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THE ROLE OF INTRACELLULAR CHLORIDE IN HYPERPOLARIZING POST-SYNAPTIC INHIBITION OF CRAYFISH STRETCH RECEPTOR NEURONES

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SUMMARY

1. The intracellular Cl^- activity (a^i_{Cl}) of isolated crayfish stretch receptor neurones was measured using liquid ion exchanger Cl^--selective micro-electrodes. The potential developed due to the difference between the normal extracellular Cl^- activity (a^o_{Cl}) and a^i_{Cl} (V_{Cl}) was compared with the simultaneously measured reversal potential of the inhibitory post-synaptic potential ($E_{i.p.s.p.}$) to further clarify the ionic basis of the i.p.s.p..

2. In normal Ringer solution, V_{Cl} ($63{\cdot}3 \pm 2{\cdot}3$ mV) was found to be close to the resting membrane potential (E_m, $62{\cdot}6 \pm 3{\cdot}9$ mV) while $E_{i.p.s.p.}$ ($74{\cdot}5 \pm 1{\cdot}9$ mV) was more negative than either. The V_{Cl} value corresponds to an apparent a^i_{Cl} of $12{\cdot}7 \pm 1{\cdot}3$ mM, which is about 4 mM more than required for a Cl^- governed $E_{i.p.s.p.}$ of 74·5 mV.

3. Reducing a^o_{Cl} caused smaller changes in V_{Cl} than predicted for passive Cl^- re-distributions. On complete removal of extracellular Cl^- (Cl^-_o), V_{Cl} increased to $84{\cdot}6 \pm 2{\cdot}7$ mV, equivalent to an apparent a^i_{Cl} of about 5 mM-Cl^-. This value can be used as an estimate of the level of intracellular interference on the Cl^--selective micro-electrode.

4. Increasing extracellular K^+ (K^+_o) decreased both V_{Cl} and $E_{i.p.s.p.}$. Decreasing K^+_o had the converse effect. The time course of the changes in V_{Cl} and $E_{i.p.s.p.}$ was much the same. The difference between V_{Cl} and $E_{i.p.s.p.}$ decreased to about 3 mV in high K^+_o, and increased to about 30 mV in low K^+_o. This variation in the difference between $E_{i.p.s.p.}$ and V_{Cl} is consistent with the assumption that anions other than Cl^- contribute to the recorded V_{Cl} rather than another ion contributes to the inhibitory current.

5. Application of 5 mM-NH_4^+ or of frusemide (6×10^{-4} M) decreased V_{Cl} and $E_{i.p.s.p.}$. The difference between V_{Cl} and $E_{i.p.s.p.}$ was also decreased.

6. We conclude that a^i_{Cl} is lower than predicted from a passive distribution and thus the chloride equilibrium potential (E_{Cl}) is more negative than E_m. If a constant intracellular interference equivalent to about 4 mM-Cl^- is assumed to contribute to the recorded V_{Cl}, E_{Cl} was approximately equal to $E_{i.p.s.p.}$ in all the experimental conditions. Therefore we suggest that the i.p.s.p. is solely generated by Cl^- ions.

INTRODUCTION

The participation of chloride ions in the generation of the inhibitory post-synaptic potential (i.p.s.p.) is well established for motoneurones (Coombs, Eccles & Fatt, 1955; Araki, Ito & Oscarson, 1961; Eccles, Eccles & Ito, 1964), cortical neurones (Kelly, Krnjević, Morris & Yim, 1969) and invertebrate preparations (Boistel & Fatt, 1958; Motokizawa, Reuben & Grundfest, 1969; Ozawa & Tsuda, 1973). However, the assumption of a passive Cl^- distribution compelled the postulate of a K^+ contribution to account for the usually hyperpolarizing nature of the i.p.s.p. (Coombs *et al.* 1955; Eccles *et al.* 1964). Since then the evidence for an intracellular Cl^- activity (a^i_{Cl}) lower than expected from a passive distribution has accumulated for *Aplysia* neurones (Brown, Walker & Sutton, 1970; Ascher, Kunze & Neild, 1976) and cat motoneurones (Lux, 1971), whereas positive evidence for a K^+ contribution is still lacking (Allen, Eccles, Nicoll, Oshima & Rubia, 1977). Direct measurements of the Cl^- equilibrium potential (E_{Cl}) and comparison with the simultaneously determined $E_{i.p.s.p.}$ should resolve this problem since the two parameters would coincide if the inhibitory current was carried solely by chloride.

The first attempt to measure E_{Cl} in motoneurones with Cl^--selective micro-electrodes indicated that V_{Cl} (the potential recorded by the Cl^--selective micro-electrode) was less negative than E_m, although the i.p.s.p. were clearly hyperpolarizing (Lux, 1974). This finding appeared to favour the argument for a K^+ contribution. But the Cl^- ion exchanger used was known to be liable to interference from other anions (Walker, 1971; Brown *et al.* 1970; Lux, 1974), and thus may have given an underestimate of E_{Cl}.

The aim of the work described in this paper was to measure V_{Cl} and $E_{i.p.s.p.}$ simultaneously and to determine the relationship between them under a variety of conditions. The relationship should provide evidence for or against a pure Cl^- dependence of the i.p.s.p.

Some of these results have been reported in preliminary form (Deisz & Lux, 1976*a*, *b*, 1978).

METHODS

General

The methods, with a few exceptions, were the same as already reported (Deisz & Lux, 1977; Aickin, Deisz & Lux, 1981). The abdominal stretch receptors of crayfish (*Astacus fluviatilis* and *A. leptodactylus*) were used since their inhibitory synapses are known to be evenly distributed over the neurone and are not restricted to the dendrites (Peterson & Pepe, 1961). Thus the possibility of different ionic gradients at the site of i.p.s.p. generation and the intracellular location of the Cl^--selective micro-electrode is largely obviated. The stretch receptors were dissected (according to Wiersma, Furshpan & Florey, 1963) and mounted in a perspex chamber (volume ~ 1 ml), maintained at 15 ± 1 °C by a circulating cooled fluid. The normal Ringer solution was a modification of that described by van Harreveld (1936) and had the following composition (mM): NaCl 200; KCl, 5·4; $CaCl_2$, 13·5; $MgCl_2$, 2·6. It was buffered to pH 7·55 with 10 mM-Tris/maleate or 10 mM-HEPES (2-*N*-2 hydroxyethyl piperazine-*N'*-2-ethanesulphonic acid)/sodium hydroxide. Ringer solution with altered K^+ or NH_4^+ had appropriately altered Na^+ to maintain the osmotic strength. Cl_o^- was lowered to 50% and 15·4% by an equimolar substitution of NaCl with Na-isethionate. Gluconate salts of K^+, Mg^{2+} and Ca^{2+} were used together with Na-isethionate in the Cl^--free Ringer solution. This solution had about one third of the normal Ca^{2+} activity, as determined by a Ca^{2+}-selective electrode.

