J. Membrane Biol. 93, 75–83 (1986)

The Journal of
Membrane Biology

Nonselective Ionic Channels in *Aplysia* Neurones

Dominique Chesnoy-Marchais and Michael G. Evans
Laboratoire de Neurobiologie, Ecole Normale Supérieure, Paris 75005, France

Summary. Single-channel recordings from outside-out patches of *Aplysia* neurones in K-free solutions revealed the presence in most membrane patches of ionic channels showing surprising selectivity properties, as deduced from reversal potential measurements. After complete substitution of external NaCl by mannitol (in the presence of internal CsCl), these channels are more permeable to Cl than to Cs, but are also slightly permeable to Cs: $P_{Cl}/P_{Cs} = 4$. Furthermore, in the presence of external NaCl, their ability to discriminate cations from anions seems lower than in external mannitol. Substitutions of external Cl by various anions showed that the channels are more permeable to NO_3 than to Cl, and that they are appreciably permeable to isethionate, SO_4 and methanesulfonate. Their elementary conductance is about 100 pS in 600 mM symmetrical Cl. However, different conductance states (usually 2 or 3) can often be detected in the same membrane patch. By using voltage ramps, we established the *I-V* curves corresponding to each of these states and found small but significant differences between the reversal potentials of each state.

Key Words single channel · chloride · selectivity · conductance state · *Aplysia* neurones

Introduction

Using the outside-out configuration of the patch-clamp technique (Hamill et al., 1981), we have observed in most membrane patches from *Aplysia* neurones a channel which has unusual selectivity properties: After substitution of the external NaCl by mannitol, it is clearly more permeable to Cl than to cations; however, the complete substitution of the external NaCl by Na isethionate has almost no effect on the reversal potential of its elementary current (*see* Chesnoy-Marchais & Evans, 1984, for a preliminary report). The present paper describes this channel which, although permeable to Cl ions, is clearly different from the Cl channels responsible for the hyperpolarization-activated current of *Aplysia* neurones (Chesnoy-Marchais, 1982, 1983; Chesnoy-Marchais & Evans, 1984).

Materials and Methods

Preparation

All experiments were performed on 'outside-out' patches (Hamill et al., 1981) from *Aplysia californica* neurones at room temperature (20 to 22°C). Ganglia were isolated and the connective tissue was carefully removed by dissection. Usually the ganglia were not treated with any enzyme, but in some experiments, trypsin (TRTPCK, Worthington) was applied (0.1 mg/ml, 10 to 20 min); no significant difference was observed. The probability of obtaining an outside-out patch with a high seal resistance (>10 GΩ) differed from neurone to neurone within a given ganglion, and the situation varied considerably from animal to animal. However, the channels studied were present in most neurones. Thus, the experiments were done on many different neurones, in the cerebral and pleural ganglia, rather than on a single-family of neurones.

The size of the capacitive current (correlated to the size of the neurone) and the very frequent spikes observed in the whole-cell configuration (before isolating the membrane patch) indicated that the outside-out patches were excised from neurones, rather than from satellite cells.

Recording and Analysis

The micropipettes were fire-polished (resistance 1 MΩ after filling with the internal solution, 0.6 M CsCl) and the shank was covered with either Sylgard® or insulating varnish. In some cases, just before trying to make a seal, the tip of the pipette was immersed in a protamine solution (*see* Chesnoy-Marchais, 1985). The results obtained with or without protamine were identical. The bath was connected to ground via an agar bridge in series with a calomel electrode. The current output of an EPC5 List Amplifier was stored on magnetic tape (Racal). The records were either played back into a chart recorder (Gould Brush 280) or digitized for computer analysis (PDP11-23) after having been filtered (8-pole lowpass Bessel filter; cutoff frequency indicated at −3 dB in the Figure legends). The *I-V* curves of elementary current were taken either from successive recordings at different membrane potentials or directly from voltage ramps. Usually identical voltage ramps were regularly applied for several minutes. After recording, the current traces obtained during voltage

Table 1. Composition of some external solutions (in mM)[a]

Solution	NaCl or substitute	$MgCl_2$	$CaCl_2$	$Mg(NO_3)_2$	$Ca(NO_3)_2$
NaCl	480	50	10	0	0
Tris-HCl	520	50	10	0	0
Mannitol/Cl	780	50	10	0	0
Mannitol/NO_3	780	0	0	50	10

[a] All external solutions were K free and were buffered at pH 7.8 with 5 mM HEPES-NaOH. 'Mannitol' is used instead of 'mannitol/Cl' when no confusion is possible.

ramps were separated into different groups: the control traces, recorded when all channels were closed, and the traces recorded during prolonged openings of channels in a given conductance state (ramps of rather long duration could be used since the opening bursts of the channel often lasted several seconds; typically ramps of 200-msec duration were applied every 500 msec). Similar traces were averaged and the mean control trace was subtracted from the other traces (or mean traces), each difference giving the *I-V* curve of elementary current of the corresponding conductance state. This method allowed us to get a complete *I-V* curve in several external solutions even when the opening frequency was very low and the lifetime of the membrane patch rather limited. It also allowed us to easily discriminate the different conductance states.

Solutions and Corrections for Junction Potentials

All internal and external solutions were K free in order to eliminate K currents. Even the agar bridge (made with the NaCl solution described in Table 1) was K free.

Table 1 indicates the composition of some of the external solutions which were used. The composition of the '$NaNO_3$', 'Na isethionate', 'Na methanesulfonate', '$NaSO_4$' and 'Na gluconate' external solutions (*not shown* in Table 1) were the same as that of the control NaCl external solution, except that 480 mM of the Na salt replaced the NaCl.

