Unidirectional K^+ Fluxes through Recombinant *Shaker* Potassium Channels Expressed in Single *Xenopus* Oocytes

PER STAMPE and TED BEGENISICH

Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, NY 14642

ABSTRACT We describe a method to evaluate the ratio of ionic fluxes through recombinant channels expressed in a single *Xenopus* oocyte. A potassium channel encoded by the *Drosophila Shaker* gene tested by this method exhibited flux ratios far from those expected for independent ion movement. At a fixed extracellular concentration of 25 mM K^+, this channel showed single-file diffusion with an Ussing flux-ratio exponent, n', of 3.4 at a membrane potential of −30 mV. There was an apparent, small voltage dependence of this parameter with n' values of 2.4 at −15 and −5 mV. These results indicate that the pore in these channels can simultaneously accommodate at least four K^+ ions. If each of these K^+ ions is in contact with two water molecules, the minimum length of the pore is 24 Å.

INTRODUCTION

There has been much recent work designed to provide a molecular description of the ion permeation and selectivity properties of K channels. Some of this work has focused on site-specific mutagenesis of amino acids in the P-region, a highly conserved loop of 21 amino acids present in all cloned K channels (e.g., Yool and Schwarz, 1991; Kirsch et al., 1992; Heginbotham et al., 1994; Kirschet al., 1995). Other efforts have been directed toward experimentally determining aspects of the physical structure of this region (Kürz et al., 1995; Lü and Miller, 1995; Pascual et al., 1995) or modeling pore structure (Durrell and Guy, 1992; Bogusz and Busath, 1992; Bogusz et al., 1992; Guy and Durrell, 1994,1995; Mackinnon, 1995).

There have also been several biophysical studies of the ion selectivity and permeation properties of cloned K channels (Newland et al., 1992; Heginbotham and Mackinnon, 1993; Pérez-Cornejo and Begenisich, 1994). The conclusion from this work is that ion permeation through *Shaker* K channels is quite complex. These results cannot be explained by ion permeation through a pore that contains at most a single ion. Rather, they are consistent with properties expected of a pore that can simultaneously be occupied by more than one ion.

As these various approaches converge toward an accurate molecular picture of ion permeation, it will be necessary to know the number of ions that occupy the pore. There is no means to directly measure this quantity, but the flux-ratio exponent, n', can give some information on this issue. This parameter is defined (Hodgkin and Keynes, 1955) in a modified form of the Ussing (1949) flux ratio test for independent ion movement:

$$\frac{m_e}{m_i} = \left[\frac{[K]_i}{[K]_o}\exp\left(\frac{VmF}{RT}\right)\right]^{n'} \quad (1)$$

where m_e and m_i are unidirectional K^+ efflux and influx, respectively; $[K]_i$ and $[K]_o$ are the intracellular and extracellular K^+ concentrations; V_m is the membrane voltage; and R, T, and F have their usual thermodynamic meanings. The flux-ratio exponent, n', is 1 for a pore in which ion movements are independent—that is, a pore that never contains more than a single ion.

Some theoretical analyses have shown that the flux-ratio exponent is equal to one more than the number of ions in the pore (Hodgkin and Keynes, 1955). Others conclude that the flux ratio may approach but cannot exceed the number of ions that can simultaneously occupy the pore (Hille and Schwarz, 1978). Thus, a determination of the flux-ratio exponent for a channel places a lower limit on the number of ions that simultaneously occupy the pore. This, in turn, places physical and electrical constraints on possible models of the pore structure.

To determine the flux-ratio exponent, measurements of unidirectional K^+ influx and efflux at a fixed membrane voltage are required. We describe here methods for measuring unidirectional fluxes through *Shaker* K channels expressed in *Xenopus* oocytes. We used these

Address correspondence to Dr. Ted Begenisich, Department of Pharmacology and Physiology, University of Rochester Medical Center, 601 Elmwood Avenue, Box 711, Rochester, NY 14642. Fax: (716) 461-3259; E-mail: tbb@crocus.medicine.rochester.edu

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methods to obtain estimates of the flux-ratio exponent for these channels. We found values near 2.4 at −5 mV and as large as 3.5 at −30 mV. These results indicate that the pore of *Shaker* K channels can simultaneously accommodate at least four ions. This finding places constraints on possible structural models for the ion selective pore in these channels. In addition, these methods, combined with site specific mutagenesis, may help to reveal detailed aspects of the molecular architecture of the permeation pathway in cloned ion channels.

A report of these results has been published in abstract form (Stampe and Begenisich, 1995).

METHODS

Chemicals

All inorganic salts and 4-aminopyridine (4-AP) were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant charybdotoxin (CTX) was produced as described by Park, Hausdorff, and Miller (1991). The ^{42}K was obtained from Dupont NEN Research Products (Boston, MA).

Solutions

The external (bath) solution used in all experiments consisted of (in mM): 25 KCl, 75 NaCl, 5 $MgCl_2$, 10 HEPES (pH 7.4).

Cloned K Channels

All the experiments described here were done with the *Shaker* channel inactivation deletion mutant, ShB Δ6-46 (Hoshi et al., 1990; kindly provided by Dr. R.W. Aldrich, Stanford University). The absence of inactivation greatly increases the amount of K^+ flux through these channels. This deletion alters only channel inactivation and not other gating or permeation properties (Hoshi et al., 1990; Heginbotham and MacKinnon, 1993).

