Single-Channel Recordings of Three K^+ -Selective Currents in Cultured Chick Ciliary Ganglion Neurons

Phyllis I. Gardner

Department of Pharmacology, University College London, and Department of Medicine, Stanford University Medical Center, Stanford, California 94305

Multiple distinct K^+ -selective channels may contribute to action potential repolarization and afterpotential generation in chick ciliary neurons. The channel types are difficult to distinguish by traditional voltage-clamp methods, primarily because of coactivation during depolarization. I have used the extracellular patch-clamp technique to resolve single-channel K^+ currents in cultured chick ciliary ganglion (CG) neurons. Three unit currents selective for K^+ ions were observed. The channels varied with respect to unit conductance, sensitivity to Ca^{2+} ions and voltage, and steady-state gating parameters. The first channel, GK_1 , was characterized by a unit conductance of 14 pS under physiological recording conditions, gating that was relatively independent of membrane potential and intracellular Ca^{2+} ions, and single-component open-time distributions with time constants of approximately 9 msec. The second channel, GK_2 , was characterized by a unit conductance of 64 pS under physiological recording conditions and gating that was affected by membrane potential but was not dependent on the activity of intracellular Ca^{2+} ions. Open-time distributions indicated 2 open states, with open-time constants of 0.09 (61%) and 0.35 (39%) msec, at +40 mV membrane potential. The third channel, $GK_{Ca^{2+}}$, was identified in isolated patch recordings in which the concentration of internal Ca^{2+} was 10^{-7} M or greater, which was an absolute prerequisite for channel opening. $GK_{Ca^{2+}}$ was characterized by a unit conductance of 193 pS in symmetrical 0.15 M KCl solutions, an open-state probability that was a function not only of $[Ca^{2+}]_i$, but also of membrane potential, and single-component open-time distribution with a time constant of 1.11 msec at -10 mV patch potential. These results suggest the presence of at least 3 distinct K^+ channel populations in the membrane of cultured chick CG neurons.

Voltage-clamp records provide evidence that several types of outward K^+ current can flow across the soma membrane of invertebrate (Connor and Stevens, 1971; Meech and Standen, 1975; Neher, 1971; Thompson, 1977) and vertebrate neurons (Adams et al., 1982; Barrett and Barrett, 1976; Barrett et al., 1980; Belluzzi et al., 1985; Dubois, 1981; Galvan and Sedlmeir, 1984; Kostyuk et al., 1981; Krylov and Makovsky, 1978; McAfee and Yarowsky, 1979). Supporting evidence from these studies

for a multiplicity of outward K^+ currents includes identification of complex gating kinetics and separation of individual current components by pharmacological manipulations. Characterization of the individual components of outward K^+ current is of fundamental interest, since K^+ conductances appear to be involved in such physiological processes as resting potential determination, action potential repolarization, afterpotential generation, and control of repetitive firing. Moreover, K^+ channels may be an important site for neurotransmitter modulation of neuronal excitability (for review, see Hartzell, 1981). However, the identification and characterization of the individual components of outward K^+ current by voltage-clamp analysis has been difficult, primarily because all components can be coactivated during depolarization. The extracellular patch-clamp technique (Hamill et al., 1981) enables the resolution of aggregate outward K^+ currents into unitary channel currents. The individual K^+ channel population can then be characterized with respect to conductance, ionic selectivity, steady-state gating kinetics, and sensitivity to Ca^{2+} and voltage.

This paper reports experiments using the patch-clamp technique to study K^+ channels in dissociated chick ciliary parasympathetic ganglion neurons maintained in primary culture. Previous conventional microelectrode studies (Bader et al., 1982) have suggested that at least 2 K^+ conductances contribute to action potential repolarization and to hyperpolarizing inhibitory afterpotentials in chick ciliary neurons in culture. These K^+ conductances, or as-yet-unidentified ones, appear to be the site of action mediating the hyperpolarizing postsynaptic responses of muscarinic ACh (Hartzell et al., 1977) and enkephalins (Katayama and Nishi, 1984), 2 transmitters that have been localized by immunohistochemical study to preganglionic nerve terminals in the chick ciliary ganglion (Erichsen et al., 1982). In the present study, 3 distinct K^+ channel populations are identified and briefly characterized by single-channel recordings.

Materials and Methods

Cell preparation

Chick CG cells were dissociated from 8- to 10-d-old chicks and grown in tissue culture by the methods of Nishi and Berg (1981). Cells were maintained in bicarbonate/ CO_2 -buffered Eagle's minimal essential medium (MEM), containing a raised KCl concentration of 25 mM, 10% calf serum, 2% chick eye extract (Nishi and Berg, 1981), 120 μ g/ml benzylpenicillin, 200 μ g/ml streptomycin, and 100 μ g/ml ampicillin, in a humidified atmosphere of 5% CO_2 in air at 37°C for 5-6 d before experiments.

Experimental conditions

During experiments, the growth medium was replaced by HEPES-buffered Ringer's solution with the following composition (in mM): NaCl, 134; KCl, 5.4; $MgSO_4 \cdot 7H_2O$, 0.8; NaH_2PO_4 , 1.1; $CaCl_2$, 1.8; $NaHCO_3$, 5; glucose, 5.5; HEPES, 10 (adjusted to pH 7.2-7.3 by NaOH). In records with isolated patches, a high K^+ solution, consisting of (in mM): KCl, 150; $CaCl_2$, 1.0; $MgCl_2$, 1.0; HEPES, 10 (pH 7.2-7.3, adjusted by KOH),

Received Oct. 31, 1985; revised Jan. 15, 1986; accepted Jan. 17, 1986.