Although the bath was connected to ground via an agar bridge, the junction potential between the ground and the bath was not exactly the same in all external solutions. The shifts induced during the change from the control NaCl external solution to any of the other external solutions were measured by a low-resistance 3 M KCl microelectrode and were taken into account for substitution experiments. These shifts were smaller than 6 mV.

The 'CsCl internal solution' usually put inside the pipettes contained 590 mM CsCl, 5 mM EGTA [ethyleneglycol-*bis*-(β-aminoethylether)-N,N'-tetraacetic acid], 0.5 mM $CaCl_2$ [internal free Ca concentration 0.5 to 1×10^{-7} M (*see* Chesnoy-Marchais, 1985)] and was buffered at pH 7.2 with 5 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-NaOH (or CsOH). The '$CsCl$-Cs_2SO_4 internal solution', used in a few experiments, contained 145 mM CsCl and 370 mM Cs_2SO_4 instead of 590 mM CsCl (other components unchanged). The junction potential between the internal solution and the external solution present in the bath before formation of the seal was measured by the voltage shift read by the microelectrode filled with the internal solution when the solution in the bath was changed from the internal solution to the external solution; for this measurement, the bath was connected to the ground directly by a calomel electrode without agar bridge. When we began the experiment in the NaCl external solution (which was usually the case) and used the CsCl internal solution, this junction potential was almost zero. It would have been simpler (for junction potentials and for permeability ratio calculations (*see* Results and Discussion)) to use Na (or Cs) on both faces of the membrane rather than Na outside and Cs inside. However, if the internal solution contained NaCl instead of CsCl, most membrane patches showed a large number of ionic channels selective for divalent cations which remained functional for several hours and prevented the study of other channels (Chesnoy-Marchais, 1985). Furthermore, the substitutions of external Na ions by Cs ions appeared to cause deterioration of the outside-out patches. Therefore, we could not use symmetrical cations. Note that during the study of the channels permeable to divalent cations, the channels reported in the present paper were also observed even though the internal solution contained Na ions instead of Cs ions. Thus, internal Cs ions are not responsible for the activation of these Cl-permeable channels (*see* McBurney et al., 1985).

E_{Cl} Calculation

In order to determine the selectivity of the channels studied, we had to compare the reversal potential value to the Cl equilibrium potential value, E_{Cl}. E_{Cl} was calculated using Nernst equation and the internal and external *concentrations* rather than *activities*. The resulting error cannot be significant when the internal and external solutions are similar, the activity coefficients being most probably very similar for both solutions. This might not be the case for experiments in which the external mannitol solution and internal CsCl solution were used. We tried to estimate the E_{Cl} calculation error done in this case. This was done thanks to Dr. Letellier from Laboratoire de Physico-chimie des Solutions (ENSCP). The method consisted in measuring directly E_{Cl} by using anion-selective membranes and an experimental setup analogous to the usual experimental configuration, i.e. two compartments, one containing the CsCl internal solution and an Ag/AgCl electrode, the second one containing the mannitol external solution and the usual agar bridge connected to the calomel electrode. The potential difference between the two compartments was measured under two different situations, i) when the junction between the two compartments was free (leaky tap) and ii) when both compartments were separated by the anion-selective membrane. The difference between the second and the first measurement gave an estimate of E_{Cl}. The result (37 mV) was very close to the calculated value (40 mV) showing that there were no serious errors in the calculation of E_{Cl}.

Results

Characterization of the Channels Studied

Figure 1 illustrates the currents studied in the present paper. The records of Fig. 1*A* were obtained in the external mannitol solution from an outside-out patch bathed with the internal CsCl solution and successively brought at different

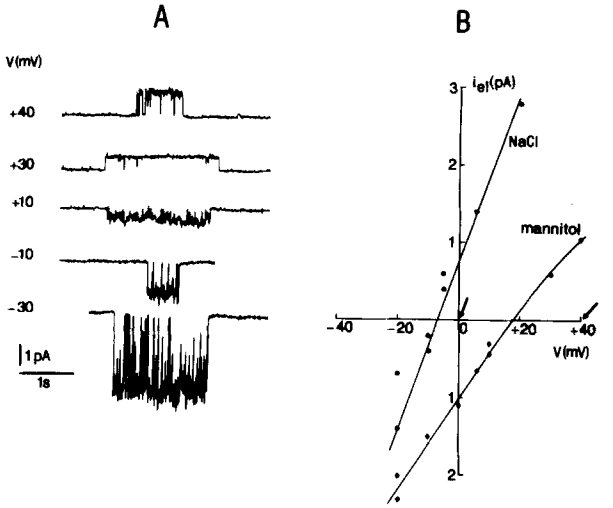

Fig. 1. (*A*) Examples of single-channel openings successively recorded at different membrane potentials from an outside-out patch bathed with the CsCl internal solution and the mannitol external solution (E_{Cl} = +40 mV). (*B*) *I-V* curves of elementary current obtained during the experiment illustrated in *A*, when the membrane patch was bathed either with the NaCl external solution (E_{Cl} = 0 mV; ●) or with the mannitol external solution (◆). For each curve, the presence of two different points for a same membrane potential indicates the existence of two different conductance states (*see also* Fig. 7 and Table 2)

