Characterization of a C-type natriuretic peptide (CNP-39)-formed cation-selective channel from platypus (*Ornithorhynchus anatinus*) venom

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1. The lipid bilayer technique is used to characterize the biophysical and pharmacological properties of a novel, fast, cation-selective channel formed by incorporating platypus (*Ornithorhynchus anatinus*) venom (*Oa*V) into lipid membranes.

2. A synthetic C-type natriuretic peptide *Oa*CNP-39, which is identical to that present in platypus venom, mimics the conductance, kinetics, selectivity and pharmacological properties of the *Oa*V-formed fast cation-selective channel. The N-terminal fragment containing residues 1–17, i.e. *Oa*CNP-39(1–17), induces the channel activity.

3. The current amplitude of the TEACl-insensitive fast cation-selective channel is dependent on cytoplasmic K^+, $[K^+]_{cis}$. The increase in the current amplitude, as a function of increasing $[K^+]_{cis}$, is non-linear and can be described by the Michaelis–Menten equation. At +140 mV, the values of γ_{max} and K_S are 63·1 pS and 169 mM, respectively, whereas at 0 mV the values of γ_{max} and K_S are 21·1 pS and 307 mM, respectively. γ_{max} and K_S are maximal single channel conductance and concentration for half-maximal γ, respectively. The calculated permeability ratios, $P_K : P_{Rb} : P_{Na} : P_{Cs} : P_{Li}$, were 1 : 0·76 : 0·21 : 0·09 : 0·03, respectively.

4. The probability of the fast channel being open, P_o, increases from 0·15 at 0 mV to 0·75 at +140 mV. In contrast, the channel frequency, F_o, decreases from 400 to 180 events per second for voltages between 0 mV and +140. The mean open time, T_o, increases as the bilayer is made more positive, between 0 and +140 mV. The mean values of the voltage-dependent kinetic parameters, P_o, F_o, T_o and mean closed time (T_c), are independent of $[KCl]_{cis}$ between 50 and 750 mM ($P > 0·05$).

5. It is proposed that some of the symptoms of envenomation by platypus venom may be caused partly by changes in cellular functions mediated via the *Oa*CNP-39-formed fast cation-selective channel, which affects signal transduction.

Envenomation by platypus (*Ornithorhynchus anatinus*) venom (*Oa*V) causes severe local effects, including intense pain, hyperalgesia and plasma extravasation (oedema) (de Plater *et al.* 1995), as well as hypotension and peripheral vasodilation in systemically administered experimental animals (Fenner *et al.* 1992). The molecular mechanisms underlying these *Oa*V-induced effects are not known. Using the lipid bilayer technique it has been shown that platypus venom (*Oa*V), which contains 19 peptide–protein components (de Plater *et al.* 1995; de Plater, 1998), formed inward anion and outward slow and fast cation-selective channels (J. I. Kourie unpublished data). The *Oa*V-formed anion channel has a maximum conductance of 857 ± 23 pS in 250/50 mM KCl *cis/trans*. The current–voltage relationship of this channel shows strong inward rectification. The channel activity undergoes time-dependent inactivation that can be removed by depolarizing voltage steps more positive than +40 mV, the equilibrium potential for chloride (E_{Cl}). The conductance values for the slow and fast channels are 22·5 ± 2·6 and 38·8 ± 4·6 pS in 250/50 mM KCl *cis/trans* and 41·38 ± 4·2 and 60·7 ± 7·1 pS in 750/50 mM KCl *cis/trans*, respectively. The kinetics of the slow ion channels are voltage dependent. The channel open probability (P_o) is between 0·1 and 0·8 at potentials between 0 and +140 mV. The channel frequency (F_o) increases with depolarizing potentials between 0 and +140 mV, whereas the mean open time (T_o) and mean closed time (T_c) decrease. The channel has conductance values of 21·47 ± 2·3 and 0·53 ± 0·1 pS in 250 mM KCl and choline chloride, respectively. The amplitude of the single channel current is dependent on cytoplasmic $[K^+]$ ($[K^+]_{cis}$) and the reversal potential (E_{rev}) value responds to increases in $[K^+]_{cis}$ by shifting to more negative voltages.

The increase in current amplitude as a function of increasing $[K^+]_{cis}$ is described by the Michaelis–Menten equation. At +140 mV, γ_{max} and K_S (maximal single channel conductance and concentration for half-maximal γ, respectively), have values of 38·6 pS and 380 mM and decline at 0 mV to 15·76 pS and 250 mM, respectively. The permeability values for $P_K : P_{Na} : P_{Cs} : P_{choline}$ are 1 : 1 : 0·63 : 0·089, respectively.

The fast cation channel has not yet been studied in detail. The aims of this study are: (a) to characterize the biophysical properties of the *Oa*V-formed fast cation-selective channel; (b) to compare these properties with those of a fast cation-selective channel formed by a synthetic C-type natriuretic peptide (*Oa*CNP-39), which has been identified as a major component of *Ornithorhynchus anatinus* venom (de Plater, 1998); and (c) to determine the role of the 17 residue N-terminal fragment, i.e. *Oa*CNP-39(1–17) of *Oa*CNP-39 in the channel activity.

METHODS

Solutions

Unless otherwise stated, the initial experimental solution for incorporating *Oa*V, *Oa*CNP-39 and *Oa*CNP-39(1–17) into the bilayers contained KCl (250 mM *cis* and 50 mM *trans*) plus 1 mM $CaCl_2$ and 10 mM Hepes buffer (pH 7·4, adjusted with 4·8 mM KOH).

Synthesis of *Oa*CNP-39 and *Oa*CNP-39(1–17) and amino acid analysis

Small synthetic peptides are powerful tools for the study of structure–function relationships of ion channel-forming proteins. The procedures for synthesis of *Oa*CNP-39 and *Oa*CNP-39(1–17) and amino acid analysis have been detailed previously (de Plater *et al.* 1998*a*, *b*).

The total sequence of *Oa*CNP-39 is shown below:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Leu-Leu-His-Asp-His-Pro-Asn-Pro-Arg-Lys-Tyr-Lys-Pro-Ala-Asn-Lys-Lys-
(extended N-terminal)

18 19 20 21 22
Gly-Leu-Ser-Lys-Gly- (N-terminal)

23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
Cys-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Thr-Ser-Gly-Leu-Gly-Cys
(loop).