Some experiments were carried out with abdominal stretch receptors of lobster (*Homarus americanus*). The physiological saline used was as described by Pantin (1969), with the addition of 10 mM-Tris/maleate adjusted to pH 7·55.

Electrodes

Conventional micro-electrodes. These were pulled from theta capillaries (Duran or Pyrex) and filled with a mixture of 0·6 M-potassium sulphate (85%) and 1·5 M-potassium chloride (15%). The electrodes used had resistances between 20 and 40 MΩ. Sulphate was chosen as the major constituent of the filling solution since it has no effect on the $E_{i.p.s.p.}$ (Araki *et al.* 1961) and causes less interference with the Cl^--selective micro-electrode than, for example, acetate. It seems unlikely that use of this filling solution caused significant error in the recorded V_{Cl} because (*a*) comparable values were obtained when the Cl^--selective micro-electrode was inserted prior to the conventional micro-electrode, and (*b*) V_{Cl} was unaffected by repeated injection of hyperpolarizing current.

Cl^--selective micro-electrodes. Pyrex capillaries (Plowden and Thompson, 1 mm o.d.) were pulled to tip diameters beyond the resolution of the light microscope. The freshly pulled micro-pipettes were silanized by immersing their tips in a solution of 5% trimethylchlorosilane in carbon tetrachloride. The pipettes were then backfilled with 100 mM-KCl and subsequent application of pressure expelled the silane and the air bubble in front of the electrolyte. The ion-exchanger was then drawn into the tip as described by Lux & Heyer (1975).

The response characteristics of these electrodes were determined in pure KCl solutions and in solutions containing a constant concentration of other anions. Both the Orion (9825) and Corning (477315) liquid Cl^- ion exchanger gave a 54–58 mV (56·5 ± 1·6 mV; $n = 24$) response to a tenfold change in Cl^- activity in pure KCl solutions (20–22 °C). This response, however, was decreased by the presence of other anions, depending upon the ion-exchanger and the species and concentration of the anion as shown in Fig. 1. Selectivity coefficients were calculated by a non-linear least-square fit using the modified Nernst–Planck equation (Nicolsky, Shultz, Belijustin & Lev, 1967):

$$V_{Cl} = E_o + \frac{R\,T}{z\,F} \ln (a_{Cl} + \Sigma K_i\, a_A),$$

where V_{Cl} is the experimentally observed potential of the Cl^--selective micro-electrode, E_o the standard potential of both ion-selective and reference half cell, a_{Cl} and a_A are the activities of Cl^- and the interfering anions, respectively, and K_i is the selectivity coefficient. R, T, z and F have their usual meaning.

The following selectivity coefficients were obtained in the presence of a constant concentration of 30 mM interfering anion (except thiocyanate at 0·3 mM), those for the Orion exchanger being given in brackets: acetate 0·27 (0·28); citrate 0·10; isethionate 0·13; propionate 0·59; sulphate 0·11 (0·40); tartrate 0·14 and thiocyanate 67. The selectivity coefficient for bicarbonate of 0·12 (0·21) is possibly an underestimate because a fractional loss via CO_2 was neglected. The selectivity coefficient for a mixture of $H_2PO_4^-/HPO_4^{2-}$ (pH 7·5) was 0·04 ($n = 3$). The effect of amino acids on the response characteristics of the Cl^--selective micro-electrode was small (alanine, aspartate, glycine and glutamate selectivity coefficents were at or below 0·04). Most of these values are in fairly good agreement with those obtained by Saunders & Brown (1977). The use of different cations (Na^+, K^+) had no effect on the response characteristics. The Corning ion-exchanger was chosen for all intracellular measurements because of its better selectivity coefficients.

The electrodes were broken from resistances around 80 G Ω down to 20 G Ω to test for tip potentials. The potential change caused by breakage was smaller than 3 mV and is satisfactorily explained by the input current ($3·5 \times 10^{-14}$ A) of the amplifier used. The resistance of the electrodes used intracellularly, measured by a calibrated ramp-pulse, was between 60 and 90 G Ω.

Electrical arrangements

The potentials of both intracellular electrodes were differentially measured against an extracellular 2 M-KCl agar bridge, using an Ancom 15B2 amplifier for the voltage electrode and an Ancom 15A71 amplifier for the Cl^--selective micro-electrode. The common mode rejection ratio was better than −66db. A bridge circuit allowed the injection of current through the voltage electrode. Passive bridge components were subtracted with a differential amplifier. Subtraction of E_m from the intracellular voltage reading of the Cl^--selective micro-electrode was carried out at a second amplification stage using a second differential amplifier (both Analog Devices 520). The potentials of both electrodes were displayed on an oscilloscope and a pen recorder.

