Anion Permeation in GABA- and Glycine-Gated Channels of Mammalian Cultured Hippocampal Neurons

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Anion permeation in GABA- and glycine-gated channels of mammalian cultured hippocampal neurons

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SUMMARY

Single-channel currents through GABA- and glycine-activated chloride channels of post-natal tissue-cultured hippocampal neurons were measured to determine their anion selectivity and their concentration dependence of permeation. Current–voltage relations for both agonists displayed rectification with single-channel conductance increasing at positive potentials. Permeabilities determined from reversal potentials were maximal for anions with a diameter of about 4 Å†. Larger diameter anions had lower permeabilities, consistent with an approximate pore diameter of 6 Å for both agonist-activated channels. The permeability for anions of similar size was greatest for those ions with a more symmetrical charge distribution (e.g. NO_3^- > Bicarbonate$^-$). The permeability sequence was $SCN^- > NO_3^- > I^- > Br^- > Cl^-$ > Formate$^-$ > Acetate$^-$ > Bicarbonate$^-$ > Gluconate$^-$ > F^- > Phosphate$^-$, whereas the conductance sequence for anion efflux was $Cl^- > Br^- > NO_3^- > I^- > SCN^-$ > Formate$^-$ > Acetate$^-$ > Bicarbonate$^-$ > Gluconate$^-$ > F^- > Phosphate$^-$. These results suggest that the ions interact with sites within the channel, with hydration forces contributing an important component to the barrier for ion entry into the channel. The spherically symmetrical halides displayed an exponential relation between relative permeability and hydration energy. Concentration dependence of conductance for Cl^- channels in symmetrical Cl^- solutions with agonist in the pipette showed an increase at positive potentials and a decrease at negative potentials. GABA- and glycine-activated channels also exhibited anomalous mole-fraction effects in a mixture of Cl^- and SCN^-. These results suggest that both agonist-activated channels act as multi-ion pathways and have similar permeation characteristics.

1. INTRODUCTION

Bicuculline-sensitive GABA- and strychnine-sensitive glycine-gated currents were observed in post-natal tissue-cultured hippocampal neurons (Fatima-Shad & Barry 1992), in contrast with previous studies in which bicuculline (Curtis *et al.* 1970) but not strychnine (Andersen *et al.* 1963) was observed as the main blocker of inhibitory inputs in the adult hippocampus *in vivo*. In our previous paper we also showed that the inhibitory transmitters of the central nervous system bind to separate receptors to activate their anionic channels.

In this paper, we describe the anion selectivity and concentration dependence of permeation in both GABA- and glycine-gated chloride channels in tissue-cultured hippocampal neurons. Our present study reveals that the relative anion permeabilities for both GABA- and glycine-gated channels were similar and were consistent with earlier studies on IPSPs (Eccles *et al.* 1977). We also observed that the concentration dependence and mole-fraction dependence of permeation were very similar for both types of channels. The estimated minimum pore diameter and the magnitude of the outward rectification for both GABA and glycine channels were also very similar in our preparation. In contrast, in pre-natal tissue-cultured spinal neurons, Bormann *et al.* (1987) found no rectification of single-channel currents of GABA- and glycine-gated channels, and found that the pore size of the open GABA-gated channel was slightly larger than glycine-gated channel. These differences could be due to the age of the preparation or any of the other reasons which we discuss later on. In this paper we describe the anion selectivity and concentration dependence of permeation in both GABA- and glycine-gated chloride channels in tissue-cultured hippocampal neurons.

† 1 Å = 10^{-10} m.

2. METHODS

(*a*) *General*

Tissue-culture preparation of post-natal hippocampal neurons and electrophysiological techniques have been described in more detail elsewhere (Fatima-Shad & Barry 1992). Briefly, one-day-old rat pups were quickly decapitated, and their hippocampi removed. Cells were mechanically dissociated in 3 ml ice-cold Puck's saline and incubated in EMEM (pH 7.4), at 37 °C in an atmosphere of humidified air with 5% CO_2. Cells were used from the third day of preparation onwards.

We used giga-seal recording methods (Hamill *et al.* 1981),

generally with the inside-out excised patch configuration in which the cytoplasmic side faces towards the bath solution. The sleeve technique (Quartararo & Barry 1987) was used to rapidly change the bath solution to determine reversal potentials of chloride and a number of other anions, and thereby evaluate the relative permeability sequences of the ions for both channels.

