J. Physiol. (1985), **365**, *pp.* 41–58
With 11 *text-figures*
Printed in Great Britain

AN INTRACELLULAR ANALYSIS OF γ-AMINOBUTYRIC-ACID-ASSOCIATED ION MOVEMENTS IN RAT SYMPATHETIC NEURONES

BY K. BALLANYI AND P. GRAFE

From the Institut für Physiologie der Universität München, Pettenkoferstr. 12, D-8000 München 2, F.R.G.

(*Received 13 December 1984*)

SUMMARY

1. Double-barrelled ion-sensitive micro-electrodes were used to measure the changes of the intracellular activities of Cl^-, K^+, and Na^+ (a^i_{Cl}, a^i_K, a^i_{Na}) in neurones of isolated rat sympathetic ganglia during the action of γ-aminobutyric acid (GABA).

2. The membrane potential of some of the neurones was manually 'voltage clamped' by passing current through the reference barrel of the ion-sensitive micro-electrode. This enabled us to convert the normal depolarizing action of GABA into a hyperpolarization.

3. A GABA-induced membrane depolarization was accompanied by a decrease of a^i_{Cl}, a^i_K and no change in a^i_{Na}, whereas a GABA-induced membrane hyperpolarization resulted in an increase of a^i_{Cl}, a^i_K and also no change in a^i_{Na}.

4. GABA did not change the free intracellular Ca^{2+} concentration, as measured with a Ca^{2+}-sensitive micro-electrode, whereas such an effect was seen during the action of carbachol. pH-sensitive electrodes, on the other hand, revealed a small GABA-induced extracellular acidification.

5. The inward pumping of Cl^- following the normal, depolarizing action of GABA required the presence of extracellular K^+ as well as Na^+, whereas CO_2/HCO_3^--free solutions did not influence the uptake process. Furosemide, but not DIDS, blocked the inward pumping of Cl^-.

6. In conclusion, our data show that only changes in intracellular activities of K^+ and Cl^- are associated with the action of GABA. Furthermore, they indicate that a K^+/Cl^- co-transport, and not a Cl^-/HCO_3^- counter-transport, may be involved in the homoeostatic mechanism which operates to restore the normal transmembrane Cl^- distribution after the action of GABA.

INTRODUCTION

Intracellular Cl^- activity (a^i_{Cl}) in rat sympathetic neurones is higher than predicted from a passive distribution of this ion. Recently this was measured by means of ion-sensitive micro-electrodes (Ballanyi, Grafe, Reddy & ten Bruggencate, 1984*a*) as well as in terms of element concentrations with an electron microprobe (Galvan, Dörge, Beck & Rick, 1984). A GABA-induced opening of Cl^- channels, therefore,

results in a Cl^- efflux, a decrease of a^i_{Cl}, and a membrane depolarization of these neurones (Adams & Brown, 1975). During and after the end of the GABA response, a Cl^- pump is activated which can restore and maintain the high a^i_{Cl}. However, no precise mechanism has been described yet to underlie this inwardly directed Cl^- pump. Previous investigations of inward Cl^- transport in frog spinal cord (Nicoll, 1978) and cat dorsal root ganglia (Gallagher, Nakamura & Shinnick-Gallagher, 1983) have characterized the pump mechanism in pharmacological terms. In these studies, the reversal potential of GABA-induced membrane polarizations was used as an indirect measure of a^i_{Cl}. The authors reported that a variety of compounds which are known to block Cl^- transport in other tissues depressed the GABA-induced membrane depolarizations. However, these drugs did not change the reversal potential of the GABA action. Therefore, Gallagher *et al.* (1983) suggested that the Cl^- pump in cat dorsal root ganglia was resistant to SITS, furosemide or bumetanide. An exception seemed to be piretanide. This compound has been reported to produce a weak reduction of the inward pumping of Cl^- in frog dorsal root ganglion cells (Wojtowicz & Nicoll, 1982).

In the present study, we have attempted to explore the ionic mechanism of GABA-activated Cl^- transport in rat sympathetic neurones by means of an intracellular study using double-barrelled micro-electrodes sensitive to Cl^-, K^+, Na^+ and Ca^{2+}. Using the reference barrel of such electrodes to pass current GABA-induced membrane hyperpolarizations as well as depolarizations could be elicited. This report describes (a) changes of intracellular ion activities associated with the action of GABA and (b) experiments designed to characterize the transport mechanism necessary to restore the GABA-induced ion movements. With respect to the latter, the effects of extracellular Na^+, K^+, Cl^-, and HCO_3^- were examined, as well as those of furosemide and DIDS, on the inward pumping of Cl^-. Furthermore, extracellular pH was recorded during the action of GABA in order to get an indirect measure of possible changes in the extracellular HCO_3^- concentration. The results indicate that a furosemide-sensitive, Na^+-dependent K^+/Cl^- co-transport may be the mechanism underlying the GABA-activated Cl^- transport. Parts of these results have been presented at a meeting of the German Physiological Society (Ballanyi, Grafe & ten Bruggencate, 1984*b*).

METHODS

Preparation and solutions

Experiments were performed on superior cervical ganglia isolated from urethane (1·5 g/kg, I.P.)-anaesthetized rats using methods which have previously been described in detail (Ballanyi *et al.* 1984*a*).

The standard Krebs solution contained (mM): NaCl, 118; KCl, 4·8; $NaHCO_3$, 25; KH_2PO_4, 1·2; $MgSO_4$, 1·2; $CaCl_2$, 2·5 and glucose, 10 (gassed with 95 % O_2, 5 % CO_2; pH 7·4). In K^+-free and K^+-rich solutions KCl was replaced by NaCl and vice versa. Na^+-free solutions were prepared with either Trizma (Tris(hydroxymethyl)aminomethane) or choline as the Na^+ substitute. Trizma solutions had the following composition (mM): Trizma HCl, 115; Trizma base, 27·8; KCl, 4·8; KH_2PO_4, 1·2; $MgSO_4$, 1·2; $CaCl_2$, 2·5. In spite of no difference in the Cl^- concentration as measured with a Cl^- meter (Eppendorf 6610), the Cl^- activity in such solutions was about 74 as compared to 94·5 mM in the normal Krebs solution. Choline-substituted solutions were made by replacing NaCl with an equimolar concentration of choline chloride. In the low-Cl^- solutions Cl^- was replaced by glucuronate (Na^+ salt). CO_2/HCO_3^--free solutions had the following composition (mM): NaCl, 118;

Na^+ isethionate, 25; KCl, 4·8; KH_2PO_4, 1·2; $MgSO_4$, 1·2; $CaCl_2$, 2·5; HEPES, 5·8 (pH adjusted to 7·4 with NaOH and gassed with 100% O_2). Drugs were added to the superfusion solutions (all chemicals in this study purchased from Sigma, München, F.R.G.).

Ion-sensitive micro-electrodes

The methods used for the construction and the calibration of the double-barrelled ion-sensitive micro-electrodes and their properties are described in detail elsewhere (Grafe, Rimpel, Reddy & ten Bruggencate, 1982; Grafe, Ballanyi & ten Bruggencate, 1985). The ligands used for the Cl^--, K^+-, Na^+-, Ca^{2+}- and pH-sensitive micro-electrodes were IE-170 (WP Instruments), Corning 477317, Fluka 71176, Fluka 21048 and Fluka 82500, respectively. All values of intracellular Cl^-, K^+ and Na^+ are given in activities (and in voltage of the difference channel output in the case of the Cl^- measurements), assuming an intracellular activity coefficient of 0·74 for these ions (Meier, Ammann, Morf & Simon, 1980). The Ca^{2+}-sensitive micro-electrodes were calibrated in terms of free ion concentrations according to calibration solutions given by Tsien & Rink (1981). The Ca^{2+}-sensitive electrodes used in our study had slopes between 26 and 30 mV at pCa 3–6, between 16 and 24 mV at pCa 6–7, and below 10 mV at pCa 7–8. The electrodes were also tested for their sensitivity against Na^+. The results resembled data given by Deitmer & Schlue (1983) and Weingart & Hess (1984). Changing Na^+ from 5 to 20 mM at pCa 6 resulted in a potential reading indicating an apparent increase of Ca^{2+} by about 3 mV. Intracellular impalements with double-barrelled micro-electrodes were performed by means of a piezo driven micromanipulator (built by M. Frankenberger, München, F.R.G.).