We obtained the ShB Δ6-46 clone in a modified pSP72 (Promega) vector. We subsequently subcloned the channel into pBluescript II KS(−) (pBS, Stratagene, La Jolla, CA). These plasmids were propagated by and purified from competent *E. coli* (JM109, Promega Corp., Madison, WI). The pBS-channel construct was linearized with Xho I and channel DNA used as a template for synthesis of capped mRNA with T7 RNA Polymerase (Promega). RNA amounts were visualized by gel electrophoresis and the amount and purity determined spectrophotometrically. Approximately 50 nl of 0.2 to 0.5 mg/ml RNA was injected into each oocyte with a Drummond microinjector (Broomall, PA).

Two-Microelectrode Voltage Clamp

The measurement of radiolabeled ion fluxes requires the use of cells in which very large numbers of channels can be expressed. The *Xenopus* oocyte is uniquely suited for this purpose. Voltage clamp of the oocytes was accomplished with a custom-designed two-microelectrode circuit. This design includes a high gain, high output voltage control amplifier (for passing current through one of the microelectrodes) and a capacity compensated voltage recording head stage. To minimize electrical coupling between the two microelectrodes, the current-passing electrode was coated with a (grounded) conducting paint layer and a second, insulating coating. Both electrodes were filled with 3 M KCl. The capacity transient of this voltage clamp system was complete in about 500 μs, sufficiently fast for the measurement of macroscopic currents and radioisotope fluxes from the ShB Δ6-46 channel. Except where noted, the ionic currents are shown without correction for leak and capacity currents.

The resistance of the current-passing electrodes were all less than 1 MΩ with an average value of 0.24 ± 0.08 MΩ (SD, $n = 20$; range 0.15 to 0.4 MΩ). To enhance the measured K^+ flux, we used oocytes that expressed large K channel currents. Fig. 1 illustrates an example of the time and voltage-dependent activation of currents through *Shaker* channels expressed in *Xenopus* oocytes. The inset contains superimposed current records from 20-ms depolarizations to −40, −30, −20, −10, and 0 mV from a holding potential of −80 mV. The current levels increased with depolarization, and channel activation kinetics became faster. The lack of inactivation in these records is characteristic of this ShB Δ6-46, inactivation deletion mutant (Hoshi et al., 1990). The main figure shows the current measured at the end of the depolarization as a function of test potential.

We used potentials of −30, −15, and −5 mV as activation voltages for measuring unidirectional fluxes. The currents at −15 and −5 mV are quite large and so there may be voltage errors caused by the presence of an electrical resistance in series with the cell membrane. We estimated the series resistance from the time constant of the capacity current (Hodgkin and Huxley,

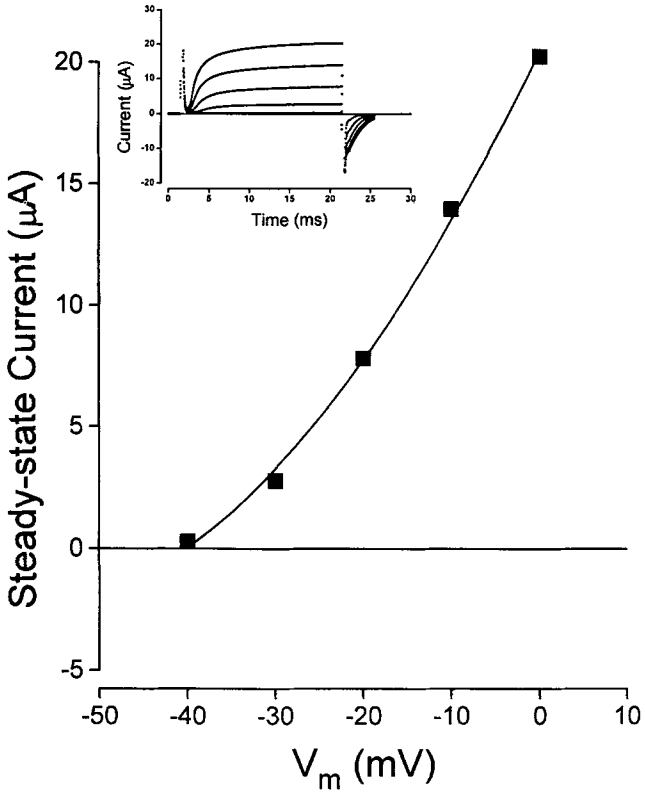

FIGURE 1. *Shaker* K^+ channel currents during step depolarizations. *Inset:* Superimposed *Shaker* channel current records in response to 20-ms depolarizations to −40, −30, −20, −10, and 0 mV from a holding potential of −80 mV. *Main figure:* Currents at the end of the 20-ms depolarizations to the indicated potentials.

1952) and obtained values from 0.3–4 KΩ. Our circuit design includes dynamic compensation for series resistance; for the experiments in this study a range of 0.2–4 KΩ was used with a mean value of 1.4 ± 1.3 KΩ (SD, n = 15). We estimated the largest uncompensated voltage error to be near 2 mV.