The reversal potentials of these GABA- and glycine-activated currents were determined by applying voltage pulses to excised inside-out patches with agonist and Cl^- in the pipette and mainly foreign anions in the bath. The currents were sampled at 10 kHz and generally filtered at 5 kHz. The permeability ratio was calculated by using the Goldman–Hodgkin–Katz equation from reversal potential shifts determined by substituting the test ions in the bath. The conductance ratios for SCN^-, I^-, NO_3^- and Br^- were determined from single-channel conductances with symmetrical substitution of each ion in the bath and the pipette solutions. For all the other polyatomic anions (formate, acetate, bicarbonate, gluconate and phosphate) and F^-, the ratio was determined by measuring the inward current at the Cl^- reversal potential, which was -72 mV (as the bath solution contained 132 mM of foreign polyatomic anion or F^- and 8 mM of Cl^-, and the pipette solution 140 mM of Cl^- and agonist). Concentration dependence of single-channel conductance was measured at five different symmetrical Cl^- concentrations for both agonists. The conductance mole-fraction dependency of channels was observed in mixtures of Cl^- and SCN^- at mole fractions of the internal SCN^- solution of 0, 0.2, 0.7 and 0.9.

(*b*) *Solutions*

The cells were bathed in mammalian Ringer containing (in millimoles per litre): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 2, Na-HEPES (*N*-[2-Hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid]) 5 (pH 7.4). For single-channel recording of excised, inside-out membrane patches, the pipette contained (in millimoles per litre): NaCl 134, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, and 2 μmol l^{-1} GABA or 5 μmol l^{-1} glycine, and the bath solution contained (in millimoles per litre); NaCl 138, $MgCl_2$ 1, EGTA 5, HEPES 10. In both solutions, the pH was adjusted to 7.4 with NaOH.

For experiments on the relative permeability of halides and polyatomic anions, the sleeve technique was used for exchanging the bath solution at the cytoplasmic side of the patch. In the bath solution the 140 mM Cl^- was replaced by 132 mM of test anion and 8 mM Cl^-. The pH of the bath solution was 7.4 except when phosphate or bicarbonate was present in the bath as a test ion, in which case the pH of the bath solution was changed to 6.0 and 8.5, respectively, to have these anions primarily in the monovalent form. For the dependence of Cl^- current on Cl^- concentration, single-channel conductances at five symmetrical Cl^- concentrations were obtained. Patches were obtained with each Cl^- concentration as the pipette solution and with the standard bath solution. After obtaining Cl^- currents, the bath was changed to symmetrical Cl^- activities of 50 mM, 105 mM, 215 mM, 405 mM and 510 mM. The mole-fraction dependency of the channels was observed by using two highly permeant anions Cl^- and SCN^-, maintaining the total anion concentration constant on both sides of the membrane. Pipette solutions remained constant at 140 mM Cl^-, whereas the bath solution was changed at the cytoplasmic side of the membrane with the sleeve technique. The four different bath compositions of Cl^- and SCN^- were, respectively (with mole fraction in parenthesis and concentrations in millimoles per litre): 140 Cl^- and 0 SCN^- (0), 112 Cl^- and 28 SCN^- (0.2), 42 Cl^- and 98 SCN^- (0.7) and 14 Cl^- and 126 SCN^- (0.9). Voltage steps were applied to obtain the current–voltage relation and hence the reversal potential at each mole fraction.

No currents were observed in inside-out excised patches when there was no agonist in the pipette, confirming that the currents passing through these anion channels were agonist activated. This was further confirmed by the fact that no single-channel currents were observed in outside-out patches exposed to standard bath solution with no agonist in bath or pipette.

Data fitting was achieved with two 'in-house' nonlinear regression-fitting programs: reversal potentials, by fitting data to a polynomial curve, and permeability, by fitting to the Goldman-Hodgkin-Katz equation. All voltages have been corrected for junction potentials by using the program JPCALC. (Further information about the program and its availability can be obtained from its author, P. H. Barry.) All voltages have been expressed as the potential of the interior membrane surface with respect to the exterior one.

3. RESULTS

Single-channel currents were measured through GABA- and glycine-activated chloride channels to determine their anion selectivity, relative conductance, and their conductance dependency on concentration and mole fraction.