In some of the experiments the reference barrel of the ion-sensitive micro-electrode was used (a) to measure the input resistance of the neurones by injection of short lasting (*ca.* 300 ms) hyperpolarizing current pulses and (b) to manually 'voltage clamp' the membrane by injection of adequate current for several minutes. These tasks required an amplifier (designed by E. Schmidbauer) with both a current source and a bridge balance on the reference side (input impedance 10^{12} Ω). In addition, 'crosstalk compensation' on the ion-sensitive side (input impedance 10^{15} Ω) was used to compensate for the voltage drop along the high resistance of the ion exchanger resulting from leakage currents originating from the reference side and flowing through the partition wall. This modification was necessary since the ratio between the resistances of the partition wall and the ion-sensitive side was sometimes only as low as 3:1. Therefore, before the impalement of a neurone and sometimes also intracellularly both bridge balance (using current pulses) and 'crosstalk compensation' (using constant current) had to be adjusted. After withdrawing the electrode from the neurone, the adjustment of these parameters was tested again. With appropriate settings, hardly any voltage shifts were observed at the output of the differential amplifier during current injections (see right part of Fig. 1). Nevertheless a transient voltage deflexion both at the beginning and at the end of a current pulse was inevitable due to the capacitative coupling between the two barrels. Typical values for the resistances of the ion-sensitive barrel (R_{ion}), reference barrel (R_{ref}), and partition wall (R_{pw}) of a K^+-sensitive micro-electrode were: R_{ion}: 1–3 GΩ; R_{ref} (1 M-Mg^{2+} acetate): 150 MΩ; R_{pw}: 10–20 GΩ. The corresponding values for a Cl^--sensitive micro-electrode were: R_{ion}: 10–30 GΩ; R_{ref} (0·5 M-K^+ sulphate): 80–100 MΩ; R_{pw}: 100–300 GΩ.

RESULTS

General observations

The mean a^i_{Cl} in neurones of the superior cervical ganglion of rats was $29·9 \pm 4·4$ mM (mean ± S.D., $n = 39$), whereas a^i_K was measured as $96·2 \pm 9·6$ mM ($n = 48$). In a preceding paper (Ballanyi *et al.* 1984*a*), these values were given as free intracellular ion concentrations. Membrane resting potentials (E_m) of these neurones were in the range of −40 to −75 mV ($-49·1 \pm 5·4$ mV) with a mean action potential amplitude of $91·8 \pm 14·1$ mV. Since, in the present study, manipulations of the membrane potential were performed (in order to evoke hyperpolarizing actions of GABA), a short description of the voltage dependency of intracellular K^+ and Cl^- will be given. An experiment in which the membrane potential of a sympathetic neurone was depolarized

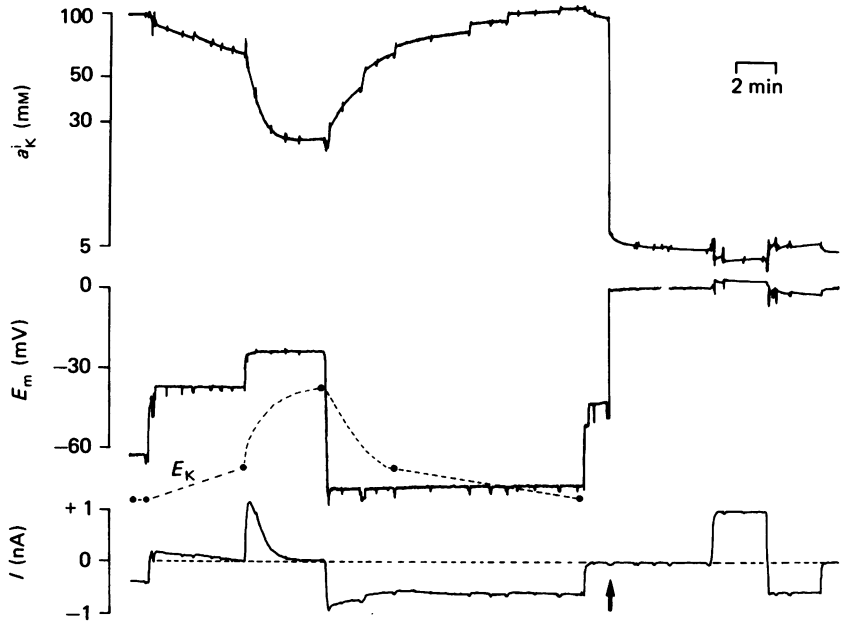

Fig. 1. Intracellular K^+ activity (a^i_K) in a manually 'voltage-clamped' sympathetic neurone. At the beginning of the pen recording turning from a hyperpolarizing to a depolarizing current (see lowermost trace) resulted in a decrease of a^i_K from 100 to about 65 mM. Further depolarization decreased a^i_K rapidly to about 20 mM. Note, that there appears to be a correlation between loss of K^+ and reduction of depolarizing current most probably due to a reduced driving force for K^+ illustrated as the potential difference between K^+ equilibrium potential (E_K) and membrane potential (E_m). (E_K was plotted under the assumption that the extracellular K^+ activity does not change significantly.) Subsequent hyperpolarization led to a rapid restoration of a^i_K. E_m, as measured after turning off the hyperpolarizing current (end of intracellular recording), was −40 mV. At the time indicated by the arrow, the electrode was withdrawn from the cell and currents similar to those used intracellularly were applied again. Note, that due to the bridge balance and 'crosstalk compensation' (see text) only very small voltage shifts on both E_m and a^i_K traces occurred. The vertical inflexions, present throughout the recording, are due to correction of the bridge balance.

from −60 to −20 mV by the injection of depolarizing current is illustrated in Fig. 1. Such a potential shift usually resulted in a rapid loss of intracellular K^+ within a few minutes. In the experiment illustrated, a drop of a^i_K from 100 to 20 mM was observed within about 5 min. Neurones 'voltage clamped' to low membrane potentials in order to elicit hyperpolarizing GABA actions therefore always had low intracellular K^+ activities. a^i_{Cl} (and the Cl^- reversal potential as a consequence) increased less rapidly during membrane depolarizations. For example, a^i_{Cl} increased from 30 to 40 mM during a period of 5 min in which the membrane potential was depolarized from −55 to −10 mV (not illustrated).

Intracellular ion activities during GABA application

We have shown previously (Ballanyi *et al.* 1984*a*) that the *depolarizing* action of GABA is accompanied by a decrease of a^i_{Cl},a^i_K and no change in a^i_{Na}. These observa-

tions are extended here by the measurement of the alterations in these intracellular ion activities during a *hyperpolarizing* action of GABA. As illustrated in the following Figures such a GABA action led to an increase of a^i_{Cl} as well as of a^i_K and again no change in a^i_{Na}. The recording illustrated in Fig. 2 was made with a Cl^--sensitive micro-electrode. At the onset of the pen recording (resting potential about −45 mV), application of GABA resulted in a membrane depolarization, decrease of input

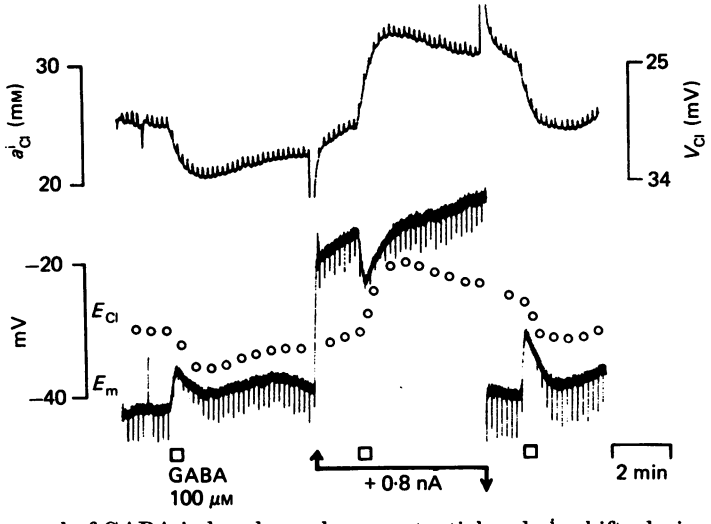

Fig. 2. Reversal of GABA-induced membrane potential and a^i_{Cl} shifts during membrane depolarization. Note that a depolarizing action of GABA is accompanied by a decrease of a^i_{Cl}, whereas a hyperpolarizing action results in an increase of a^i_{Cl}. Constant current pulses (−0·1 nA, 300 ms) were passed through the reference channel every 10 s to test for GABA-induced changes in membrane conductance, and are seen as hyperpolarizing, vertical inflexions. The Cl^- equilibrium potential ($E_{Cl} = 60 \log(a^i_{Cl}/a^o_{Cl})$) was calculated and plotted. V_{Cl} in this and the subsequent Figures is the difference voltage of the Cl^--sensitive micro-electrode ($V_{Cl} = E_{ion} - E_{ref}$).

resistance, and a diminution of a^i_{Cl} from 25 to 21 mM. Afterwards, 0·8 nA of constant current passed through the reference barrel of the electrode resulted in a membrane depolarization of 25 mV. Beside a transient, artificial potential shift (capacitive coupling as described in Methods) no change of a^i_{Cl} was observed. However, application of GABA now led to a membrane hyperpolarization and an increase of a^i_{Cl} from 25 to 33 mM. After the end of the constant current injection, the GABA-induced membrane potential change as well as the GABA-evoked shift of a^i_{Cl} were in the same direction as during the control application.