*Oa*CNP-39 and its fragments are routinely synthesized at the Centre for Molecular Structure and Function at the Australian National University, using an Applied Biosystems 470A solid phase peptide synthesizer (de Plater *et al.* 1998*a*). Disulphide bridges are formed using a 10-fold molar excess of EKATHIOX™ resin (Ekagen, Palo Alto, CA), stirred at room temperature for 4 h. The peptide is purified by preparative RP-HPLC (BioRad Gradient Module 850 M), using a Dynamax C8 column (300A, 21·4 mm × 25 cm, Ranin Associates) and analysed by amino acid analysis (see below) and TOF-MALDI (VG Analytical, Fisons) mass spectrometry to confirm authenticity. Peptide concentrations are determined by quantitative amino acid analysis (de Plater, 1998). A lyophilized sample is subjected to gas-phase hydrolysis under N_2 at 110 °C for 20 h, using 6 N HCl with 0·1 % phenol. Analyses are performed using an H-P AminoQuant Series II (Hewlett-Packard) amino acid analyser. Amino acids are subjected to pre-column derivitization with *ortho*-pthalaldehyde (OPA with 3-mercaptopropionic acid in 400 mM sodium borate, pH 10·4) and 9-fluorenylmethyl chloroformate, and derivitized amino acids are detected using an HP1046 (Hewlett-Packard) fluorescence detector.

Lipid bilayer membranes and incorporation of *Oa*V, *Oa*CNP-39 and *Oa*CNP-39(1–17)

Bilayers were formed across a 150 μm hole in the wall of a 1 ml delrin™ cup, using a mixture of palmitoyl-oleoyl-phosphatidylethanolamine, palmitoyl-oleoyl-phosphatidylserine and palmitoyl-oleoyl-phosphatidylcholine (5 : 3 : 2, by volume) (Miller & Racker, 1976; Kourie *et al.* 1996), obtained from Avanti Polar Lipids (Alabama). The lipid mixture was dried under a stream of N_2 and redissolved in *n*-decane at a final concentration of 50 mg ml^{-1}. *Oa*V, *Oa*CNP-39 and *Oa*CNP-39(1–17) were incorporated into the lipid bilayer by addition to the *cis* chamber up to a final peptide concentration of 0·1–1 μg ml^{-1}. The side of the bilayer to which the *Oa*V, *Oa*CNP-39 and *Oa*CNP-39(1–17) were added is defined as *cis*, and the other side as *trans*. The experiments were conducted at room temperature (between 20 and 25 °C).

Recording single channel activity

The pCLAMP6 program (Axon Instruments Inc.) was used for voltage command and acquisition of Cl^- current families with an Axopatch 200 amplifier (Axon Instruments Inc.). The current was monitored on an oscilloscope and stored on a compact disc recorder (CD-R). The *cis* and *trans* chambers were connected to the amplifier head stage by Ag–AgCl electrodes in agar salt-bridges containing the solutions present in each chamber. Voltages and currents were expressed relative to the *trans* chamber. Data were filtered at 1 kHz (4-pole Bessel, −3 dB) and digitized *via* a TL-1 DMA interface (Axon Instruments Inc.) at 2 kHz. The optimal bilayers that were formed had specific capacitance values larger than 0·42 μF cm^{-2} but lower than the ~1·0 μF cm^{-2} value for biological membranes, because the bilayer area includes some of the thick film of the annulus, which has much lower capacitance than is found in biological membranes (Kourie, 1996).

Data analysis

CHANNEL 2 (developed by P. W. Gage & M. Smith, at The John Curtin School of Medical Research, Australian National University), an in-house analysis program (Kourie *et al.* 1996*a*), was used to measure the parameters of single channel activity (Colquhoun & Hawkes, 1983). These include: (a) mean open time, T_o (the total time that the channel was not closed and including openings to all conductance levels, divided by the number of events); (b) mean closed time, T_c (the total time that the channel was closed divided by the number of events); (c) frequency of the channel opening, F_o; (d) the open probability, P_o (the sum of all open times as a fraction of the total time). The value of the current amplitude was obtained by measuring the distance (in pA) between two lines. The first line was set on the maximum baseline noise of the closed level, where the current amplitude was considered 0 pA, and the second was set on the noise of the majority of distinct open events longer than 0·5 ms. The threshold level for the detection of single channel events was set at 50 % of the current amplitude (Patlak, 1993). The reversal potential was corrected for the liquid junction potential by using the JPCalc software (Barry, 1994). Assuming that the only permeant ions in the system were K^+ and the substituting cation, X^+, this shift ($\Delta E_{rev} = E_X - E_K$) can be used to estimate the selectivity from the following equation (Hodgkin and Katz, 1949):

$$\Delta E_{rev} = 59 \log P_x[X^+]_{cis}/P_K[K^+]_{cis}.$$

Statistics

Unless otherwise stated, each ion channel was used as its own control and the comparison was made between biophysical

Table 1. Parameters of cation-selective channels recorded in 250/50 mM KCl *cis*/*trans* and clamped at +80 mV

Channel-forming peptide	Conductance (pS)	Open probability	Selectivity sequence $P_K : P_{Rb} : P_{Na} : P_{Cs} : P_{Li}$
*Oa*V	38·8 ± 4·6 ($n = 11$)	0·52 ± 0·11 ($n = 10$)	—
*Oa*CNP-39	37·6 ± 4·3 ($n = 5$)	0·47 ± 0·09 ($n = 5$)	1 : 0·64 : 0·28 : 0·13 : 0·06 ($n = 1$)
*Oa*CNP-39(1–17)	41·1 ± 4·7 ($n = 8$)	0·45 ± 0·13 ($n = 7$)	1 : 0·76 : 0·21 : 0·09 : 0·03 ($n = 2$)

parameters of the channel before and after changing the *cis* or *trans* solutions. Data are reported as means ± S.E.M. (see Table 1).

RESULTS

One hundred and seventy-four fast channels were recorded after the incorporation of *Oa*V (65 channels), *Oa*CNP-39 (62 channels) and *Oa*CNP-39(1–17) (47 channels) into bilayers of specific capacitance $>0{\cdot}42$ pF cm^{-2}. These channels were recorded at different voltages ranging between −140 and +160 mV and in solutions of different composition and concentrations. The lifespan of the channel was, on average, 30 min and varied between less than 1 min to 2 h. The activity of these channels was lost, mainly because of bilayer breakage, after: (a) application of large voltages, particularly positive voltages; (b) electronic mixing of the *cis* and/or *trans* solutions after the addition of a treatment; (c) perfusion of the *cis* or *trans* solutions with new solutions. Also, to a lesser extent, loss of activity was due to the bilayer thickening that results from the increase in the volume of the solvent separating the monolayers of the painted artificial bilayer. The *Oa*V-formed channels were stable and irreversibly associated with those lipid bilayers that maintained their specific capacitance of $>0{\cdot}42$ pF cm^{-2}. This indicates that the formed channels are not likely to be