(*a*) *Anion selectivity*

Halide permeabilities, determined from reversal potentials, had a sequence of $SCN^- > NO_3^- > I^- > Br^- > Cl^- > F^-$, and were maximal for anions with a diameter of about 4 Å (e.g. SCN^-, NO_3^-, I^-), suggesting that the interactions of these larger anions with the channel are more favourable than those of smaller anions. Relative permeabilities of other anions were similarly measured in excised inside-out patches from reversal potential shifts of single-channel current–voltage relations after test anion substitution in the bath (cytoplasmic) solution ($n = 15$ for both agonists). Table 1 shows the relative permeabilities for both halides and polyatomic anions. Larger diameter ions had lower permeabilities, consistent with a minimum pore diameter of 6 Å for both GABA- and glycine-activated channels as both receptor channels were permeable to gluconate and phosphate. The permeability sequence for both channels was $SCN^- > NO_3^- > I^- > Br^- > Cl^-$ > Formate$^-$ > Acetate$^-$ > Bicarbonate$^-$ > Gluconate$^-$ > F^- > Phosphate$^-$. Figure 1*a* shows the permeabilities of the halide and other polyatomic anions relative to Cl^-, plotted against their diameters for both GABA- and glycine-gated channels. As shown in this figure, the permeability for anions of similar size was largest for those ions with a more symmetrical charge distribution (e.g. NO_3^- is many times more permeable than bicarbonate$^-$ even though both ions are of similar size). However, for the spherically symmetrical halides the relative permeability is directly related to their diameter. A plot of relative permeabilities as a function of relative hydration energy in units of RT (figure 1*b*) displays an exponential relation between relative permeability and hydration energy (e.g. F^- is the smallest halide with

Table 1. *Permeability ratios for both GABA- and glycine-gated Cl^- channels were calculated by using the Goldman-Hodgkin-Katz equation from reversal potential shifts that result from substituting 132 mM of test ion in the bath along with 8 mM of Cl^-, the pipette solution remaining constant with 140 mM Cl^- plus agonist*

(The conductance ratio was determined from single-channel conductances in the presence of the symmetrical substitution of halides. For all the other polyatomic anions and F^-, the conductance ratio was determined by measuring the inward current at the Cl^- reversal potential (−72 mV), when the Cl^- in the bath solution was substituted by 132 mM of test anion and 8 mM Cl^-. Such currents were compared with a similarly obtained value for Br^- (bath substitution of 132 mM Br^- and 8 mM Cl^- at −72 mV), to obtain the relative conductance γ_x/γ_{Br} and was multiplied by the Br^-:Cl^- conductance ratio, γ_{Br}/γ_{Cl}, to determine the conductance of these anions relative to Cl^-. Given the large rectification ratio for Cl^- conductance at ±100 mV (2.14 for GABA and 2.23 for glycine), the above data suggest that for the halides the relative influxes at positive potentials are greater than the effluxes at negative potentials. The errors shown represent the s.e.m.)

	GABA			glycine		
Anions	P_x/P_{Cl}	γ_x/γ_{Cl} (−100 mV)	γ_x/γ_{Cl} (+100 mV)	P_x/P_{Cl}	γ_x/γ_{Cl} (−100 mV)	γ_x/γ_{Cl} (+100 mV)
SCN^-	4.25±0.06	0.38±0.01	0.40±0.01	4.3±0.05	0.41±0.05	0.42±0.01
NO_3^-	2.7±0.05	0.58±0.02	0.76±0.02	2.8±0.06	0.65±0.03	0.80±0.02
I^-	2.4±0.05	0.57±0.02	0.46±0.01	2.3±0.03	0.61±0.02	0.47±0.01
Br^-	1.4±0.04	0.63±0.03	0.80±0.01	1.5±0.05	0.71±0.03	0.89±0.02
Cl^-	1	1	1	1	1	1
Formate$^-$	0.55±0.02	0.31±0.009[a]	—	0.6±0.02	0.3±0.005[a]	—
Acetate$^-$	0.49±0.02	0.3±0.008[a]	—	0.5±0.01	0.29±0.009[a]	—
Bicarbonate$^-$	0.44±0.02	0.26±0.008[a]	—	0.4±0.02	0.25±0.007[a]	—
Gluconate$^-$	0.11±0.03	0.22±0.005[a]	—	0.1±0.04	0.21±0.01[a]	—
F^-	0.09±0.02	0.49±0.015[a]	—	0.09±0.01	0.054±0.001[a]	—
Phosphate$^-$	0.009±0.01	0.048±0.001[a]	—	0.006±0.01	0.053±0.001[a]	—

[a] Relative conductances for these anions were obtained at −72 mV (E_{Cl}).

most negative hydration energy and lowest permeability). The fact that, in both GABA and glycine receptor channels, permeability sequences are dominated by hydration energies suggests that, in both channel types, ion–channel interaction is relatively weaker than ion–water interactions. This implies the presence of weak field strength sites (see, for example, Eisenman & Horn 1983) in both agonist-activated channels.