I extend my thanks to Dr. David Colquhoun for his excellent guidance and instruction during my tenure in his laboratory. I also thank Chris Magnus for his expert instruction on tissue culture techniques and Joan Rosel for her skilled assistance in manuscript preparation.

This work was supported initially by a fellowship from the Muscular Dystrophy Association of America, and subsequently by a grant-in-aid from the American Heart Association, with funds contributed in part by the Santa Clara County Chapter of the AHA, California Affiliate, and in part by the AHA, Florida Affiliate.

Correspondence should be addressed to Phyllis I. Gardner, M.D., Cardiovascular Research Center, 291, Department of Medicine, Stanford University Medical Center, Stanford, CA 94305

Copyright © 1986 Society for Neuroscience 0270-6474/86/072106-11\$02.00/0

Figure 1. Single-channel currents recorded from 8 d chick embryo ciliary ganglion cells dissociated and maintained in tissue culture. Recordings were from a "cell-attached" patch depolarized 60 mV from resting membrane potential, yielding an absolute patch potential of 0 mV. The patch pipette contained a physiological solution with 134 mM NaCl and 5.4 mM KCl. The outward current is *upward*. The presence of 2 step sizes indicates that 2 channel types are active in this patch.

was used in the circumstances stated in the text. In other indicated circumstances, KCl was replaced by equimolar amounts of NaCl. Solutions of variable ionized Ca^{2+} were obtained by use of appropriate Ca-EGTA buffers, taking 10^{-7} M as the apparent dissociation constant (Marty, 1981). Solutions with varying levels of Ca^{2+} were made as follows: $< 10^{-9}$ M [Ca^{2+}] = 0.0 mM CaCl_2 , 2 mM MgCl_2 , and 1.1 mM EGTA, pH 7.3; estimated 10^{-8} M [Ca^{2+}] = 0.1 mM CaCl_2 , 2 mM MgCl_2 , and 1.1 mM EGTA, pH 7.3; estimated 10^{-7} M [Ca^{2+}] = 0.55 mM CaCl_2 , 2 mM MgCl_2 , and 1.1 mM EGTA, pH 7.3; estimated 5×10^{-7} M [Ca^{2+}] = 0.92 mM CaCl_2 , 2 mM MgCl_2 , and 1.1 mM EGTA, pH 7.3; and estimated 10^{-6} M [Ca^{2+}] = 1 mM Ca^{2+} , 2 mM MgCl_2 , and 1.1 mM EGTA, pH 7.3. Solutions were buffered to pH 7.3 by 10 mM HEPES and adjusted by KOH.

During experiments, plates were mounted on an aluminum holder incorporated into a microscope fitted with phase-contrast optics and viewed with a $20\times$ long-working-distance objective. Experiments were performed at room temperature, which ranged from 18–22°C.

Patch-clamp recording

The techniques for high resistance (10–100 G Ω) pipette-membrane seal formation, excision of membrane patches, and single ion-channel recording are as described by Hamill et al. (1981). The recording configurations of "cell-attached" patch, "inside-out" patch, and "outside-out" patch were employed. Patch-clamp circuitry (List EPC-7, List-Electronic, Pfungstadt Strasse 18-20, 6100 Darmstadt-Eberstadt, FRG) was similar to that described in Hamill et al. (1981). Patch pipettes were fabricated from Pyrex glass with microfilament insertion (GC 150F, Clark Electromedical Instruments, Pangbourne, Reading, UK) on a vertical 2-stage pipette puller (pipette puller model 720, David Kopf Instruments, Tujunga, CA) to form resistances of 10–15 M Ω . Patch pipettes were coated at the tip with Sylgaard (Sylgaard 184 silicone elastomer kit, Dow Corning Corp., Midland, MI 48640) and fire-polished prior to use.

Data analysis

Patch-clamp currents were recorded on magnetic tape at tape speed $7\frac{1}{2}$ ips on a Racal Store 4DS FM tape recorder (Racal Recorders Ltd., Bracknell, Berkshire, UK). Recordings were digitized at 0.1 msec time intervals (at tape speed 3.75 ips, yielding a 20 kHz sampling frequency) and stored on a PDP 11-34 computer (Digital Equipment Corporation, 10 Forbes Road, Northboro, MA) after appropriate low-pass filtering at 3 kHz (-3 dB point, Bessel characteristic, 48 dB octave rolloff). The digitized record was used to measure the amplitude and duration of individual current pulses and the interval between single pulses by a semiautomatic procedure (Colquhoun and Sigworth, 1983). After the measurement, a value was chosen for the minimum resolvable duration, and the record was revised by concatenation of adjacent gaps and openings separated by intervals less than this chosen resolution. The minimum resolvable event was consistently between 100 and 200 μsec in

these records. The data from the idealized record were used for construction of histograms to display the distribution of current amplitudes (excluding open events too brief to reach full amplitude under recording conditions) and of open and shut durations, and to fit probability density functions by the method of maximum likelihood to the continuous distribution of data over the entire measured range. Multicomponent exponential fits were assessed by eye. Probability of channel openings (P_{open} , equal to the ratio of the total time spent in the open state to the total length of record) and opening frequency (O_{freq} , equal to the number of channel openings per second of closed time) were also determined from the records. Further details on these methods are given by Colquhoun and Sakmann (1981) and Colquhoun and Sigworth (1983).