A similar reversal was seen in the direction of GABA-related K^+ movements. Usually, as shown in the left column of the recording illustrated in Fig. 3, the GABA-induced membrane depolarization was accompanied by a diminution of a^i_K. However, at a depolarized potential, an increase of a^i_K consistent with the GABA-induced membrane hyperpolarization was observed (central column in Fig. 3). Another example of the correlation between K^+ movements and GABA-related potential shifts is illustrated in Fig. 4. In this neurone injection of current was used before the start of the illustrated record to depolarize the membrane. This

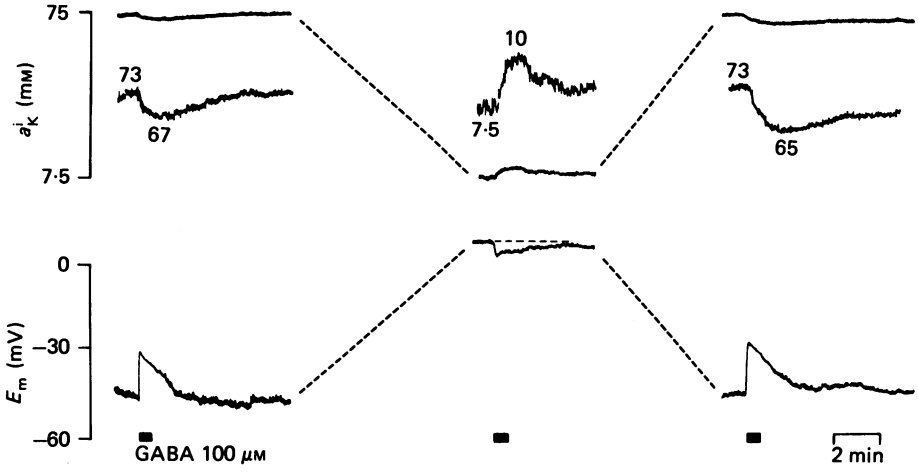

Fig. 3. Reversal of GABA-induced membrane potential and a^i_K shifts during membrane depolarization. After a control application (left column), GABA was applied again at a depolarized membrane potential. This depolarization was produced by current injection through the reference barrel as shown in Fig. 1. At such potentials, a GABA-induced membrane hyperpolarization was accompanied by an increase of a^i_K. Repolarization of the membrane restored the high a^i_K as well as the depolarizing GABA action (right column). Three insets show the changes of a^i_K during GABA with higher resolution. The numbers at the beginning and in the middle of the traces refer to a^i_K values at these times.

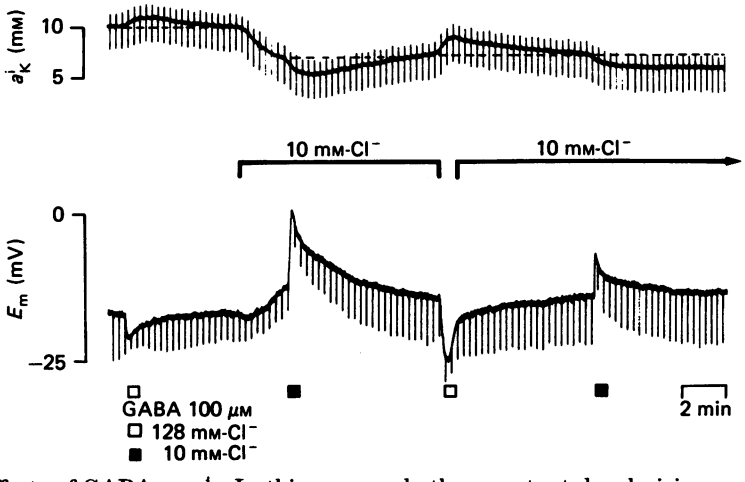

Fig. 4. Effects of GABA on a^i_K. In this neurone both a constant depolarizing current, as well as low-Cl^- solutions were used to alternate between hyperpolarizing and depolarizing actions of GABA. At the beginning of the recording from this artificially depolarized cell, a hyperpolarizing action of GABA was accompanied by an increase of a^i_K. The ganglion was then superfused with a low-Cl^- solution (10 mM). Under these conditions, GABA in low extracellular Cl^- evoked a membrane depolarization, whereas GABA during a short application in normal extracellular Cl^- (128 mM; note gap in the bar indicating low extracellular Cl^-) induced a membrane hyperpolarization. Note the clear correlation between a GABA-induced membrane *hyperpolarization* and an *increase* in a^i_K, whereas a *depolarization* was accompanied by a *decrease* of a^i_K. Constant hyperpolarizing current (−0·1 nA; 300 ms) was passed through the reference barrel every 15 s to illustrate the changes in membrane conductance during GABA application.

depolarization was accompanied by a depletion of a^i_K as already shown in Fig. 1 (left part). After the end of the current injection membrane potential and intracellular K^+ activity reached relatively constant values of -20 mV and 10 mM, respectively. In this situation (Fig. 4), application of GABA resulted in a membrane hyperpolarization and an increase of a^i_K. A low-Cl^- solution was then used to shift Cl^- equilibrium potential (E_{Cl}) towards a more depolarized level. Under these circumstances, GABA in low extracellular Cl^- led to a membrane depolarization and a decrease of a^i_K, whereas GABA in normal extracellular Cl^- induced a hyperpolarization and an increase of a^i_K.

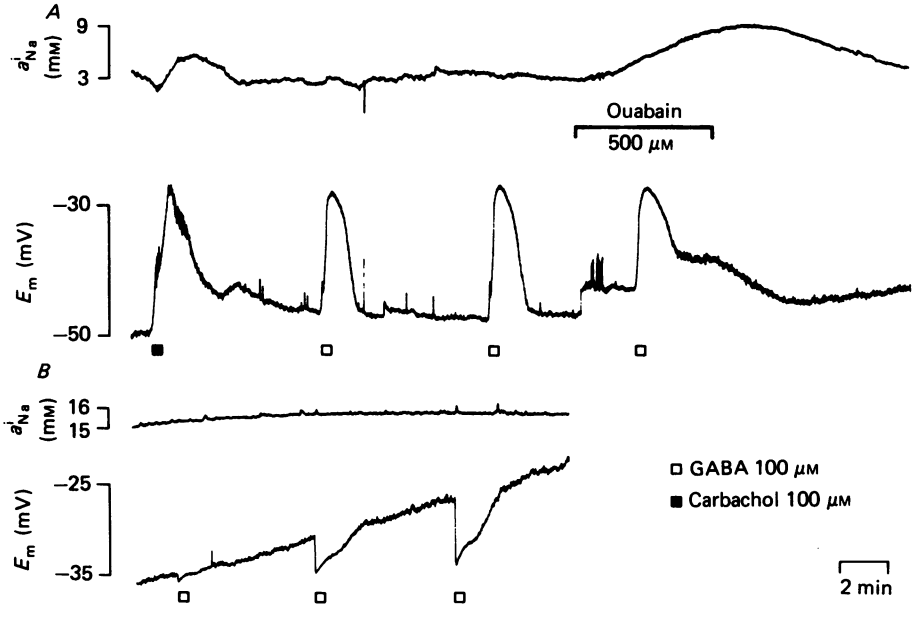

Fig. 5. Effects of GABA on intracellular Na^+ activity (a^i_{Na}). In the neurone illustrated in *A*, effects of carbachol, GABA, and ouabain on a^i_{Na} were investigated. It is visible, that carbachol and ouabain induced a rise in a^i_{Na}, whereas GABA (even in the presence of ouabain, see text) had no such effect. *B*, this neurone spontaneously drifted to a depolarized state. Under these circumstances hyperpolarizing GABA actions were elicited, which also did not alter a^i_{Na}.