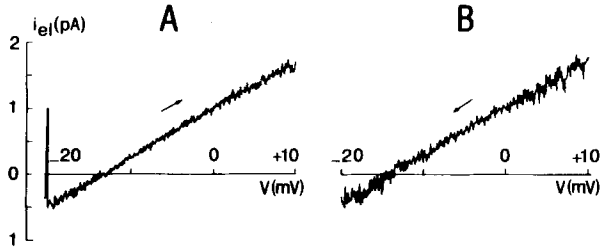

Fig. 2. Negative reversal potential precisely measured by voltage ramps while E_{Cl} is 0 mV. The two *I-V* curves of elementary current (*A* and *B*) were obtained in a membrane patch bathed with the NaCl external solution and the CsCl internal solution (E_{Cl} = 0 mV) by using voltage ramps of opposite direction. The arrows indicate the direction of the ramps. In both cases, the holding potential was +10 mV and voltage ramps of 30-mV amplitude and 200-msec duration were applied every 500 msec. In *A*, the voltage ramp was an increasing ramp and was superimposed on a negative square-voltage jump of 30-mV amplitude and 200-msec duration. In *B*, the voltage ramp was a decreasing ramp from +10 mV to −20 mV and was applied alone. The protocol (*A* or *B*) was changed during the same long opening burst [so that the two *I-V* curves correspond to the same conductance state (*see* Fig. 7)]. This was repeated several times during the same experiment and the reversal potential was always independent of the protocol, a result which has been confirmed in another membrane patch. The slight difference in noise between *A* and *B* results from the difference in the number of traces which have been averaged during channel openings (30 in *A*, 8 in *B*; 20 control traces averaged in both cases; 1 kHz filter and 2.5 kHz sampling frequency). The reversal potential is found close to −13 mV in *A* and close to −14 mV in *B*. Thus, independently of the method used, the reversal potential can be quite negative in the control NaCl solution

membrane potentials. Figure 1*B* shows the two *I-V* curves of elementary current obtained from this membrane patch, one in the external NaCl solution (E_{Cl} = 0 mV, ●), the other in the external mannitol solution (E_{Cl} = +40 mV, ◆). In the external mannitol solution, the reversal potential E_r is clearly positive, which shows that the channel is more permeable to Cl ions than to the cations present (mainly internal Cs ions).

There is, however, a discrepancy between the reversal potential of the current E_r and the Cl equilibrium potential E_{Cl}: E_r is more negative than E_{Cl}. The difference is particularly marked in the external mannitol solution and can neither be attributed to errors in the estimation of membrane potential (junction potential modifications are small and have been taken into account) nor to the E_{Cl} calculation (*see* Materials and Methods). Thus, the channel appears to be permeable to both anions and cations, and if we use the Goldman equation, the reversal potential measured in mannitol (close to +20 mV and confirmed by several similar experiments) leads to a permeability ratio for Cl and Cs ions (P_{Cl}/P_{Cs}) close to 4. (We did not try to estimate the permeability ratio for Cl and divalent cations. However, the external divalent cation concentration is only 60 mM while the internal Cl concentration is 600 mM; thus, a moderate permeability of the channel for divalent cations would not change our conclusions.)

In the external NaCl solution as well, E_r was systematically more negative than E_{Cl}. In the experiment illustrated by Fig. 2, the reversal potential was precisely measured in the NaCl solution by using voltage ramps and was found close to −13 mV. (The same value was obtained with voltage ramps of opposite directions.) Such negative reversal potentials were observed in many experiments (*see* Table 2). This result is surprising and indicates that the selectivity of the channel for Cl over cations is less pronounced in the NaCl solution than in the mannitol solution (*see* Discussion).

The elementary conductance of the channel studied, about 100 pS in the NaCl external solution, is clearly larger than the elementary conductance of the hyperpolarization-activated Cl channel observed under identical conditions (10 to 15 pS; *see* Chesnoy-Marchais & Evans, 1984). Furthermore, the channel studied in the present paper can be activated in the whole voltage-range tested (between −80 and +40 mV) and the voltage-dependence of its

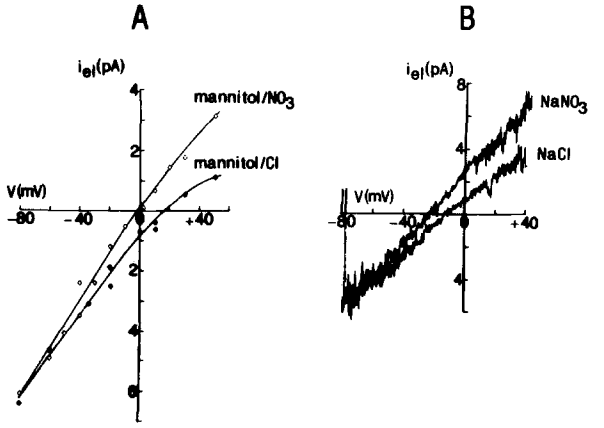

Fig. 3. External Cl-NO_3 substitutions. (*A*) *I-V* curves of elementary current obtained from the same membrane patch in the mannitol/Cl (◆) and mannitol/NO_3 (◇) external solutions. (*B*) *I-V* curves of elementary current obtained from another membrane patch by using voltage ramps in the NaCl and $NaNO_3$ external solutions. The holding potential was +30 mV in NaCl and +33 mV in $NaNO_3$. In both cases, an increasing voltage ramp of 120-mV amplitude and 200-msec duration, superimposed on a negative square-voltage jump of 110-mV amplitude and same duration, was applied every 500 msec. For comparison, the $NaNO_3$ curve has been shifted to the right by 3 mV (*see* the artifacts corresponding to the beginning of the ramp). The NaCl curve is the difference between the average of four traces recorded during channel openings and the average of 35 control traces. The NO_3 curve is the difference between the average of 15 traces recorded during channel openings and the average of eight control traces. In both experiments, the Cl-NO_3 substitution induced a shift of the reversal potential to the left and an increase of the outward currents, which shows that the channel is more permeable to NO_3 ions than to Cl ions