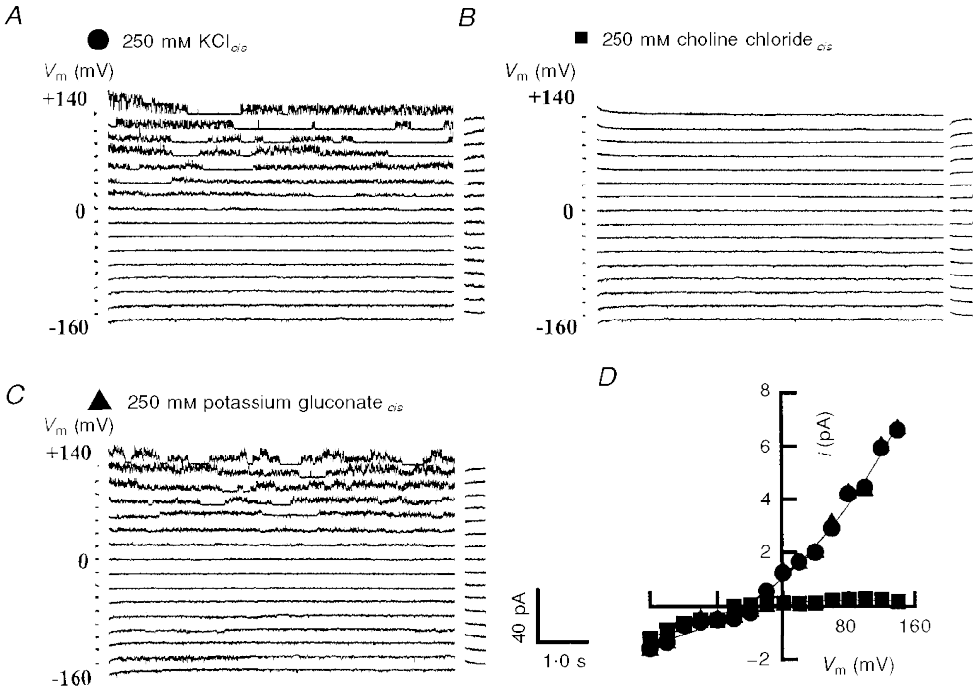

Figure 1. The cationic nature of the *Oa*V-formed fast ion channels

Representative families of current traces illustrating the activity of fast channels recorded from a voltage-clamped optimal bilayer, i.e. specific bilayer capacitance >42 μF cm^{-2}. The *cis* solutions were 250 mM KCl (*A*), 250 mM choline chloride (*B*) and 250 mM potassium gluconate (*C*) . The *trans* solution was 50 mM KCl in *A*–*C*. Membrane voltages between −160 and +140 in +20 mV steps are shown on the left of the current traces in *A*–*C*. Following convention, the upward deflections denote activation of outward potassium current, i.e. potassium ions moving from the *cis* chamber to the *trans* chamber. For a better display, the data are filtered at 1 kHz, digitized at 2 kHz and reduced by a factor of five. The current traces are separated by a 10 pA offset. *D*, current–voltage relationships for the treatments in *A*–*C*. For these treatments the values for E_{rev} were −44·2 mV (KCl), −5·6 mV (choline chloride) and −43·8 mV (potassium gluconate); mean of 1–3 experiments. KCl, ●; choline chloride, ■ and potassium gluconate, ▲.

due to peptide-induced structural disturbances of the bilayer. Furthermore, *Oa*V and *Oa*CNP-39, when inactivated by being boiled in 250 mM KCl, failed to form channels in the lipid bilayer ($n = 3$ and 5, respectively), when used at concentrations and for periods similar to those in which the active peptides produced channels. This indicates that certain natural features of the bioactive peptides in the venom are needed for channel formation. In order to ascertain that this was not due to a low probability of peptide incorporation into the lipid bilayer, liposomes (Arispe *et al.* 1993) were used to detect the incorporation of the peptide into the bilayer by monitoring the specific capacitance of the lipid bilayer. Although the liposomes were incorporated as indicated by the increase in the specific capacitance, no channel activities were observed for *Oa*CNP-39 and *Oa*V ($n = 7$).

Cationic nature of the *Oa*V-formed fast ion channel

To determine whether the outward current was the result of an efflux of cations or an influx of anions, the *cis* cytoplasmic side of the bilayer was successively exposed to 250 mM KCl, 250 mM choline chloride and 250 mM potassium gluconate, while the *trans* side of the bilayer remained exposed to 50 mM KCl. A voltage protocol was then used to obtain families of currents under these different ionic compositions. From an initial holding potential of 0 mV, the membrane potential (V_m) was stepped to voltages ranging from −160 to +140 mV, in steps of +20 mV, for periods lasting 6·25 s. Figure 1*A* shows typical current traces recorded for the *Oa*V-formed fast channels at voltages between −160 and +140 mV. The activity of this channel is characterized by 'bursts' in the outward currents at voltages between 0 and +140 mV. These bursts are separated by long periods of channel inactivation or closure. The time course of the single channel activity is voltage independent. It is apparent that when the 250 mM KCl in the *cis* chamber was totally replaced by 250 mM choline chloride the outward currents were eliminated (Fig. 1*B*). Furthermore, replacement of choline chloride with potassium gluconate led to the recovery of the outward currents at all voltages between −20 and +140 mV (Fig. 1*C*). The current–voltage relationships constructed from these current families show the changes in the current amplitude and E_{rev} (Fig. 1*D*). In contrast to choline chloride, substituting potassium gluconate for KCl had relatively little effect on either channel kinetics (Fig. 1*B* and *C*) or the current–voltage (*i–V*) relationships (Fig. 1*D*), suggesting that the cation, rather than the anion, carries the current.

Potassium-dependence of the *Oa*V-formed fast ion channel

The dependence of the fast outward current on $[K^+]_{cis}$ was examined at several concentrations between 50 and 750 mM. The *cis* solution was successively perfused with 50, 150, 250, 350, 450, 550, 650 and 750 mM K^+_{cis} and families of current traces were obtained at voltages between −160 and +140 mV (e.g. Fig. 2). The time course of the single channel activity was independent of $[K^+]_{cis}$ where the burst characteristics of the channel activity remained unaffected.