Data Acquisition

Membrane currents were acquired with a 12-bit analogue digital converter. The voltage-clamp pulses were generated by a 12-bit digital/analogue converter. The data acquisition and pulse generation protocols were controlled by our custom software.

Calculation of the flux-ratio exponent, n′. As described in Introduction, the flux ratio exponent, n', can provide a measure of the number of ions that may occupy a pore. This parameter was computed from a rearranged form of Eq. 1:

$$n' = \frac{RT}{(V_m - V_K)F} \ln \frac{m_e}{m_i} \qquad (2)$$

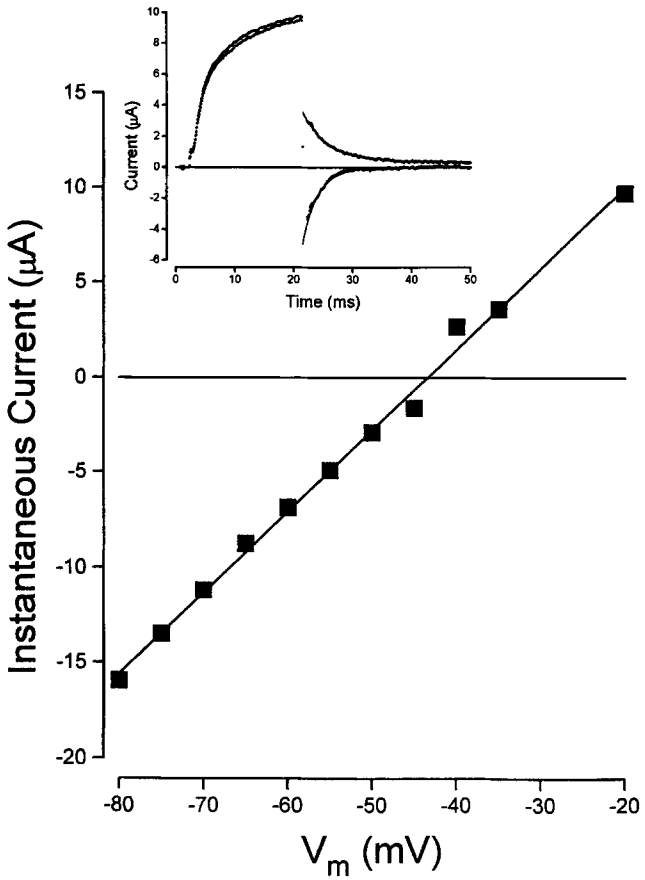

FIGURE 2. Instantaneous *Shaker* K^+ channel currents. *Inset: Shaker* channel records recorded at repolarization potentials of −55 and −35 mV after a 20-ms depolarization to −20 mV. A P/4 subtraction protocol was used. Superimposed (*lines*) are fits of a single exponential time function to the currents during repolarization. *Main figure:* Instantaneous current–voltage relation. Currents obtained by extrapolation of the exponential fits to the time of repolarization. The line is a least-squares linear fit to the current-voltage data. The interpolated K^+ equilibrium potential, V_K was −43 mV.

Thus, n' is inversely proportional to the difference between the membrane voltage, V_m, and the K^+ equilibrium potential, V_k. It is proportional to the logarithm of the ratio of the unidirectional fluxes m_e and m_i. The calculation of n' requires an estimate of V_k and at the test potential of −30 mV is particularly sensitive to the accuracy of this determination. Fig. 2 illustrates a method for obtaining an accurate value for V_k. The inset contains two superimposed current records, each in response to a 20-ms depolarization to −20 mV. A repolarization to −55 mV produced inward current and repolarization to −35 mV produced outward current. This indicates that V_k was between these two values. To obtain an accurate value of the instantaneous current, we fit a single exponential time function to the tail current (inset, *solid line*) and plotted the zero time amplitude as a function of repolarization potential. As seen in the figure, the instantaneous current–voltage relation was reasonably linear over a substantial voltage range. The K^+ equilibrium potential can be estimated by interpolation and was found to be −43 mV in the example of Fig. 2.

The linearity of the instantaneous current–voltage relation allows use of a simpler method of determining V_k (Connor and Stevens, 1971). For this method only one steady state current and one tail current value are required. Use of this method has the additional value of testing for any K^+ accumulation and depletion that might occur during the long pulses used to promote ^{42}K fluxes. We used this method with various test pulse voltages and with repolarizations to −80 mV. Values of V_k determined with 20-ms pulses to −40 mV averaged −40.8 ± 0.9 mV (SD, n = 8); V_k determined from 20-ms pulses to −30 mV was −39.2 ± 1.5 mV (SD, n = 6); for 300-ms pulses to −15 mV, V_k was −40.1 ± 3.6 mV (SD, n = 4). Thus, all methods produced the same estimated value near −40 mV. With our external K^+ concentration of 25 mM, this value indicates that the internal K^+ concentration was ~124 mM, which is within the range of values determined by a variety of methods (see Dascal, 1987).