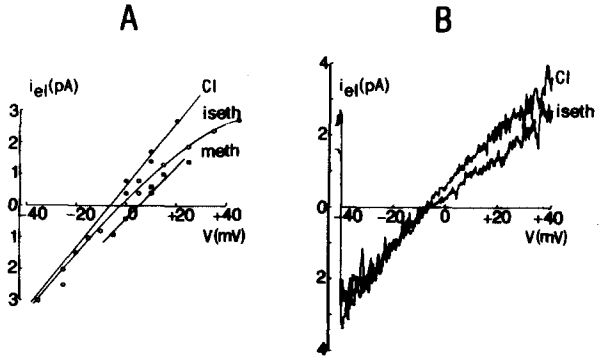

Fig. 4. External Cl-isethionate and Cl-methanesulfonate substitutions. (*A*) *I-V* curves of elementary current successively obtained from the same membrane patch in the NaCl (●), Na isethionate (○) and Na methanesulfonate (■) external solutions (CsCl internal solution). (*B*) *I-V* curves of elementary current obtained from another outside-out patch in the NaCl and Na isethionate solution using voltage ramps. The holding potential was +20 mV and an increasing voltage ramp of 80-mV amplitude and one-sec duration, superimposed on a negative square-voltage jump of 60 mV amplitude and same duration, was occasionally applied during channel openings as well as in the absence of openings. The *I-V* curve for the NaCl solution is the difference between one trace during a channel opening and the average of two control traces; the curve for the Na isethionate solution is the difference between the average of two traces during channel openings and the average of two control traces (100-Hz filter and 2-msec sampling frequency). In both experiments, the Cl-isethionate substitution did not much affect the reversal potential nor the inward elementary currents carried below these potentials, but this substitution did appreciably reduce the outward currents. Note that in *A*, the Cl-methanesulfonate substitution induced only a small shift (of about 10 mV) while the E_{Cl} shift was of +40 mV

activation, if any, is quite different from that of the hyperpolarization-activated Cl channel.

The channel studied showed flickering long openings often lasting for several seconds (*see* Fig. 1*A*); in nine experiments where long duration (30 to 60 sec) voltage jumps were regularly applied between two membrane potentials in the range −80 to +40 mV, the fraction of the time spent in these noisy long openings did not appear very voltage dependent. However, we did not investigate the possible voltage dependence of the flickering.

External Anion Substitutions

As shown above, the channel studied can be permeable to both anions and cations and shows some selectivity for Cl ions in the presence of external mannitol. The following section describes the results of substitution experiments which were done in order to further study the permeability of this channel to anions, in particular in the presence of external Na ions.

Cl-NO_3 Substitutions

The effects of an external Cl-NO_3 substitution on the *I-V* curve of elementary current are illustrated first in the absence of external Na ions by using the mannitol/Cl and mannitol/NO_3 external solutions (Fig. 3*A*). The fact that the reversal potential was clearly more negative in NO_3 than in Cl shows that this channel is more permeable to NO_3 ions than to Cl ions.

The effects of another kind of external Cl-NO_3 substitution, performed in the presence of external Na ions by using the NaCl and $NaNO_3$ solutions, are illustrated in Fig. 3*B*. These effects are essentially the same as in the experiment of Fig. 3*A*: i) a shift of E_r towards more negative membrane potentials (E_r was −12 mV in control and −22 mV in the

$NaNO_3$ external solution) and ii) an increase in the elementary conductance (corresponding to a clear increase of the outward elementary current carried by the influx of external anions).

In another experiment using voltage ramps, the reversal potential was −7 mV in control and −21 mV after the NaCl-$NaNO_3$ substitution.

Thus, both in the absence and in the presence of external Na ions, the channel is permeable to anions, and more permeable to NO_3 than to Cl.

Substitutions of External Cl Ions by Much Larger Anions

In this series of experiments, we obtained the *I-V* curves of elementary current (either by successive recordings at different membrane potentials or by using voltage ramps) before and after substitution of the external NaCl by a Na salt of isethionate, methanesulfonate, SO_4 or gluconate. Each of these substitutions corresponds to a positive shift of the Cl equilibrium potential of about 40 mV. The results are presented by Figs. 4, 5, and 6.

Figure 4 illustrates the effects of the Cl-isethionate and Cl-methanesulfonate substitutions. Surprisingly, whatever the method used (*compare A* and *B*), neither the reversal potential of this channel nor the inward current carried below this potential were strongly affected by the Cl-isethionate substitution. The E_r shift did not exceed 5 mV (five experiments). The outward elementary current was, however, appreciably reduced by this substitution, which shows that isethionate ions enter less easily than Cl ions.

The Cl-methanesulfonate substitution had more pronounced effects than the Cl-isethionate substitution (Fig. 4*A*), although the reversal potential shift was still not very large (about 10 mV, a result confirmed by two additional experiments performed using voltage ramps), and was certainly smaller than the shift induced by the NaCl-mannitol substitution (*see* Fig. 1*B*).

Figure 5 shows that the Cl-SO_4 substitution also shifts the reversal potential to the right. This shift (about 6 mV) is again much smaller than the shift induced by the NaCl-mannitol substitution. In two other experiments using voltage ramps, the reversal potential was found close to −12 and −13 mV in the NaCl external solution and was shifted by 13 and 10 mV, respectively, by the Cl-SO_4 substitution. In one of these experiments, the *I-V* curve could be obtained both in the Na_2SO_4 and in the Na-methanesulfonate solution, and was very similar in both solutions.