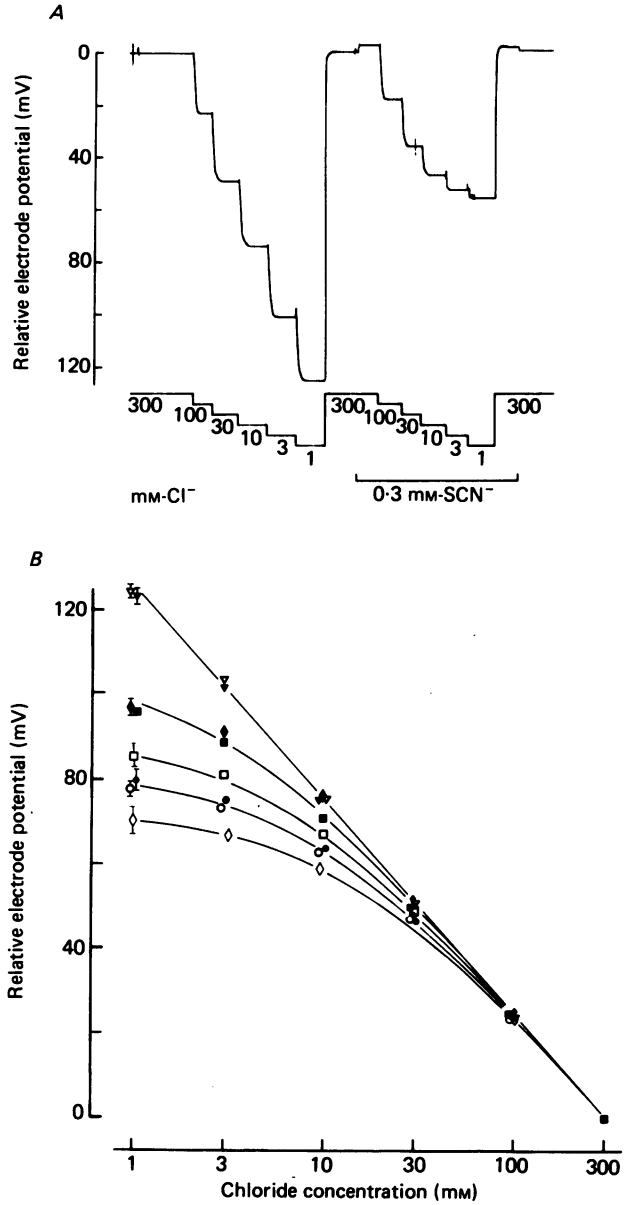

Fig. 1. *A*, pen recording of the response of a Cl^--selective micro-electrode to changes in Cl^- concentrations in the presence and absence of 0·3 mM-SCN^-. *B*, calibration graphs of two Cl^--selective ion exchangers (Corning 477315, filled symbols; Orion 9825, open symbols). The response to pure Cl^- solutions (▽, ▼) was decreased in the presence of 30 mM-acetate (○, ●), bicarbonate (□, ■) and sulphate (◇, ◆). The standard deviation, represented by the vertical bars, is only shown for the measurements in 1 mM-Cl^- where the deviation was greatest. The lines were drawn according to a non-linear least squares fit corresponding to an interference equivalent to, from top to bottom, 0·02 mM, 3·3 mM, 6·2 mM, 8·1 mM and 12·9 mM extra Cl^-.

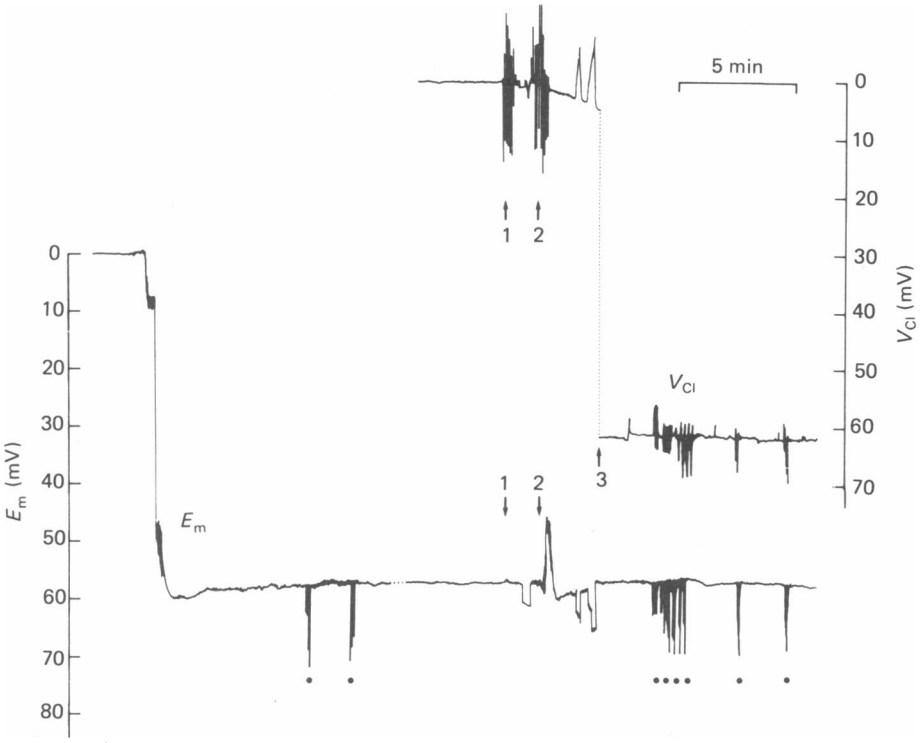

Fig. 2. Pen recording of the beginning of an experiment illustrating the procedure used to measure a^i_{Cl}. Following penetration with the voltage electrode, E_m stabilized at about 60 mV as shown in the lower trace. Current steps were applied to hyperpolarize the membrane, while i.p.s.p.s were elicited. This allowed determination of $E_{i.p.s.p.}$ (indicated by dots). The progressive hyperpolarization produced by current injection and spontaneous action potentials following penetration with both electrodes have been trunkated by the low frequency response of the pen recorder. The first attempts to impale the neurone with a Cl^--selective micro-electrode (at the time indicated by arrow 1) failed, since the hyperpolarization induced by injecting current through the conventional micro-electrode was not recorded by the Cl^--selective micro-electrode. The second attempts (arrow 2) were successful as judged from the Cl^--selective micro-electrode also recording the current-induced hyperpolarization. Following subtraction of E_m (arrow 3), the potential of the Cl^- selective micro-electrode (top trace) gives a direct recording of V_{Cl}. Due to the relative slow response of the Cl^--selective micro-electrode (time constant about 200 ms) fast components of changes in E_m are incompletely suppressed. The break in the E_m record was for a period of about 10 min while the Cl^--selective micro-electrode was placed in the bath.

Experimental procedure and analysis

Current steps (duration usually 150 ms) of increasing amplitude were used to hyperpolarize the membrane progressively while i.p.s.p.s were elicited at about 1·5 Hz. Recordings where the electrodes deviated from an ohmic behaviour during the passage of increasing current steps were discarded from determination of $E_{i.p.s.p.}$ and R_m. The changes of E_m and i.p.s.p. amplitude during 6–12 current steps were photographed from the oscilloscope screen (e.g. see Figs. 6*A* and 7*A*). E_m, i.p.s.p. amplitudes and injected current were manually analysed. Linear regression of E_m against i.p.s.p. amplitude (measured with a resolution of 0·25 mV) was used to determine $E_{i.p.s.p.}$, and that of E_m against the injected current amplitude (resolution 0·1 nA) to determine the neuronal input resistance (R_m).

