A puff of NaCl and GABA (200 μM) produced trace 8. The shapes of the current–voltage curves for the leakage and influx currents had a similar dependence upon the identities of the intracellular ions. See text. Extracellular solutions contained 0·5 mM picrotoxin.

When the patch pipette contained CsCl (Fig. 10*C*), a puff of Na^+ produced a current that was inward at hyperpolarized potentials and asymptotically approached zero at depolarized potentials (trace 5). For comparison, a puff of Na^+ and GABA produced a current (trace 6) that also asymptotically approached zero at depolarized potentials (compare with Fig. 4*D*). Similar results were observed when the intracellular solution contained LiCl (three cells), KCl (three cells) or CsCl (two cells).

When the patch pipette contained NaCl (Fig. 10*D*), a puff of Na^+ produced a

current that was inward at hyperpolarized potentials and outward at depolarized potentials (trace 7). For comparison, a puff of Na^+ and GABA produced a current (trace 8) that also had an outward component at depolarized potentials (compare with Fig. 4*B*). An outward component in the leakage and influx currents depended on the presence of intracellular NaCl (three cells).

The collected results in Fig. 10 indicate that the leakage current shared two properties with the influx current. First, the apparent reversal potential (range of +20 to +40 mV in six cells) did not correspond to the Nernst potential of any ion, but was similar to the potential at which the influx current crossed the voltage axis (see Fig. 10*D*). Second, the polarity of the leakage and influx currents observed at a depolarized voltage had the same dependence upon the identity of intracellular ions. The currents were inward when the intracellular solution contained either Cl^- or an alkali metal cation (but not both), approached zero when the intracellular solution contained both Cl^- and an alkali metal cation other than Na^+, and was outward when the intracellular solution contained both Cl^- and Na^+. However, unlike the influx current, which required extracellular Cl^- and Na^+, an inward leakage current was observed with any extracellular alkali metal cation and did not require extracellular Cl^-. In addition, it was not inhibited by nipecotic acid.

A simple test indicated that the leakage current flowed through the transporter. The experiment required two pipettes. A cell was continuously superfused with a Na^+-free solution (Na^+ replaced by choline). When a voltage clamp was established with a patch pipette that contained NaCl, a puff of extracellular Na^+ produced a leakage current (Fig. 11, trace 1) that was inward at a hyperpolarized potential, e.g. between −70 and −80 mV. In contrast, after the membrane occluding the tip of the second pipette was ruptured and GABA entered the cell, a subsequent puff of Na^+ produced an outward current (Fig. 11, trace 2) that asymptotically approached zero between −70 and −80 mV (four cells). This current could be the sum of two components if a puff of Na^+-rich solution now activated both leakage and efflux. However, the asymptotic behaviour of the observed current (trace 2) at hyperpolarized potentials would require that the two components fortuitously always summed to zero between −70 and −80 mV (see also Fig. 7). This unlikely possibility was excluded by an observation that relied upon the pharmacological action of nipecotic acid. We previously observed that nipecotic acid blocked influx (p. 84) and efflux (Fig. 5, trace 3) without affecting leakage (Fig. 5, trace 1). When we continued the experiment in Fig. 11, a puff of Na^+ and nipecotic acid did not produce a current change (Fig. 11, trace 3). If trace 2 were produced by two components, we would expect that blocking efflux with nipecotic acid would reveal a leakage component. However, the entire change produced previously in trace 2 was blocked by the addition of nipecotic acid. Thus, nipecotic acid did not unmask a leakage component that might be produced by Na^+ alone. Consequently, all of the current initiated by extracellular Na^+, after GABA was introduced, can be attributed to efflux. We conclude that trace 2 was produced entirely by efflux and that leakage stopped when GABA was introduced into the cytoplasm. In brief, the generation of an efflux current stopped the leakage current.

A parsimonious interpretation is that leakage, influx and efflux are all mediated by one transporter. Now it is possible to return to Fig. 5 and understand why traces 2

and 3 are not symmetrical. The difference current produced by the addition of GABA to the cytoplasm (trace 2) included an outward component that reflects the cessation of leakage. After GABA was introduced into the cytoplasm, leakage stopped. A subsequent puff of nipecotic acid inhibited only an efflux current (trace 3).

