Journal of Physiology (1990), **424**, *pp.* 167–185
With 6 figures
Printed in Great Britain

CURRENTS CARRIED BY MONOVALENT CATIONS THROUGH CYCLIC GMP-ACTIVATED CHANNELS IN EXCISED PATCHES FROM SALAMANDER RODS

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(*Received 31 October 1989*)

SUMMARY

1. Ionic selectivity and affinity for monovalent cations of channels activated by guanosine 3′,5′-cyclic monophosphate (cyclic GMP) were studied in excised inside-out patches of plasma membrane from retinal rods of the tiger salamander. Channels were activated by addition of cyclic GMP to the medium bathing the cytoplasmic side of the membrane. The ionic solution at the cytoplasmic side was rapidly changed using the method of Nunn (1987*a*).

2. Permeability ratios were calculated with the Goldman–Hodgkin–Katz potential equation from reversal potential measurements for alkali monovalent cations in bi-ionic conditions. The permeability sequence was: $Li^+:Na^+:K^+:Rb^+:Cs^+ = 1{\cdot}14:1:0{\cdot}98:0{\cdot}84:0{\cdot}58$.

3. The selectivity sequence obtained from macroscopic current measurements in bi-ionic conditions at +100 mV was: $Na^+:K^+:Rb^+:Li^+:Cs^+ = 1:1:0{\cdot}67:0{\cdot}36:0{\cdot}25$.

4. The organic cations tetramethylammonium (TMA^+), choline and tetraethylammonium (TEA^+) were not permeant through the cyclic GMP-activated channels and caused a reduction of the Na^+ inward current. At −100 mV the current ratio for inward current was $1:0{\cdot}75:0{\cdot}58:0{\cdot}2$ in the presence, at the cytoplasmic side, of 110 mM-Na^+, TMA^+, choline or TEA^+ respectively.

5. The concentration dependence of the macroscopic current and the reversal potential was studied by changing the internal concentration of Na^+ or K^+ or Li^+ from 5 mM to 500 mM. The permeability ratios were nearly constant regardless of the permeant ion concentration.

6. The current as a function of internal ion activity could be described by a Michaelis–Menten relation with a half-saturating activity, K_m, at +90 mV equal to 249, 203 and 160 mM for Na^+, K^+ and Li^+ respectively. The ratio of the extrapolated saturating current I_{max} at +90 mV was $1:0{\cdot}86:0{\cdot}26$ for Na^+, K^+ and Li^+ respectively.

7. The outward currents and the reversal potentials measured in different mixtures of Na^+ and Li^+ were monotonic function of the mole fraction.

The experiments were performed in the laboratory of Dr Brian J. Nunn, at Duke University, after his tragic death.

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8. These results can be explained by assuming that, at least in a narrow region, the cyclic GMP-activated channel is a one-ion channel, possibly with other poorly voltage-dependent binding sites in a large inner vestibule.

INTRODUCTION

The membrane of the outer segment of rods from the vertebrate retina mainly contains one type of channel: the channel activated by guanosine 3′,5′-cyclic monophosphate (cyclic GMP; Fesenko, Kolesnikov & Lyubarsky, 1985; Haynes, Kay & Yau, 1986; Zimmerman & Baylor, 1986) and experimental evidence suggests that this channel is the light-sensitive channel (Matthews & Watanabe, 1987; Nakatani & Yau, 1988*b*; see for a review Yau & Baylor, 1989). Similar channels are also present in cones from the vertebrate retina (Haynes & Yau, 1985). Channels gated by cyclic GMP have also been observed in olfactory receptor cilia (Nakamura & Gold, 1987), but the physiological function of these channels is not yet known.

The selectivity of the light-sensitive channel, measured as the efficacy for carrying inward current, has been studied in intact cells (Yau & Nakatani, 1984; Hodgkin, McNaughton & Nunn, 1985; Nakatani & Yau, 1988*a*; Menini, Rispoli & Torre, 1988*b*) by changing solutions at the extracellular side of the rod.

The aim of this paper is to determine the effect of changing internal monovalent cations on the cyclic GMP-activated currents in retinal rods. The results were obtained with the patch-clamp technique in the excised inside-out patch configuration (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). It is shown that this channel is not very selective among alkali monovalent cations and that the organic cations TMA^+, choline and TEA^+ are not permeant but, when present at the internal side, they reduce the inward current carried by Na^+. The ionic selectivity of the channel was measured both as permeability ratios, calculated by changes of reversal potential (as in Fesenko *et al.* 1985, and Nunn, 1987*b*) and as the sequence of efficacy in carrying macroscopic outward currents. This last sequence is different from that obtained from the light-sensitive channels in intact rods because Li^+, which is the alkali cation carrying the highest inward current in intact cells, carries only about one-third of the outward current measured in membrane patches in the presence of Na^+.

The dependence of the cyclic GMP-activated current on ion activity of Na^+, K^+ or Li^+ is very well described by a simple Michaelis–Menten equation, and the permeability ratios do not depend on internal ion activity. These results, together with experiments with mixtures of Na^+ and Li^+, are consistent with a simple model of the cyclic GMP-activated channel consisting of a one-ion pore, as has already been suggested (Menini *et al.* 1988*b*; Zimmerman & Baylor, 1988).

Some of the early experiments were performed in collaboration with Dr V. Torre, Preliminary results have already been presented in abstract form (Menini, Nunn & Torre, 1988*a*; Menini & Torre, 1989).

METHODS

Preparation

Cyclic GMP-activated channels were studied in excised inside-out patches (Hamill *et al.* 1981) from the plasma membrane of rods, obtained from the retina of the tiger salamander *Ambystoma tigrinum* after decapitation. The animals were supplied by Lawrence Waterdog Farm (Oklahoma, USA). The dissection and preparation of rods was similar to that described by Menini *et al.* (1988*b*). In earlier experiments the salamanders were dark adapted, the dissection was carried out under dim red light and the experiments were performed in room light but subsequently, since similar results were obtained with animals kept in the light, all the operations were performed in room light.

Current recording

Patch pipettes were pulled from borosilicate glass micropipettes (7087-44 Brandt, FRG) using a Flaming–Brown puller. The tip of the pipette was not fire-polished: bubble numbers in methanol were about 4·0 and electrode resistances were 10–20 MΩ when filled with 110 mM-NaCl. Rod outer segments were first sucked into a suction pipette, as described in Menini *et al.* (1988*b*), and then a patch pipette was moved close to the outer segment. The position of the stage of the microscope was then moved so that the suction pipette holding the cell and the patch pipette were both in front of a tube through which a solution identical to the solution filling the patch pipette was flowing (see description of the perfusing system). The pipette potential was then adjusted to give zero current between the pipette and the bathing solution. This procedure also minimized possible contamination of the solution inside the patch pipette when it was necessary to apply a negative pressure for the purpose of forming a tight seal. Seals of resistance between 1 and 10 GΩ were usually obtained simply by touching the patch pipette against the membrane of the outer segment, often even with a slight positive pressure inside the patch pipette. Excised inside-out patches were obtained by quickly withdrawing the patch pipette.