Results

Currents were recorded from embryonic chick ciliary ganglion cells after 5–6 d in culture using gigaseal patch-clamp recording techniques. With single-channel records obtained from cell-attached patches with electrodes containing 5.4 mM KCl, channel activity was infrequent at zero applied voltage (resting membrane potential, V_m , 58 ± 2 mV, 10 samples) and at hyperpolarized potentials. Depolarization of the patch usually revealed at least 2 channel types mediating outward current in the cell-attached patches (Fig. 1). When the K⁺ concentration on the extracellular side of the membrane patch [K^+]_o was raised to levels between 11 and 150 mM, these 2 channel types were seen to be active at resting membrane potential, and inward current steps were observed at hyperpolarizing voltages. Most patches studied contained both channel types. In addition, at very depolarized voltages (80–120 mV from resting potential) a third type of unitary outward current, consisting of rare brief events of very large amplitude, could infrequently be discerned in cell-attached patches exposed to pipettes with 5.4 mM KCl. These events rarely reached full amplitude at the recording bandwidth of 3 kHz. When the membrane patches were isolated in the "inside-out" recording configuration and exposed to 10^{-7} M or greater levels of Ca^{2+} on the intracellular membrane surface, the third current became readily apparent as single-channel events of very large unit amplitude.

Thus, 3 distinct channels mediating outward current are suggested by patch-clamp recordings of CG neurons. The experimental results describing these channels—referred to as GK₁, GK₂, and GK_{Ca(2+)}—follow. Evidence that GK₁, GK₂, and GK_{Ca(2+)} are all predominantly K⁺-selective but differ in unitary conductance, steady-state gating kinetics, and Ca^{2+} and voltage sensitivity is presented.

Figure 2A. Conductance and selectivity properties of single GK_1 channels. Examples of GK_1 outward currents recorded from a "cell-attached" patch at various depolarized patch membrane potentials. The current-recording pipette contained a physiological solution with 134 mM NaCl and 5.4 mM KCl. The numbers at the left of each trace indicate the shift in patch potential (in mV) with respect to the cell's resting potential (V_r) of -60 mV; for example, +20 mV indicates a patch potential depolarized 20 mV from V_r , yielding an absolute patch potential of -40 mV.

Conductance properties of GK_1

The channel identified as GK_1 can be identified as the unitary current event of smaller amplitude in Figure 1. This channel is characterized by well-defined square-pulse events that increase in amplitude when the membrane potential of the patch is shifted to potentials more positive than V_r (Fig. 2A). Figure 2B shows the relationship between unitary current amplitude and membrane potential in cell-attached patches with $[K^+]_o$ of 5.4 mM. The I - V relationship is essentially linear, indicating a voltage-

Figure 2B. Current-voltage relationship of single GK_1 channel currents obtained from 7 patches as in A. The mean amplitude of current step sizes is plotted as a function of patch potential. Each circle represents the cumulative results of the 7 patches. The mean V_r was approximately -60 mV (range, -58 to -63 mV). The line represents a linear-regression line fitted to the data points. The slope conductance is 14 pS, and the extrapolated zero-current, or reversal, potential is -95 mV.

Figure 2C. Zero-current potentials as a function of $[K^+]_o$. The data represent the results of 16 experiments. Each symbol represents the average extrapolated zero-current potential from 2-7 experiments. These values are plotted as a function of the logarithm of $[K^+]_o$ and fitted by a straight line with a slope of 63.5 mV per 10-fold change in $[K^+]_o$.

Figure 3A. Occurrence of GK₁ current sublevels. Example of an outward GK₁ current step recorded at absolute patch potential of +20 mV (depolarized 80 mV from V_r), recorded with 5.4 mM KCl in the pipette. The current wave shape recording digitized at 10 KHz is represented by the dots. The waveform opens to the full current amplitude predicted by single-channel conductance, enters an intermediate level of current, and then returns to the main level before closure.

independent single-channel slope conductance of 14 pico-Siemens (pS) and an extrapolated zero-current potential of -95 mV.

The negative zero-current potential suggests that GK₁ is predominantly K⁺ selective. This is further indicated by Figure 2C, which depicts the semilogarithmical plot of zero-current potential vs external K⁺ concentration. The straight line obtained by least-squares fit has a slope of 63.5 mV change in zero-current potential for a 10-fold increase in [K⁺]_o. The predicted change in zero-current potential for a 10-fold change in [K⁺]_o, which is derived from the Nernst equation with an average temperature of 20°C (293.2 K), is 58.2 mV. Within the limitation of experimental error, the actual 63.5 mV change in zero-current potential is very close to this predicted value. It is concluded that GK₁ is highly K⁺-selective.

In addition to the main conductance state, GK₁ appears to adopt a substate of conductance. In approximately half the patches studied, events of one-half to two-thirds full GK₁ current amplitude (Fig. 3A) were seen, both contiguous with and isolated from full GK₁ currents. A correlation was seen between the occurrence of these events, suggesting that the small event was due to a substate of the fully conducting GK₁ channel; however, an entirely unrelated channel could not be absolutely ruled out. Distributions of GK₁ current amplitudes (Fig. 3B) often showed, in addition to the main peak, clusters of events approximately 0.5–0.6 the amplitude of the main current peak and were infrequent relative to the frequency of occurrence (calculated from the area under the curve) of the main current peak. Figure 3C shows the composite *I*-*V* relationship obtained from 9 experiments in which "sublevels" were seen. The slope of the linear regression line fitted to the data points provides an estimate of conductance of 9 pS, which compares to a conductance of 14 pS for the fully conducting state. Similar substates of conductance have been described for other K⁺-selective channels, including the inward-rectifying K⁺ channel in ventricular cells (Sakmann and Trube, 1984a) and the slow-potential-sensitive K⁺ channels in longitudinal smooth muscle cells (Benham and Bolton, 1983).