The effect of GABA on intracellular Na^+ and Ca^{2+} activities was also investigated. The neurone shown in Fig. 5*A* was impaled with a Na^+-sensitive electrode. At the beginning of the recording, carbachol (100 μM) was applied for 30 s. Carbachol induced an increase of a^i_{Na} from 3 to about 6 mM. However, subsequent applications of GABA (100 μM) did not change a^i_{Na}. The lack of such an effect was also evident in the presence of ouabain, which was added in order to prevent a possible outward pumping of Na^+ which might have obscured a GABA-induced a^i_{Na} increase. The steady increase of a^i_{Na} seen in this part of the recording is due to the Na^+-pump inhibition induced by ouabain. Also hyperpolarizing actions of GABA did not change a^i_{Na}, which can be seen in the recording illustrated in Fig. 5*B*. This neurone shifted spontaneously to a depolarized state.

Although the Na^+ ligand shows considerable Ca^{2+} interference, it is not likely that

an increase in the intracellular free Ca^{2+} concentration contributes significantly to the potential reading of the Na^+-sensitive micro-electrode during the action of carbachol or ouabain. A calculation based on a selectivity ratio for $Ca^{2+}:Na^+:K^+ = 1{\cdot}6:1:0{\cdot}005$ (Meier, Lanter, Ammann, Steiner & Simon, 1982) reveals that a voltage change of only 1·2 mV would result from a carbachol-induced increase in the intracellular free Ca^{2+} concentration from 0·4 to 1 μM (see Fig. 6; background 120 mM-K^+; 10 mM-Na^+). In fact, the Na^+-sensitive micro-electrode showed a potential reading of 14 mV according to a rise of a^i_{Na} from 3 to 9 mM.

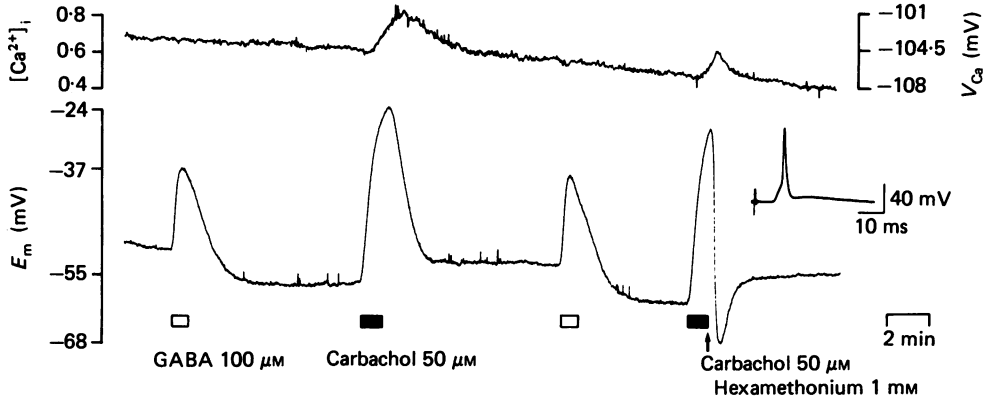

Fig. 6. Lack of effect of GABA on free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). In this neurone, a Ca^{2+}-sensitive micro-electrode was used to measure the effects of GABA and carbachol on intracellular Ca^{2+}. Only in the presence of carbachol, was a rise of the $[Ca^{2+}]_i$ observed, whereas GABA was without effect. Ca^{2+}-sensitive micro-electrodes were calibrated as voltage of the difference signal ($V_{Ca} = E_{ion} - E_{ref}$) and as free ion concentrations. The inset shows an action potential recorded in this neurone elicited by stimulation of the preganglionic nerve trunk. After the second application of carbachol, hexamethonium was used to block the nicotinic receptors. This experimental procedure, which is supposed to abbreviate passive ion fluxes at the time of carbachol wash-out (Brown, Brownstein & Scholfield, 1972) accelerated the post-carbachol hyperpolarization.

Fig. 6 illustrates a typical example of observations made with Ca^{2+}-sensitive micro-electrodes. Whereas carbachol induced a clearly visible rise of the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), application of GABA did not induce such an effect. However, it remains possible that part of the voltage change seen by the Ca^{2+}-sensitive micro-electrodes in the presence of carbachol is due to the rise in intracellular Na^+ (due to the Na^+ sensitivity of the Ca^{2+} ligand; see Deitmer & Schlue, 1983; Weingart & Hess, 1984). Nevertheless, the electrodes would have been sensitive enough to detect a possible GABA-induced rise of $[Ca^{2+}]_i$.

GABA and extracellular pH

Extracellularly positioned pH-sensitive micro-electrodes were used in order to gain insight in possible changes of the extracellular HCO_3^- concentration induced by the action of GABA. In such experiments, changes of extracellular pH (pH_o) during repetitive synaptic stimulation and the application of carbachol and GABA were compared. A typical result is illustrated in Fig. 7. Synaptic stimulation resulted,

parallel to a rise of the extracellular K^+ activity, in a biphasic, alkaline-going followed by an acid-going shift of pH_o as previously described in the cerebellar cortex (Kraig, Ferreira-Filho & Nicholson, 1983). Carbachol, in this example, induced an extracellular acidification. In some ganglia, however, an alkaline-going shift was seen at the beginning of a response to carbachol. In contrast, in the case of GABA, always a pure acidification, which did not exceed 0·04 pH units in CO_2/HCO_3^--buffered solutions, was observed.

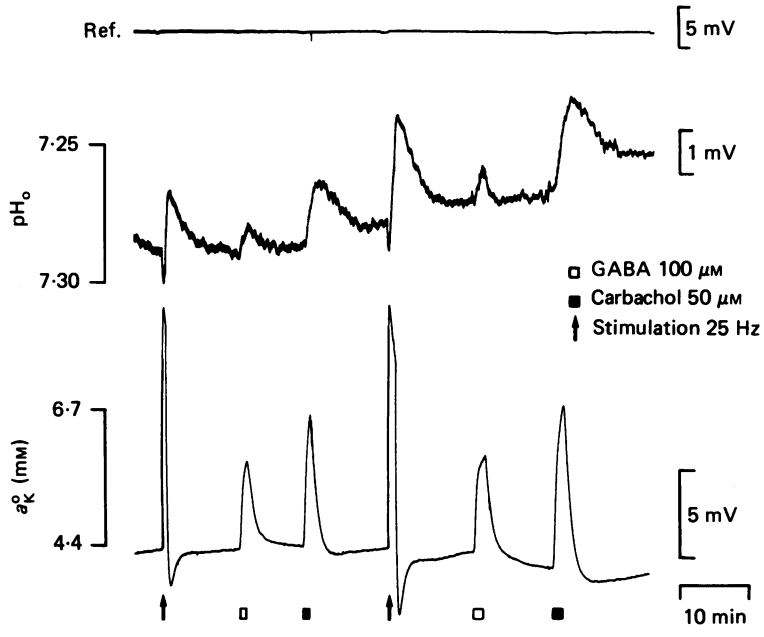

Fig. 7. Effects of GABA on extracellular K^+ and pH (a^o_K and pH_o). In this experiment, a K^+- and a pH-sensitive micro-electrode were positioned nearby each other in the extracellular space of the ganglion. The effects of synaptic stimulation, carbachol, and GABA on these parameters were compared (for further explanation see text).