Channels were activated by the addition of cyclic GMP (disodium salt, G6129 Sigma) to the bathing solution. When the perfusing solution was nominally Na^+ free, cyclic GMP in the free-acid form was used (G7504 Sigma). Current recordings were made with an Axopatch 1-B amplifier (Axon Instruments, USA). Currents were filtered through a low-pass filter at 1 kHz and digitized on-line at a sampling interval of 0·6 ms by using a CED 1401 interface (Cambridge Electronic Design, UK) and stored and analysed on an IBM-AT computer with a program written in ASYST by Brian J. Nunn. Data were also stored on videotape after conversion into digital form with a modified (Lamb, 1985) pulse-code-modulation device (Sony PCM-701ES).

In all experiments, the current and potential are presented with the usual sign convention: currents flowing from the previously intracellular side of the membrane patch (bath solution) to the extracellular side (pipette solution) are positive, and are plotted upwards.

Cyclic GMP-activated currents were obtained as the difference of recordings in the presence of cyclic GMP at the cytoplasmic side of the membrane and in the absence of cyclic GMP. Sometimes a droop in the activated current was observed. This effect was studied by Zimmerman, Karpen & Baylor (1988) and is caused by restricted diffusion of ions, due to intracellular material that adheres to the surface membrane. It was found, during the experiments described in this paper, that this droop was present only when it had been necessary to apply a strong negative pressure to the patch pipette in order to obtain a seal. When the seal was obtained simply by touching the membrane, it was possible to record large currents (more than 1 nA) without the appearance of a significant droop (see Fig. 4*A*). The experiments presented in this paper are only those in which no droop in the current was present.

Currents had different values in each patch, probably depending on the surface area of the membrane patch and on the channel density in the part of the rod from which the patch was excised. In 110 mM-NaCl symmetrical solutions, currents at +100 mV varied from 50 pA to 1 nA. In order to compare values from different patches the value of the current measured in symmetrical NaCl at $V = +100$ mV was arbitrarily assigned as 1 and all the other values were scaled according to this convention.

Solutions and perfusing system

The ion composition of the solution filling the patch pipette was usually 110 mM-NaCl, no added divalent salts, 50 μM-EDTA and 10 mM-HEPES buffered to pH 7·6 with tetramethylammonium hydroxide (TMA-OH) (in some experiments, see Fig. 3*C*, NaCl was completely substituted by TMA-Cl). The ion composition of the bath solutions was similar except that 110 mM-NaCl was substituted as specified in the figure legends. The cytoplasmic side of the membrane patch was exposed to the different solutions by using the perfusing system described in Nunn (1987*a*) and in Menini & Nunn (1990). Four different solutions were flowing simultaneously side-by-side so as to allow several rapid solution changes to be made. In all experiments complete recovery of the current was checked by measuring, before and after each test solution, the current in symmetrical NaCl.

When the solution inside the patch pipette was different from the bathing solution a junction potential was present at the tip of the pipette. Liquid junction potentials were measured after the experiments by comparing the zero-current voltage in symmetrical NaCl solutions, and after replacement of the bath solution with the various solutions used in the experiments. A KCl agar bridge was employed in the bath to eliminate changes in the reference electrode potential.

Ion activities were calculated either directly from tables of activity coefficients (Robinson & Stokes, 1965) for solutions containing only one electrolyte or from tabulated coefficients of the electrolytes as described in Pitzer (1979) when mixtures of ions were present. The temperature was 20–23 °C in all experiments.

RESULTS

The results presented in this paper were obtained by measuring under voltage-clamp conditions the macroscopic current activated by cyclic GMP in excised inside-out patches from the plasma membrane of rod outer segments. Currents and reversal potentials were measured when the NaCl of the bathing medium was replaced by various salts at different concentrations.

Reversal potential in bi-ionic conditions

Reversal potentials were measured in bi-ionic conditions. The solution filling the patch pipette, at the extracellular side of the patch, contained 110 mM-NaCl, nominally 0 Ca^{2+} and 0 Mg^{2+}, as described in the Methods, and the fluid bathing the cytoplasmic side of the patch had the same composition except that 110 mM-NaCl was replaced by 110 mM-LiCl, KCl, RbCl or CsCl. Voltage pulses of 100 ms duration from a holding potential of 0 mV, increasing and decreasing in steps of ± 2 mV, were given in the absence and in the presence of cyclic GMP. The traces shown in Fig. 1*A* are cyclic GMP-activated currents recorded from one patch in the presence of 100 μM-cyclic GMP at the cytoplasmic side of the membrane. Each trace was obtained by subtraction of records in the presence and in the absence of cyclic GMP. The dashed line indicates the zero-current level.

Figure 1*B* shows the steady-state current from the experiment illustrated in *A*, plotted as a function of the membrane potential, after correction for junction potentials as described in the Methods. Reversal potentials, V_{rev}, were obtained by a linear interpolation of the currents recorded in response to voltage steps of ± 2 mV around the reversal potential. The mean values and standard deviations of reversal potential from many patches are plotted in Fig. 1*C* as a function of the inverse of the Pauling radius (Eisenmen & Horn, 1983) for alkali cations. Data were obtained at two different concentrations of cyclic GMP: 100 μM (□) and 5 μM (○). Permeability ratios were calculated from the measured reversal potentials using the Goldman–

Hodgkin–Katz (GHK) potential equation (eqn (1)). When the only permeant cation present at the external side of the membrane patch is Na^+ and the only permeant cation present at the intracellular side is X^+, the permeability ratio, P_X/P_{Na}, defined in the GHK potential equation (Goldman, 1943; Hodgkin & Katz, 1949), is:

$$\frac{P_X}{P_{Na}} = \frac{[Na^+]_o}{[X^+]_i} e^{-\frac{FV_{rev}}{RT}}, \quad (1)$$

where R is the gas constant, F the Faraday constant, T the absolute temperature, $[Na^+]_o$ the activity of Na^+ at the external side and $[X^+]_i$ the activity of the cation X^+ at the internal side of the membrane.

The permeability ratio sequence, calculated using eqn (1) from the average values of reversal potentials measured in the presence of 100 μM-cyclic GMP, was:

$$P_{Li}:P_{Na}:P_K:P_{Rb}:P_{Cs} = 1{\cdot}14:1:0{\cdot}98:0{\cdot}84:0{\cdot}58. \quad (2)$$

The permeability sequence (2) was approximately the same when the concentration of cyclic GMP was 5 μM. From Fig. 1*C* and eqn (1) it is seen that the permeability ratio decreases with increasing ion radius. The sequence (2) is different from the sequence of mobilities in water, indicating that the cyclic GMP-activated channels are not simple water-filled pores, and it corresponds to Eisenman sequence XI, which is to be expected if cations bind to a strong-field-strength site during their permeation process (Eisenman & Horn, 1983).