Voltage independence and steady-state kinetic properties of the GK₁ channel

The effect of steady-state voltages on GK₁ channel gating parameters was analyzed from single-channel records in which the holding voltage was maintained constant for 30–40 sec. Channel

Figure 3B. Distribution of current levels from a recording of 300 single-current steps at the same membrane potential and [K⁺]_o. In addition to the main peak with mean amplitude of 1.9 pA, there was a small cluster of events around 1.0 pA amplitude that may have represented the sublevel amplitude.

behavior was studied in patches in which no more than one GK₁ channel was open at any one time during the period of observation in order to maximize the probability that there was only one channel in the membrane patch. (The presence of 2 or more channels that do not coincidentally open during the period of observation cannot be ruled out; in such a case, estimates obtained would be too high for single-channel open-state probability.) Individual channels appeared as relatively long openings interrupted by rapid closings and separated by longer closed periods. The fraction of time spent in the open state P_{open} (calculated as total open time over total length of record) was approximately 0.10, independent of the patch potential studied. Thus, no clear voltage dependence of either unitary conductance or open-state probability of GK₁ could be demonstrated. The voltage independence was further analyzed by measuring the open time of the channel and the interval between successive openings from continuous recordings for more than 10 sec at two different patch potentials. Probability density histograms of the open time measured at patch potentials depolarized 50 and 100 mV from V_r are shown in Figure 4A, B. In each case, the histogram is well fitted by a single exponential with a time constant of 9.7 and 8.6 msec, respec-

Figure 3C. *I*-*V* relationship for sublevels observed in cell-attached patches with [K⁺]_o = 5.4 mM. Data from 9 different experiments are shown. Each symbol represents the mean ($\pm$ SD) sublevel amplitude from at least 5 observations, with the exception of data points without SD bars (only 1 observation). The slope conductance from the line fitted by linear regression to the data points was 9 pS.

Figure 4A. Distribution of open times of GK_1 , plotted as probability density. Frequency vs open-time histogram from 300 single-channel events at a patch potential of -10 mV (depolarized 50 mV from V_r). The histogram of the channel open times was fitted by a single exponential (curved line) with a time constant of 9.7 msec. The coincidentally determined probability of opening (P_{open} ; measured as open time divided by total record duration, which included the time segment containing the 300 openings) was 0.1 , and the opening frequency (O_{freq} = number of openings per second of closed time) was 11.6 /sec, as indicated in the upper right-hand corner.

Figure 4B. Frequency vs open-time histogram from 370 single-channel events at a patch potential of $+40$ mV (depolarized 100 mV from V_r). The histogram of channel open times at this potential was fitted by a single exponential (curved line) with a time constant of 8.6 msec. The coincidentally determined P_{open} and O_{freq} were 0.1 and 10.4 /sec, respectively.

tively, for -10 and $+40$ mV. The mean channel lifetime, channel opening frequency, and fraction of time spent in the open state were very similar at these 2 voltages, further suggesting that GK_1 is relatively voltage-insensitive.

The open-state probability of the GK_1 channel appeared to be unaffected by varying internal Ca^{2+} concentration from $<10^{-9}$ to 10^{-6} M in experiments with inside-out patches. (At higher levels of $[Ca^{2+}]_i$, the presence of GK_1 was difficult to determine secondary to the presence of large Ca^{2+} -activated K^+ channels.) Similarly, the fraction of time spent in the open state did not appear to change substantially during maintained depolarizations; that is, second-to-second open-state probabilities varied randomly without consistent changes.

Conductance and selectivity of GK_2

The channels that are termed GK_2 are the unitary events of larger amplitude seen in Figure 1. Examples of GK_2 currents recorded from a cell-attached patch at various depolarized patch potentials are seen in Figure 5A. The average amplitude at each potential was calculated from openings of sufficient duration to achieve full amplitude with a square-pulse conformation (open events too rapid to be resolved, thus appearing as foreshortened triangles in digitized records, were not used in mean amplitude calculations). The relation between mean amplitude and holding potential is plotted in Figure 5B. This I - V relation is essentially linear, indicating voltage-independent unitary conductance. The linear-regression line fitted to the data points provides an estimate of single-channel conductance of 64 pS and an extrapolated zero-current potential of -85 mV, near the predicted K^+ equilibrium potential.

Relatively high K^+ selectivity of GK_2 , which is suggested by the negative zero-current potential under "physiological" recording conditions, is confirmed by experiments with variable $[K^+]_o$. The semilogarithmic plot of zero-current potential vs external K^+ concentration (Fig. 5C) provides an estimate of 55.4 mV shift in zero-current potential for a 10-fold increase in extracellular K^+ concentration very close to the theoretical value of 58.2 mV predicted by the Nernst equation of a K^+ selective channel at 20°C . This suggests that GK_2 , like GK_1 , is highly selective for K^+ ions.

Voltage dependence and steady-state gating kinetics of GK_2

Steady-state mean open-channel open times and voltage dependence were assessed from records of cell-attached patches during maintained depolarizations for 10–15 sec, as previously described. Probability-density histograms of the open and closed times measured from a patch depolarized 50 and 100 mV from V_r are shown in Figure 6. The open-time histogram at $+40$ mV (Fig. 6B) consists of 2 exponentials, a fast component with a time constant of 0.09 msec (this component represents 61% of total area of the open-time distribution, corresponding to 475 fast openings, the vast majority of which would be too short to resolve with a fixed minimum resolvable open duration of 0.1 msec) and a slower component of 0.35 msec (39% of the area). This suggests that the GK_2 channel may have 2 distinct open states with similar conductances but dissimilar lifetimes. In contrast, the open-time distribution at -10 mV (Fig. 6A) is well fitted by a single exponential with a time constant of 0.26 msec. This may indicate that the presence of 2 open states is potential-dependent. Alternatively, the faster component may not have been adequately resolved at the more negative potential under these recording conditions, in which a minimum resolvable open duration of 0.1 msec was found.