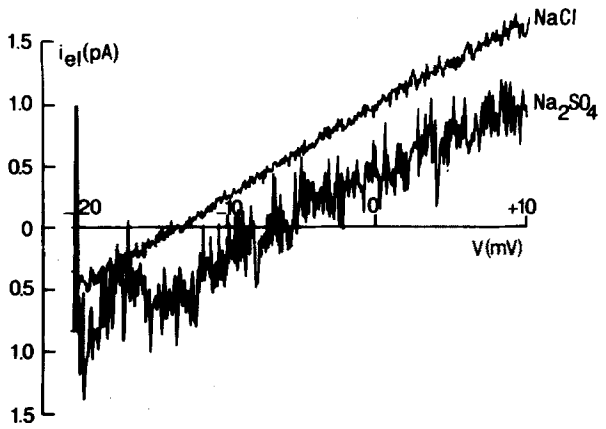

Fig. 5. External Cl-SO_4 substitution. *I-V* curves of elementary current obtained from the same membrane patch in the NaCl and Na_2SO_4 external solutions (CsCl internal solution; the NaCl curve was already shown in Fig. 2). The holding potential was +10 mV, and every 500 msec, a voltage jump to −20 mV and a simultaneous increasing voltage ramp of 30-mV amplitude were applied for 200 msec. The difference in noise between the two traces results from the difference in the number of current traces which have been averaged during channel openings (30 in the NaCl solution, 2 in the Na_2SO_4 solution). In both cases, 20 control current traces were averaged in the absence of channel openings. 1-kHz filter and 2.5-kHz sampling frequency. The reversal potential shift induced by the Cl-SO_4 substitution is rather small compared to the 40-mV shift of E_{Cl} and to the shift induced by the NaCl-mannitol substitution

Figure 6*A* compares the *I-V* curves shown in Fig. 4*A* to an *I-V* curve of elementary current obtained after the Cl-gluconate substitution. It is clear that the Cl-gluconate substitution altered the elementary current more than the other anionic substitutions tested. Nonetheless, the Cl-gluconate substitution did not affect the reversal potential as much as one would expect from the NaCl-mannitol substitution experiments. The fact that the reversal potential was less positive in the Na gluconate solution than in the mannitol solution (*compare* Figs. 6*A* and 1*B*) was confirmed in another experiment where the *I-V* curves of elementary current in both solutions were obtained from the same membrane patch.

The interpretation of the substitution experiments described above in terms of relative permeabilities of the various ions tested is not as simple as it could be in the case of a channel permeable to anions only (or to cations only). The results suggest, however, that isethionate, methanesulfonate and sulfate are all appreciably permeant and the following qualitative sequence of relative permeabilities can be proposed: NO_3 > Cl > isethionate > SO_4, methanesulfonate > gluconate.

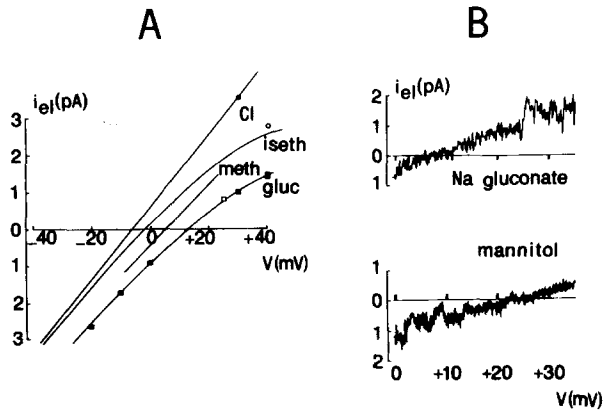

Fig. 6. External Cl-gluconate substitution. (*A*) Comparison of the *I-V* curve of elementary current obtained in the Na gluconate external solution (CsCl internal solution) with those obtained from another membrane patch in the NaCl, Na isethionate and Na methanesulfonate external solutions (CsCl internal solution; three curves already presented with the corresponding experimental points in Fig. 4*A*). (●): elementary current value obtained at +30 mV in the NaCl external solution from the same membrane patch as the Na gluconate curve (■). A few points derived from a third membrane patch in the Na isethionate (○) and Na gluconate (□) solutions are also indicated (at +40 mV, the symbols □ and ■ are superimposed) and confirm (by comparison on a same membrane patch) the difference between the Na isethionate and Na gluconate curves. The Cl-gluconate substitution clearly induces more pronounced effects than the two other anion substitutions. The reversal potential in Na gluconate was about +13 mV, which is more positive than values usually obtained in the Na isethionate, Na_2SO_4 or Na methanesulfonate solutions, and indicates that isethionate, SO_4 and methanesulfonate are all appreciably permeant. The reversal potential, however, seemed smaller in the Na gluconate external solution than in the mannitol external solution [*see also* (*B*)]. (*B*) Comparison of the *I-V* curves of elementary current obtained from the same membrane patch in the Na gluconate and mannitol external solutions by using voltage ramps. The holding potential was 0 mV and an increasing voltage ramp of 35-mV amplitude and 200-msec duration was applied every 500 msec. Each curve is the difference between the average of two current traces recorded during channel openings and the average of 20 control current traces. The reversal potentials measured in both solutions are clearly different, the difference exceeding 10 mV. 1-kHz filter and 2.5-kHz sampling frequency. The sign of this difference (reversal potential more positive in the mannitol external solution, that is in the absence of external Na ions) is opposite to what would be expected if the channel was not permeant to gluconate and had the same permeability ratio for cations and Cl ions in both solutions. (Note that only one conductance state was observed in this experiment.)