RESULTS

Measurement of V_{Cl} and $E_{i.p.s.p.}$

Because determination of V_{Cl} requires correct subtraction of E_m from the voltage recorded intracellularly by the Cl^--selective micro-electrode, it was initially ensured that two micro-electrodes measured the same potential. Conventional micro-electrodes indeed recorded the same potential to within 2 mV. It is generally assumed that ion-selective micro-electrodes record the same E_m as KCl-filled electrodes, but sulphate-filled electrodes have been reported to record a lower potential in snail neurones than KCl electrodes (Thomas, 1977) while this difference is below 2 mV in sheep heart Purkinje fibres (Vaughan-Jones, 1979). The use of the K_2SO_4/KCl mixture in the experiments may therefore cause an underestimate of V_{Cl}. However this will not affect the comparison of V_{Cl} and $E_{i.p.s.p.}$ since both are derived from the measured E_m.

The procedure adopted for measurement of V_{Cl} is illustrated in Fig. 2. The soma of the slowly adapting stretch receptor neurone was usually impaled first with the voltage electrode, seen in the downward deflexion of the voltage trace. Neurones with an R_m of less than 2 M Ω and E_m of less than 56 mV were discarded, as were those in which there was serious polarization during passage of current through the intracellular electrode. Comparable $E_{i.p.s.p.}$ values obtained in later experiments using separate current and voltage electrodes (Deisz & Lux, 1977; Deisz, Aickin & Lux, 1979; Aickin *et al.* 1981) suggest that the criteria for bridge balance were adequate. The stimulating electrodes were then positioned to evoke the i.p.s.p. and the antidromic action potential, at least of 70 mV amplitude in acceptable neurones. The soma was then impaled with the Cl^--selective micro-electrode. The potential change recorded by this electrode on penetration was very small (1–2 mV), and therefore changes in E_m, induced by current injection were used to ascertain its position. Following a successful penetration E_m was subtracted electronically from the potential of the Cl^--selective micro-electrode to give V_{Cl} (see Fig. 2). R_m often fell below the acceptable limit on insertion of the Cl^--sensitive micro-electrode and the neurone had to be discarded. In cases of no detectable i.p.s.p., but acceptable E_m and R_m, the experiment was continued.

Of about 300 neurones tried only eleven fulfilled all the above criteria. The mean values (given here and throughout the Results as mean ± S.D. of an observation) were E_m 62·6 ± 3·9 mV, $E_{i.p.s.p.}$ 74·5 ± 1·9 mV and V_{Cl} 63·3 ± 2·3 mV. The mean V_{Cl} yields an apparent a^i_{Cl} of 12·7 mM (average slope of 55·5 mV per decade change a_{Cl} at 15 °C and a^0_{Cl} of 175 mM). Thus a^i_{Cl} seems considerably higher than anticipated from the Nernst-equation for a Cl^- dependent $E_{i.p.s.p.}$ (8·6 mM).

The difference between $E_{i.p.s.p.}$ and V_{Cl} is similar to, although smaller than, that found in cat spinal motoneurones (Lux, 1974). It could be due either to ions other than Cl^- contributing to the i.p.s.p. or to other anions adding to the potential developed by the Cl^--selective micro-electrode. These alternatives can be tested by inducing changes in $E_{i.p.s.p.}$ or V_{Cl} since in the former case an approximately constant voltage deviation between $E_{i.p.s.p.}$ and V_{Cl} might be anticipated, and in the latter an increasing deviation with an increasing $E_{i.p.s.p.}$.

Variation in extracellular Cl^-.

Complete replacement of Cl_o^- transiently decreased E_m by up to 15 mV, while the firing of action potentials persisted. V_{Cl} increased with a half-time of about 1 min to a mean value of 84·6±2·7 mV ($n = 5$). I.p.s.p.s were initially depolarizing and initiated action potentials, but then rapidly dwindled to an undetectable size. The reversible abolition of i.p.s.p. in the presence of maintained firing of action potentials

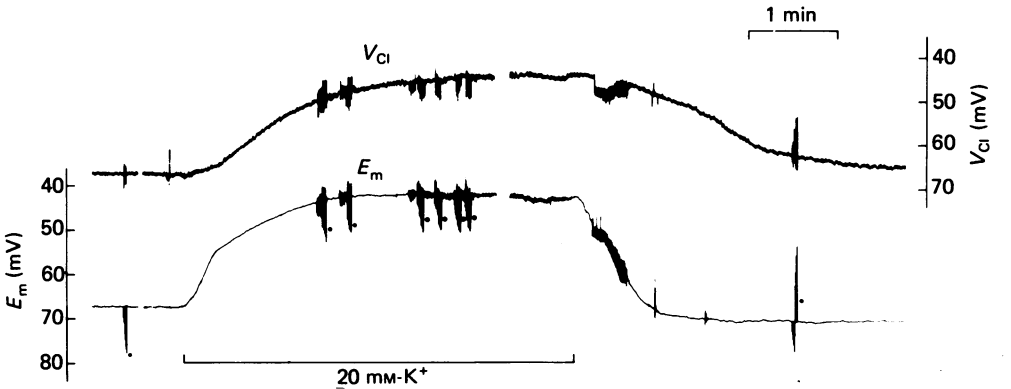

Fig. 3. Pen recording of part of an experiment showing the effects of 20 mM-K^+ on E_m, V_{Cl} and $E_{i.p.s.p.}$ (indicated by dots). On return to normal Ringer solution, the neurone began to fire action potentials spontaneously. The breaks in the records were for 3 min before and 1·5 min during 20 mM-K^+ application.

seems unlikely to have been mediated by conduction failure at the efferent nerve. Reducing Cl_o^- to 50% had no effect on E_m but V_{Cl} increased from 63 mV to 74 mV with a half time of about 1 min (the predicted V_{Cl} for a proportional decrease would be 79 mV). $E_{i.p.s.p.}$ transiently fell but recovered to close to the control values (72·4±0·4 mV compared with 73·9+0·5 mV in normal Ringer solution, $n = 6$). In six neurones without detectable i.p.s.p.s, decreasing Cl_o^- to 15·4% of normal increased V_{Cl} to 76·9±2·8 mV (the predicted V_{Cl} for a proportional decrease would be 108·4 mV).