Cl^- transport mechanism

Effects of extracellular K^+. In a series of ten experiments, effects of K^+-free solutions (KCl replaced by NaCl) were tested on the Cl^- uptake after the action of GABA. In all cases an almost complete blockade of the a^i_{Cl} recovery was observed. A typical example is illustrated in Fig. 8. At the beginning of this recording, GABA (100 μM) was applied for 45 s in the standard Krebs solution (extracellular K^+ concentration ($[K^+]_o$) = 6 mM). This resulted in a reversible decrease of a^i_{Cl} from 28 to 25 mM. The ganglion was then superfused with a K^+-free solution. An initial membrane hyperpolarization was followed by a slowly developing depolarization, and a fall of a^i_{Cl} by about 2 mM was then observed. A quantitative analysis of such measurements revealed that a^i_{Cl} decreased within 5 min by $2·6 \pm 0·37$ mM (mean ± S.D.), in ten cells tested. At this time, a^i_{Cl} was still above equilibrium. Application of GABA in a K^+-free solution (Fig. 8) resulted in an enhanced fall of a^i_{Cl}. The post-GABA a^i_{Cl} recovery,

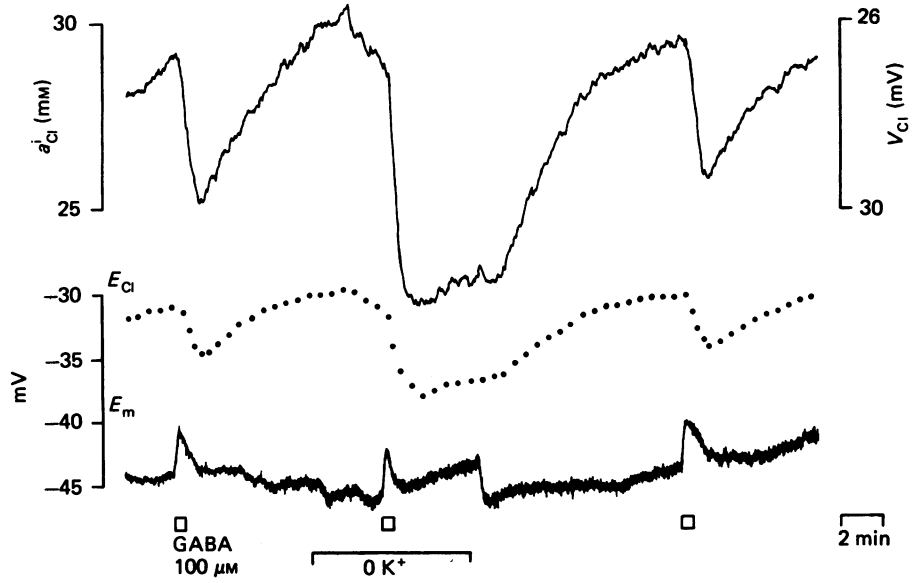

Fig. 8. Effects of a K^+-free solution on the post-GABA a^i_{Cl} recovery. Note that the a^i_{Cl} recovery, usually seen after the end of a GABA application, was significantly slowed down in the absence of extracellular K^+.

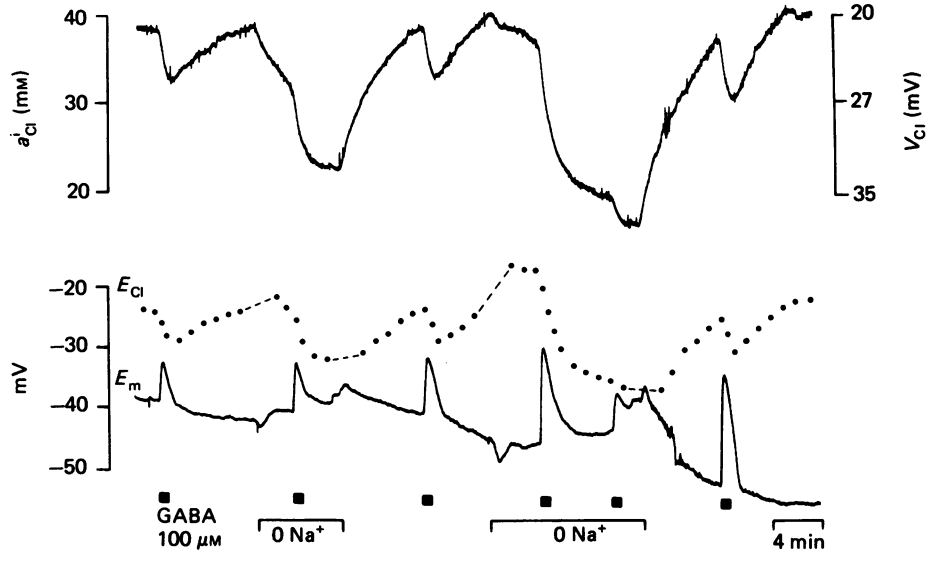

Fig. 9. Effects of Na^+-free solutions on the post-GABA a^i_{Cl} recovery. This Figure illustrates a continuous recording from a neurone impaled with a Cl^--sensitive micro-electrode. The normal bathing solution was replaced twice for short periods by a Na^+-free (Trizma) solution. In Na^+-free solution a complete blockade of the post-GABA a^i_{Cl} recovery was observed. Dashed lines on the E_{Cl} plot indicate that the decrease of the Cl^- activity in Trizma solutions was taken into account.

however, was significantly slowed down. After readdition of 6 mM-K^+ to the bathing solution, a^i_{Cl} returned to its resting value with kinetics similar to those usually seen after GABA applications in normal extracellular K^+.

Effects of extracellular Na^+. To explore the Na^+ dependency of the Cl^- transport, the effects of Na^+-free solutions were tested on a^i_{Cl}. In thirteen experiments Na^+ was replaced by Trizma base/HCl (Tris(hydroxymethyl)aminomethane), whereas in three cases choline was used as the Na^+ substitute. In all these recordings, a block of the post-GABA a^i_{Cl} recovery was observed. In the experiment illustrated in Fig. 9 the normal bathing solution was replaced twice with a Na^+-free solution ($NaHCO_3$ and NaCl replaced by Trizma base/HCl; bubbled with 5% CO_2/95% O_2, pH = 7·4).

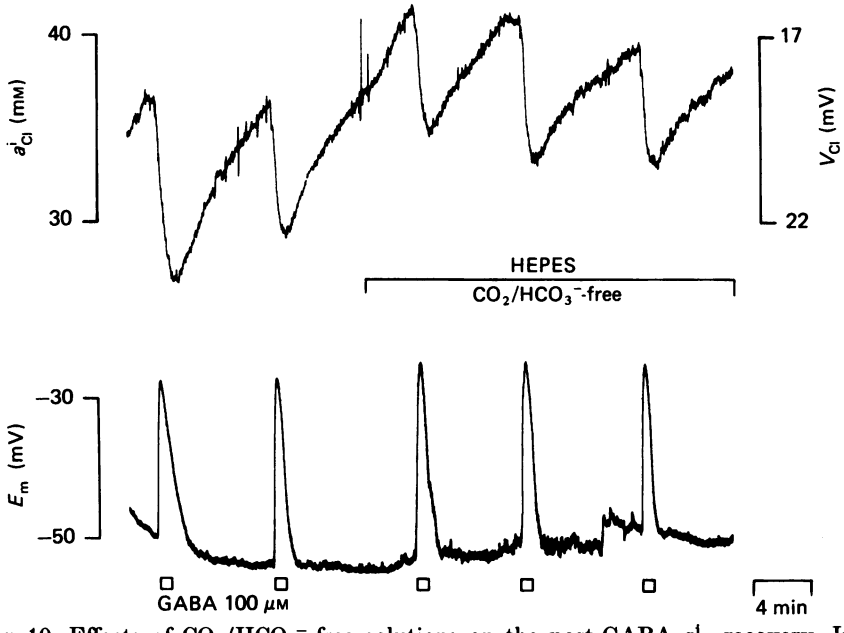

Fig. 10. Effects of CO_2/HCO_3^--free solutions on the post-GABA a^i_{Cl} recovery. In this experiment the normal bathing solution was replaced by a HEPES-buffered 100% O_2-gassed solution. This procedure did not alter the kinetics of the post-GABA a^i_{Cl} recovery.

In this solution the a^i_{Cl} base line was decreased and a blockade of the post-GABA a^i_{Cl} recovery was observed. In each case this effect was readily reversible. However, in spite of a nominally equimolar Cl^- concentration, Trizma solutions had a decreased Cl^- activity. Extracellular Cl^- activity (a^o_{Cl}) was about 74 mM in Trizma as compared to 94·5 mM in the normal bathing solution. However, this lack of 20 mM-Cl^- activity alone is insufficient to explain the observed effects since solutions in which 20 mM-Cl^- was substituted by glucuronate did not change the post-GABA a^i_{Cl} recovery (not illustrated). Nevertheless, the change of the a^o_{Cl} was taken into account for the calculation of the Cl^- equilibrium potential (E_{Cl}, note dashed lines in Fig. 9). Such calculations always revealed a good correlation between the amplitude of the GABA-induced depolarizations and E_{Cl}.

As an alternative to Trizma, choline (118 mM) was used as a Na^+-substitute in three

further experiments. These recordings were difficult in view of the excitatory cholinomimetic effects of this compound. Therefore, the nicotinic antagonist hexamethonium (1 mM) was added to the low-Na^+, choline-containing solutions. In choline-substituted solutions an inhibition of the post-GABA a^i_{Cl} recovery was observed (not illustrated).