Conductance ratios determined from single-channel conductance with symmetric substitution of SCN^-, I^-, NO_3^-, Br^-, and single-sided bath (cytoplasmic) substitution of polyatomic anions and F^-, suggest that the anion influx at positive potentials is greater than the anion efflux at negative potentials. The permeability and conductance ratios (table 1) for both GABA- and glycine-activated channels are similar. In addition, for most of the highly permeant halides, the permeability ratio is in a reverse sequence to the conductance ratio.

(*b*) *Concentration dependency of Cl channel conductance*

The dependency of channel conductance on Cl^- concentration was observed in inside-out patches with symmetrical Cl^- solutions and agonist in the pipette. Single-channel conductances at five Cl^- activities are shown in figure 2*a* for both GABA and glycine. At all the Cl^- concentrations, the conductance was greater at positive than at negative voltages. The relation between conductance and Cl^- activity exhibits a lower conductance and K_D (activity for half-maximal response) at negative than positive potentials for both GABA- and glycine-activated channels (figure 2*b*). A Michaelis-Menten fit of the conductances at +80 mV gave an apparent maximal conductance of 686 pS for GABA and 576 pS for glycine, and K_D values of 633 mM and 498 mM, respectively. In contrast, at −80 mV, the values for maximal conductance were 102 pS for GABA and 77 pS for glycine, and K_D values were 254 mM and 186 mM for GABA and glycine, respectively.

(*c*) *Mole fraction dependency*

Multiple occupancy within the transmitter-activated Cl^- channels was suggested by the anomalous mole-fraction behaviour of Cl^- and SCN^- (e.g. spinal cord neurons (Bormann *et al.* 1987)). Results of similar experiments are shown in figure 3, except that in our experiments the pipette solution remained constant at 140 mM Cl^-, and the bath solution was changed from pure Cl^- to a mixture of Cl^- and SCN^-. The Cl^- concentration, in inside-out patches at the external side of the membrane interface and in outside-out patches at the internal side of the membrane, was kept constant during the measurements of mole fraction dependency of GABA- and glycine-activated channels from both sides of the membrane.

The single-channel conductance was measured at four mole fractions of SCN^-: 0, 0.2, 0.7 and 0.9, and was found to be lowest at a mole fraction of 0.2. Under bi-ionic conditions, SCN^- is more permeant than Cl^-, whereas the Cl^- conductance was found to be greater than that of SCN^-. However, when the mole fraction of SCN^- was 0.2, the membrane conductance becomes smaller (figure 3*b*) and the reversal potential becomes