Effect of Cl^- replacement on the reversal potential

The possibility that Cl^- might contribute to the cyclic GMP-activated current was first studied by measuring the reversal potential in solutions where 110 mM-internal NaCl was replaced by 110 mM-sodium isethionate (data not shown). Isethionate was chosen because it is a large organic anion. If the cyclic GMP-activated channel is permeable to anions and if, because of its size, isethionate is much less permeant than Cl^-, then the reversal potential should show a large shift (110 mM-NaCl was present at the external side of the membrane patch). No shift in reversal potential was observed (three patches), indicating that Cl^- does not carry current through the cyclic GMP-activated channel. In one patch NaCl was also substituted by sodium propionate and the reversal potential did not change. Moreover, as will be shown later, measurements of the reversal potentials at various concentrations of NaCl, KCl and LiCl also suggest that Cl^- is not permeant. However, the inward and outward currents measured in the presence of isethionate or propionate were about 10% lower than in the presence of Cl^-. It will be shown in a later section that organic cations also have a blocking effect on the channel.

Current–voltage relations in bi-ionic conditions

Figure 2*A* shows the current–voltage relations from -100 mV to $+100$ mV in the presence of 100 μM-cyclic GMP, obtained under the same bi-ionic conditions as described in the first section. These results were obtained from two patches and, since each patch contained a different number of channels, the amplitude of the current flowing in symmetrical 110 mM-NaCl solutions at an applied potential $V = +100$ mV was normalized to unity (see Methods).

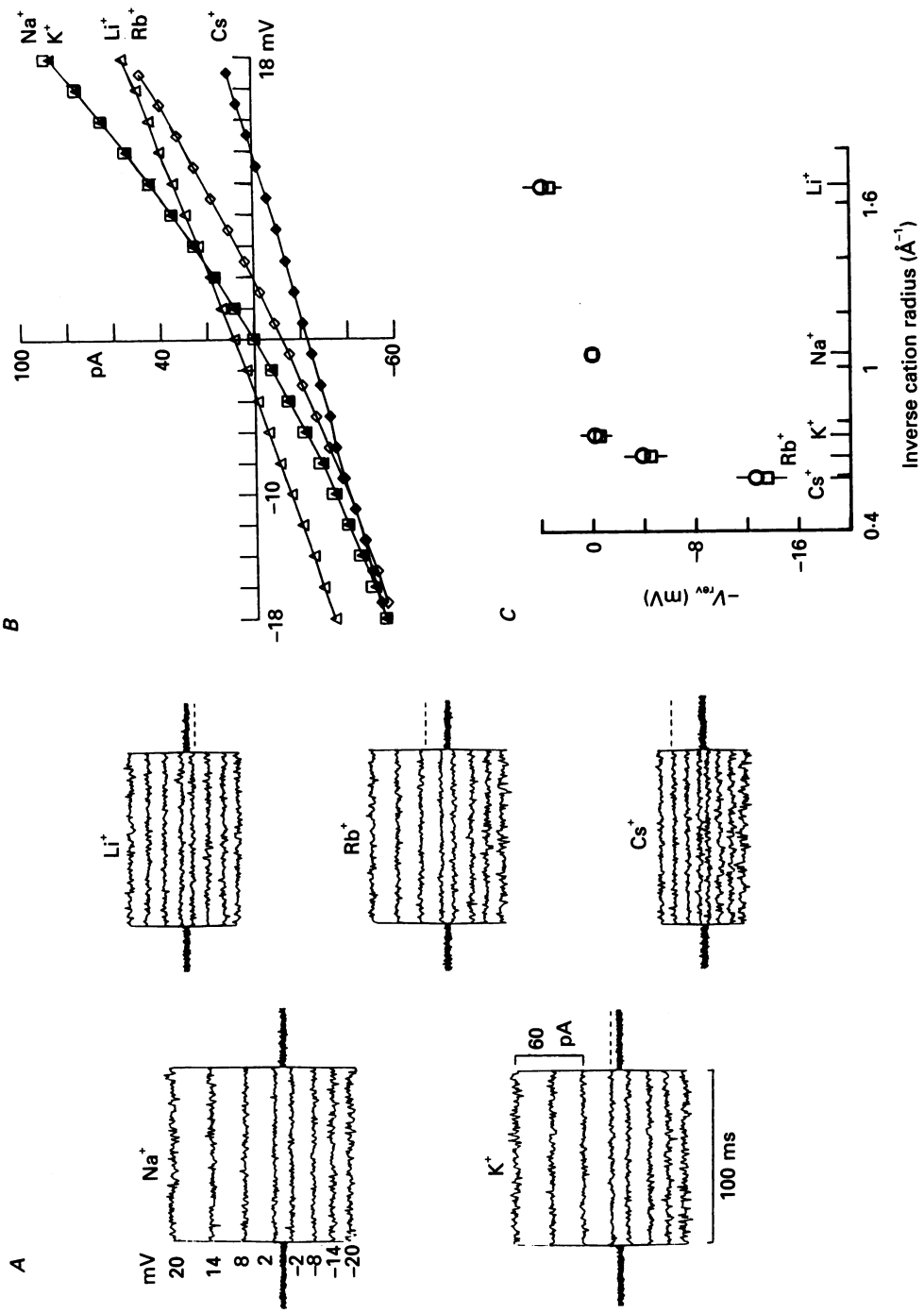

Fig. 1. For legend see facing page.

The current–voltage relation in a symmetrical Na^+ solution has an outward rectification (see Figs 1*A* and 2*A*), which may reflect properties of the single channel or changes in the number of open channels with voltage. Experiments on single channels in similar conditions indicate that the current–voltage relation is symmetrical (Haynes *et al.* 1986; Zimmerman & Baylor, 1986) and moreover, analysis of the gating of the cyclic GMP-activated channel in Na^+-symmetrical solutions (Karpen, Zimmerman, Stryer & Baylor, 1988) indicates that the outward rectification of the macroscopic currents is caused by a weak voltage dependence of the gating process, so that fewer channels are open at negative than at positive potentials.

By comparing the outward current carried by alkali ions at a fixed potential a selectivity sequence was obtained, different from that obtained for the permeability ratio (sequence 2). Na^+ and K^+ have similar values, but Li^+, which has a permeability ratio with respect to Na^+ close to 1, carries only about one-third of the current carried by Na^+ or K^+ at +100 mV. The selectivity sequence for outward currents, obtained as the average from ten patches, was:

$$I_{Na}:I_K:I_{Rb}:I_{Li}:I_{Cs} = 1:1:0{\cdot}67:0{\cdot}36:0{\cdot}25, \quad (3a)$$

at $V = +100$ mV, and:

$$I_{Na}:I_K:I_{Rb}:I_{Li}:I_{Cs} = 1:0{\cdot}85:0{\cdot}5:0{\cdot}46:0{\cdot}15, \quad (3b)$$

at $V = +40$ mV.