Effects of extracellular HCO_3^-. The effects of CO_2/HCO_3^--free, HEPES-buffered solutions were examined in nine experiments to investigate whether a Cl^-/HCO_3^- exchange is involved in the post-GABA a^i_{Cl} recovery. Such solutions have been

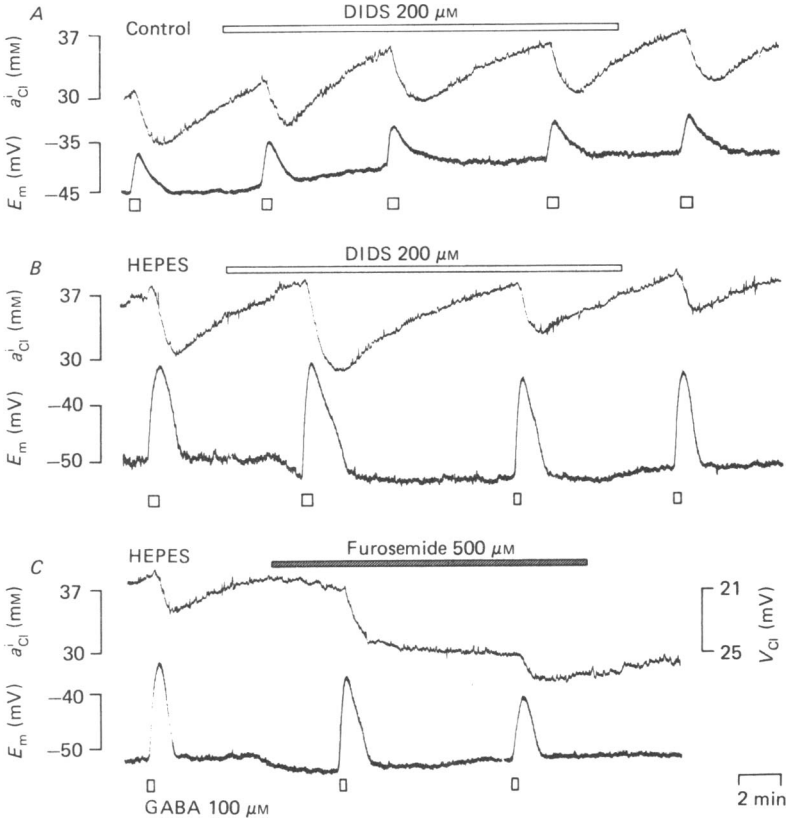

Fig. 11. Pharmacology of the post-GABA a^i_{Cl} recovery. This Figure shows a continuous recording from a single neurone with a Cl^--sensitive micro-electrode. Note that DIDS, even in a HEPES-buffered solution, did not alter the a^i_{Cl} recovery following the application of GABA (*A*, *B*), whereas such an effect was seen immediately after the addition of furosemide (*C*). There was a 15 min break between traces *A* and *B*.

reported to slow Cl^- reuptake in heart and smooth muscle cells (Vaughan-Jones, 1979; Aickin & Brading, 1984). In rat sympathetic ganglion cells, however, no differences with respect to the normal bathing solution were observed. In the experiment illustrated in Fig. 10, two control applications of GABA (100 μM) were followed by three applications of GABA in a HEPES-buffered solution. No change in the kinetics of the a^i_{Cl} recovery in such HCO_3^--free solutions was observed. The small increase

of the a^i_{Cl} base line visible in this cell was not a consistent finding. In the other neurones CO_2/HCO_3^--free solutions had no influence on the a^i_{Cl} base line.

Pharmacological aspects. The disulphonic stilbene derivatives are well established inhibitors of Cl^-/HCO_3^- counter-transport. We have used DIDS to further investigate a participation of anion exchange in the a^i_{Cl} recovery after the action of GABA. Fig. 11 shows a continuous recording from a single cell. First DIDS (200 μM) was added to a bicarbonate-buffered solution (Fig. 11*A*). It can be seen that DIDS and later on HEPES-buffered solutions (Fig. 11*B*) did not affect the kinetics of the a^i_{Cl} recovery after GABA. However, addition of furosemide (500 μM) to the HCO_3^--free solution resulted in a decrease of a^i_{Cl} base line and a blockade of the a^i_{Cl} recovery after GABA (Fig. 11*C*). Furosemide has been demonstrated to be an inhibitor of $(Na^+)/K^+/Cl^-$ co-transport in a variety of tissues (see Discussion for references) and we have previously shown that it blocks the reuptake of both Cl^- and K^+ in sympathetic neurones bathed in HCO_3^--buffered solutions (Ballanyi *et al.* 1984*a*).

DISCUSSION

GABA-associated ion movements

The Cl^- movements associated with the action of GABA can be sufficiently explained by the opening of receptor-activated Cl^- channels. At the resting potential an a^i_{Cl} higher than that expected from the passive distribution of this ion was observed. The consequence of a GABA-induced increase in Cl^- conductance is therefore a net Cl^- efflux according to the outwardly directed driving force (Adams & Brown, 1975; Gallagher, Higashi & Nishi, 1978). At low membrane potentials, an E_{Cl} more negative than the membrane potential was found. Application of GABA under these circumstances, therefore, resulted in a net Cl^- influx observable as an increase of a^i_{Cl} due to the inwardly directed driving force.

The decrease of a^i_K seen during the depolarizing action of GABA could be the consequence of either a GABA receptor-coupled K^+ channel, voltage-dependent K^+ channels gated by membrane potential changes, and/or by K^+ fluxes through leak channels. In the latter case, the increase in the outwardly directed driving force ($E_m - E_K$) would promote the K^+ efflux. A GABA-induced elevation of $[K^+]_o$ has been previously observed in rat sympathetic ganglia (Förstl, Galvan & ten Bruggencate, 1982), frog spinal cord (Kudo & Fukuda, 1976; Sykova, 1979), rat dorsal root ganglia (Deschenes & Feltz, 1976) and rat pituitary cells (Loeffler, Desaulles, Demeneix & Feltz, 1982). At the moment, we cannot differentiate between these mechanisms. However, it might also be possible to manually 'voltage clamp' the neurones during the action of GABA and to measure changes in intracellular ion activities. This should enable one to separate receptor-, voltage-, and leak-dependent effects. In the case of the hyperpolarizing GABA response, we favour the idea that the decrease of the outwardly directed driving force in this situation can result in a net uptake of K^+ assuming a constant, Na^+/K^+ pump-mediated K^+ uptake. A K^+ uptake due to a membrane hyperpolarization, indicated by a decrease of the extracellular K^+ activity, can be clearly seen in the retina during light-induced hyperpolarization of the receptors (Shimazaki & Oakley, 1984). If this interpretation is correct, application

of ouabain should remove the hyperpolarization-induced increase in a_K^i; such experiments have not yet been performed in the present study.

The lack of changes in the intracellular activities of Na^+ and Ca^{2+} indicates that GABA, in contrast to carbachol, does not directly increase the membrane conductance for these ions. An indirect effect via an activation of voltage-dependent ion channels apparently is also of little importance in rat sympathetic ganglia. However, it has been reported that GABA can decrease the extracellular Ca^{2+} concentration in the rat pituitary (Loeffler *et al.* 1982).

GABA and cell volume

In the present study measurements of intracellular ion activities were used to estimate transmembrane ion movements. In such a context, however, one has to consider possible variations in cell volume and the consequences of such alterations on intracellular ion activities. Since no direct information is available on changes in cell volume during the GABA response, we can only estimate the most likely situation. The assumptions of such a calculation are: total intracellular osmolarity = 300 mosmol/l; intracellular concentration of K^+ = 130 mM; intracellular concentration of Cl^- = 40 mM; cell membrane freely permeable for water. During a depolarizing action of GABA, KCl is supposed to leak out of the cells as a 300 mosmol solution, i.e. the neurone will be losing K^+ at a concentration of 150 mM, and also Cl^- at 150 mM. After a loss of, say, 10% of the cell volume, the remaining ions will distribute within 90% of the original cell volume. This will result in a final intracellular concentration of 127·8 mM-K^+ and 27·8 mM-Cl^-, respectively. This calculation shows that volume changes can compensate differentially for a decrease in the amount of intracellular ions. In spite of the same GABA-induced decrease in the amount of K^+ and Cl^-, a_K^i will be less affected as compared to a_{Cl}^i. In other words and with respect to the following discussion, we conclude that cell volume changes associated with the GABA response do not interfere importantly with the interpretation of changes in a_{Cl}^i as being due to transmembrane Cl^- movements.