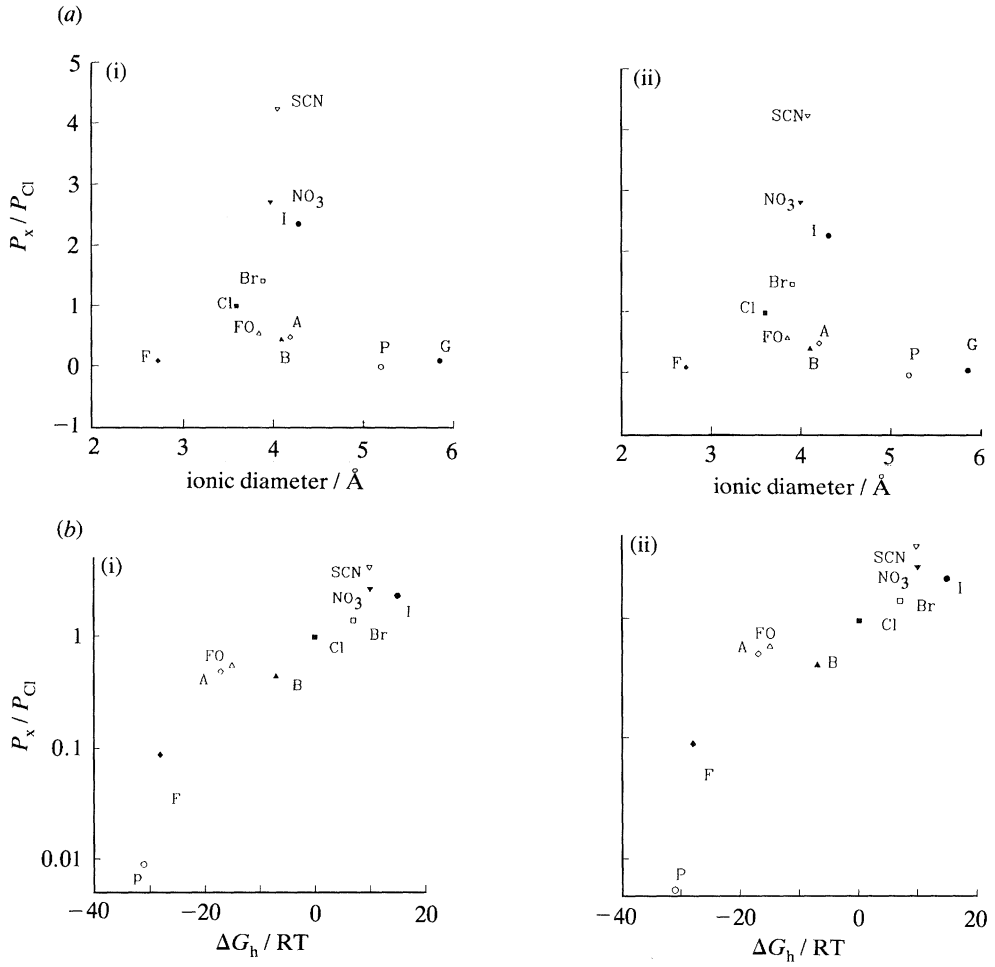

Fig. 1. Anion selectivity determined by measuring the reversal potentials of single-channel currents through (i) GABA- and (ii) glycine-activated channels. (*a*) Relative permeabilities of spherically symmetrical halides and polyatomic anions plotted against their ionic diameter. Reversal potentials were determined by applying voltage pulses to excised inside-out patches with agonist and 140 mM Cl^- in the pipette and 132 mM of test anions and 8 mM Cl^- in the bath. Permeability values are obtained from table 1. Relative permeabilities determined for both GABA- and glycine-activated channels were very similar. Both channels were permeable to gluconate and phosphate, implying that each pore diameter is of similar size and is about 6 Å. Ionic diameters were from Robinson & Stokes (1965), Halm & Frizzell (1992) and Dwyer *et al.* (1980). (*b*) A semi-log plot of relative permeabilities from table 1 plotted as a function of free energies of hydration relative to Cl^- in units of RT (where R is the gas constant and T the temperature in K). Free energies of hydration were obtained from Wright & Diamond (1977) and Vasil'ev *et al.* (1960). In the above figures, (A) refers to acetate, (B) refers to bicarbonate, Fo refers to formate, P refers to phosphate and G refers to gluconate.

more negative (figure 3*a*) than in either pure solution. The former observations can be explained in terms of multiple occupancy of anions in the Cl^- channels, with interactions of Cl^- and SCN^- within the conduction pathway.

4. DISCUSSION

Our present study of the ionic selectivity, concentration and anomalous mole fraction dependence of permeation for GABA and glycine-activated Cl^- channels supports our previous experiments in which we demonstrated that both GABA and glycine receptor channels are present in post-natal tissue-cultured hippocampal neurons and displayed similar permeation characteristics.

Outward rectification of the single-channel current–voltage relation, a characteristic feature of these GABA- and glycine-activated Cl^- channels (see figure 3 in Fatima-Shad & Barry (1992) for a sample of current records and I–V curves), could be due to a number of factors. First, rectification could result from the filtering of rapid opening and closing kinetics of these channels, and the observed single-channel conductance could be reduced by the filtering characteristics of the recording system, as observed in the L-type Ca^{2+} channel of excitable cells (Prod'hom *et al.* 1987). Secondly, an asymmetric distribution of surface charges between the internal and external side of the channel protein could differentially change the local concentration of permeant ions near both entrances of the channel, thereby altering the availability of ions for permeation (Dani & Eisenman 1987). Thirdly, structural features of the channel interior may cause a voltage dependence for their passage through the pore. Fourthly, access-limited diffusion could be limiting the flow of Cl^- ions towards the opening on the cytoplasmic side of the channel. However, whatever the structural