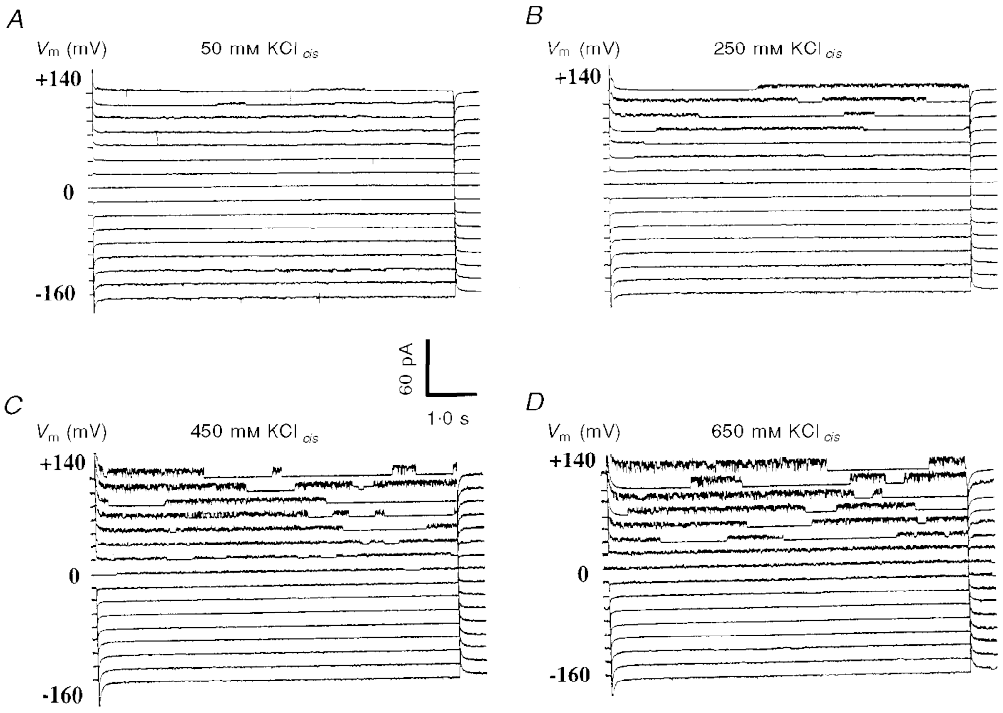

Figure 2. Dependence of the *Oa*V-formed fast cation-selective channels on $[KCl]_{cis}$ between 50 and 750 mM

For clarity, only four families of current traces are shown. *A*, 50/50 mM KCl; *B*, 250/50 mM KCl; *C*, 450/50 mM KCl; *D*, 650/50 mM KCl. The current traces are separated by a 15 pA offset. The rest of the conditions for data display are as in Fig. 1.

However, it is apparent that the amplitude of the outward current increased as a function of increasing $[K^+]_{cis}$.

The current–voltage relationships constructed from these current families show outward rectification and confirm the increase in current amplitude at depolarizing voltages (Fig. 3*A*). The current reversal potential (E_{rev}) responded to changes in $[K^+]_{cis}$ (Fig. 3*B*) in a manner similar to E_K, suggesting that this fast outward current is carried by K^+. For example, the reversal potentials of the *Oa*V-formed fast cation-selective channels in 50/50, 250/50 and 750/50 mM KCl *cis/trans* are 0, −44·2, and −75·7 mV, close to E_K values of 0, −41·2 and −69·4 mV, respectively. The increase in current amplitude as a function of increasing $[K^+]_{cis}$ was non-linear (Fig. 3*C*), indicating K^+ binding to sites in the channel pore. The outward current is described by the Michaelis–Menten equation:

$$\gamma_S = \gamma_{max,S}/1 + K_S/[S],$$

where K_S is the concentration for half-maximal γ. At +140 mV, the values of γ_{max} and K_S are 63·1 pS and 169 mM whereas at 0 mV the values of γ_{max} and K_S are 21·1 pS and 307 mM, respectively. It is thought that saturation is due to a binding and an unbinding step of K^+ to the channel protein which makes the permeation rate limiting at high ionic concentration (Hille, 1992).

Kinetics of the *Oa*V-formed single fast ion channels

The kinetic parameters of the *Oa*V-formed fast channels were obtained by analysing channel activity within bursts at positive voltages and $[KCl]_{cis}$ ranging between 50 and 750 mM (Fig. 4). The probability of the channel being open, P_o, is voltage dependent, with mean values between 0·15 and 0·75 at voltages between 0 and +140 mV, respectively (Fig. 4*A*). This increase is mainly due to increases in the mean values of the average open time, T_o (Fig. 4*C*), rather than to increases in the values of F_o (Fig. 4*B*) or changes in the mean values of the mean closed time, T_c (Fig. 4*D*). In contrast, the channel frequency, F_o, decreased with depolarization of the bilayer. The mean values of F_o were between 357 and 155 events s^{-1} for voltages between 0 and +140 mV, respectively. For the same voltage range, the mean values of the mean open time, T_o, were between 0·75 and 4·5 ms, while no clear pattern emerged for the values of the mean closed time, T_c, at voltages between 0 and +140 mV. The voltage dependency of P_o was not affected by $[KCl]_{cis}$ (Fig. 4*A*). This implies that the increase in the probability of opening of the outward cation-selective channels at depolarizing voltages will allow the efflux of K^+ at a wide range of $[KCl]_{cis}$. Like P_o, the values of F_o, T_o and T_c were independent of $[KCl]_{cis}$ ranging between 50 and 750 mM ($P > 0·05$). The kinetic parameters of the *Oa*CNP-39-formed channels were also obtained at the steady state by analysing channel activity at different depolarizing voltages and for different $[K^+]_{cis}$ (data not shown; cf. Figure 5). The kinetic parameters, voltage- and potassium-dependence of the *Oa*CNP-39-formed channels ($n = 5$) were similar to those of the *Oa*V-formed channels (see also Table 1).

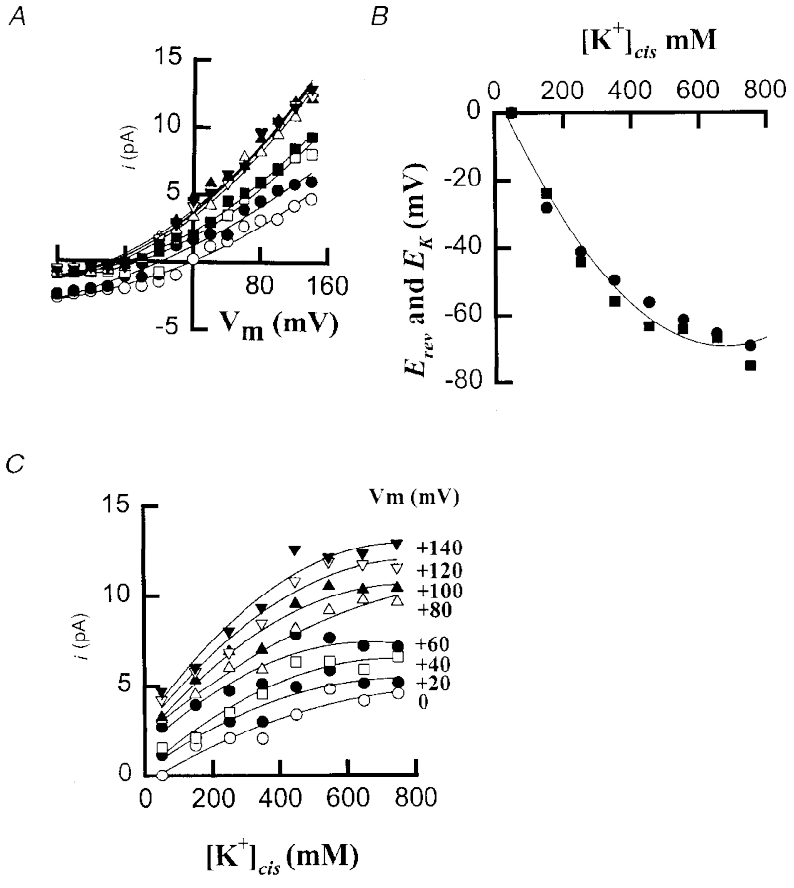

Figure 3. Voltage dependence of the current amplitude of the *Oa*V-formed fast cation-selective channel