The degree of voltage sensitivity could be approximated from the single-channel records in patches in which no more than one GK_2 channel was open at any one time during the period of observation. In contrast to the findings with GK_1 , GK_2 appeared to have a voltage-sensitive P_{open} , which increased with membrane depolarization. The voltage dependence of P_{open} resides in both longer open durations and higher opening frequencies at positive potentials. For example, P_{open} increased from 0.002 at -10 mV ($V_r + 50$ mV) to 0.01 at $+40$ mV ($V_r + 100$ mV). Channel-opening frequency, coincidentally, increased from 5.1 to 31.0 /sec, while the slow-time constant of open time increased from 0.26 to 0.35 msec (Fig. 6A, B). In contrast, there was no evidence that P_{open} was affected by changes in Ca^{2+} concentration from 10^{-9} to 10^{-6} M in experiments with inside-out patches. The presence of GK_2 was difficult to determine at higher levels of $[Ca^{2+}]_i$, due to the presence of large, Ca^{2+} -activated K^+ channels, as described for GK_1 . As noted with GK_1 , there was no evidence of any time-dependent inactivation process during maintained depolarizations of 10–60 sec; this was not explored in detail, however.

Figure 5A. Conductance and selectivity properties of single GK₂ channels. Examples of GK₂ outward currents recorded from a chick CG neuron "cell-attached" patch at various depolarized patch-membrane potentials. The current-recording pipette contained a physiological solution with NaCl, 134 mM, and KCl, 5.4 mM. The numbers at the left of each trace represent displacement from V_r .

Conductance and selectivity properties of GK_{Ca(2+)}

In addition to GK₁ and GK₂ channels identified in recordings from cell-attached patches, a K⁺-selective, Ca²⁺- and voltage-dependent current of very large unit conductance was seen in records from inside-out patches exposed to variable levels of internal (previously intracellular) Ca²⁺ concentration. Figure 7A shows unitary outward membrane currents from a cell-free inside-out membrane patch in symmetrical high-K⁺ solutions (KCl, 150 mM, with 1.0 mM CaCl₂), depolarized from absolute zero mV to 5 different positive membrane potentials. Single-channel events characteristic of GK_{Ca(2+)} were of very large unit amplitude, which depended on membrane potential. The *I*-*V* relationship for GK_{Ca(2+)} obtained from 5 inside-out patches in symmetrical 0.15 M KCl solutions is shown in Figure 7B. The linear-regression line fitted to the data points provides an estimate of single-channel conductance of 193 pS. The zero-current potential was about 0 mV, as expected with identical solutions on both sides of the membrane face. The zero-current potential of isolated patches (with constant 5.4 mM [K⁺]_i) de-

Figure 5B. The *I*-*V* relationship of GK₂ channel currents obtained from 5 cell-attached patches. Mean amplitude of current step sizes is plotted as a function of membrane potential. The slope conductance obtained from a linear-regression line fitted to the data points was 64 pS, with an extrapolated zero-current potential of -84 mV.

pendent on the logarithm of [K⁺]_o, as illustrated in Figure 7C. The slope of the regression line fitting the data in semilogarithmic coordinates provides an estimate of a 60.3 mV shift in zero-current potential for a 10-fold increase in external K⁺ concentration. This value indicates that the current is highly selective for K⁺ ions, since it is very close to the predicted value of the Nernst equation (58.2 mV at 20°C). In addition, it should be noted that unitary outward currents were never observed when inside-out patches were perfused with internal NaCl solutions.

Figure 5C. Zero-current potentials as a function of [K⁺]_o. The data points represent the results of 13 experiments. Each symbol represents the average extrapolated zero-current potential from 2-4 experiments. These values are plotted as a function of the logarithm of [K⁺]_o and are fitted by a straight line with a slope of 55.4 mV per 10-fold change in [K⁺]_o.

Figure 6A. Distribution of open times of GK_2 , plotted as probability density. Frequency vs open-time histogram from 328 single-channel events at a patch potential of -10 mV (depolarized 50 mV from V_r). The histogram of the channel open time was fitted by a single-exponential curve with a time constant of 0.26 msec. The coincidentally determined P_{open} equaled 0.002 , and O_{freq} equaled $5.8/\text{sec}$.

Figure 6B. Frequency vs open-time histogram from 500 single-channel events at a patch potential of $+40$ mV (depolarized 100 mV from V_r). The histogram of the channel open times was fitted by a double-exponential curve with time constants of 0.09 msec (representing 61% of the area under the curve) and 0.35 msec (39% of the area). The coincidentally determined P_{open} equaled 0.01 , and O_{freq} equaled $31.0/\text{sec}$.

Dependence of $GK_{Ca(2+)}$ opening on cytoplasmic Ca^{2+} and voltage

$GK_{Ca(2+)}$ activation was dependent on the internal Ca^{2+} concentration. Ionized Ca^{2+} concentrations were obtained by the use of appropriate Ca-EGTA buffers described in Materials and Methods. There was no evidence of channel activation with internal Ca^{2+} concentrations of 10^{-8} M or less. $[Ca^{2+}]_i$ of 10^{-7} M produced small increases in channel-opening probability at depolarized voltages ($P_{open} = 0$ at -50 mV; $P_{open} = 0.3$ at 0 mV; $P_{open} = 0.4$ at $+50$ mV). An increase of $[Ca^{2+}]_i$ to 10^{-3} M produced a dramatic voltage-dependent increase in P_{open} that equaled 0.5 at approximately -45 mV membrane potential and approached 1.0 from potentials more positive than -10 mV at millimolar Ca^{2+} concentrations (Fig. 8A). The channel disappeared when the internal membrane surface was reperused with nominally Ca^{2+} -free solutions. Since pipette solutions containing 1.0 mM Ca^{2+} were used for these inside-out patches, Ca^{2+} ions appeared to be ineffective in producing channel openings from the outside of the membranes.