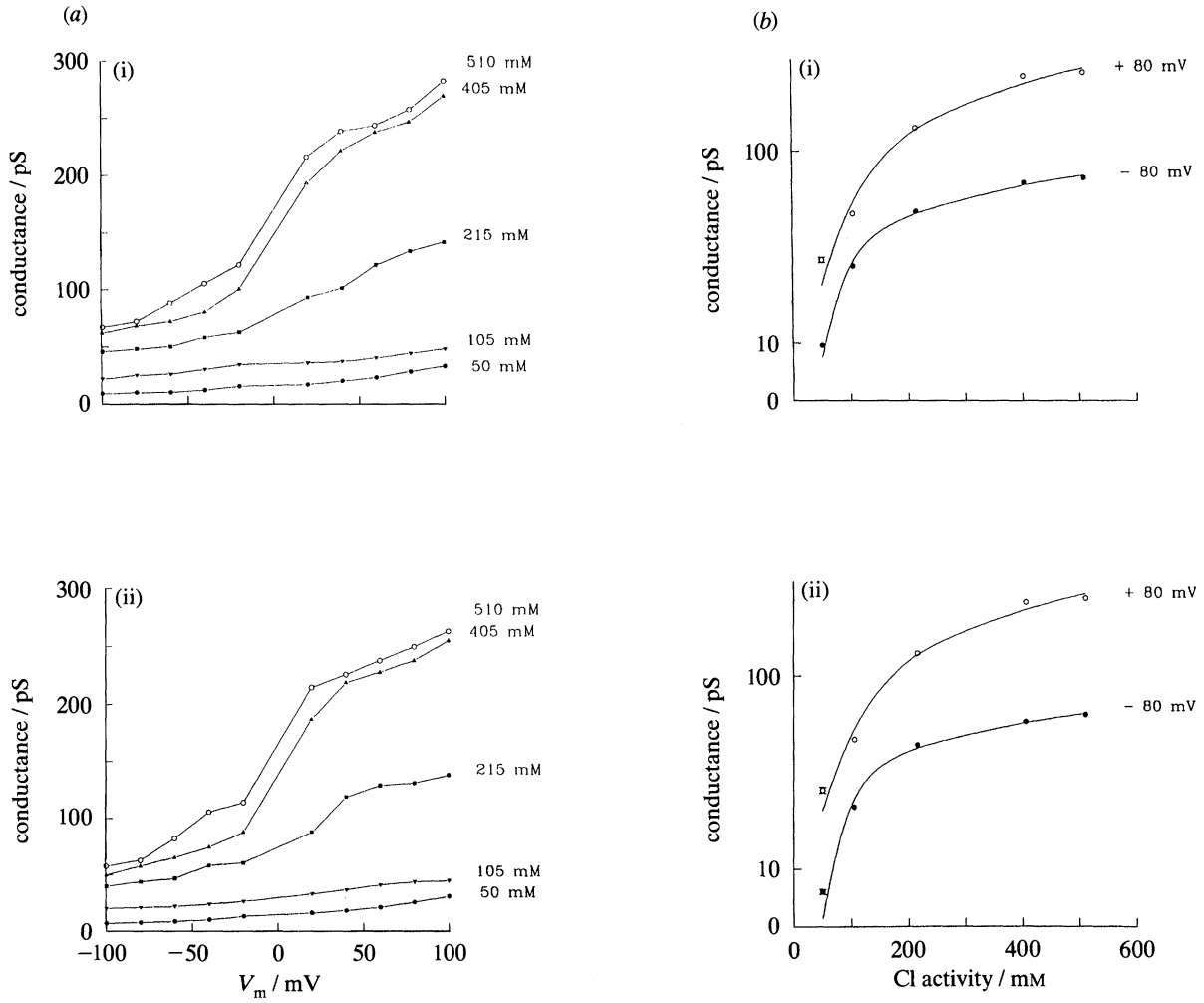

Fig. 2. Concentration dependence of conductances for (i) GABA- and (ii) glycine-activated Cl^- channels in symmetrical Cl^- concentrations. (*a*) Chord conductances were calculated from single-channel current–voltage curves at five different Cl^- activities and were plotted against their membrane potential for both agonists. The degree of rectification determined by the ratio of conductances at ± 100 mV for the GABA-activated Cl^- channel was 3.5 ± 0.12, ($n = 5$; 50 mM), 2.1 ± 0.01, ($n = 25$; 105 mM), 3.04 ± 0.01, ($n = 7$; 215 mM), 4.3 ± 0.02, ($n = 7$; 405 mM) and 4.19 ± 0.02, ($n = 5$; 510 mM). For the glycine-activated Cl^- channel the conductance ratio values at ± 100 mV were 4.38 ± 0.01, ($n = 5$; 50 mM), 2.2 ± 0.01, ($n = 24$; 105 mM), 3.4 ± 0.012, ($n = 7$; 215 mM) 5.08 ± 0.02, ($n = 7$; 405 mM) and 4.5 ± 0.01, ($n = 5$; 510 mM). (*b*) A semi-log plot of conductance against Cl^- activity at ± 80 mV for both agonist-activated channels. Michaelis-Menten fits gave higher values of maximal conductances and K_D at positive potentials than at negative potentials for both GABA- and glycine-activated channels.

details, it is clear that rectification of current flow requires an asymmetry in the free energy profile experienced by Cl^- in the channels or their adjacent regions.