A, current–voltage relationships in: 50/50 mM KCl (○); 150/50 mM KCl (●); 250/50 mM KCl (□); 350/50 mM KCl (■); 450/50 mM KCl (△); 550/50 mM KCl (▲); 650/50 mM KCl (▽) and 750/50 mM *cis/trans* KCl (▼). The values for E_{rev} were 0 mV (50); −23·9 mV (150); −44·2 mV (250); −56·0 mV (350); −63·4 mV (450); −64·0 mV (550); −67·0 mV (650) and −75·5 mV (750); mean of 1–5 experiments. *B*, E_K (●) and E_{rev} (■) plotted against $[KCl]_{cis}$. *C*, Michaelis–Menten curves for the single-channel current amplitude as a function of $[KCl]_{cis}$ at different voltages between 0 (bottom) and +140 mV (top) in +20 mV steps. The continuous lines are drawn to a second-order polynomial fit.

Voltage-dependence of the *Oa*CNP-39 formed ion channel

*Oa*CNP-39 is a major component of platypus venom and thus its ability to form ion channels was examined to see whether it is involved in the formation of channels similar to those of the *Oa*V-formed channels. It was found that, like *Oa*V, the incorporation of synthetic *Oa*CNP-39 into lipid bilayer membranes in 250/50 mM KCl *cis/trans* produced bursts of channel activity. The time course of channel activity showed no inactivation even after periods of up to 15 min (data not shown). A voltage protocol was used to examine the voltage dependence of single-channel currents in 250/50 mM KCl *cis/trans*. From an initial holding potential of 0 mV, the membrane potential (V_m) was stepped to voltages ranging from −70 mV to +80 mV, in steps of +10 mV, for periods lasting 6·25 s.

There was no delay in the channel activity and the channel opened immediately after the application of the depolarizing voltage steps (Fig. 5*A*). Current–voltage relationships were constructed to examine the voltage dependence of the conductance of the *Oa*CNP-39-formed ion channel conductance (Fig. 5*D*). The current–voltage relationship exhibited weak outward rectification that is characteristically fitted with two exponentials. The reversal potential (E_{rev}) of −44·2 mV is close to the E_K value of −41·2, calculated from the Nernst equation (Hodgkin & Katz, 1949), which indicates that under these experimental conditions the outward current is primarily due to the movement of K^+. Previously, it was shown that the activity of the *Oa*V-formed fast cation-selective channel was not affected by 100 mM $[TEACl]_{trans}$ (a blocker of outwardly rectifying K^+ channels) (J. I. Kourie unpublished data), nor by 50 mM $[TEACl]_{cis}$ (data not shown). Similarly, neither the conductance of the *Oa*CNP-39-formed fast cation-selective channel nor the E_{rev} were affected by 50 mM $[TEACl]_{trans}$ or/and 50 mM $[TEACl]_{cis}$ (Fig. 5*B–D*). Several other agents also failed to affect the channel conductance (see below).

The role of the N-terminus of *Oa*CNP-39 in the formation of the fast cation-selective channel

To ascertain the role of the *Oa*CNP-39 components which formed the fast cation channel, the 17 residues of the N-terminal fragment, i.e. *Oa*CNP-39(1–17) and the fragment of amino acids from residue 18 to 39, i.e. *Oa*CNP-39(18–39), of the *Oa*CNP-39 were synthesized and incorporated into lipid bilayer membranes. It was found that the *Oa*CNP-

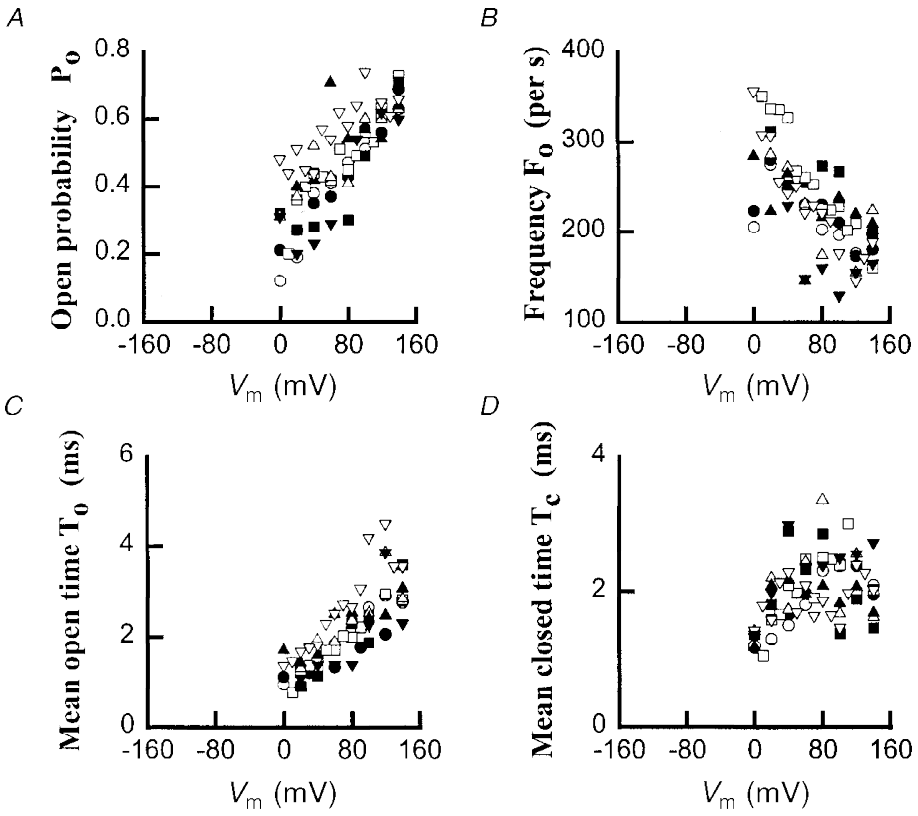

Figure 4. Voltage- and $[K^+]_{cis}$-dependence of the kinetic parameters of *Oa*V formed fast cation-selective channel

A, open probability (P_o); *B*, frequency (F_o); *C*, mean open time (T_o); *D*, mean closed time (T_c). 50/50 mM KCl, (○); 150/50 mM KCl, (●); 250/50 mM KCl, (□); 350/50 mM KCl, (■); 450/50 mM KCl, (△); 550/50 mM KCl, (▲); 650/50 mM KCl (▽) and 750/50 mM KCl *cis/trans* (▼). The threshold for channel detection was set at 50 % of the current amplitude

39(18–39) forms a large cation channel (Kourie, 1999) which is different in its conductance, selectivity and kinetics from the fast cation-selective channel formed by either the parent peptide, *Oa*CNP-39, or *Oa*V. On the other hand, the N-terminal *Oa*CNP-39(1–17) formed fast cation-selective channels (Fig. 6*A*) that have properties similar to those of the channel formed by both the parent peptide, *Oa*CNP-39, and *Oa*V (see Table 1). The cationic nature of the channel was also determined by successively exposing the *cis* (cytoplasmic) side of the bilayer to 250 mM KCl, 250 mM choline chloride and 250 mM potassium gluconate. In contrast to choline chloride, substituting potassium gluconate for KCl had no effect on either channel kinetics or the *i–V* relation (data not shown). These findings suggest that the cation rather than the anion carries the current through the *Oa*CNP-39(1–17)-formed fast channel.