Effects of alterations in K_o^+

Alterations of K_o^+ are known to affect both $E_{i.p.s.p.}$ (Motokizawa *et al.* 1969) and V_{Cl} (Ascher *et al.* 1976). On application of Ringer solution containing 20 mM-K^+, V_{Cl} declined to values between 40 and 47 mV (43·0±2·6 mV, $n = 9$) with a half-time of about 1 min (see Fig. 3). The neurones depolarized fairly rapidly (half-time about 20 s) and reached a mean stable E_m of 41·3±2·7 mV ($n = 9$), with a range from 38–46 mV. During the depolarization, the neurones usually started to fire action potentials of progressively declining amplitude, and when they had become quiescent again, antidromic action potentials had a greatly reduced amplitude. R_m fell by an average of 70% to values between 0·9 and 2·0 M Ω. Only five out of nine neurones still had i.p.s.p.s of detectable amplitude but $E_{i.p.s.p.}$ was consistently more negative than E_m by 3–5 mV and had a mean value of 45·0±1·9 mV. All parameters recovered within 10 min on return to normal Ringer solution.

Removal of K_o^+ increased V_{Cl} to values between 68 and 79 mV (74·2 ± 3·5 mV, $n = 12$) with a half-time of about 1 min (see Fig. 4). E_m increased by 15–34 mV (half time about 20 s) and stabilized at values between 80 and 100 mV (88·8 ± 7·7 mV, $n = 12$). During this increase in E_m, i.p.s.p.s transiently reversed their polarity (not shown in Fig. 4) but became hyperpolarizing again within 3 min. $E_{i.p.s.p.}$ stabilized at a mean value of 104·7 ± 3·4 mV ($n = 6$) with a range of 100–110 mV.

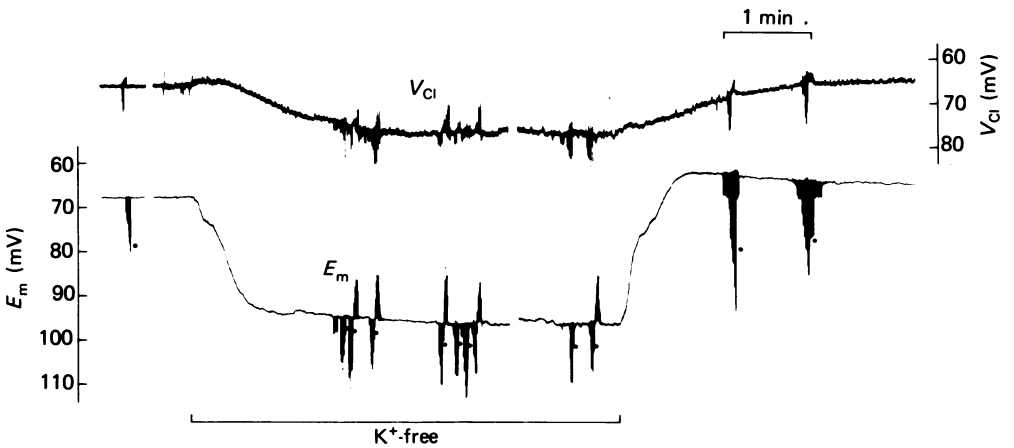

Fig. 4. Pen recording of part of an experiment showing the effects of removal of K_o^+ on E_m, V_{Cl} and $E_{i.p.s.p.}$ The breaks in the records were for 1·2 min before and 2·5 min during K^+-free Ringer application.

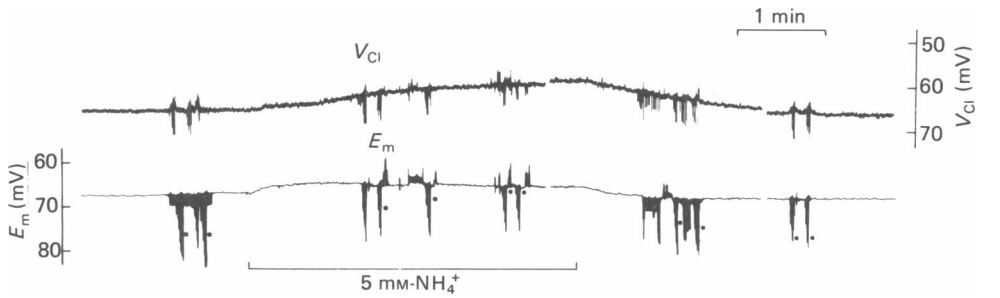

Fig. 5. Pen recording of part of an experiment illustrating the effects of 5 mM-NH_4^+ application on E_m, V_{Cl} and $E_{i.p.s.p.}$ The breaks in the records were for 3 min during and after NH_4^+ application. During the first sequences of current steps both in the presence of 5 mM-NH_4^+ and after its removal maintained stimulus parameters failed to elicit i.p.s.p.s. However, a slight increase in the intensity of the stimulation re-evoked the i.p.s.p.

Effects of NH_4^+-application

NH_4^+ is well known to reduce post-synaptic inhibition in many preparations (Lux, Loracher & Neher, 1970; Lux, 1971; Llinás, Baker & Precht, 1974; Meyer & Lux, 1974; Raabe & Gumnit, 1975; Nicoll, 1978; Iles & Jack, 1980) except cat hippocampus (Allen *et al.* 1977). The effect of 5 mM-NH_4^+ application is illustrated in Fig. 5. E_m depolarized fairly rapidly by 1·5–4 mV. V_{Cl} decreased considerably more slowly by 4–8 mV (6·4 ± 1·8 mV, $n = 13$) and was finally less negative (56·5 ± 3·7 mV) than the mean E_m (61·3 ± 4·8 mV). The decrease of V_{Cl} corresponded to an increase in apparent

a^i_{Cl} to 16·8 mM, hence above the value expected from a passive distribution. Concomitant with the decline in V_{Cl}, $E_{i.p.s.p.}$ fell by 8–12 mV (10·6 ± 1·7 mV, $n = 7$), but remained more negative than E_m in five out of seven neurones. In the remaining two neurones $E_{i.p.s.p.}$ was equal to E_m. The mean reduction in i.p.s.p. driving force ($E_m - E_{i.p.s.p.}$) was by 79·2 ± 17·6 % ($n = 7$). R_m decreased by 16–29 % (20·0 ± 5·5 %, $n = 7$). The slight depolarization and the decrease in R_m, also reported by Hino (1979), are probably attributable to the K^+-like effects of NH_4^+ (Hille, 1973).