Unidirectional flux measurements. We have modified some of our earlier methods for measuring radiolabeled fluxes through ion channels (Begenisich and De Weer, 1977; 1980; Vestergaard-Bogind et al., 1985) for the oocyte system. The determination of n' from Eq. 2 requires values for both unidirectional influx and efflux. It is impractical to obtain both from the same oocyte, but it is possible to measure a single tracer flux and the net flux (current) and compute the remaining unidirectional flux through the following equation:

$$I_{Net} = F(m_e - m_i) \qquad (3)$$

These are called the EFFLUX and INFLUX methods (Begenisich and De Weer, 1980) named after the unidirectional flux that is directly measured. The unidirectional fluxes were obtained from gamma counting (Gamma 4000, Beckman) of trace amounts of ^{42}K. The standard pulse protocol consisted of repetitive (200–300 ms) voltage steps from the holding potential (−80 mV) to a test voltage. The depolarizing pulses were applied at 1 Hz for 4 min (240 pulses).

For the EFFLUX method the oocytes were loaded with ^{42}K by soaking them for 8–12 hours in a high-specific-activity solution. After a thorough wash, the oocyte was transferred to the experimental chamber. The external solution was perfused at ~1 ml/min by peristaltic pumps and the outflow routed to a fraction collector. One-minute samples were collected for counting of ^{42}K.

The unidirectional efflux and the time integral of measured membrane current (net flux) were computed. The unidirectional influx was computed by subtraction of these two values.

The short half-life of the isotope makes it unrealistic to wait for equilibrium to occur between ^{42}K in the soaking solution and the oocytes. In the absence of this equilibrium, we would need accurate determinations of oocyte water volume and internal K^+ concentration to calculate the internal K^+ content. Whereas the internal K^+ concentration can be determined from the measured K^+ equilibrium potential, no sufficiently accurate method is available for measuring the water content of individual oocytes. Thus, we could not obtain intracellular ^{42}K specific activity and were unable to directly calculate the unidirectional efflux. Instead, we measured tracer efflux and net current at two voltages. One voltage was to a potential (−5 mV) at which the driving force ($V_m - V_k$) was sufficiently large such that the net efflux (current) approached the unidirectional efflux.[1] This allowed the determination of a calibration factor relating measured tracer to unidirectional K^+ efflux. At a less depolarized second voltage (−30 mV) we measured ^{42}K flux and used the calibration factor to convert this to unidirectional K^+ efflux. This value and the integrated net current was used to compute unidirectional K^+ influx.

The INFLUX method required a rather different experimental design. The oocyte was placed in a very low volume chamber and voltage clamped in a solution without radiotracer. The oocyte was then allowed a period of time to recover from the electrode impalements, after which a radiolabeled solution was introduced into the chamber. After a 4-min experimental period, the oocyte was washed extensively with the membrane voltage maintained near −80 mV. Only after removing the radiotracer solution was the oocyte unclamped, the electrodes removed, and the amount of ^{42}K in the oocyte determined.

To determine the amount of ^{42}K radioactivity not associated with influx through K channels, we performed three types of control experiments. We measured ^{42}K influx in (*1*) oocytes injected with *Shaker* RNA and maintained only at the −80 mV holding potential for 4–6 min.; (*2*) oocytes not injected with *Shaker* RNA but subjected to the same voltage protocol as injected cells; and (*3*) uninjected oocytes maintained at the −80 mV holding potential but without pulsing. The results of these various experiments are summarized in Fig. 3 (*open symbols*) where the counts/minute (cpm) for each oocyte is shown as a function of the solution ^{42}K specific activity (in cpm/mol K^+). The radioactive counts in these control oocytes may be the result of flux through pathways other than expressed K channels or (more likely) may represent residual ^{42}K adhering to the oocyte.

The filled symbols in Fig. 3 are data from oocytes injected with *Shaker* RNA and stimulated to the potentials indicated. The counts from these oocytes were 3.5–15 times larger than those from the control oocytes. To compute the K influx through *Shaker* K channels, these values were corrected by subtracting the control counts at the experimental specific activity by using the regression line fit to the control data. The correction had only a limited impact on the calculated value of n' (less than 5% in the experiment with the biggest subtraction).

[1]With an n' value of 1.0, unidirectional efflux would be 80% of net flux; for n' values of 2.0 and 3.0 this becomes 94% and 97%, respectively.

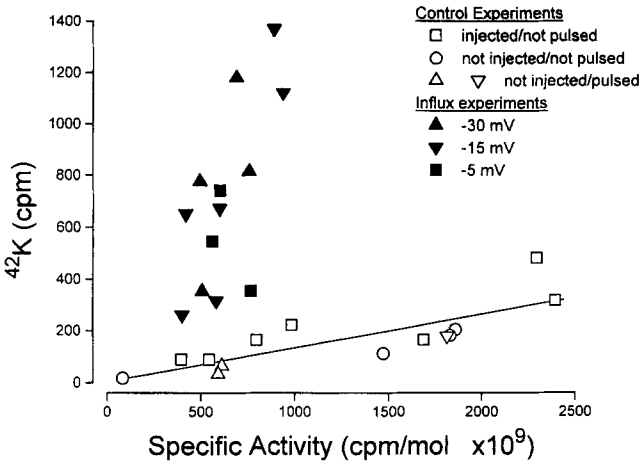

FIGURE 3. ^{42}K influx under several types of experimental conditions. Oocyte radioactivity (in counts per minute, cpm, corrected for radioactive background) as a function of solution specific activity (see Methods). *Open symbols:* Data from control oocytes including those not injected with *Shaker* RNA and maintained at −80 mV (○), non-injected oocytes repetitively pulsed to −5 mV (△) or −15 mV (▽), and oocytes (□) injected with *Shaker* RNA and maintained at a constant potential of −80 mV. *Filled symbols:* Radioactivity of oocytes injected with *Shaker* RNA and pulsed to −5 (■), −15 (▼), and −30 mV (▲). The line is a linear regression fit to the control data.