Ion selectivity of the *Oa*CNP-39(1–17)-formed fast cation-selective channel

The ion selectivity of the channel was determined in ion substitution experiments that involved the use of the *Oa*CNP-39(1–17), because of its availability and easier synthesis. The 250 mM KCl in the *cis* chamber was totally replaced by 250 mM of each of NaCl, CsCl, RbCl and LiCl, and families of current traces were obtained at voltages between −160 and +140 mV. The current–voltage relationships constructed from these current families show changes in the current amplitude and E_{rev} (Fig. 6*B*). The conductance of the channel decreases with increasing dehydration energy of the permeant monovalent cations in the order $K^+ > Rb^+ > Na^+ > Cs^+ > Li^+$. The shift in the reversal potential for the single unitary currents to more negative values when Rb^+, Na^+, Li^+or Cs^+ is substituted for K^+ indicates that these ions are less permeable than K^+ (Fig. 6*B*). The calculated relative permeability values for $P_K : P_{Rb} : P_{Na} : P_{Cs} : P_{Li}$ were 1 : 0·76 : 0·21 : 0·09 : 0·03, respectively (see also Table 1).

Negative data

The conductance of the *Oa*CNP-39(1–17)-formed a fast cation-selective channel and the E_{rev} were affected by 25–50 mM $[TEACl]_{trans}$ or/and $[TEACl]_{cis}$ (each $n = 5$). Furthermore, the addition to the *cis* solution of 2·5 mM neflumic acid; 0·36 mM cAMP; 20 μM IP_3 or IP_4; 40 μg ml^{-1} prostigmin; 20 μl anti-cGMP; 20 μl putrescine; 20 μM ATP; 10 mM $AlCl_3$ or 50 mM NH_4Cl, failed to affect the conductance of the *Oa*CNP-39(1–17)-formed channel ($n = 2$–8).

DISCUSSION

Characteristics of *Oa*V, *Oa*CNP-39(1–17) and *Oa*CNP-39-formed fast channels

The results presented in this study show that platypus venom forms a fast cation-selective channel. The activity of this channel is characterized by bursts in the outward currents at voltages between −20 and +140 mV in 250/50 mM KCl *cis*/*trans*. The bursts are separated by long

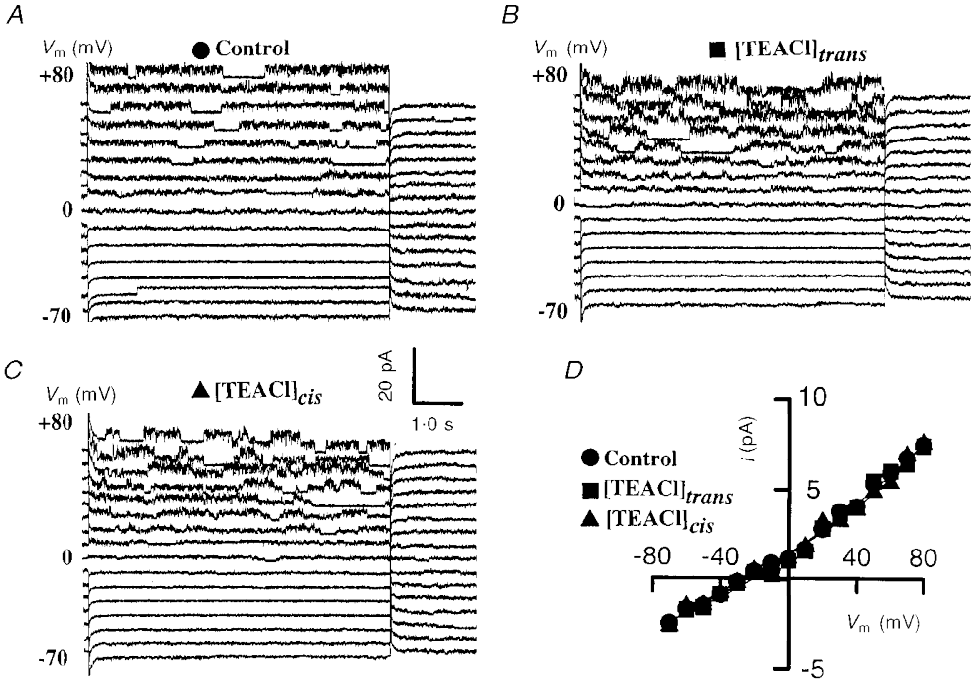

Figure 5. Effects of TEACl on synthetic *Oa*CNP-39-formed channel

Effects of TEACl on synthetic *Oa*CNP-39 (a major peptide found in the platypus venom *Oa*V)-formed fast cation channel: control (*A*), 50 mM $[TEACl]_{trans}$ (*B*) and 50 mM $[TEACl]_{cis}$ (*C*). *D*, current–voltage relationships control (●), 100 mM $[TEACl]_{trans}$ (■) and 50 mM $[TEACl]_{cis}$ (▲). The current traces are separated by a 5 pA offset. Similarly, TEACl had no effect on the *Oa*V-formed fast cation channel.