The decrease in V_{Cl} (or increase in a^i_{Cl}) on application of NH_4^+ is consistent with the proposed inhibition of a Cl^- extrusion mechanism (Lux, 1971). However, the observed decrease in R_m may represent an increased Cl^- influx, thus yielding the higher a^i_{Cl}. Picrotoxin (PTX) was applied in an attempt to inhibit Cl^- influx since it is known to block the inhibitory synaptic channels (Iwasaki & Florey, 1969; Takeuchi & Takeuchi, 1969; Aickin *et al.* 1981) and has been suggested to block extrasynaptic Cl^- channels (Ozeki, Freeman & Grundfest, 1966). Application of PTX 10^{-4} M increased V_{Cl} by 2 mV, indicating a considerable Cl^- influx via PTX-sensitive channels. However, application of 5 mM-NH_4^+ in the presence of PTX caused a similar decrease in V_{Cl} to that observed in the absence of PTX. This suggests that the NH_4^+-induced increase in a^i_{Cl} is not via an increase in the PTX-sensitive Cl^- conductance. It is worth mentioning that application of diphenylhydantoin (10^{-4} M) causes a similar reduction in R_m to application of 5 mM-NH_4^+, in this case through potentiation of spontaneous synaptic events, but only slightly reduced $E_{i.p.s.p.}$ (Deisz & Lux, 1977; Aickin *et al.* 1981).

Effects of inhibitors of Cl^- transport

The above results clearly show that the discrepancy between $E_{i.p.s.p.}$ and V_{Cl} does not remain constant but decreases with decreasing potentials and *vice versa*. This, together with the apparent a^i_{Cl} of 5·2 mM (V_{Cl} of 84·6 mM) in Cl^--free Ringer solution, is consistent with anions other than Cl^- contributing to the intracellularly recorded V_{Cl}. This implies that a^i_{Cl} is lower than expected from a passive distribution and hence suggests the presence of a mechanism extruding Cl^- against the prevailing electrochemical gradient. In addition to the NH_4^+ effects described above, we therefore have tested a variety of substances reported to inhibit Cl^- transport.

(*a*) *Effect of acetazolamide.* Acetazolamide, an inhibitor of carbonic anhydrase, has been reported to affect Cl^- transport in rabbit ileum (Nellans, Frizzell & Schultz, 1975) and frog skin (Watlington, Jessee & Baldwin, 1977). Application of acetazolamide at 10^{-4} M left E_m and V_{Cl} unaltered. In two of the four neurones tested, i.p.s.p.s could be elicited and $E_{i.p.s.p.}$ was found to be unaltered. This suggests that carbonic anhydrase does not play a major role in the maintenance of the hyperpolarizing Cl^- gradient.

(*b*) *Effect of SCN^-.* SCN^-, a long known inhibitor of the Cl^- transport involved in gastric acid secretion (Davenport, 1940), has been reported to block Cl^- transport in various preparations (Zadunaisky, Candia & Chiarandini, 1963; Epstein, Maetz & DeRenzis, 1973). As shown in Fig. 6, application of 5·4 mM-SCN^- caused a rapid and reversible fall in V_{Cl} of 16·2 ± 3·1 mV ($n = 5$), but neither E_m, $E_{i.p.s.p.}$ nor R_m were consistently affected. It should be noted that due to the extraordinarily high selectivity coefficient for SCN^- (67), this apparent increase of a^i_{Cl} of about 12 mM could have been produced by only about 0·2 mM intracellular SCN^-.

(*c*) *Effect of frusemide.* Fig. 7 illustrates the effect of application of frusemide

(6×10^{-4} M) known to inhibit Cl^- transport in a variety of preparations (Burg, Stoner, Cardinal & Green, 1973; Candia, 1973; Cousin & Motais, 1976; Boron, Russell, Brodwick, Keifer & Ross, 1978). In seven out of nine neurones tested E_m decreased slowly by up to 3 mV, stabilizing at a mean of $63{\cdot}0 \pm 3{\cdot}0$ mV compared with $64{\cdot}4 \pm 3{\cdot}2$ mV in normal Ringer solution. E_m was unaffected in the other two neurones. V_{Cl}

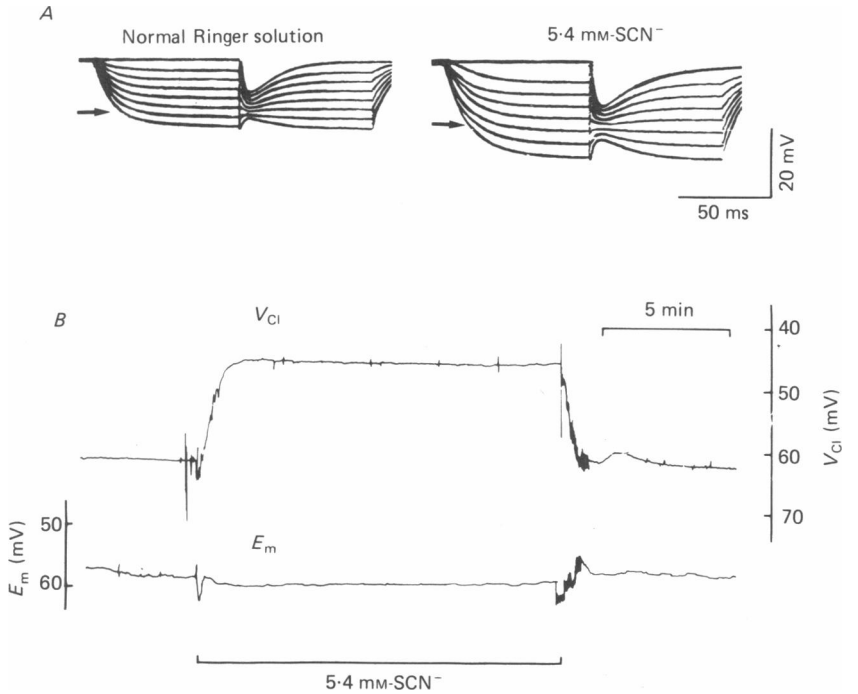

Fig. 6. *A*, oscillographs showing the lack of effect of 5·4 mM-SCN^- on $E_{i.p.s.p.}$ (indicated by arrows). E_m was 59 mV during SCN^- application. Current steps (not shown) were each of 0·5 nA. *B*, pen recording of part of another experiment illustrating the effect of 5·4 mM-SCN^- application on V_{Cl} and E_m.

slowly decreased by 2–5 mV to become less negative than E_m, reaching a mean of $59{\cdot}1 \pm 3{\cdot}5$ mV. Concomitant with the decline in V_{Cl}, $E_{i.p.s.p.}$ decreased by 4–12 mV to stabilize at $67{\cdot}6 \pm 2{\cdot}7$ mV ($n = 9$). In neurones where frusemide induced a small change in $E_{i.p.s.p.}$ (4–6 mV) the V_{Cl} change was also small (2–3 mV). The i.p.s.p. driving force ($E_m - E_{i.p.s.p.}$) was decreased by $57{\cdot}6 \pm 11{\cdot}2$ %. R_m was not significantly affected.