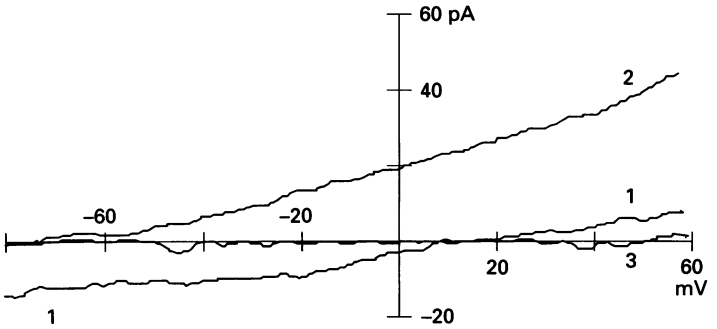

Fig. 11. Initiation of an efflux current stopped the leakage current. The experiment required two pipettes. A voltage clamp was established with a pipette that contained NaCl and lacked GABA. The cell was continuously superfused with a Na^+-free solution (NaCl was replaced with choline chloride). A puff of Na^+-rich solution produced a leakage current (trace 1). Next, GABA (20 mM) was introduced into the cell from a second patch pipette. Now, a second puff of Na^+-rich solution produced an efflux current (trace 2). A puff of Na^+-rich solution with added nipecotic acid (100 μM) did not produce a current change (trace 3). Extracellular solutions contained 0·5 mM picrotoxin.

DISCUSSION

We have investigated the identity of ions required for the production of current by a GABA transporter. As expected, both influx and efflux currents required the simultaneous presence of GABA, Na^+ and Cl^- on the same (*cis*) side of the membrane. However, the identity of ions required on the *trans* side was not anticipated. Any alkali metal cation was sufficient. In addition, an influx current also occurred in the absence of an intracellular alkali metal cation if Cl^- was present.

The *trans* conditions for influx (either Cl^- or an alkali metal cation is required) might suggest the existence of two transporters, each having a different ion binding site at its intracellular surface. This conjecture is easily dismissed for the effects of *trans* Cl^- and alkali metal cations are not additive. At depolarized potentials each supports an inward current, but the presence of both produces zero current. Hence, anion and cation act on one transporter. Either enables influx, and the presence of both alters the behaviour of the transporter at a depolarized potential.

Ion gradients for Na^+ and Cl^- do not determine the magnitudes of the influx and efflux currents. For example, Na^+ was always required on the *cis* side but could be exchanged with any other alkali metal cation on the *trans* side without significantly altering the magnitude of either the influx or efflux current. Moreover, no current occurred when the *trans* side lacked Cl^- and an alkali metal cation, but large currents were observed when both the *cis* and *trans* solutions contained approximately equal concentrations of NaCl.

The GABA transporter mediates both influx and efflux. Unexpectedly, three remarkable properties indicate that movement in one direction is not simply the

reverse of steps required for movement in the opposite direction. First, the *trans* ions required for influx and efflux currents are not the same. Second, the amplitudes of the influx and efflux currents are not determined simply by ion concentration gradients. Third, the influx current can, at depolarized potentials, be inward, zero or outward depending upon the composition of the *trans* solution. These properties may

TABLE 1. Ions required for electrogenic function

Mode	Outside	Inside
Influx	Na^+ and Cl^-	X^+ or Cl^-
Efflux	X^+	Na^+ and Cl^-
Leakage	X^+	Not required

X^+ is any alkali metal cation.

be explained if *trans* ions produce different allosteric effects on each side of the membrane or if efflux is not the reverse of influx.

We have been careful to isolate influx and efflux currents (with the exception of traces 5 and 8 in Fig. 6) that are produced when GABA is delivered to only one side of the membrane. Both currents are voltage dependent. Within the physiological range, depolarization increases efflux and decreases influx. However, the net current produced when GABA is present on both sides of the membrane may not be the simple sum of two unidirectional currents that have been individually and separately identified. The movement of GABA in one direction may significantly alter the simultaneous flux in the opposite direction (as indeed indicated by trace 8 in Fig. 6).

Often, transport is assumed to be tightly coupled. However, cations appear to slip through the transporter in the absence of GABA. In contrast, GABA, a neutral molecule at physiological pH, only translocates with a charge movement. A simple model would describe a continuous flux of ions whose transit time is decreased by the co-ordinated translocation of GABA. At non-saturating concentrations of GABA, both leakage and unidirectional flux are expected. The relative magnitudes of these two modes will determine the efficiency of transport.

Because (i) the influx and efflux currents are not symmetric, (ii) influx and efflux interact and, therefore, are not additive, and (iii) the relative amplitudes of leakage and transport may not be fixed, thermodynamic predictions are at present, perhaps, unwise.

Our experiments have identified ions that enable GABA transport. Many important issues remain unresolved. Ions may bind and allosterically influence transport or they may be required to move through the transporter. Although a net positive charge must move, the number and identity of the ions and the stoichiometry (coupling) between GABA and charge are still uncertain. Radioactive Cl^- has been reported to be transported during influx (Keynan & Kanner, 1988). We do not know whether Na^+ always carries the excess charge. It is conceivable that Na^+ binds at the *cis* side and enables any available alkali metal cation to translocate with GABA and Cl^-. In this scenario, Na^+ would act at a selective allosteric site, but permeation would be relatively non-selective. Moreover, we do not know if alkali metal cations translocate from the *trans* side.

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