The considerable similarities in anion selectivity between these GABA- and glycine-activated channels could be due to the structural similarities of their subunits (Grenningloh *et al.* 1987). The halide selectivity sequence, dominated by hydration forces, indicates the presence of weak field strength sites within the channel (see, for example, Eisenman & Horn 1983). A possible structure for such interaction sites within the channel would be that of a fixed charge site with a large radius or a relatively weak dipole site (Wright & Diamond 1977). Fixed positive charges in amino acid residues in proteins can be found on the side chains of lysine, arginine and histidine, and all of these charged groups are relatively large in size. In addition, many amino acids have polar side groups that might contribute to a weak interaction site: serine, threonine, tyrosine, asparagine and glutamine. The peptide bond with dipolar groups could be oriented to form an anion interaction site.

The deviation of NO_3^- and SCN^- from the exponential relation in figure 1*b* suggests that several groups coordinate with the anion at a selectivity site, which then favours anions with an appropriately arranged charge distribution. A similar interpretation might also explain why acetate and formate permeate faster than would be predicted from their hydration energies. For the large polyatomic anions, in addition to their having a more negative hydration energy, the steric hindrance of the minimal pore region becomes a dominant factor in reducing their permeability and conductance. The permeation of various anions suggests that the minimum pore diameter of the channel reaches a value of 5.5–6.0 Å. However, highly permeant anions have a lower relative single-channel