Figure 2*B* compares the current carried at +100 mV by different monovalent cations at two different cyclic GMP concentrations: 100 or 5 μM; recordings were from the same patch. The selectivity sequences obtained by measuring the steady-

Fig. 1. Currents activated by 100 μM-cyclic GMP in bi-ionic conditions. The ionic solution filling the patch pipette was: 110 mM-NaCl, 50 μM-EDTA, no added divalent salts and 10 mM-HEPES buffered at pH 7·6 with TMA-OH. The solution at the cytoplasmic side of the membrane patch had the same composition, but NaCl was substituted by 110 mM-LiCl, KCl, RbCl or CsCl, as indicated in the figure. These solutions were rapidly changed using the method of Nunn (1987*a*). Between the exposure to each test solution, the current in symmetrical Na^+ was measured to evaluate the recovery of the current. Voltage steps of ± 2 mV were given from a holding potential of 0 mV. The step duration was 100 ms. *A*, each trace is obtained as the difference between the current in the absence of cyclic GMP and the current in the presence of 100 μM-cyclic GMP. All traces are from the same patch. Outward currents are upward. Numbers adjacent to the traces indicate the membrane potential in millivolts before correction for junction potentials. The dashed line represents the zero-current level. *B*, current–voltage relations corrected for junction potentials for the alkali metal cations Na^+ (□), K^+ (▲), Li^+ (△), Rb^+ (◇) and Cs^+ (◆) from the results of the experiments shown in *A*. Continuous lines were drawn by hand. *C*, reversal potentials in bi-ionic conditions are plotted as a function of the inverse of the Pauling radius for alkali cations at two different concentrations of cyclic GMP: 100 μM (□) and 5 μM (○). V_{rev} is the average reversal potential, after correction for junction potentials. Collected data are plotted as mean ± S.D. Number of experiments in the presence of 100 μM (5 μM) cyclic GMP was: Li^+, 15 (12); K^+, 20 (10); Rb^+, 8 (7); Cs^+, 9 (7).

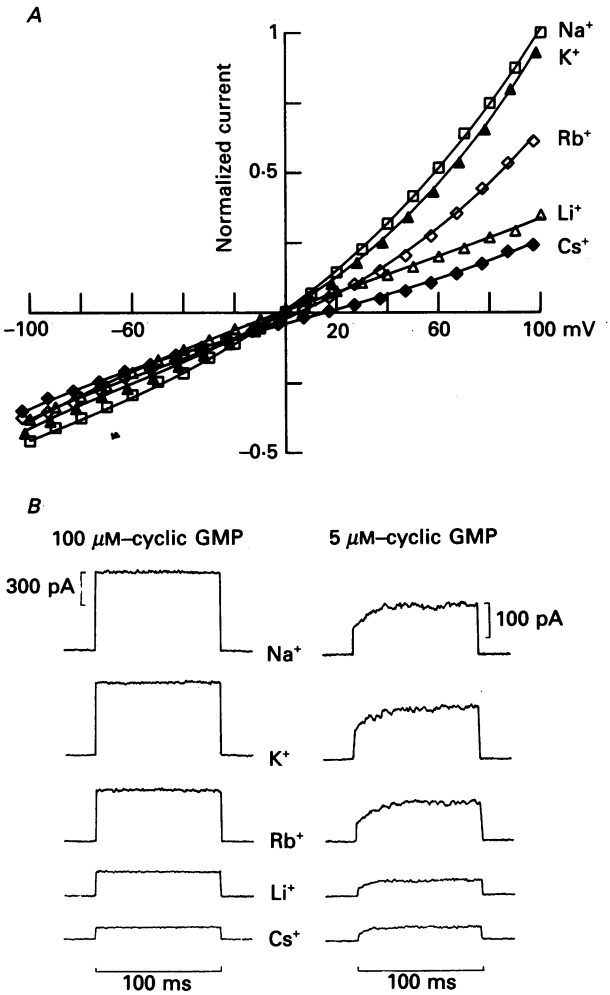

Fig. 2. *A*, current–voltage relations of channels activated by 100 μM-cyclic GMP in the same bi-ionic conditions of Fig. 1. Currents, obtained from two patches, were measured in a voltage range from -100 to $+100$ mV and normalized to unity in NaCl-symmetrical solutions at $+100$ mV as explained in the Methods. Continuous lines were drawn by hand. *B*, currents from the same patch at $V = +100$ mV, activated by 100 μM or 5 μM-cyclic GMP as indicated.

state current activated by 5 μM-cyclic GMP were approximately the same as the sequences in eqns (3*a* and *b*).

The effect of the presence of different alkali cations at the intracellular side on the inward currents, carried by external Na^+ ions, was also analysed (see Fig. 2*A*). At -100 mV the inward Na^+ currents, activated by 100 μM-cyclic GMP, were in the ratio 1:1:0·95:0·84:0·77 in the presence of internal Na^+, K^+, Rb^+, Li^+ and Cs^+ respectively. It is seen that, even at very negative potentials, Cs^+ and, to a lesser extent, Li^+ affect the Na^+ inward current.

Effect of organic cations

The analysis of the permeation of monovalent cations was extended to some organic cations. It was found that TMA^+, TEA^+, choline, glucosamine, lysine and arginine are impermeant, and that they also reduce the inward current carried by Na^+.

Figure 3*A* and *C* illustrates an experiment, performed on the same patch, in which the bathing medium was changed from 110 mM-NaCl to 110 mM-TMA-Cl, choline chloride or TEA-Cl. When NaCl in the internal solution was entirely substituted by any one of the organic cations, the outward currents at positive voltages were replaced by small inward currents, as would be expected if an internal permeant ion were substituted by an impermeant one. It is seen that the organic cations do not carry any outward current and that also the inward Na^+ current is strongly reduced when internal Na^+ is substituted by the organic cations. The mean values of the relative inward Na^+ currents at -100 mV, obtained as the average of four experiments, were 1 : 0·77 : 0·75 : 0·58 : 0·2 in the presence of internal Na^+, glucosamine, TMA^+, choline or TEA^+, respectively.

Figure 3*B* and *D* illustrates an experiment in which the patch pipette was filled with a solution containing 110 mM-TMA-Cl. After obtaining an excised patch, the bathing medium was changed to a solution containing 110 mM-NaCl and 100 μM-cyclic GMP. After an outward current activated by cyclic GMP was observed, the bathing medium was replaced with a solution identical to the solution filling the patch pipette with and without cyclic GMP. No cyclic GMP-activated current was observed when TMA^+ was present on both sides of the patch, confirming that this cation is impermeant.

The dependence of current on ion activity

This section describes experiments designed to measure currents through the channels activated by 100 μM-cyclic GMP, when the internal activity of permeant ions was changed.

Currents were first measured with internal Na^+ at five different concentrations: 5, 27·5, 55, 110 and 500 mM-NaCl. The same experiment was repeated with internal K^+ and Li^+, while the external solution always contained 110 mM-NaCl. The osmolarity was kept constant by adding dextrose, except in the case of 500 mM, where the bathing solution was hypertonic. The ionic strength of the bathing medium in these experiments was not constant, since it was decided not to use ions such as choline, TEA^+ or TMA^+ to substitute Na^+, because they block the cyclic GMP-activated channel as shown in the previous section (see Fig. 3*A* and *C*).