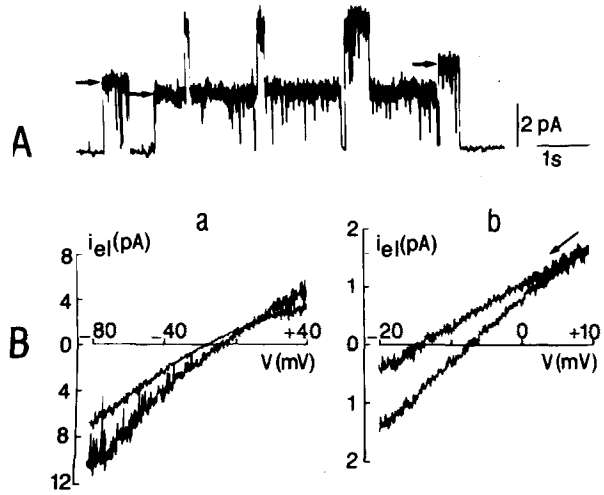

Fig. 7. Different conductance states corresponding to slightly different reversal potentials. (*A*) Chart record of a current trace obtained at +30 mV in the TRIS-HCl external solution and CsCl-Cs_2SO_4 internal solution, showing three different conductance states indicated by the arrows. (*B*) The *I-V* curves of elementary current of two different conductance states are compared in two voltage ramp experiments (*a* and *b*) performed with the external NaCl and internal CsCl solutions. In both experiments, voltage ramps of 200-msec duration were applied every 500 msec. In *a*, the holding potential was −40 mV, the voltage ramps were increasing ramps of 120-mV amplitude and were superimposed on negative square-voltage jumps of 40-mV amplitude and identical duration, giving the *I-V* curve between −80 and +40 mV. In *b* (same experiment as for Figs. 2 and 5), the holding potential was +10 mV, and decreasing voltage ramps of 30 mV amplitude were applied alone, giving the *I-V* curve between +10 and −20 mV. The arrow indicates the direction of the voltage ramps. The number of traces averaged were in *a*, 25 during channel openings to the less conductive state, 2 during channel openings to the more conductive state and 8 control traces; in *b*, 8 during channel openings to the less conductive state, 9 during channel openings to the more conductive state and 20 control traces; 1-kHz filter and 2.5-kHz sampling frequency in both cases. In each case, the two different conductance states correspond to two different reversal potentials. The elementary conductances (measured around the reversal potential) and reversal potentials for the two states are (85 pS, −13 mV) and (116 pS, −5 mV) in *a*, (75 pS, −14 mV) and (117 pS, −7 mV) in *b*

Different Conductance States Corresponding to Slightly Different Reversal Potentials

As already illustrated in Figs. 1*B*, 3*A* and 4*A*, successive channel openings observed under identical conditions in the same membrane patch often led to two different elementary current values for a given membrane potential. In a few cases even three different elementary current values were observed; this is illustrated in Fig. 7*A*. These results indicate the existence of 2 or 3 different conductance states.

By using voltage ramps, which allowed us to obtain a precise *I-V* curve of elementary current from a single opening corresponding to a single conductance state, we systematically found small but significant differences between the reversal potentials of each of the conductance states. This result is illustrated in Fig. 7*B* by two different experiments

performed with voltage ramps in the CsCl internal solution and NaCl external solution. In one case (Fig. 7*Ba*), the applied voltage ramps increased from −80 to +40 mV (600 mV/sec). In the other case (Fig. 7*Bb*, same experiment as Fig. 2), the ramps decreased from +10 to −20 mV (150 mV/sec). In both cases, two different values of E_r can be obtained according to which channel openings were selected: −13 and −5 mV in Fig. 7*Ba*, −14 and −7 mV in Fig. 7*Bb*. In the experiment of Fig. 7*Bb*, a third intermediate conductance state was also occasionally observed and corresponded to a third intermediate reversal potential (−10 mV).

Similar results were obtained in eight similar experiments performed in the CsCl internal solution and NaCl external solution. These experiments are presented in Table 2. In six of them, two conductance states whose reversal potentials differed by about 7 mV were observed; in two of these six experiments, a third conductance state with an intermediate reversal potential could also be observed. In the remaining two experiments, the two conductance states observed had reversal potentials differing by only about 4 mV. The less conductive state always had the more negative reversal potential so and the *I-V* curves of elementary current corresponding to different conductance states cross each other above their reversal potentials.

The fact that the different conductance states correspond to slightly different reversal potentials explains the slight variability in reversal potential which may have been noticed from the data presented above. (For example, E_r was equal to −13 mV in Fig. 2 and to −8 mV in Fig. 4*B*.)

Different conductance states corresponding to slightly different reversal potentials have also been observed occasionally with external solutions other than the NaCl solution. In one experiment, two conductance states corresponding to reversal potentials of 0 and +8 mV were observed in the Na methanesulfonate external solution while only one state corresponding to a reversal potential of −12 mV was detected in the control NaCl solution. In another experiment (partly described in Fig. 5), two conductance states corresponding to reversal potentials of −7 and +1 mV were observed in the Na_2SO_4 external solution while three conductance states corresponding to reversal potentials of −13, −10 and −7 mV were observed in control. (The curves shown in Fig. 5 correspond to the more negative reversal potentials.) Even the most positive values of the reversal potential in these Cl-substituted solutions remained clearly smaller than the smallest values obtained in the mannitol solution. In the same way, the reversal potential observed in the Na isethionate solution was never clearly positive.