periods of channel inactivation or closure. The conductance values of the fast cation-selective channel in lipid bilayer membranes were $38{\cdot}8 \pm 4{\cdot}6$ and $60{\cdot}7 \pm 7{\cdot}1$ pS in 250/50 and 750/50 mM *cis/trans*, respectively. The current reversal potential of −44 mV in 250/50 mM KCl *cis/trans* is closer to the E_K value than that of E_{Cl}, indicating that this channel is more selective for cations than for anions. The E_{rev} responded to changes in $[K^+]_{cis}$ in a manner similar to E_K, suggesting that this fast outward current is carried by K^+. The increase in current amplitude as a function of increasing $[K^+]_{cis}$ was non-linear and can be described by the Michaelis–Menten equation. At +140 mV, the values of γ_{max} and K_S are 63·1 pS and 169 mM, whereas at 0 mV, the values of γ_{max} and K_S are 21·1 pS and 307 mM, respectively. On the other hand, the conductance values for the slow channels are $22{\cdot}5 \pm 2{\cdot}6$ pS and $41{\cdot}38 \pm 4{\cdot}2$ pS in 250/50 and 750/50 mM KCl *cis/trans*, respectively. At +140 mV, γ_{max} and K_S values were 38·6 pS and 380 mM and decreased to 15·76 pS and 250 mM at 0 mV, respectively. These conductance values are much smaller than the previously reported value of 546 ± 23 pS for the synthetic human CNP-22 and the *Oa*CNP-39(18–39) venom. The cationic selectivities of the *Oa*V-, *Oa*CNP-39(18–39)- and *Oa*CNP-39(1–17)-formed channels were $P_K : P_{Rb} : P_{Na} : P_{Cs} : P_{Li}$, $1 : 0{\cdot}76 : 0{\cdot}21 : 0{\cdot}09 : 0{\cdot}03$, respectively. By comparison, the values for the *Oa*V-formed slow cation channel were $P_K : P_{Na} : P_{Cs} : P_{choline}$, $1 : 1 : 0{\cdot}63 : 0{\cdot}089$, respectively and for the CNP-22 and *Oa*CNP-39(18–39)-formed cation channel were $P_K : P_{Na} : P_{Cs} : P_{choline}$, $1 : 0{\cdot}88 : 0{\cdot}76 : 0{\cdot}13$, respectively. These findings suggest that the selectivity filter of this fast cation-selective channel discriminates on the basis of charge, size and energy required for dehydration of monovalent cations. However, the amino acids in the oxygen rings-based filter (Eisenman & Dani, 1987) of the *Oa*CNP-39(1–17) have yet to be determined.

The time courses of the current transitions of the *Oa*V-, *Oa*CNP-39-, and *Oa*CNP-39(1–17)-formed channels, unlike those of the CNP-22 and *Oa*CNP-39(18–39)-formed channels, reveal no inactivation at voltages between −160 and +140 mV. The kinetic parameters of the *Oa*V-, *Oa*CNP-39(18–39)-, and *Oa*CNP-39(1–17)-formed channels reported here are different from those of the CNP-22- and

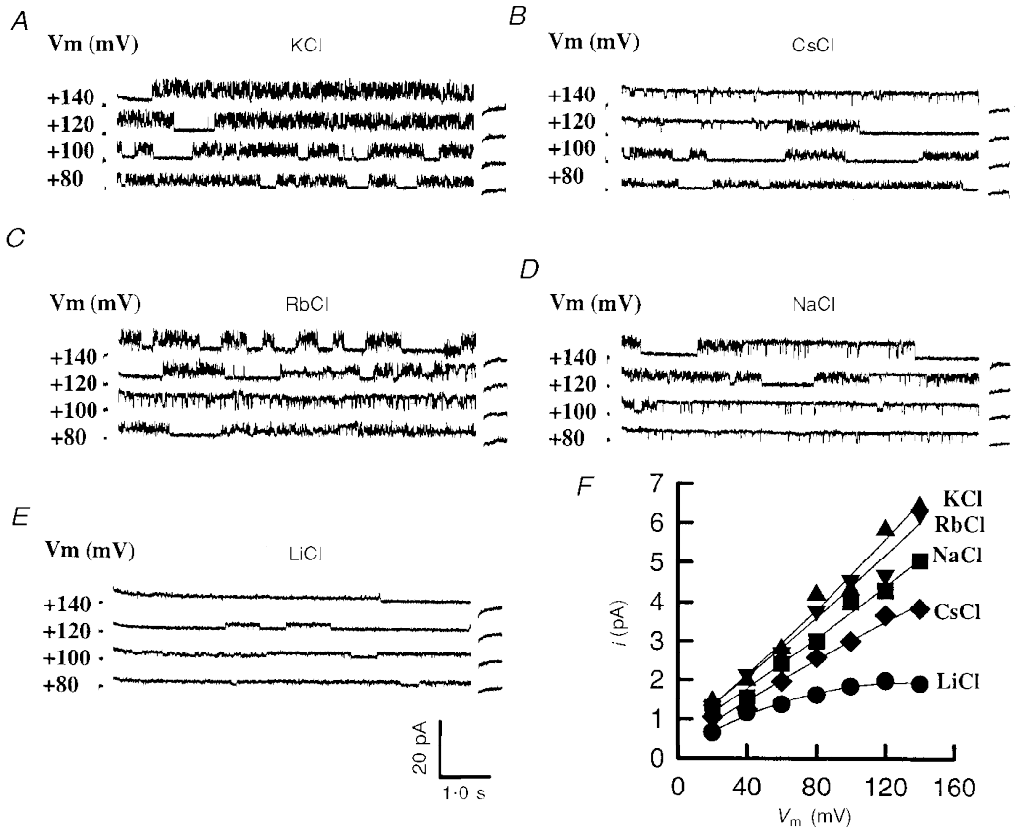

Figure 6. Monovalent cation selectivity of the *Oa*CNP-39(1–17) (a fragment of the parent peptide *Oa*CNP-39 found in *Oa*V)-formed fast cation-selective channel at voltages between −160 and +140 mV

For clarity, only examples of the single channel currents activated at +80, +100, +120 and +140 mV are shown in: *A*, 250 mM $[KCl]_{cis}$; *B*, 250 mM $[CsCl]_{cis}$; *C*, 250 mM $[RbCl]_{cis}$; *D*, 250 mM $[NaCl]_{cis}$ and *E*, 250 mM $[LiCl]_{cis}$. The current traces are separated by a 10 pA offset. *F*, current–voltage relationships for the monovalent cations Li^+ (●), Na^+ (■), K^+ (▲), Rb^+ (▼), and Cs^+ (◆).

*Oa*CNP-39(18–39)-formed channels. At positive voltages between 0 and +80 mV, the probability of the CNP-22- or *Oa*CNP-39(18–39)-formed channels being open, P_o, was virtually constant and had a value of 1. At negative voltages between −70 and −10 mV, the P_o of the CNP-22 and *Oa*CNP-39(18–39)-formed channels had a bell-shaped curve with a peak at −60 mV. The channel frequency, F_o, decreased from 14·96 events s^{-1} at −70 mV to 0·8 events s^{-1} at +80 mV. The mean open time, T_o, increased from 65 ms at −70 mV to 201 ms at −50 mV and to 335 ms at −30 mV. In contrast, the mean closed time, T_c, decreased exponentially from 16·4 ms at −70 mV to 0·5 ms at −20 mV. The potassium independence of the kinetic parameters of the fast channel indicates that voltage rather than K^+ underlies the conformational changes in the amino acids of the channel proteins controlling the gating mechanism.