Figure 4*A* and *B* shows currents measured on the same patch, with Na^+ or Li^+ as the internal cation, respectively. The dashed line indicates the zero-current level. Voltage steps, in increments of ± 20 mV, were given from a holding potential of 0 mV, over the range -100 to $+100$ mV. The experiments were then repeated with different concentrations of K^+, and traces similar to those presented in Fig. 4*A* for Na^+ were obtained. Figure 4*C* and *D* shows currents as a function of membrane potential, corrected for junction potentials, from the experiments in Fig. 4*A* and *B*. The presence of a saturation of the current is evident: an increase of about tenfold

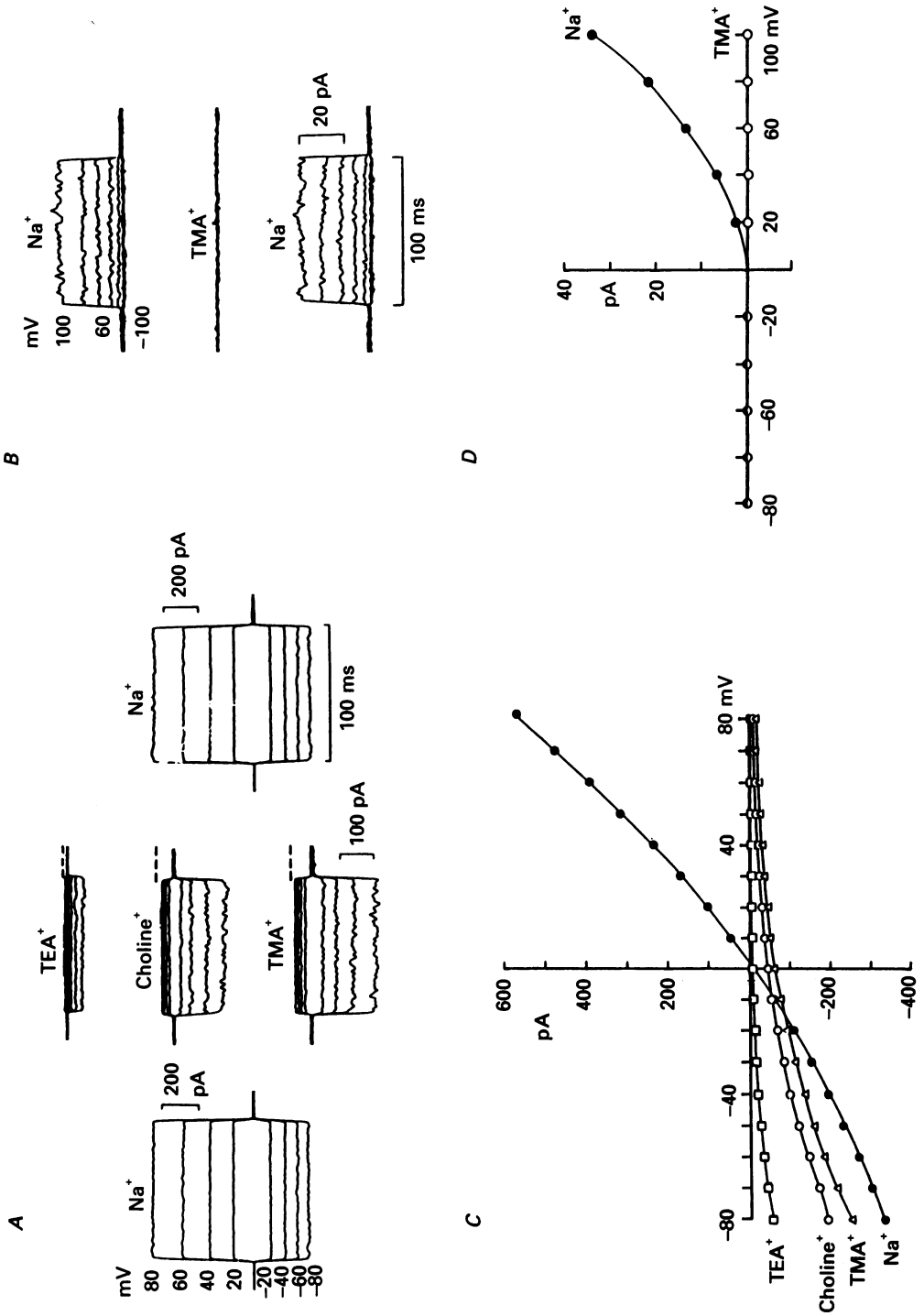

Fig. 3. For legend see facing page.

in internal Na^+ concentration produces an increase of about threefold the outward current flowing at +90 mV.

Reversal potentials were calculated from linear interpolation of the currents around reversal and the results from several experiments are shown in Fig. 5, where mean values (±S.D.) of reversal potentials are plotted as a function of the internal cation activity for Na^+, K^+ and Li^+. Each point is the average from at least five patches. The continuous line in Fig. 5 represents the theoretical reversal potential *versus* internal cation activity calculated from the GHK equation (eqn (1)) at 20 °C with $P_X/P_{Na} = 1$. The permeability ratios for Li^+ and K^+ with respect to Na^+ at a concentration of 110 mM were close to 1 (see sequence 2) and Fig. 5 shows that this value was approximately the same in the range of concentrations from 5 to 110 mM. Reversal potentials deviated slightly from the value expected from the GHK equation only at a concentration of 500 mM for Na^+ and Li^+. This behaviour cannot be explained by including a permeability to Cl^- in eqn (1), since this would give a deviation of opposite sign of the one observed. The good fit obtained with the GHK potential equation (eqn (1)), assuming that Cl^- is impermeant, confirms that the cyclic GMP-activated channel is impermeable to Cl^- and shows that permeability ratios do not depend on the internal ion activity of Na^+, Li^+ or K^+.

Figure 6*A* shows the average steady-state current (±S.D.) measured at +90 mV as a function of the internal activity of Na^+, K^+ or Li^+, from experiments similar to those presented in Fig. 4. Each point is the average from at least five patches. The currents were normalized to unity in symmetrical 110 mM-NaCl at $V = +100$ mV. In Fig. 6*A* it is shown that the current saturates as the ionic activity increases. The GHK current equation (see Hille, 1975, 1984) cannot describe this result, since a linear dependence of current on ion activity is expected. Figure 6*A* shows that the experimental points at +90 mV could be very well fitted by a Michaelis–Menten equation:

$$I_X = I_{max,X} \frac{[X^+]_i}{[X^+]_i + K_{m,X}}, \tag{4}$$

where I_X is the current carried by the ion X^+, $[X^+]_i$ is its activity at the intracellular side of the membrane patch, $I_{max,X}$ is the saturating current and $K_{m,X}$ is the half-

Fig. 3. *A*, currents activated by 100 μM-cyclic GMP in the presence of organic cations at the cytoplasmic side. The solution filling the patch pipette was always 110 mM-NaCl. At the intracellular side 110 mM-NaCl was substituted by 110 mM-TMA-Cl, choline chloride or TEA-Cl. Dashed line indicates zero-current level. Numbers adjacent to traces indicate membrane potentials in millivolts. Currents in symmetrical NaCl were measured before (traces on the left) and after (traces on the right) exposure to solutions containing organic cations to test the recovery. *B*, the filling solution in the patch pipette contained 110 mM-TMA-Cl. At the intracellular side the solution contained 110 mM-NaCl or 110 mM-TMA-Cl. No currents in symmetrical TMA-Cl were observed in the presence of 100 μM-cyclic GMP. The current showed a complete recovery after exposure to the TMA-Cl solution (compare top and bottom traces) when NaCl replaced TMA-Cl. *C*, current–voltage relations from the experiment shown in *A*. Continuous lines were drawn by hand. *D*, current–voltage relations from the experiment shown in *B*. Continuous lines were drawn by hand.