Several investigators have described long-lived but infrequently occurring closed states for Ca^{2+} -activated K^+ -selective channels exposed to millimolar levels of internal Ca^{2+} concentrations (Barrett et al., 1982; Latorre et al., 1982). This has been suggested to be the result of channel blockade by Ca^{2+} ions (Vergara and Latorre, 1983). While Ca^{2+} ion blockade of $GK_{Ca(2+)}$ in CG neurons may occur at millimolar internal Ca^{2+} concentrations, no definitive evidence of this was found in the present study. Infrequent, nonsustained closures to baseline occasionally occurred, but estimates of open-state probability were greater than 0.9 at positive potentials during 10 – 15 sec maintained depolarizations. Longer periods of depolarization may have provided evidence of a decreased P_{open} , indicating channel blockade.

Channel open-state probability was dependent on voltage as well as internal Ca^{2+} concentration. As illustrated in Figure 8A, channel open-state probability is a sigmoidal function of voltage that increases as the channel is depolarized for any given level of internal Ca^{2+} concentration. This finding is similar to the results of investigators on large-conductance, Ca^{2+} -activated channels in several preparations. As with GK_2 , the increase in P_{open} at more positive potentials resides in both higher channel-opening frequencies and longer effective channel open times. Steady-state gating kinetic analysis during maintained depolarizations was performed as previously described. Probability

density histograms of open time measured at -10 and -60 mV absolute patch potential are shown in Figure 8B, C. The open-time histogram at each potential revealed a single-exponential distribution with a time constant of 1.11 msec at -10 mV and 0.76 msec at -60 mV. The more depolarized potential was characterized not only by longer mean channel open time, but also by higher opening frequency, as indicated in the upper right-hand corner ($O_{freq} = 3750/\text{sec}$ at -10 mV; $O_{freq} = 790/\text{sec}$ at -60 mV). It should be noted that several investigators (Barrett et al., 1982; Magleby and Pallotta, 1983a, b; Moczydlowski and Latorre, 1983) have reported 2-component open-time distributions for large-conductance, Ca^{2+} - and voltage-activated K^+ channels in other preparations. It is possible that a second, faster component was not resolved under current recording conditions.

Discussion

The single-channel current records in the present study definitively identify 3 distinct K^+ conductances in the membrane of cultured chick ciliary ganglion neurons. While all 3 channels appear to be highly selective for K^+ ions, they differ with respect to unitary conductance, mean channel open times, opening frequency, probability of opening, and sensitivity to voltage and intracellular Ca^{2+} ions. At present, there are relatively few single-channel data on K^+ currents in nerves with which to compare the results. Comparisons will thus be made to patch-clamp studies of K^+ channels in a variety of tissues.

GK_1 is characterized by a low unitary conductance (14 pS) associated with high selectivity. While many highly selective low-conductance K^+ channels have been described in the literature (for review, see Latorre and Miller, 1983), most are voltage-dependent, with either rectifying unitary conductances or gating kinetics that are sensitive to membrane potential. GK_1 differs from these in its apparent voltage insensitivity with respect to either conductance or gating properties. For instance, 2 examples of single neuronal K^+ channel studies, which coupled low conductance with high selectivity, are the delayed rectifier of squid axon (Conti and Neher, 1980; Llano and Bezanilla, 1983) and the reconstituted lobster axon delayed rectifier (Coronado et al., 1984), both of which exhibit strongly voltage-dependent gating properties. On the other hand, patch-clamp studies of the anomalous rectifier K^+ channel in tunicate eggs (Fukushima, 1981, 1982), in rat myotubes (Ohmori et al., 1981), in atrioventricular and sinoatrial nodal cells of the rabbit heart

Figure 7A. Conductance and selectivity properties of GK_{Ca(2+)} currents. Examples of Ca²⁺-activated outward currents recorded from a cell-free "inside-out" membrane patch isolated from a chick ciliary neuron. The recording solutions in the bath and pipette were symmetrical, with the following composition (in mM): KCl, 150; CaCl₂, 1.0; MgCl₂, 1.0; KHEPES, 5.0, pH 7.3. The numbers at the left of each trace indicate displacement of the patch potential from 0 mV (positive numbers indicate depolarization). The lines at the left of each trace indicate zero-current level.

(Sakmann et al., 1983), in guinea pig ventricular cells (Kameyama et al., 1983; Sakmann and Trube, 1984a, b; Trube et al., 1981), and in bullfrog atrial cells (Momose et al., 1983) indicate that these highly K⁺-selective channels have low unitary conductances that are strongly voltage-dependent, exhibiting strong inward rectification at positive potentials. Low-conductance, highly selective K⁺ channels in human erythrocytes (Grygorczyk et al., 1984; Hamill, 1981) and *Helix pomatia* neurons (Lux et al., 1981) have been described; these 2 channels, however, show both voltage- and Ca²⁺-sensitive gating properties. Thus, GK₁ appears to be somewhat novel with respect to its apparent voltage insensitivity with respect to conductance and gating.