Measurement of V_{Cl} and $E_{i.p.s.p.}$ in lobster stretch receptor neurones

The effect of interfering anions on the measured V_{Cl} is obviously smaller at higher Cl^- activities (see Fig. 1). We therefore attempted to measure V_{Cl} in lobster stretch receptor neurones where a higher a^i_{Cl} might be anticipated from the higher Cl_o^-. From a larger number of neurones only two were acceptable on the criteria given in the Methods. One of these neurones was impaled with a Cl^--selective micro-electrode. E_m was 57 mV while the $E_{i.p.s.p.}$ was 63 mV and V_{Cl} 59 mV. The other neurone was impaled with an additional current electrode to facilitate $E_{i.p.s.p.}$ determinations. The difference between E_m and $E_{i.p.s.p.}$, although smaller than in crayfish neurones (5 mV), was reduced by about 50 % with frusemide and 100 % by 5 mM-NH_4^+.

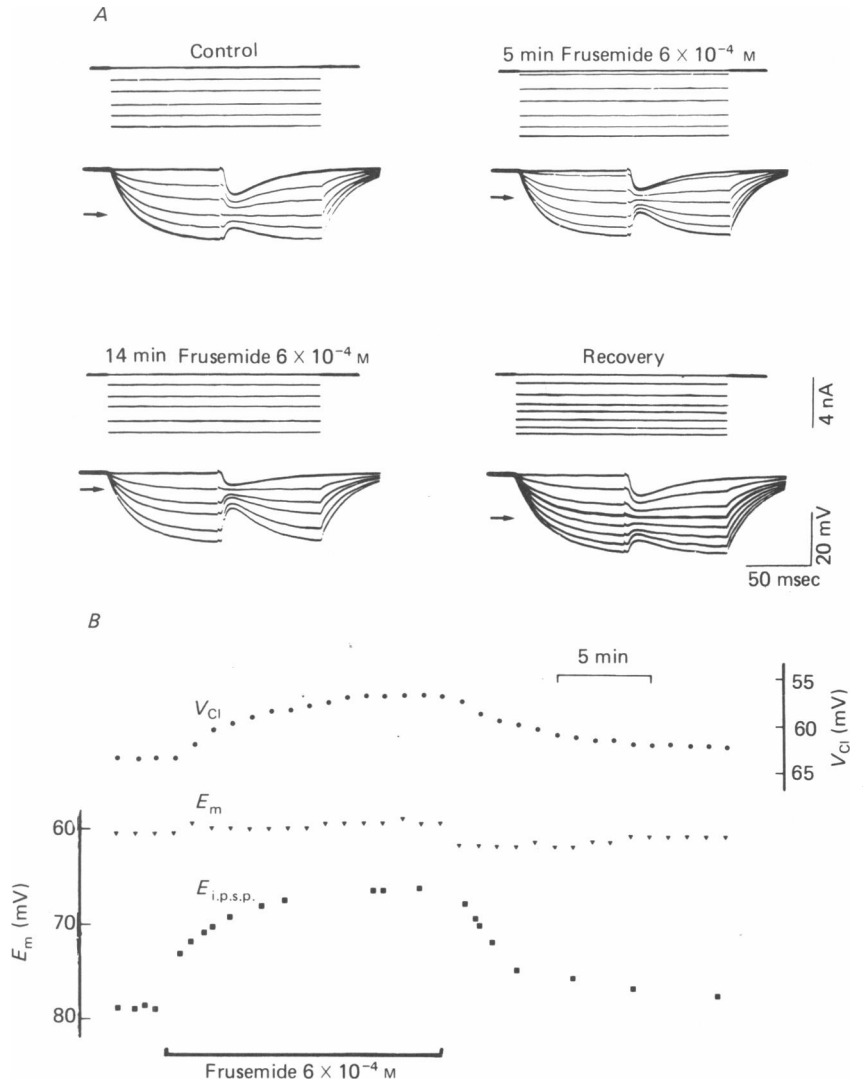

Fig. 7. *A*, oscillographs showing the effects of frusemide (6×10^{-4} M) application on the i.p.s.p. elicited at various membrane potentials obtained by progressively increasing the amplitude of the injection current shown at the top of each pair of records. E_m was 61·5, 61, 60·5 and 6·25 mV in the preceding control, at 5 and 14 min after frusemide application and 5 min after its removal, respectively. $E_{i.p.s.p.}$ is indicated by the arrows. *B*, time course of changes of *E*m (▼), V_{Cl} (●) and $E_{i.p.s.p.}$ (■) on application and removal of frusemide 6×10^{-4} M.

DISCUSSION

At first sight the similarity between V_{Cl} and E_m would be consistent with a passive Cl^- distribution, and, together with the disparity between V_{Cl} and $E_{i.p.s.p.}$, with a K^+ component in the generation of the i.p.s.p. (Eccles *et al.* 1964; Allen *et al.* 1977; Brown, Ottoson & Rydqvist, 1978). However, V_{Cl} will only equal E_{Cl} if the Cl^--selective

micro-electrode gave a Nernstian response and no other anions contributed to the potential recorded by the electrode. First, the electrodes used produced less than the Nernstian response in pure KCl solutions. Hence a V_{Cl} less negative than E_m would indicate a passive Cl^- distribution. But V_{Cl} was more negative than E_m in normal Ringer solution. Secondly, the electrodes showed considerable interference from other anions which, if present intracellularly, would further decrease V_{Cl}. Since total