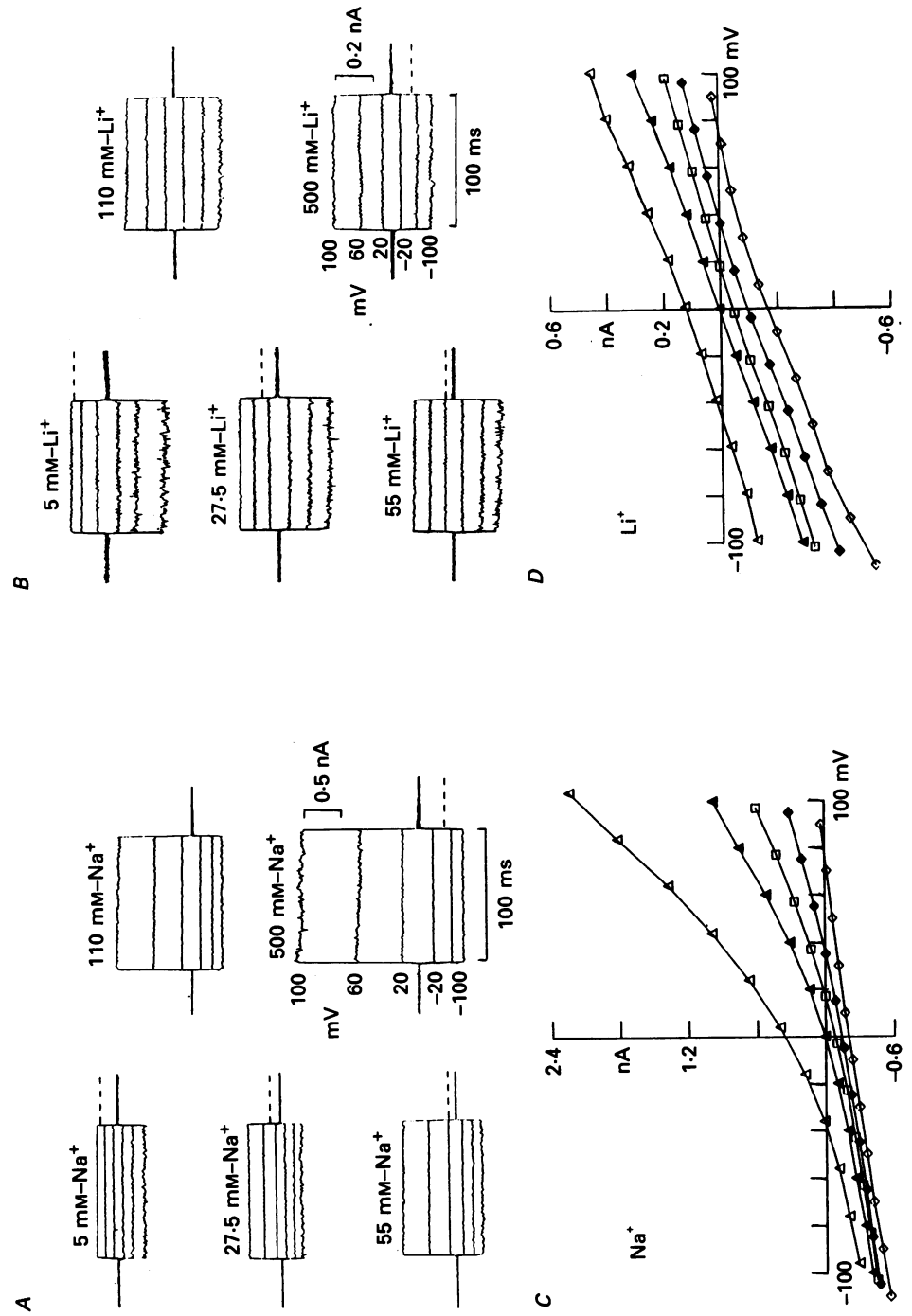

Fig. 4. For legend see facing page.

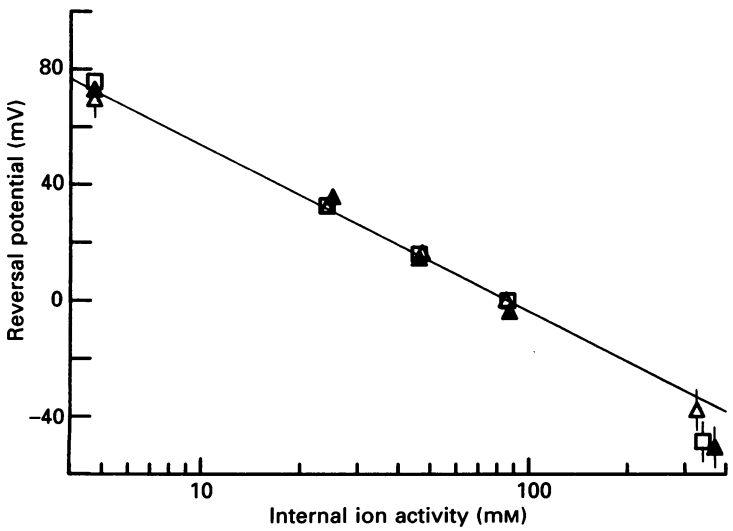

Fig. 5. Reversal potential of the current activated by 100 μM-cyclic GMP as a function of internal ion activity for Na^+ (□), Li^+ (▲) or K^+ (△). Each point is the average (±S.D.) from at least five patches. Continuous line was obtained from the GHK equation (eqn (1)) with $P_X/P_{Na} = 1$. Ion activities were calculated as described in the Methods.

saturating activity of the ion X^+. The values for $K_{m,X}$ and $I_{max,X}$ usually depend on the transmembrane potential. Equation (4) can be obtained by assuming that during ionic permeation the permeating ion jumps across energy barriers from one well to another (Eyring, Lumry & Woodbury, 1949; Hille, 1975, 1984).

The continuous curves in Fig. 6*A* show the fit of eqn (4) to the data with values: $K_{m,Na} = 249$ mM, $K_{m,K} = 203$ mM and $K_{m,Li} = 160$ mM and for the saturating currents: $I_{max,Na} = 3{\cdot}5$, $I_{max,K} = 3$ and $I_{max,Li} = 0{\cdot}9$ ($I = 1$ corresponds to the current measured in 110 mM-NaCl symmetrical solutions at $V = +100$ mV). These results show that Li^+ has a slightly higher affinity for the channel than Na^+ or K^+ and that the saturating current measured in the presence of Li^+ is much lower than the one obtained with Na^+ or K^+.

Mixtures of Na^+ and Li^+

The currents in solutions containing mixtures of Na^+ and Li^+ were also investigated. In these experiments the total concentration of Na^+ and Li^+ at the

Fig. 4. Currents activated by 100 μM-cyclic GMP in the presence of different amounts, indicated in figure, of NaCl (*A*) or LiCl (*B*) at the cytoplasmic side. The osmolarity was kept constant by adding dextrose with the exception of the solution containing 500 mM of salt in which the osmolarity was not compensated. The dashed line indicates the zero-current level. Voltage steps of ±20, 60, 100 mV from a holding potential of 0 mV as indicated by the numbers adjacent to the traces. All results were obtained from the same patch and the solution filling the patch pipette contained 110 mM-NaCl. *C* and *D*, current–voltage relations corrected for junction potentials from the experiments shown in *A* and *B* respectively. Symbols indicate the intracellular concentration: 5 mM (◇), 27·5 mM (◆), 55 mM (□), 110 mM (▲) and 500 mM (△).