A potential problem for the INFLUX (but not the EFFLUX) method is the presence of "tail" currents at the end of the voltage clamp pulses. Any ^{42}K influx that is part of the tail current should not be included in the computation for n' at the test potential. An accurate estimate of the tail influx can be obtained from the integral of the tail current. If necessary, the measured influx can be corrected for the amount of influx that occurs during the tail current. We found that the tail influx, even in 25 mM external K^+, was a very small fraction of the measured ^{42}K influx. This error has the largest effect at −5 mV, where the influx is minimized by this rather depolarized potential. In our three influx experiments at −5 mV, the tail flux was 17% of the measured influx. If a correction were applied for this effect, the computed n' value would increase by less than 5%. Because corrections would be even smaller for the more negative voltages, we have chosen to not apply any correction for this effect.

RESULTS

Fig. 4 illustrates an example of the measurement of ^{42}K efflux through *Shaker* K channels expressed in a single oocyte. The external solution flowing past a ^{42}K-loaded oocyte was collected in the tubes of a fraction collector. The amount of ^{42}K in each sample was determined by gamma counting and corrected for background radiation. This quantity (ordinate of Fig. 4) reflects the amount of unidirectional efflux that occurred during the sample period (1 min in these experiments). In the experiment illustrated in Fig. 4, the membrane potential was maintained for ~12 min at −80 mV, during

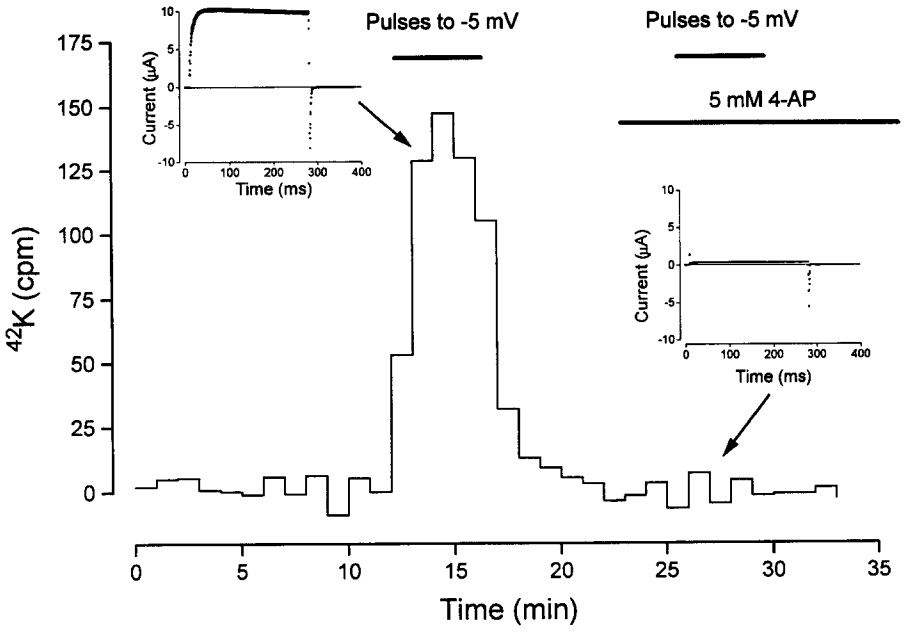

FIGURE 4. K^+ Efflux and *Shaker* K^+ channel current. ^{42}K efflux determined as the number of counts in 1-min samples corrected for radioactive background as described in Methods. The oocyte membrane potential was maintained at −80 mV except for the periods indicated during which 280-ms pulses to −5 mV were applied at 1 Hz. 5 mM 4-AP was applied as indicated. Each inset contains two records: the average of the first 40 ionic currents collected and the average of the last 40. These currents were obtained before and during the application of 4-AP, as indicated.

which there was only a very small baseline efflux. During the (4-min) period indicated, 280-ms-duration pulses to −5 mV were applied at 1 Hz. The associated ^{42}K efflux increased substantially during this period as the K channels were repetitively opened. Essentially all the increased efflux occurred through expressed *Shaker* K channels as indicated by the lack of flux in the presence of 5 mM of the K channel inhibitor 4-aminopyridine (4-AP).

We recorded all the K^+ currents during the stimulation periods of the experiment shown in Fig. 4. The inset associated with the first period (the absence of 4-AP) includes two superimposed raw current records—one is an average of 40 records obtained at the beginning of the stimulation period and the other is an average of the last 40 records. These data show that the repetitive pulses had little or no effect on the magnitude of K channel current. The other inset includes two similar superimposed average currents obtained during stimulation in the presence of 4-AP. These data show that this concentration of 4-AP eliminated almost all the time-dependent K channel current and the current reduction was correlated with an almost complete inhibition of ^{42}K efflux.