Table 2. Elementary conductances and reversal potentials of the different conductance states[a]

	Experiment number							
	1	2	3	4	5	6	7	8
	113	143	125	116	117	119		
	−6	−3	−6	−5	−7	−4		
γ (pS)					90	90	93	121
E_r (mV)					−11	−7	−8	−10
	78	94	103	85	75	79	81	100
	−14	−10	−13	−13	−14	−11	−12	−14

[a] This Table presents the results of eight experiments in which at least two different conductance states were observed. The elementary conductance γ (measured around the reversal potential) and the reversal potential E_r of each individual state are given superimposed. In experiments 5 and 6, three different conductance states were observed while the other experiments showed only two different conductance states. In experiments 1 to 6, two states corresponding to reversal potentials differing by about 7 mV were observed. The mean values of the elementary conductance and reversal potential of each of these two states are (−5.2 ± 1.3 mV (6); 122 ± 10 pS (6)) and (−12.5 ± 1.5 mV (6); 86 ± 10 pS (6)) (mean ± variance$^{1/2}$ (n)).

Furthermore, Cl-NO_3 substitutions always induced a negative shift of the reversal potential, whether the control E_r value was −13 or −7 mV. Thus, none of the qualitative conclusions which have been proposed above can be questioned because of the existence of reversal potential differences between different conductance states.

Discussion

Permeability to Both Anions and Cations

The permeability of the channel described to *anions* was demonstrated by the positive shift of the reversal potential induced during the substitution of external NaCl by mannitol (Fig. 1), as well as by the effects of various external anion substitutions, which in particular clearly affected the outward elementary current flowing through these channels (Figs. 3, 4, 5 and 6*A*).

The simultaneous permeability of this channel to *cations* was first suggested by the clear difference (of about 20 mV) between the reversal potential and E_{Cl} in the mannitol external solution (*see* Chesnoy-Marchais & Evans, 1984, and Fig. 1*B*). It was confirmed by the systematic observation of a negative reversal potential in the NaCl external solution when E_{Cl} was 0 mV.

Modulation of the Permeability Ratio for Cl and Cations by the External Ions

In the NaCl external solution the reversal potential was more negative than one would expect from its value in the mannitol solution. Indeed, according to the Goldman equation, if the permeability ratio for Cl and Cs ions was the same in the mannitol and NaCl external solutions [that is $P_{Cl}/P_{Cs} = 4$ (*see* Results)], in the NaCl solution the reversal potential should be -1 mV if Na and Cs ions were equally permeant or -6 mV if Na ions were impermeant. To explain that reversal potentials more negative than -6 mV were reproducibly observed in the external NaCl solution, one has to assume that the permeability ratio P_{Cl}/P_{Cs} is smaller in the NaCl solution than in the mannitol solution, and thus depends on the external ions.

The observation that the reversal potential was more positive in the mannitol solution than in the Na gluconate solution (Fig. 6*B*) supports this conclusion. It is very unlikely that an anion as large as gluconate could be permeant. Thus, replacing Na gluconate by mannitol (that is suppressing external Na ions without changing the concentrations of permeant anions) should shift the reversal potential to negative values, because of the cationic permeability of the channel. The difference between this prediction and the result can be explained if the selectivity for Cl over cations is less pronounced in Na gluconate than in mannitol. (The Goldman equation with $P_{Na} = P_{Cs}$ and $E_r = 9$ to 13 mV in Na gluconate would lead to a value of $P_{Cl}/P_{cations}$ close to 1, that is clearly smaller than the ratio found in mannitol.)

Thus, in the presence of external Na ions, the channel described seems to have comparable permeabilities to anions and cations. We cannot, however, give a reliable quantitative estimate of the permeability ratios for Cl and Cs or Na ions, in particular because the permeability of the channel for Na and Cs ions might be different. For the conductance states having a reversal potential more negative than -6 mV in the NaCl external solution, it is not possible to find a positive permeability ratio for cations and Cl which satisfies the Goldman equation if we suppose $P_{Na} = P_{Cs}$; rather we have to suppose $P_{Na} < P_{Cs}$.

Cl-permeable channels slightly permeable to small cations (like Na or K) have been described recently in several other preparations; their permeability to small cations was reported to be about 5 times smaller than their permeability to Cl ions [*see* Gray et al. (1984) and Schwarze & Kolb (1984) for channels of large conductance (350 to 450 pS) which are similar to those previously described in muscle cells (Blatz & Magleby, 1983) and to the VDAC channels of the outer membrane of mitochondria (Schein et al., 1976) *see also* Blatz & Magleby (1985) for rat skeletal muscle channels of 45 to 60 pS conductance].

The even lower selectivity of the *Aplysia* channel in the external NaCl solution can be understood if these channels do not show permanent charges. The fact that they become more selective for Cl ions in the external mannitol solution could be explained by the polarizability of the channel.

It would be very difficult to show convincingly the existence of nonselective ionic channels without doing *single-channel* recordings. Thus, it is quite possible that nonselective channels are present in many preparations even though such channels have not yet been frequently described.

Weak Discrimination Between Different Anions

The anion substitution experiments showed that the channel studied is more permeable to NO_3 than to Cl and is less permeable to isethionate, methanesulfonate and SO_4 than to Cl. However, the reversal potential shifts induced when replacing Cl by these less permeant anions were never as large as the shifts induced by the Cl-gluconate or NaCl-mannitol substitutions. Thus, it seems that this channel is appreciably permeant to isethionate, methanesulfonate and SO_4. For example, if one considers that gluconate is impermeant and that $P_{Na} = P_{Cs}$, which leads to the ratio $P_{Cl}/P_{cations} = 1$ (*see above*), the ratio $P_{methanesulfonate}/P_{Cl}$ would be 0.75 or 0.34, depending on whether the reversal potential in the Na methanesulfonate solution was 0 or +5 mV. If $P_{Cl}/P_{cations}$ was taken larger, these large anions would appear still more permeant.