Cl^- transport mechanism

No changes of the membrane potential were recorded in our experiments during the phase of the a_{Cl}^i recovery. This observation is already sufficient to restrict the discussion about the GABA-activated Cl^- transport in rat sympathetic neurones to two main principles. Since the lack of a change in membrane potential indicates electroneutrality, Cl^- can either enter the cell in exchange for an anion leaving the cell or be coupled to cation(s) also entering the cell (Hoffmann, 1982). Both types of transport mechanisms have been previously described in nervous tissues. Astroglial cells and glioma cells in culture, for example, seem to possess a Cl^-/HCO_3^- exchange to maintain an a_{Cl}^i higher than predicted from a passive equilibration (Kimelberg, 1981; Wolpaw & Martin, 1984). Snail and crayfish neurones use such a transport mechanism participating in their pH-regulating system (Moody, 1981; Roos & Boron, 1981; Thomas, 1977, 1984). Furthermore, an *in vivo* study has revealed the presence of a fast Cl^-/HCO_3^- exchange between brain cells and brain extracellular fluid in respiratory acidosis (Ahmad & Loeschcke, 1982).

There have also been reports of Cl^- transport linked to cations in nervous tissue.

A furosemide-sensitive K^+/Cl^- co-transport has been described which maintains a *low* intracellular Cl^- activity (and a hyperpolarizing inhibitory post-synaptic potential as a consequence) in crayfish stretch receptor neurones (Aickin, Deisz & Lux, 1982, 1984; Deisz & Lux, 1982). A K^+/Cl co-transport has also been reported to underlie the *high* a^i_{Cl} in squid axon (Russell, 1983) and in glioma cells (Wolpaw & Martin, 1984). For the latter mechanism, a source of energy is required to transport K^+ and Cl^- against their outwardly directed driving forces. Russell (1983) found that the coupling of the K^+/Cl^- transport to Na^+ and its inwardly directed driving force could provide this energy. Such a $Na^+/K^+/Cl^-$ co-transport has been previously described in a variety of non-nervous tissues (Geck, Pietrzyk, Burckhardt, Pfeiffer & Heinz, 1980; Aiton, Chipperfield, Lamb, Ogden & Simmons, 1981; Haas, Schmidt & McManus, 1982; Greger, Schlatter & Lang, 1983; Duhm & Göbel, 1984). It may well have a functional significance in maintaining the cell volume in hypertonic media (Kregenow, 1981).

The prerequisites for the a^i_{Cl} recovery which follows the depolarizing action of GABA on rat sympathetic ganglion cells was also examined in this study. One possible mechanism of the a^i_{Cl} recovery, described above, would be an efflux of HCO_3^- in exchange for an influx of Cl^- (Vaughan-Jones, 1979; Aickin & Brading, 1984). However, no changes in the kinetics of the a^i_{Cl} recovery after GABA were seen in nominally HCO_3^-- and CO_2-free, HEPES-buffered solutions (gassed with 100 % O_2). In such a solution the intracellular HCO_3^- concentration would then be too low to support such an equimolar exchange with Cl^-. However, the tissue itself might produce some CO_2, and thus small effects of HCO_3^- movements cannot be completely excluded on the basis of this observation (see also Aickin & Brading, 1984). Experiments with the well known anion-exchange blocker DIDS were therefore performed. Such recordings did not show any impairment of the a^i_{Cl} recovery. Thus it can be concluded that HCO_3^- is probably not important for the post-GABA a^i_{Cl} recovery.

The lack of importance of HCO_3^- for the a^i_{Cl} recovery was confirmed by pH_o recordings. If HCO_3^- leave the cell in exchange for Cl^- entering the cell an extracellular alkaline-going shift would be produced. However, this was not the case (see Fig. 7). In fact, an acidification was seen, which might be related to a metabolically linked lactate release (Kraig *et al.* 1983).

The presence of extracellular K^+ was clearly a prerequisite for the a^i_{Cl} recovery since low-K^+ solutions inhibited the post-GABA inward pumping of Cl^- (see Fig. 8). However, a transport mechanism with simple coupling of K^+ and Cl^- would need a source of energy since in rat sympathetic ganglia both ions need to be pumped against their outwardly directed driving forces. This energy appears to be the Na^+ gradient. Our data revealed a block of the GABA-associated Cl^- transport in Na^+-free media (see Fig. 9). A possible role for a linked Na^+ and K^+/Cl^- movement to maintain a high a^i_{Cl} has been previously described in squid axon (Russell, 1983). However, in spite of a dependency of the a^i_{Cl} recovery on extracellular Na^+, we have no evidence that a Na^+ *transport* is linked to the K^+/Cl^- uptake. Even in the presence of ouabain, no GABA-induced increase of a^i_{Na} was seen. However, it still remains a possibility that some other ouabain-insensitive mechanism (e.g. Na^+/Ca^{2+} exchange) closely regulates a^i_{Na} and so obscures the predicted increase. It may also be that Na^+ is

necessary for an allosteric binding site at the external surface of the presumed K^+/Cl^- carrier.

Taken together, our data indicate that a Na^+-dependent K^+/Cl^- co-transport may be the mechanism underlying the GABA-activated Cl^- transport in rat sympathetic ganglion neurones. It is also possible, that the same mechanism contributes to the regulation of the steady-state transmembrane Cl^--distribution (see effects of Na^+-free and K^+-free solutions on a^i_{Cl} base line). The effects of furosemide are consistent with this view, since furosemide is known to block this particular type of Cl^- transport in a variety of cell types. However, it should be noted that cat dorsal root ganglia and frog primary afferent terminals seem to possess an inwardly directed Cl^- pump that is resistant to furosemide (Nicoll, 1978; Gallagher *et al.* 1983). These authors reported a block of the GABA-associated Cl^- channel within 5 min of the application of furosemide. In our hands, this application time was too short to lower a^i_{Cl}. Furthermore, several subsequent GABA applications were necessary to decrease a^i_{Cl} to values which could be measured on the basis of reversal potential recordings (see also Wojtowicz & Nicoll, 1982). It therefore seems possible that furosemide has effects on both the Cl^- channel as well as on the Cl^- pump.

A final question concerns the possible mechanism underlying the outward pumping of Cl^- which follows the hyperpolarizing actions of GABA. Unfortunately, it was not possible to explore the ionic dependency and pharmacology of this transport, since neurones could not be recorded long enough in such a depolarized state. However, there seems no reason to assume that the mechanism responsible for outward transport of Cl^- following a GABA-induced hyperpolarization is different from that responsible for the inward transport studied. There is increasing evidence for reversibility of ion transport mechanisms of many different types. In this context, it has been shown already that in crayfish stretch receptor neurones the outward pumping of Cl^- may be also linked to K^+ (Aickin *et al.* 1982).

We would like to thank Professor G. ten Bruggencate for constant encouragement and helpful discussions during the course of this project. We are grateful to Dr N. T. Slater for reading the manuscript. Ms G. Schneider, Mrs C. Müller and Ms I. Englmaier provided expert technical and secretarial assistance. The work was supported by the Deutsche Forschungsgemeinschaft (SFB 220).

REFERENCES

ADAMS, P. R. & BROWN, D. A. (1975). Actions of γ-aminobutyric acid on sympathetic ganglion cells. *Journal of Physiology* **250**, 85–120.

AHMAD, H. R. & LOESCHCKE, H. H. (1982). Fast bicarbonate–chloride exchange between brain cells and brain extracellular fluid in respiratory acidosis. *Pflügers Archiv* **395**, 293–299.

AICKIN, C. C. & BRADING, A. F. (1984). The role of chloride–bicarbonate exchange in the regulation of intracellular chloride in guinea-pig vas deferens. *Journal of Physiology* **349**, 587–606.

AICKIN, C. C., DEISZ, R. A. & LUX, H. D. (1982). Ammonium action on post-synpatic inhibition in crayfish neurones: implications for the mechanism of chloride extrusion. *Journal of Physiology* **329**, 319–339.

AICKIN, C. C., DEISZ, R. A. & LUX, H. D. (1984). Mechanisms of chloride transport in crayfish stretch receptor neurones and guinea pig vas deferens: implications for inhibition mediated by GABA. *Neuroscience Letters* **47**, 239–244.