An example of the strong correlation between K channel current and ^{42}K efflux can be seen in the data of Fig. 5. In this experiment we determined the amount of ^{42}K efflux before applying 1 μM of CTX, during the application and again after washing out the toxin. We also recorded all the K channel currents stimulated during these periods. The figure illustrates the linear relationship between ^{42}K flux and the integral of the associated K channel current. The fact that the line passes near the origin indicates that essentially all the measured efflux was through expressed K channels.

Fig. 6 presents an example of the use of the EFFLUX method to determine the flux-ratio exponent, n'. After a baseline period during which the oocyte was maintained at a constant potential (−80 mV), efflux was elicited by 280-ms pulses (at 1 Hz) to −30 mV. All the currents during this period were recorded, examples of which are given in the inset. As described in Methods,

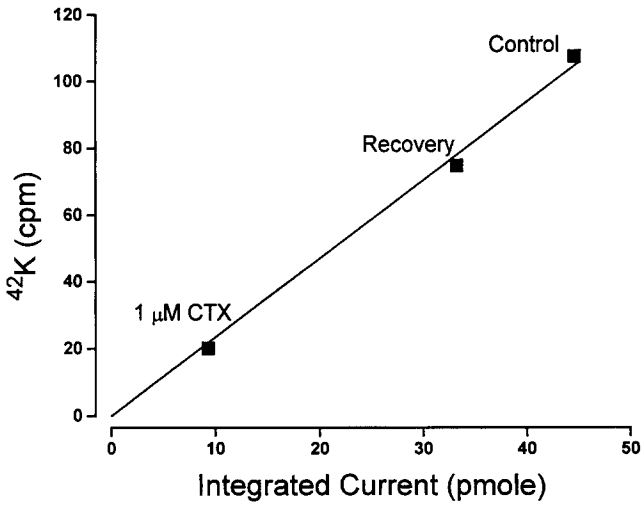

FIGURE 5. Efflux is linearly related to integrated ionic current. ^{42}K efflux and the integral of ionic current determined before (*Control*), during (*1 μM CTX*), and after (*Recovery*) application of 1 μM charybdotoxin. Line represents a best fit linear relation passing through the origin. All solutions for experiments with CTX contained 25 μg/ml of bovine serum albumin.

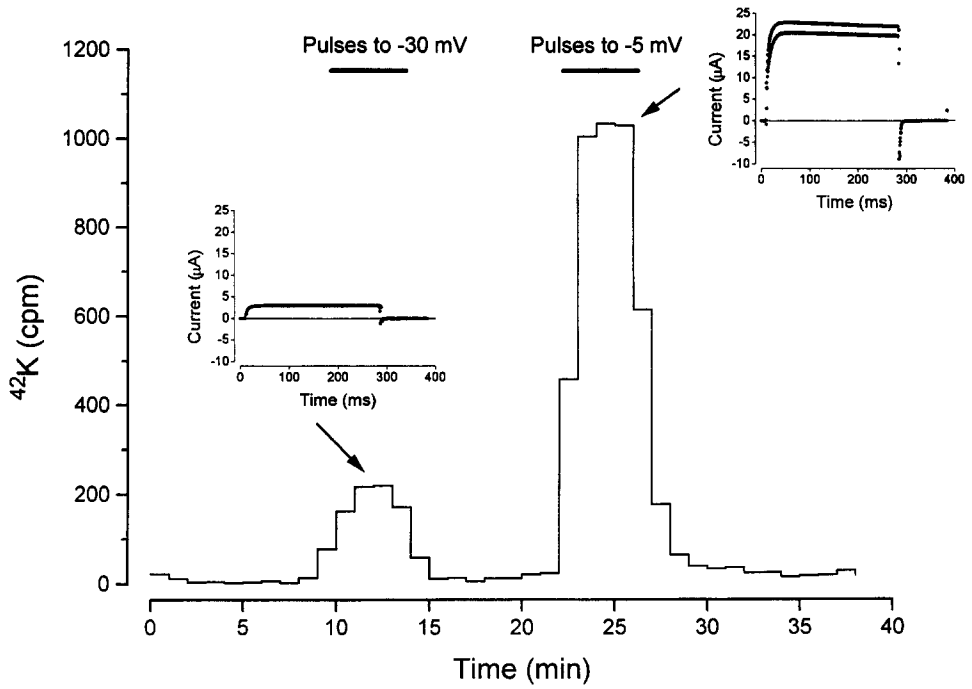

FIGURE 6. Efflux method for determining flux-ratio exponent. Oocyte membrane potential maintained at −80 mV except for indicated periods during which 280-ms pulses to −30 or −5 mV were applied for 4 min at 1 Hz. As in Fig. 4, each inset contains the average of the first and last 40 consecutive current records. The small difference between the two averages collected at −5 mV was not a typical finding (e.g., Fig. 4).

the EFFLUX method requires a second, large depolarization for calibrating ^{42}K tracer flux with ionic current. The second stimulation period in the figure illustrates the large efflux and ionic current associated with repetitive depolarizations to −5 mV. From these data, a value of n' of 4.3 was obtained. An average n' value of 3.5 ± 0.6 (SEM) at −30 mV was obtained from three such experiments.