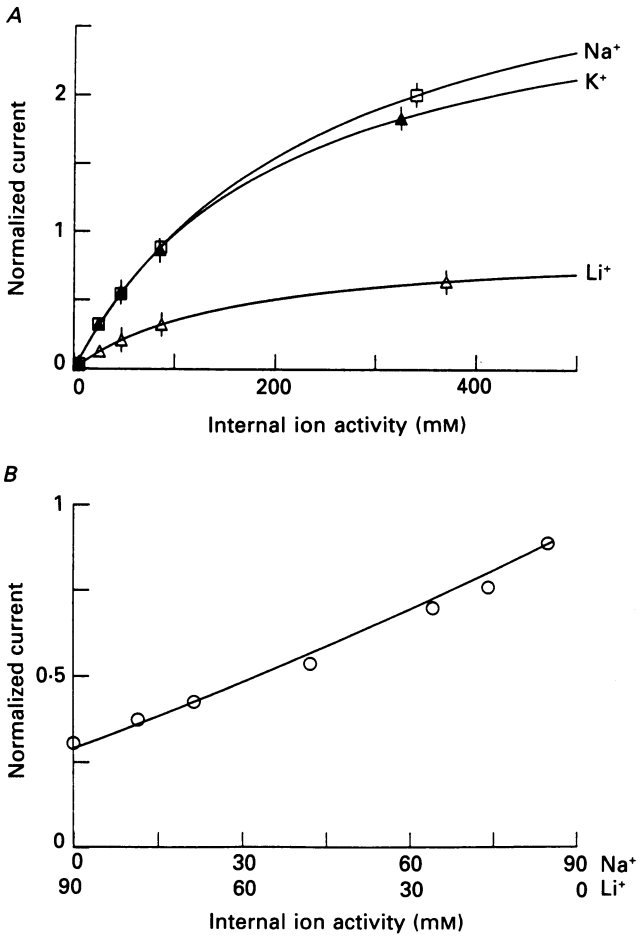

Fig. 6. *A*, dependence of the current activated by 100 μM-cyclic GMP at +90 mV on the ionic activity of Na^+ (□), K^+ (▲) and Li^+ (△). The solution filling the patch pipette contained 110 mM-NaCl. Each point is the average of the current at the steady state from at least five patches. Bars indicate standard deviation. Currents in *A* and *B* were normalized and ion activities were calculated as described in the Methods. Continuous lines were obtained from a Michaelis–Menten equation (eqn (4)) with $K_{m,X}$ at +90 mV equal to 249, 203 and 160 mM for Na^+, K^+ and Li^+ respectively. The extrapolated ratios for $I_{max,X}$ at +90 mV are 1:0·86:0·26 for Na^+, K^+ and Li^+ respectively. *B*, cyclic GMP-activated currents measured as a function of the mole fraction of Na^+ and Li^+ at +90 mV. The total concentration of Na^+ and Li^+ was 110 mM. Extracellular solution always contained 110 mM-NaCl. The continuous line is the relation expected if Na^+ and Li^+ compete for the same binding site, as described by eqn (5), with values for $I_{max,X}$ and $K_{m,X}$ for Na^+ and Li^+ as in *A*.

cytoplasmic side of the membrane was 110 mM (activity *ca* 90 mM) and the relative concentration of the two permeating ions was changed. The reversal potentials of these mixtures were a monotonic function of ion concentration varying from −4 mV, in the presence of 110 mM-LiCl, to 0 mV, when only NaCl (110 mM) was present in solution (data not shown).

In Fig. 6*B* the amplitude of the average outward current from three patches at +90 mV is plotted *versus* Na^+ and Li^+ activity. Currents were normalized as described in the Methods. It is seen that the amplitude of the outward current increased monotonically with Na^+ activity. Monotonicity of the reversal potential and of the current in mole fraction experiments is one of the features usually taken to indicate that the channel contains only one ion at a time (Hille, 1984). An anomalous mole-fraction effect (e.g. the reversal potential or the current going through a maximum or a minimum) would imply that the channel contains more than one ion at a time, as for example in Ca^{2+} channels (Almers & McCleskey, 1984; Hess & Tsien, 1984).

If two ions, such as Na^+ and Li^+, are present in the solution at the internal side, and they compete for the same site within the channel, the total outward current (Hille 1975, 1984) is:

$$I = \frac{I_{\max,Na}\frac{[Na^+]_i}{K_{m,Na}} + I_{\max,Li}\frac{[Li^+]_i}{K_{m,Li}}}{1 + \frac{[Na^+]_i}{K_{m,Na}} + \frac{[Li^+]_i}{K_{m,Li}}}, \quad (5)$$

where $[Na^+]_i$ and $[Li^+]_i$ are respectively the Na^+ and Li^+ activities at the intracellular side and $I_{max,Na}$, $I_{max,Li}$, $K_{m,Na}$ and $K_{m,Li}$ are as defined in eqn (4), with X^+ being Na^+ or Li^+.

A further test to see whether the cyclic GMP-activated channel is usually a single ion channel is to use the values of $I_{max,X}$ and $K_{m,X}$ obtained in the experiments illustrated in Fig. 6*A* to fit the results obtained with mixtures of Na^+ and Li^+. By using the values of $K_{m,Na}$ = 249 mM, $K_{m,Li}$ = 160 mM, $I_{max,Na}$ = 3·5 and $I_{max,Li}$ = 0·9 in eqn (5), the continuous line in Fig. 6*B* is obtained, which provides a good fit for the experimental data.

DISCUSSION

The aim of the experiments described in this paper was to determine the monovalent cation transport properties of the cyclic GMP-activated channel in excised patches from retinal rods of the tiger salamander. The results obtained allow several inferences concerning the structure of this channel.

The experiments on the ion activity dependence of the current for Na^+, K^+ and Li^+ showed that the permeability ratios for these ions are independent of ion activity and are well described by the GHK potential equation (eqn (1); Fig. 5), and further that the outward current at +90 mV saturates as the ion activity is increased (Figs 4 and 6*A*). This last result is not compatible with the GHK current equation, from which a linear dependence of current on ion activity is expected. Saturation of the current is usually taken as evidence that the 'independence principle' (i.e. the assumption that the movement of one ion is not influenced by other ions) is not valid, and that the channel contains at least one binding site (Hille, 1975, 1984). Ions must move in single file and bind to sites inside the pore during the permeation process. Channels are often divided in two main categories: one-ion or multi-ion channels, depending on how many ions they may bind at the same time. One-ion channels have the following characteristics: (1) the permeability ratios among different ions are

constants, independent of ion activity; (2) the dependence of the current on the activity of the permeating ion is a simple saturating function described by the usual Michaelis–Menten equation; and (3) the current and the reversal potential are monotonic functions of mole fraction when measured in mixtures of two permeating ions.