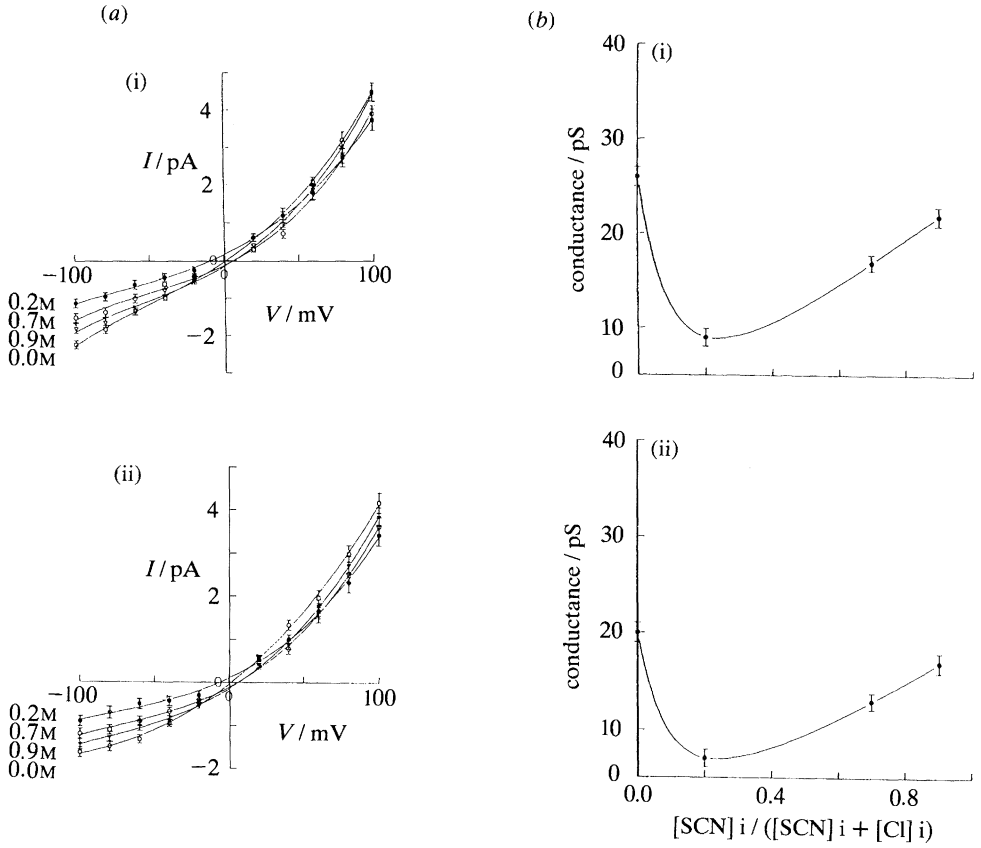

Fig. 3. Interaction of Cl^- and SCN^- within (i) GABA- and (ii) glycine-activated channels. (*a*) Dependence of single-channel current–voltage relations in inside-out excised patches activated by (i) GABA and (ii) glycine on mole fraction of SCN^- ($n = 15$ for each agonist). In both cases, the pipette solution remained constant and equal to 140 mM Cl^- plus agonist. In contrast, the bath solution at the cytoplasmic side of the membrane was changed from pure Cl^- to a mixture of Cl^- and SCN^-. Current–voltage relations for both channels show a more negative value of reversal potential at 0.2 mole fraction of SCN^-. (*b*) Single-channel conductance as a function of internal mole fraction of SCN^- and Cl^- at −70 mV for both (i) GABA- and (ii) glycine-activated channels. Contrary to the expectation that the resultant conductances should change somewhat linearly between the two extreme solutions, both agonist-activated channels showed a minimum conductance at a SCN^- mole fraction of 0.2. These observations suggest that both GABA- and glycine-activated Cl^- channels are capable of being occupied by two or more anions at the same time.

conductance. This is consistent with the principle that can arise in channels in which the more strongly bound ions tend to have a much lower mobility within the channel (see, for example, Quartararo *et al.* (1987) for permeation through acetylcholine channels). In such a situation, the sequence of conductances in pure symmetrical solutions tend to be mainly dependent on the ionic mobilities within the channel and to be somewhat inversely proportional to the binding constants of the sites, whereas the permeability sequence in bi-ionic solutions should be dependent on both binding constants and ionic mobilities, but may be dominated by the binding constants.

The increased ionic strength occurring with the symmetrical increases in Cl^- concentration (figure 2) would be expected to screen surface charges and diminish any ion rearrangement near the mouth of the channel. The similar magnitude of rectification at different concentrations suggests that surface charge effects was not a dominant factor in producing single-channel rectification. However, the concentration dependence of the current in this situation did exhibit nonlinear behaviour. The lower values of K_D and conductance at negative potentials compared with positive potentials could be due to the presence of a positively charged intracellular loop at the M2 region of these receptors. The concentration dependence of Cl^- channel conductances and the differences between relative permeability and the conductance ratios (table 1) suggest that anions may reside in the channel long enough to interact with other permeating anions. A minimum conductance of these channels in the presence of a 0.2 mole fraction of SCN^- implies the presence of multiple interaction sites within the channel, such that when both a SCN^- and a Cl^- ion occupy separate sites within the channel the conductance of the channel is reduced compared with when the sites were only occupied by either Cl^- or by SCN^- ions. This must be because the interionic repulsion between a SCN^- and a Cl^- ion is less than between two SCN^- or two Cl^- ions occupying the channel. From the reversal potential data it is also clear that this blockage is somewhat directional, because in such a situation (0.2 mole fraction of SCN^-) ion permeation is reduced from the solution containing a mixture of SCN^- and Cl^- compared with the solution on the other side of the membrane containing only Cl^- (and is independent of the patch configuration).

The above-mentioned channel behaviour of GABA- and glycine-activated Cl^- channels in post-natal tissue-

cultured hippocampal neurons suggests that the structural details of these anionic channels are probably similar to those described for some of the cationic channels, such as L-type calcium channels (Tsien *et al.* 1987), the delayed rectifier K^+ channels (Yellen 1987) and the TTX-sensitive sodium channels (Begenisich 1987).

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