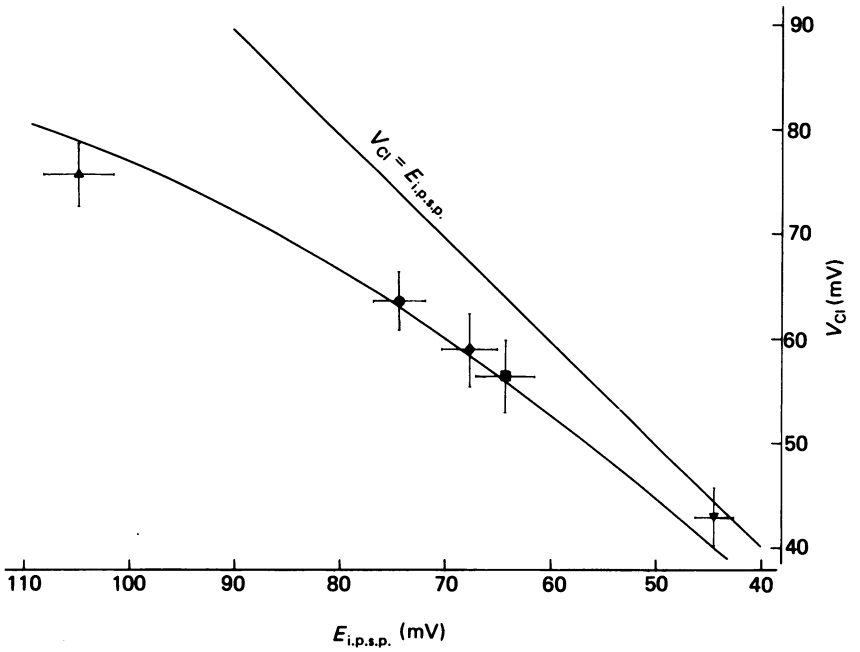

Fig. 8. Graph showing the relationship of the simultaneously determined V_{Cl} and $E_{i.p.s.p.}$ in different experiment conditions. The mean values have been plotted (K^+-free ▲, normal Ringer solution ●, frusemide ◆, NH_4^+ ■ and 20 mM-K^+ ▼) and the vertical and horizontal bars denote the standard deviation. The curve was drawn with the assumption of $E_{Cl} = E_{i.p.s.p.}$ and a constant intracellular interference on the Cl^- selective micro-electrode equivalent to 4 mM-Cl^-.

removal of Cl_o^- resulted in a rapid fall in a_{Cl}^i, little Cl^- would be anticipated to remain intracellular, but V_{Cl} indicated a level equivalent to 5·2 mM-Cl^-. Similar apparent levels of a_{Cl}^i in Cl^--free Ringer solutions have been reported in a variety of preparations and have generally been assumed to represent anionic interference (Thomas, 1977; Spring & Kimura, 1977; Vaughan-Jones, 1979; Aickin & Brading, 1980). If this value is subtracted from the apparent a_{Cl}^i measured in normal Ringer solution, the calculated E_{Cl} was approximately equal to the $E_{i.p.s.p.}$, consistent with a Cl^--dependent i.p.s.p. Further evidence against a K^+-contribution to the i.p.s.p. is provided by the rapid disappearance of i.p.s.p.s in Cl^--free Ringer solution.

If the apparent a_{Cl}^i in Cl^--free Ringer solution does represent intracellular interference and the interference remains approximately constant, V_{Cl} would show an increasing divergence from E_{Cl} as a_{Cl}^i itself decreased (see Fig. 1). The fact that this divergence was observed between V_{Cl} and $E_{i.p.s.p.}$ further suggests that E_{Cl} and

$E_{i.p.s.p.}$ may be equal. The experimental data, summarized in Fig. 8, fit reasonably well to the curve drawn under the assumption that E_{Cl} is equal to $E_{i.p.s.p.}$ and that there is a constant interference equivalent to 4 mM-Cl^-. The slight deviation of the data from the theoretical curve could be accounted for by a decrease in the activity of interfering anions at high a^i_{Cl} and an increase at low a^i_{Cl}. Such a compensatory change seems reasonable from electroneutrality considerations. It is noteworthy that a similar degree of interference in the cat motoneurone would convert the depolarizing V_{Cl} recorded (Lux, 1974) into a hyperpolarizing E_{Cl}.

Thus the present results suggest that E_{Cl} approximates $E_{i.p.s.p.}$ and that a^i_{Cl} is lower than predicted from a passive distribution. This is at variance with previous measurements of a^i_{Cl} in the crayfish stretch receptor neurone. Brown *et al.* (1978) reported that a^i_{Cl} was considerably higher than predicted from a passive distribution, and since i.p.s.p.s are usually reported to be hyperpolarizing in this preparation (Meyer & Lux, 1974) they concluded that i.p.s.p.s must be generated by both Cl^- and K^+ ions. However, they used fairly blunt 3M-KCl electrodes (5–10 MΩ) known to cause a significant increase in a^i_{Cl} in snail neurones (Thomas, 1977). The agreement of data with both liquid ion exchanger electrodes and Ag/AgCl electrodes (Brown *et al.* 1978), was probably coincidental because the latter produce an offset of up to 27 mV when intracellular (Neild & Thomas, 1974).

A lower a^i_{Cl} than predicted from a passive distribution implies the presence of a Cl^- extrusion mechanism. The elevation of a^i_{Cl} in the presence of NH_4^+ (also seen in *Aplysia* neurones: Ascher *et al.* 1976; Russell, 1978) may reflect an inhibition of this mechanism as originally proposed by Lux (1971). Similarly the increase in a^i_{Cl} on application of frusemide (6×10^{-4} M) probably represents a decreased net outward transport of Cl^- ions. If Cl^- dependence of the hyperpolarizing i.p.s.p. is common to all neurones, this action may underlie the decline in $E_{i.p.s.p.}$ seen with NH_4^+ in motoneurones of cat (Lux *et al.* 1970; Llinás *et al.* 1974; Iles & Jack, 1980) and frog with both substances (Nicoll, 1978). The apparent increase in a^i_{Cl} on application of SCN^- in absence of any change in $E_{i.p.s.p.}$, however, does not readily fit the argument for equality of E_{Cl} and $E_{i.p.s.p.}$. Nevertheless, the Cl^--selective electrode had an extraordinarily high selectivity coefficient for SCN^- over Cl^- of about 67. Thus the apparent increase in a^i_{Cl} may simply reflect an increase in interference. It is worth noting that the apparent increase in a^i_{Cl} of 12 mM in the presence of 5·4 mM-SCN^- could have been caused by 0·2 mM-SCN^-, which is less than the amount predicted from a passive distribution.

In conclusion these results show that Cl^- ions are not in equilibrium across the neuronal membrane, E_{Cl} being more negative than E_m and possibly equal to $E_{i.p.s.p.}$. This removes the primary reason for a proposal of a K^+ contribution to the hyperpolarizing inhibitory current.

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