The unitary conductance of the fast channel was not affected by 50 mM $[TEACl]_{cis}$ or 100 mM $[TEACl]_{trans}$. The *Oa*V-, *Oa*CNP-39(18–39)-, and *Oa*CNP-39(1–17)-formed fast channels reported here, like the CNP-22-formed channel (Kourie, 1999) but unlike the *Oa*V-formed slow cation channel, are TEACl insensitive. These biophysical properties of the fast channel point to a novel channel that is different from the previously reported peptide- or toxin-formed, voltage-dependent cation channels. These include the staphylococcal δ-toxin-induced 70–100 and 450 pS channels (Mellor *et al.* 1988), the tetanus toxin-induced 89 pS channel (500 mM KCl) (Gambale & Montal, 1988) and the *Clostridium botulinum* C2-II toxin-induced 55 pS channel (100 mM KCl) (Schmid *et al.* 1994).

Mechanisms involved in *Oa*CNP-39-channel formation

The possibility that natriuretic peptides actually form ion channels, as opposed to merely regulating them, has not previously been examined. The structures of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) incorporate a 17-residue intramolecular disulphide loop (between residues 6 and 22). The amino acid sequence of the loop is identical in ANP and BNP, whilst CNP is the same, except for two residues (Kojima *et al.* 1990; Sudoh *et al.* 1990; Komatsu *et al.* 1991; Mukoyama *et al.* 1991; Suga *et al.* 1992; 1993). In contrast to ANP and BNP, which possess C- and N-terminal sequence extensions beyond this loop, CNP extends only N-terminally (Suga *et al.* 1993; de Plater *et al.* 1995; 1998*a*). The results reported here suggest that the components of the C-type natriuretic peptides can form ion channels via different mechanisms. Previously, it was reported that acidic conditions play an important role in toxin-formed ion channel formation. It is thought that acidic conditions allow some domains to dock onto and insert into the membrane, as suggested for colicin E1 (Merrill *et al.* 1997) and Bcl-2-formed channels (Minn *et al.* 1997). The fact that *Oa*CNP-39 forms cation-selective channels in negatively charged lipid bilayer membranes at pH 7·4 and voltages between −80 and +80 mV suggests that acidic conditions are not required for conformational changes.

Under conditions of ion channel formation, the regions most likely to bind to the negatively charged bilayer are those containing lysine and arginine. Similarly, the charged polar residues will line the channel pore in contact with aqueous solvent. The unitary conductance of this channel is likely to be determined by rings of negatively charged amino acids, i.e. aspartate and histidine residues. The large number of positive charges, i.e. arginine and lysine, may have a role in gating the channel by sensing the electrical field across the bilayer in a manner similar to that suggested for the S4 segment of voltage-dependent channels (for review see Catterall, 1992). The most likely regions that will be in the interior of the channel protein or hidden in the membrane lipid away from the aqueous solvent are those containing proline, leucine and alanine. These peptides contain the cysteines needed to form disulphide bridges, which are used for joining separate peptide chains or linking two cysteines in the same chain. The significance of the disulphides in channel activity is not yet known. We are currently using NMR and ion channel-recording techniques to examine the physicochemical and structural properties of *Oa*CNP-39(1–17) such as polarity, basicity, bulk, ability to hydrogen bond, structural propensities and conformational flexibility, and to relate these properties to the electrophysiological properties of the channel (P. Pallaghy & J. I. Kourie, unpublished data).

Pathophysiological significance of *Oa*V-formed ion channels

Here we report that the C-type natriuretic peptide, which has been identified as *Oa*CNP-39, a component of platypus venom, can function directly by forming novel voltage-gated cation-selective channels in artificial lipid bilayer membranes. The N-terminal fragment *Oa*CNP-39(1–17) of this peptide is involved in forming this fast cation channel. Although we have characterized the biophysical properties of the fast cation-selective channel, its *in vivo* function remains to be determined. It is known that C-type natriuretic peptides are distributed widely in the mammalian central nervous system, the brain, endothelial cells, the lower part of the gastro-intestinal tract and the kidney (Sudoh *et al.* 1990; Komatsu *et al.* 1991; Suga *et al.* 1992; 1993). They form, together with ANP and BNP, a family of peptides, which exhibit potent natriuretic, diuretic, hypotensive and vaso-relaxant properties (Kojima *et al.* 1990; Sudoh *et al.* 1990; Komatsu *et al.* 1991; Suga *et al.* 1992; 1993; Hama *et al.* 1994; de Plater *et al.* 1995). There is evidence to suggest that C-type natriuretic peptides may act as potentially potent toxins. They are found in the venom of the South American pit viper (*Bothrops jararaca*) (Murayama *et al.* 1997). It has also been shown that the *Oa*CNP-39 from platypus venom is associated with sustained tonic relaxation of the rat uterus *in vitro* (de Plater *et al.* 1998*a*, *b*). There is also evidence to suggest that C-type natriuretic peptides have a pathological role in cytokine-associated disorders, septic shock and renal failure (Suga *et al.* 1992; Murayama *et al.* 1997).

The molecular mechanisms underlying the action of these peptides are not well known. It is thought that they act via the ANP_B receptor, particulate guanylate cyclase-B (Koller *et al.* 1991; Suga *et al.* 1992), leading to an increase in the level of cGMP (Stingo *et al.* 1992), which regulates ion transport pathways (Solomon *et al.* 1992; White *et al.* 1993; Wei *et al.* 1994; Kelley, Cotton & Drumm, 1997; 1998). In muscle cells, the cGMP regulation of ion transport pathways results in the inhibition of muscle contraction, vasodilatation and modification of fluid and electrolyte homeostasis (Sudoh *et al.* 1990; Morita *et al.* 1992; de Plater *et al.* 1995). The findings reported here indicate that, in addition to their known cGMP-mediated interaction with intrinsic ion transport mechanisms (see Kourie & Rive 1999), C-type natriuretic peptides may also exert their effects on signal transduction by directly forming ion transport pathways. This mechanism of action may explain the potent actions of these peptides. Ion channels are the most efficient ion transport pathways, capable of catalysing the permeation of 10^6 to 10^8 ions per second (Hille, 1992). The *Oa*CNP-39-formed channels could mediate their effects via changes in V_m and second messenger systems (e.g. Ca^{2+} homeostasis). It is reasonable to propose that the findings reported here indicate that the symptoms of envenomation by platypus venom may be partly due to channel formation affecting signal transduction. The findings support previous suggestions that both the *Oa*V-formed slow outward cation channel and the large conductance channel formed by CNP-22 may exert their pathological effects by rapidly depleting the ionic and osmotic gradients across the membrane (Kourie & Rive, 1999; Kourie, 1999).

In conclusion, data obtained using the lipid bilayer technique show that *Oa*V and *Oa*CNP-39, which is a major component peptide in platypus venom, form (in 250/50 mM KCl *cis*/*trans*) a TEACl-insensitive fast cation-selective channel. The N-terminal fragment (1–17 amino acids) of this 39 amino acid, C-type natriuretic peptide forms this channel. These findings are important to the development of strategies for therapies of clinical cases of both platypus venom-induced symptoms (e.g. pain and oedema) and CNP-associated pathologies.

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