GK₂ is a highly K⁺-selective channel with a moderate (64 pS) conductance. It does not require Ca²⁺ ions for activation, but does exhibit strongly voltage-dependent gating properties, with an open-state probability that increases with membrane depolarization, due to increases in both channel open duration and

channel-opening frequency. At least 4 other non-Ca²⁺-activated, K⁺-selective channels with similar conductances and voltage sensitivities have been studied by single-ion channel recordings. Gruol (1984) describes a 100 pS channel, which exhibits an increase in opening frequency and open duration on depolarization of the membrane of cultured Purkinje neurons. Clapham and DeFelice (1984) have described a 62 pS K⁺-selective channel with open-state probability that increases at positive membrane potentials in embryonic chick heart sarcolemma. A 50 pS K⁺-selective channel that also has an increasing open-state probability on depolarization has been found in the membrane of smooth muscle cells (Benham and Bolton, 1983). The serotonin-sensitive K⁺ channel in *Aplysia* sensory neurons has an outwardly rectifying unitary conductance of 55 pS and gating that is moderately affected by membrane potential (Siegelbaum et al., 1982).

Large-conductance "maxi" K⁺-selective channels activated

Figure 7B. Closed circles indicate the current-voltage relationship of single $GK_{Ca(2+)}$ channel currents obtained from 5 patches in symmetrical 0.15 M KCl, as in A. Slope conductance determined from the linear-regression line fitted to the data points is 193 pS, and extrapolated reversal potential is close to 0 mV, as expected in symmetrical solutions.

by micromolar concentrations of Ca^{2+} at the intracellular surface and by membrane depolarization have been described in a variety of preparations (for review, see Latorre and Miller, 1983). Other than the kinetic findings cited in the text, $GK_{Ca(2+)}$, which is characterized by a very large conductance (approximately 200 pS in 0.15 M KCl solutions) despite high selectivity for K^+ ions and by an open-state probability that is a function of both internal Ca^{2+} concentration and membrane potential, does not differ substantially from reports of these channels in other preparations.

As noted, the present study does not attempt to address the time dependence of channel gating. While no definitive evidence of inactivation processes was seen for any of the 3 channels, as judged by a decline in open-state probability during maintained voltage steps, the methods employed in this study could give only an approximate expression of time and voltage dependence due to the random behavior of single channels. The present study highlights the difficulties of interpreting non-steady-state

Figure 7C. Zero-current potential as a function of $[K^+]_o$. The data include the results from 4 different "inside-out" patches, which have the indicated K^+ concentrations in the pipette and face an internal solution with K^+ concentration of 5.4 mM. The values of zero-current potential, determined by linear-regression analysis from the current-voltage relationships, are plotted as a function of the logarithm of $[K^+]_o$. The experimental points are fitted by a straight line with a slope of 60.3 mV per 10-fold change in $[K^+]_o$.

Figure 8A. Channel-opening kinetics as a function of membrane voltage. Probability of a channel being in the open state as a function of membrane potential, $P_o(V)$, at a fixed interval (previously intracellular) Ca^{2+} concentration of 10^{-3} M. $P_o(V)$ was determined as the sum of open-state dwell times divided by the total time of the single-channel records at each potential. Records at all potentials were obtained from one inside-out patch with a single Ca^{2+} - and voltage-activated K^+ channel, as confirmed during maximal activation by 10^{-3} M Ca^{2+} (Marty, 1981). All experiments were carried out in the presence of symmetrical 0.15 M KCl solutions. The potential across the membrane patch was calculated by taking the external (previously extracellular) side of the membrane as reference; that is, negative potentials indicate hyperpolarization.

kinetic analysis without selective pharmacological blockade. Two possible experimental approaches, whole-cell aggregate current analysis or averaged unitary current responses to repetitive voltage jumps, will be contaminated by overlap of channel populations coactivated during depolarization.

The major purpose of the present study was to confirm the presence of multiple K^+ channel populations underlying the outward current activated by depolarization of chick ciliary ganglion neurons. Each of these channels represents a potential site for modulation of cellular excitability by neurotransmitters or hormones.

Figure 8B. Frequency vs open-time histogram from 300 single $GK_{Ca(2+)}$ channel events at an isolated patch potential of -10 mV. The histogram of channel open times was fitted by a single exponential (curved line) with a time constant of 1.11 msec. The coincidentally determined P_{open} equaled 0.89, and O_{freq} equaled 3750/sec of closed time.

Figure 8C. Frequency vs open-time histogram from 300 single GK_{Ca(2+)} channel events at an isolated patch potential of $-60\ mV$. The histogram of channel open times was fitted with a single exponential with a time constant of $0.76\ msec$. The coincidentally determined P_{open} equaled 0.33 , and O_{freq} equaled $790/sec$ of closed time.