We determined n' values at −30, −15, and −5 mV with the INFLUX method. As described in Methods, there are several sources of error associated with this technique. The magnitude of the errors depends on several parameters including the level of the test voltage and the level of influx. To test for a consistent bias in our results, in Fig. 7 we show n' values obtained at the three potentials as a function of the measured influx. These data indicate no consistent dependence of n' on oocyte influx and little dependence on membrane potential. Average n' values at −30, −15, and −5 mV were 3.4 ± 0.26 (SEM, n = 4), 2.4 ± 0.20 (SEM, n = 6), and 2.4 ± 0.25 (SEM, n = 3), respectively. The values at −15 and −5 mV are significantly different (t test) from the −30 mV data at the$P$ = 0.05 level but not at the P = 0.01 level.

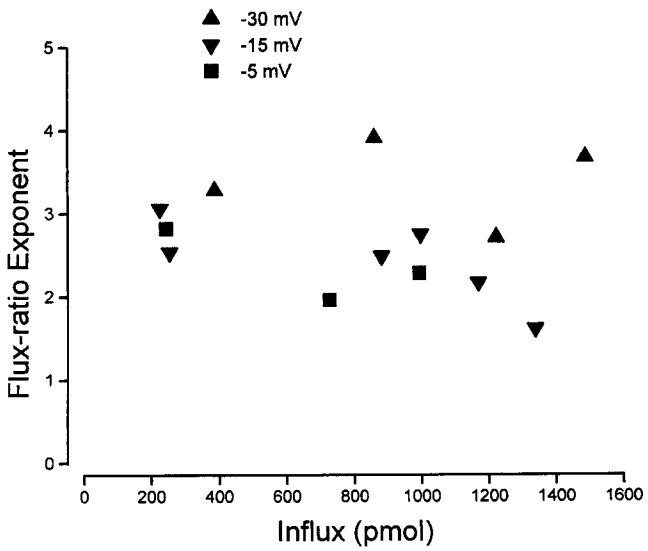

FIGURE 7. Flux-ratio exponent and measured ^{42}K influx. Flux-ratio exponent from INFLUX method as a function of measured influx values. Data obtained at −5 (■), −15 (▼), and −30 mV (▲).

We summarize the results of our determinations of the flux-ratio exponent, n', in Fig. 8. Both the influx (○) and efflux methods (■) were used at −30 mV and yielded the same value of 3.4. The n' values from influx data at −15 and −5 mV suggest a slight decrease with membrane voltage. The line in Fig. 8 is an empirical description of similar data obtained from squid axon K channels (Begenisich and De Weer, 1980). The n' values for *Shaker* channels are quite similar to those of the squid axon channels and display a similar slight voltage dependence.

DISCUSSION

We have described methods for measuring unidirectional, radiolabled fluxes through a single voltage-clamped *Xenopus* oocyte expressing cloned ion channels. We used these methods to determine unidirec-

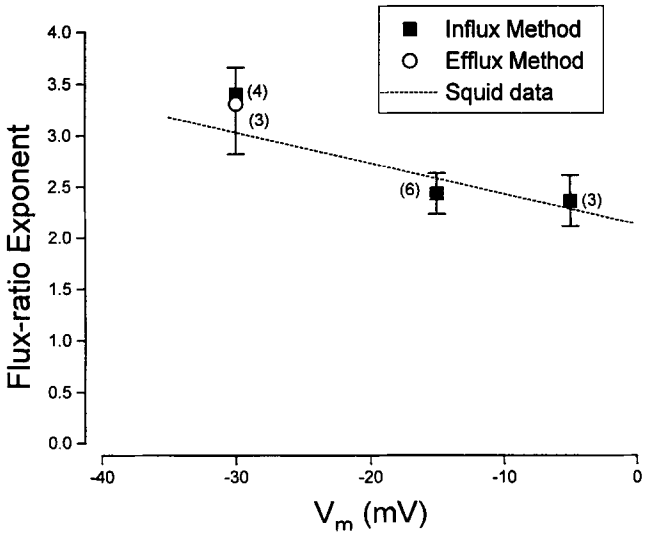

FIGURE 8. Flux-ratio exponent values. Flux-ratio exponent data obtained from INFLUX (■) and EFFLUX (○) methods. Shown are mean values and SEM limits; number of experiments indicated. Also shown is a line that represents flux-ratio data from squid axon K channels (Begenisich and De Weer, 1980).

tional K^+ fluxes through voltage-gated *Shaker* K channels. Although our results here were obtained with *Shaker* channels, the methods should be applicable to other ion channels expressed in the *Xenopus* oocyte system.

We analyzed our results in terms of the flux–ratio exponent, n' (Eq. 2), introduced by Hodgkin and Keynes (1955). We found n' values as large as 3.5—much larger than the value of 1.0 expected for independent ion movement (Ussing, 1949; Hodgkin and Keynes, 1955).

There have been few previous determinations of the flux-ratio exponent and none for cloned ion channels. Hodgkin and Keynes (1955) measured unidirectional K^+ fluxes through K channels in cuttlefish axons and obtained an n' value near 2.5. Begenisich and De Weer (1980) measured unidirectional K^+ fluxes through delayed rectifier K channels of voltage-clamped squid giant axons. The flux-ratio exponent for these channels had a weak voltage dependence and attained a value as large as 3.3 at −40 mV. Horowicz et al. (1968) and Spalding et al. (1981) studied K^+ fluxes through inward rectifier K channels in skeletal muscle. They found that the flux-ratio exponent was near 1.0 at low external K concentrations and increased to 2 for high concentrations. Vestergaard-Bogind et al. (1985) examined K^+ fluxes through Ca^{2+}-activated K channels in red blood cells. These channels exhibit an n' value of 2.7 that is independent of membrane voltage and external K concentration.