AITON, J. F., CHIPPERFIELD, A. R., LAMB, J. F., OGDEN, P. & SIMMONS, N. L. (1981). Occurrence of passive frusemide-sensitive transmembrane potassium transport in cultured cells. *Biochimica et biophysica acta* **646**, 389–398.

BALLANYI, K., GRAFE, P., REDDY, M. M. & TEN BRUGGENCATE, G. (1984*a*). Different types of potassium transport linked to carbachol and γ-aminobutyric acid actions in rat sympathetic neurons. *Neuroscience* **12**, 917–927.

BALLANYI, K., GRAFE, P. & TEN BRUGGENCATE, G. (1984*b*). GABA-action and chloride transport in rat sympathetic ganglia. *Pflügers Archiv* **400**, R 37.

BROWN, D. A., BROWNSTEIN, M. J. & SCHOLFIELD, C. N. (1972). Origin of the after-hyperpolarization that follows removal of depolarizing agents from the isolated superior cervical ganglion of the rat. *British Journal of Pharmacology* **44**, 651–671.

DEISZ, R. A. & LUX, H. D. (1982). The role of intracellular chloride in hyperpolarizing post-synaptic inhibition of crayfish stretch receptor neurones. *Journal of Physiology* **326**, 123–138.

DEITMER, J. W. & SCHLUE, W. R. (1983). Intracellular Na^+ and Ca^{2+} in leech retzius neurones during inhibition of the Na^+-K^+-pump. *Pflügers Archiv* **397**, 195–201.

DESCHENES, M. & FELTZ, P. (1976). GABA-induced rise of extracellular potassium in rat dorsal root ganglia: an electrophysiological study in vivo. *Brain Research* **118**, 494–499.

DUHM, J. & GÖBEL, B. O. (1984). Role of the frusemide-sensitive Na^+-K^+ transport system in determining the steady-state Na^+ and K^+ content and volume of human erythrocytes in vitro and in vivo. *Journal of Membrane Biology* **77**, 243–254.

FÖRSTL, J., GALVAN, M. & TEN BRUGGENCATE, G. (1982). Extracellular K^+ concentration during electrical stimulation of rat isolated sympathetic ganglia, vagus and optic nerves. *Neuroscience* **7**, 3221–3229.

GALLAGHER, J. P., HIGASHI, H. & NISHI, S. (1978). Characterization and ionic basis of GABA-induced depolarizations recorded *in vitro* from cat primary afferent neurones. *Journal of Physiology* **275**, 263–282.

GALLAGHER, J. P., NAKAMURA, J. & SHINNICK-GALLAGHER, P. (1983). The effects of temperature, pH and Cl^--pump inhibitors on GABA responses recorded from cat dorsal root ganglia. *Brain Research* **267**, 249–259.

GALVAN, M., DÖRGE, A., BECK, F. & RICK, R. (1984). Intracellular electrolyte concentrations in rat sympathetic neurones measured with an electron microprobe. *Pflügers Archiv* **400**, 274–279.

GECK, P., PIETRZYK, C., BURCKHARDT, B. C., PFEIFFER, B. & HEINZ, E. (1980). Electrically silent co-transport of Na^+, K^+ and Cl^- in Ehrlich cells. *Biochimica et biophysica acta* **600**, 432–447.

GRAFE, P., BALLANYI, K. & TEN BRUGGENCATE, G. (1985). Changes of intracellular free ion concentrations, evoked by carbachol or GABA, in sympathetic neurons. In *Recent Advances in the Theory and Application of Ion Selective Electrodes in Physiology and Medicine*, ed. KESSLER, M. Berlin, Heidelberg, New York: Springer (in the Press).

GRAFE, P., RIMPEL, J., REDDY, M. M. & TEN BRUGGENCATE, G. (1982). Lithium distribution across the membrane of motoneurons in the isolated frog spinal cord. *Pflügers Archiv* **393**, 297–301.

GREGER, R., SCHLATTER, E. & LANG, F. (1983). Evidence for electroneutral sodium chloride co-transport in the cortical thick ascending limb of Henle's loop of rabbit kidney. *Pflügers Archiv* **396**, 308–314.

HAAS, M., SCHMIDT, W. F. & MCMANUS, T. J. (1982). Catecholamine-stimulated ion transport in duck red cells. *Journal of General Physiology* **80**, 125–147.

HOFFMANN, E. K. (1982). Anion exchange and anion-cation co-transport systems in mammalian cells. *Philosophical Transactions of the Royal Society* B **299**, 519–535.

KIMELBERG, H. K. (1981). Active accumulation and exchange transport of chloride in astroglial cells in culture. *Biochimica et biophysica acta* **646**, 179–184.

KRAIG, R. P., FERREIRA-FILHO, C. R. & NICHOLSON, C. (1983). Alkaline and acid transients in cerebellar microenvironment. *Journal of Neurophysiology* **49**, 831–850.

KREGENOW, F. M. (1981). Osmoregulatory salt transporting mechanisms: Control of cell volume in anisotonic media. *Annual Reviews of Physiology* **43**, 493–505.

KUDO, Y. & FUKUDA, H. (1976). Alteration of extracellular K^+-activity induced by amino acids in the frog spinal cord. *Japanese Journal of Pharmacology* **26**, 385–387.

LOEFFLER, J. P., DESAULLES, E., DEMENEIX, B. A. & FELTZ, P. (1982). Electrophysiological study with K^+- and Ca^{2+}-sensitive micropipettes of GABA receptors in the rat neurointermediate lobe in vitro. *Neuroscience Letters* **34**, 271–276.

MEIER, P. C., AMMANN, D., MORF, W. E. & SIMON, W. (1980). Liquid-membrane ion-sensitive electrodes and their biomedical applications. In *Medical and Biological Applications of Electrochemical Devices*, ed. KORYTA, J., pp. 13–91. New York: J. Wiley & Sons Ltd.

MEIER, P. C., LANTER, F., AMMANN, D., STEINER, R. A. & SIMON, W. (1982). Applicability of available ion-selective liquid-membrane microelectrodes to intracellular ion-activity measurements. *Pflügers Archiv* **393**, 23–30.

MOODY, W. J. (1981). The ionic mechanisms of intracellular pH regulation in crayfish neurones. *Journal of Physiology* **316**, 293–308.

NICOLL, R. A. (1978). The blockade of GABA mediated responses in the frog spinal cord by ammonium ions and furosemide. *Journal of Physiology* **283**, 121–132.

ROOS, A. & BORON, W. F. (1981). Intracellular pH. *Physiological Reviews* **61**, 296–434.

RUSSELL, J. M. (1983). Cation-coupled chloride influx in squid axon. Role of potassium and stoichiometry of the transport process. *Journal of General Physiology* **81**, 909–925.

SHIMAZAKI, H. & OAKLEY, B. (1984). Reaccumulation of $[K^+]_o$ in the toad retina during maintained illumination. *Journal of General Physiology* **84**, 475–490.

SYKOVA, E. (1979). GABA-induced changes of extracellular K^+-activity in the frog spinal cord. *Physiologia bohemoslovenica* **27**, 189–192.

THOMAS, R. C. (1977). The role of bicarbonate, chloride and sodium ions in the regulation of intracellular pH in snail neurones. *Journal of Physiology* **273**, 317–338.

THOMAS, R. C. (1984). Experimental displacement of intracellular pH and the mechanism of its subsequent recovery. *Journal of Physiology* **354**, 3–22*P*.

TSIEN, R. Y. & RINK, T. J. (1981). Ca^{2+}-selective electrodes; a novel PVC-gelled neutral carrier mixture compared with other currently available sensors. *Journal of Neuroscience Methods* **4**, 73–86.

VAUGHAN-JONES, R. D. (1979). Regulation of chloride in quiescent sheep-heart purkinje fibres studied using intracellular chloride and pH-sensitive micro-electrodes. *Journal of Physiology* **295**, 111–137.

WEINGART, R. & HESS, P. (1984). Free calcium in sheep cardiac tissue and frog skeletal muscle measured with Ca^{2+}-selective micro-electrodes. *Pflügers Archiv* **402**, 1–9.

WOJTOWICZ, J. M. & NICOLL, R. A. (1982). Selective action of piretanide on primary afferent GABA responses in the frog spinal cord. *Brain Research* **236**, 173–181.

WOLPAW, E. W. & MARTIN, D. L. (1984). Cl^- transport in a glioma cell line: evidence for two transport mechanisms. *Brain Research* **297**, 317–327.