Many of the results presented in this paper can be accounted for by assuming that the cyclic GMP-activated channel is a one-ion channel. The good fit of the data (Fig. 6*A*) by a simple Michaelis–Menten equation, the independence of permeability ratios from internal ion activity and the absence of any anomalous mole-fraction effect between Na^+ and Li^+ (Fig. 6*B*) indicate that a one-ion channel is adequate for a description of these experiments. Half-saturating activities at +90 mV calculated from the best fit of eqn (4) to the data (Fig. 6*A*) were 249, 203 and 160 mM for Na^+, K^+ and Li^+, respectively. Since the half-saturating activity is inversely proportional to the affinity of the permeating ions for a binding site, Li^+ has a higher affinity than Na^+ and K^+. Moreover, these ions, when present together in the internal solution, compete for this site, as suggested by the fit of eqn (5) to the data obtained in mixtures of Na^+ and Li^+ (Fig. 6*B*).

A very useful model for describing the permeation of ions through channels is the rate-theory or Eyring barrier model (Eyring *et al.* 1949; Hille, 1974). In this model the channel is represented by an energy profile, having maxima (barriers) and minima (wells or binding sites), encountered by an ion crossing the channel. From the data presented in this paper it is possible to obtain some information about the energy profile of the cyclic GMP-activated channel. In fact, in a one-ion pore, the permeability ratios are determined only by the height of the energy barriers encountered by the ion and not by the depth of wells, whilst the saturation of the current as a function of ion activity is determined by well depth (Hille, 1975; Eisenman & Horn, 1983). Reversal potential measurements are rather sensitive tests of barrier heights and are independent of the number of open channels. The results obtained for the permeability ratios (sequence 2) indicate that barriers for Li^+, Na^+ and K^+ have similar height, while barriers for Rb^+ and Cs^+ are higher. The affinity of Li^+, Na^+ and K^+ for the channel indicate that the well is deeper for Li^+ than for Na^+ and K^+.

The analysis of the inward Na^+ currents showed that they also were reduced, at very negative potentials (−100 mV), by the presence of various cations at the cytoplasmic side of the membrane patch (Figs. 2*A* and 3*C*). The relative currents at −100 mV were 1:0·84:0·77:0·77:0·75:0·58:0·2 in the presence of Na^+, Li^+, Cs^+, glucosamine, TMA^+, choline and TEA^+ respectively. Two explanations are possible: (i) these cations, or some of them, modify the number of channels opening, or (ii) a large vestibule with weakly voltage-dependent binding sites, which can be occupied by a permeating or a non-permeating ion, is present at the cytoplasmic side of the channel. In the former case the current would be reduced because fewer channels are open in the presence of these cation. In the latter case the structure of the cyclic GMP-activated channel in retinal rods would consist of a narrow portion, behaving as a one-ion pore, and a large vestibule at the cytoplasmic side, where cations bind in a manner which is not very sensitive to membrane potential.

Comparison with light-sensitive channels in intact rods

Several experimental observations (see Yau & Baylor, 1989 for a review) indicate that the cyclic GMP-activated channel is identical to the light-sensitive channel in intact rods. The reversal potential of the light-sensitive current in intact rods in physiological conditions is close to zero, and this result has been interpreted as suggesting that K^+ has a high permeability (Bader, McLeish & Schwartz, 1979; Baylor & Nunn, 1986). An initial comparison between the experiments presented in this paper and those on intact rods can be made by assuming that in physiological conditions the dark level of cyclic GMP is only a few micromolar (Nakatani & Yau, 1988*b*) and that $[Na^+]_o = [K^+]_i = 110$ mM. Experiments obtained in excised patches in these ionic conditions and using 5 μM-cyclic GMP to activate channels gave a value close to zero for the reversal potential (Fig. 1*C*) in agreement with the results from intact rods.

In intact rods the cation selectivity has been determined from measurements of the light-sensitive inward current after replacement of extracellular Na^+ with other alkali cations (Yau & Nakatani, 1984; Hodgkin *et al.* 1985). The potential in these experiments was not clamped and it was probably around -40 mV. Reversal potentials were not studied. The selectivity sequence obtained by Hodgkin *et al.* (1985) was:

$$I_{Li}:I_{Na}:I_K:I_{Rb}:I_{Cs} = 1{\cdot}4:1:0{\cdot}8:0{\cdot}6:0{\cdot}15.$$

These values of selectivity are in general agreement with those obtained in this work for outward currents, with the important exception of Li^+. In the present work the following sequence (3*b*) was found in excised patches at $V = +40$ mV.

$$I_{Na}:I_K:I_{Rb}:I_{Li}:I_{Cs} = 1:0{\cdot}85:0{\cdot}5:0{\cdot}46:0{\cdot}15.$$

In the experiments on intact rods, replacement of external Na^+ by Li^+ increased the inward current, whilst in excised patches replacement of internal Na^+ by Li^+ decreased the outward current (Fig. 2). Different explanations are possible for this apparent discrepancy. In intact rods and excised patches, the amplitude of the measured macroscopic current is determined both by the number of open channels and by the amplitude of current through a single channel. It is possible that the permeant cations influence the gating of the channel (i.e. the probability of a channel being open) and that such influence is both cation-specific and side-specific. In addition, it is possible that ion transport through the open pore is asymmetric, and that from the outside Li^+ carries more current than Na^+ through a single open channel, while from the inside Na^+ carries more current than Li^+. Further experiments will be necessary in order to understand this point, and to separate gating from permeation.

It is also known that a variety of divalent cations permeate the light-sensitive channel (Yau & Nakatani, 1984; Hodgkin *et al.* 1985; Nakatani & Yau, 1988*a*; Menini *et al.* 1988*b*). Preliminary experiments have shown that the same cations also permeate the cyclic GMP-activated channel (Colamartino, Menini & Torre, 1989; Colamartino, Menini, Spadavecchia & Torre, 1990).

Comparison with other channels

The cyclic GMP-activated channel is a non-selective cation channel: it is highly selective for cations over anions but it does not select appreciably between different monovalent cations, particularly between the physiologically most significant K^+ and Na^+ ions. This channel appears to be quite different from the other channels. It has the same permeability sequence for alkali cations as neuronal Na^+ channels (Hille, 1975) and L-type cardiac Ca^{2+} channels (Hess, Lansman & Tsien, 1986) but the selectivity of those channels between cations is much higher. On the other hand the acetylcholine receptor channel is not very selective among alkali cations, but its permeability sequence (Adams, Dwyer & Hille, 1980) is diametrically opposite to that of the cyclic GMP-activated channel, suggesting that the lack of selectivity of these two channels is realized at a molecular level in a very different way.

It is a pleasure to acknowledge Professor John W. Moore, who greatly contributed to the realization of this work with all kinds of help. I am grateful to Dr Vincent Torre for assistance in some of the early experiments and for critically reading the manuscript. I thank Drs Franco Conti and Franco Fambale for comments on the manuscript. I appreciate the help of Mr Srinivas Gidugu in computer analysis and the expert secretarial assistance of Miss Cristina Rosati. Clive Presst kindly checked the English. This research was supported by grant EY07106 from the National Institutes of Health.

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