Our results are quite similar to those of Begenisich and De Weer (1980). Indeed, the solid line in Fig. 8 is a representation of the average squid axon data and is also a reasonable description of our *Shaker* K channel results. The close similarity of these results may not be surprising, since there is considerable homology among K channels cloned from squid (Perri et al., 1994; Pattonet al., 1994; Silva and Bezanilla, 1994) and the *Shaker* locus (Kamb et al., 1987; Tempel et al., 1987).

A variety of approaches have been taken to provide an interpretation of large values of the flux-ratio exponent. Hodgkin and Keynes (1955) showed with both mechanical and mathematical models that pores completely filled with several K^+ ions exhibit a flux-ratio exponent value equal to one more than the number of ions in the pore. They also considered a multi-site, mathematical pore model with occasional vacancies and, under these conditions, the flux-ratio exponent becomes equal to the number of sites.

Hille and Schwarz (1978) examined models based on absolute reaction rate theory. They found that the flux-ratio exponent may approach but cannot exceed the number of ions that can simultaneously occupy the pore. They also showed that only certain free energy profiles were consistent with large n' values. In agreement with the conclusion reached by Heckmann (1972) they found that large n' values require high energy barriers at each end of the pore with a high mobility of the ions within the pore. One consequence of this condition is that a single deep energy well or single rate-limiting barrier that reduces ion mobility would not be consistent with large n' values.

Similar conclusions have been reached by a variety of other theoretical analyses including calculations using a continuum approach (reviewed by Levitt, 1986), Brownian dynamic simulations (Bek and Jakobsson, 1994), and the "shaking stack" model (Schumaker, 1992). In particular the flux-ratio exponent in all these calculations approaches the number of ions that simultaneously occupy the pore.

Thus, regardless of the particular form of the theoretical construct, our finding of a value of n' as large as 3.5 implies that the pore of *Shaker* K channels can simultaneously accommodate at least four ions. This conclusion that the *Shaker* K channel contains a multi-ion pore is consistent with other recent results. Newland et al. (1992) showed that internal and external TEA ions interact through the channel pore. Pérez-Cornejo and Begenisich (1994) found concentration-dependent ion selectivity in *Shaker* channels and Cs^+ block of these channels with an effective valence as large as 1.3. Both of these results are inconsistent with single ion pores. Heginbotham and MacKinnon (1993) showed that these channels exhibited another manifestation of multi-ion occupancy: an anomalous mole-fraction effect (Hagiwara et al., 1977) for current carried by NH_4^+ and K^+ ions.

In our experiments the external and internal K^+ concentrations were 25 mM and 125 mM, respectively. If, under these conditions, the pore contained the maximum possible number of ions, then the channel flux (or conductance) would be expected to be constant or decrease (Hille and Schwarz, 1978) with further increases in external and internal K^+ concentration. However, Heginbotham and MacKinnon (1993) found that the conductance of the *Shaker* channel increases with symmetrical K^+ concentration up to ~1.2 M. This indicates that the pore of *Shaker* K channels may be able to accommodate at least an additional K^+ ion beyond our estimate of four, increasing the maximum occupancy to five or more.

The fact that the pore in *Shaker* K channels can simultaneously accommodate so many ions places constraints on the length of the pore region. Four, fully dehydrated K ions span only 5.3 Å. However, complete dehydration of a single K^+ ion requires 85 kcal/mol of energy, so the pore length is likely much larger than this minimal estimate. With two water molecules between each K^+ ion the minimum length would be 24 Å. There is, however, considerable electrostatic repulsion between ions even with this longer length. Levitt (1978) calculated that placing even three ions in a 25-Å pore with no fixed charges (with one ion at each end) requires 12 kcal/mol; adding a fourth would add substantially to this level. So either the K channel pore is much longer than 25 Å or it contains negative charges (or both). There are several candidate amino acids for providing negative charges in the K channel P-region (see Begenisich, 1994) including the highly conserved aspartate residue at *Shaker* position 447 (Kirsch et al., 1995).

A large body of evidence (reviewed in Begenisich, 1994) has demonstrated that many properties of the K channel pore are associated with the 21 amino acid P-region located between the fifth and sixth membrane-spanning domains (S5 and S6). The results of a recent study (Lü and Miller, 1995) designed to provide some structural information on the P-region indicates three or four of these amino acids may be outside the narrow part of the channel pore. The remaining amino acids appear to be accessible to the aqueous solution consistent with an α-helical structure. They conclude that the P-region forms at most a 10-Å-long narrow pore. MacKinnon (1995) argues that the P-region may form an even shorter structure. In either case, the ability of the K channel pore to accommodate as many as four or five ions is inconsistent with such a short structure. Mutations of amino acids in S6 and in the S4-S5 linker region alter some K channel permeation properties (Choi et al., 1993; Slesinger et al., 1993; Lopez et al., 1994) These or other parts of the K channel may contain the additional structural elements to accommodate the necessarily large number of K^+ ions.

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