The fact that only small reversal potential shifts were induced by the complete substitution of the external NaCl by a Na salt of some large anions (like isethionate) does not result only from the permeability of the channel to these large anions; it also results from the permeability of the channel to small cations, which in the presence of external Na ions is not negligible.

The 400-pS anionic channels described in other preparations have also been reported to be permeable to large anions like methylsulfate, SO_4 and isethionate (Gray et al., 1984; *see also* Nowak et al., 1983 for isethionate). The contribution of a nonselective anionic current to the resting potential of the perfused squid giant axon has been recently proposed from experiments performed after blockade of Na and K conductances (Inoue, 1985).

Differences Between Different Conductance States

The channels described often showed 2 or 3 different conductance states which appeared to have slightly different reversal potentials (Fig. 7 and Table 2). These differences, observed for example with CsCl inside and NaCl outside, suggest that the selectivity for small cations and Cl ions may be slightly different for different conductance states. Many ionic channels have different conductance states and in the case of the gramicidin channel, it was even shown that the shape of the *I-V* curve of elementary current can be different for different conductance states (Busath & Szabo, 1981). However, differences in reversal potential had not been reported previously.

The initial data leading to the observation of different conductance states were obtained by successive recordings at different membrane potentials (*see* Fig. 7*A*) and were first misunderstood (Chesnoy-Marchais & Evans, 1984) since we did not imagine immediately that there could be reversal potential differences between different conductance states. The observation at some membrane potentials of elementary current values which differed only very slightly and the observation at other potentials of elementary current values differing by a factor close to 2 had been interpreted as resulting from the existence of a few very similar "full states" combined with the existence of a "half state." These observations actually result from the existence of different states (probably 3) corresponding to more gradual differences in both the conductance and the reversal potential.

Could Such Nonselective Channels be Active in Intact Cells?

Previous studies in intact *Aplysia* neurones did not demonstrate the existence of a large resting anionic conductance (*see* Ascher et al., 1976 and discussion of earlier studies). The channels described above, very poorly selective, not strongly voltage dependent and present in high density in the membrane, might, however, contribute to the resting conductance. The spontaneous and rapid entry of NO_3 ions in *Aplysia* neurones, which occurs even when the hyperpolarization-activated Cl channels are not activated [P. Ascher and A. Brisson (*personal communication*); *see also* Fig. 7 in Chesnoy-Marchais, 1983] also points towards an appreciable resting anionic conductance through NO_3-permeable channels, such as those described. We cannot exclude, however, that in an intact cell these channels would be usually in an inactive state, being activated only occasionally.

We acknowledge the support of the Université Pierre et Marie Curie, of the C.N.R.S. (UA 04-295; ATP 960028) and of the M.R.T. (81 E 1382). M.G.E. was supported by the Royal Society-C.N.R.S. exchange program. We are very grateful to Professor P. Ascher for helpful discussions and useful advice.

References

Ascher, P., Kunze, D., Neild, T.O. 1976. Chloride distribution in *Aplysia* neurones. *J. Physiol. (London)* **256:**441–464

Blatz, A.L., Magleby, K.L. 1983. Single voltage-dependent chloride-selective channels of large conductance in cultured rat muscle. *Biophys. J.* **43:**237–241

Blatz, A.L., Magleby, K.L. 1985. Single chloride-selective channels active at resting membrane potentials in cultured rat skeletal muscle. *Biophys. J.* **47:**119–123

Busath, D., Szabo, G. 1981. Gramicidin forms multi-state rectifying channels. *Nature (London)* **294:**371–373

Chesnoy-Marchais, D. 1982. A Cl^- conductance activated by hyperpolarization in *Aplysia* neurones. *Nature (London)* **299:**359–361

Chesnoy-Marchais, D. 1983. Characterization of a chloride conductance activated by hyperpolarization in *Aplysia* neurones. *J. Physiol. (London)* **342:**277–308

Chesnoy-Marchais, D. 1985. Kinetic properties and selectivity of calcium-permeable single channels in *Aplysia* neurones. *J. Physiol. (London)* **367:**457–488

Chesnoy-Marchais, D., Evans, M.G. 1984. Two types of chloride channels in outside-out patches from *Aplysia* neurones. *J. Physiol. (London)* **357:**64P

Gray, P.T.A., Bevan, S., Ritchie, J.M. 1984. High conductance anion-selective channels in rat cultured Schwann cells. *Proc. R. Soc. London B.* **221:**395–409

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100

Inoue, I. 1985. Voltage-dependent chloride conductance of the squid axon membrane and its blockade by some disulfonic stilbene derivatives. *J. Gen. Physiol.* **85:**519–537

McBurney, R.N., Smith, S.M., Zorec, R. 1985. Extracellular caesium ions (Cs^+) activate a chloride (Cl^-) permeability in rat cultured spinal cord neurones. *J. Physiol. (London)* **367:**68P

Nowak, L., Ascher, P., Berwald-Netter, Y., Couraud, F. 1983. Single channel currents of cultured mammalian astrocytes studied using excised membrane patches. *Neurosci. Lett.* **14:**52–64

Schein, S.J., Colombini, M., Finkelstein, A. 1976. Reconstitution in planar lipid bilayers of a voltage-dependent anion-selective channel obtained from Paramecium mitochondria. *J. Membrane Biol.* **30:**99–120

Schwarze, W., Kolb, H.A. 1984. Voltage-dependent kinetics of an anionic channel of large unit conductance in macrophages and myotube membranes. *Pfluegers Arch.* **402:**281–291

Received 11 March 1986; revised 16 May 1986