References

- Adams, P. R., D. A. Brown, and A. Constanti (1982) M-currents and other potassium currents in bullfrog sympathetic neurones. *J. Physiol. (Lond.)* 330: 537–572.
- Bader, C. R., D. Bertrand, and A. C. Kato (1982) Chick ciliary ganglion in dissociated cell culture. II. Electrophysiological properties. *Dev. Biol.* 94: 131–141.
- Barrett, E. F., and J. N. Barrett (1976) Separation of two voltage-sensitive potassium currents, and demonstration of a tetrodotoxin-resistant calcium current in frog motoneurons. *J. Physiol. (Lond.)* 255: 737–774.
- Barrett, E. F., J. N. Barrett, and W. E. Crill (1980) Voltage sensitive outward currents in cat motoneurons. *J. Physiol. (Lond.)* 304: 251–276.
- Barrett, J. N., K. L. Magleby, and B. S. Pallotta (1982) Properties of single calcium-activated potassium channels in cultured rat muscle. *J. Physiol. (Lond.)* 331: 211–230.
- Belluzzi, O., O. Sacchi, and E. Wanke (1985) A fast transient outward current in the rat sympathetic neurone studied under voltage-clamp conditions. *J. Physiol. (Lond.)* 358: 91–108.
- Benham, C. D., and T. B. Bolton (1983) Patch-clamp studies of slow potential-sensitive potassium channels in longitudinal smooth muscle cells of rabbit jejunum. *J. Physiol. (Lond.)* 340: 469–486.
- Clapham, D. E., and L. J. DeFelice (1984) Voltage-activated K⁺ channels in embryonic chick heart. *Biophys. J.* 45: 40–42.
- Colquhoun, D., and B. Sakmann (1981) Fluctuations in the microsecond time range of currents through the acetylcholine receptor ion channels. *Nature* 294: 464–466.
- Colquhoun, D., and F. Sigworth (1983) Fitting and statistical analysis of single channel records. In *Single Channel Recording*, E. Neher and B. Sakmann, eds., pp. 191–263, Plenum, New York.
- Connor, J. A., and C. F. Stevens (1971) Voltage clamp studies of a transient outward membrane current in gastropod neural somata. *J. Physiol. (Lond.)* 213: 21–30.
- Conti, F., and E. Neher (1980) Single channel recordings of K⁺ currents in squid axons. *Nature* 285: 140–143.
- Coronado, R., R. Latorre, and H. G. Mautner (1984) Single potassium channels with delayed rectifier behavior from lobster axon membranes. *Biophys. J.* 45: 289–299.
- Dubois, J. M. (1981) Evidence for the existence of three types of potassium channels in the frog Ranvier node membrane. *J. Physiol. (Lond.)* 318: 297–316.
- Erichsen, J. T., A. Reiner, and H. J. Karten (1982) Co-occurrence of substance P-like and leu-enkephalin-like immunoreactivities in neurones and fibres of avian nervous system. *Nature* 295: 407–410.
- Fukushima, Y. (1981) Single-channel potassium currents of the anomalous rectifier. *Nature* 294: 368–371.
- Fukushima, Y. (1982) Blocking kinetics of the anomalous potassium rectifier of tunicate egg studied by single channel recording. *J. Physiol. (Lond.)* 331: 311–331.
- Galvan, M., and C. Sedlmeir (1984) Outward currents in voltage-clamped rat sympathetic neurones. *J. Physiol. (Lond.)* 356: 115–133.
- Gruol, D. L. (1984) Single-channel analysis of voltage-sensitive K⁺ channels in cultured Purkinje neurons. *Biophys. J.* 45: 33–55.
- Grygorczyk, R., W. Schwarz, and H. Passow (1984) Ca²⁺-activated K⁺ channels in human red cells. *Biophys. J.* 45: 693–698.
- Hamill, O. P. (1981) Potassium channel currents in human red blood cells. *J. Physiol. (Lond.)* 319: 97P–98P.
- Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* 391: 85–100.
- Hartzell, H. C. (1981) Mechanisms of slow postsynaptic potentials. *Nature* 291: 539–544.
- Hartzell, H. C., S. W. Kuffler, R. Stickgold, and D. Yoshikami (1977) Synaptic excitation and inhibition resulting from direct action of acetylcholine on two types of chemoreceptors on individual amphibian parasympathetic neurones. *J. Physiol. (Lond.)* 271: 817–846.
- Kameyama, M., T. Kiyosue, and M. Soejima (1983) Single-channel analysis of the inward rectifier K current in the rabbit ventricular cells. *Jpn. J. Physiol.* 33: 1039–1056.
- Katayama, Y., and S. Nishi (1984) Sites and mechanisms of actions of enkephalin in the feline parasympathetic ganglion. *J. Physiol. (Lond.)* 351: 111–121.
- Kostyuk, P. G., N. S. Veselovsky, S. A. Fedulova, and A. Y. Tsyndrenko (1981) Ionic currents in the somatic membrane of rat dorsal root ganglion neurons. III. Potassium currents. *Neuroscience* 6: 2439–2444.
- Krylov, B. V., and V. S. Makovsky (1978) Spike frequency adaptation in amphibians' sensory fibres is probably due to slow K-channels. *Nature* 275: 549–551.
- Latorre, R., and C. Miller (1983) Conduction and selectivity in potassium channels. *J. Membr. Biol.* 71: 11–30.
- Latorre, R., C. Vergara, and C. Hidalgo (1982) Reconstitution in planar lipid bilayers of a Ca²⁺-dependent K⁺ channel from transverse tubule membrane isolated from rabbit skeletal muscle. *Proc. Natl. Acad. Sci. USA* 79: 805–809.
- Llano, I., and F. Bezanilla (1983) Bursting activity of potassium channels in the cut-open axon. *Biophys. J.* 41 (2, pt.2): 38a.
- Lux, H. D., E. Neher, and A. Marty (1981) Single channel activity associated with calcium-dependent outward current in *Helix pomatia*. *Pfluegers Arch.* 389: 293–295.
- Magleby, K. L., and B. S. Pallotta (1983a) Calcium dependence of open and shut interval distributions from calcium-activated potassium channels in cultured rat muscle. *J. Physiol. (Lond.)* 344: 585–604.
- Magleby, K. L., and B. S. Pallotta (1983b) Burst kinetics of single calcium-activated potassium channels in cultured rat muscle. *J. Physiol. (Lond.)* 344: 605–623.
- Marty, A. (1981) Ca-dependent K channels with large unitary conductance in chromaffin cell membranes. *Nature* 291: 497–500.
- McAfee, D. A., and P. J. Yarowsky (1979) Calcium-dependent potentials in the mammalian sympathetic neurone. *J. Physiol. (Lond.)* 290: 507–523.
- Meech, R. W., and N. B. Standen (1975) Potassium activation in *Helix aspersa* neurones under voltage clamp: A component mediated by calcium influx. *J. Physiol. (Lond.)* 249: 211–239.
- Moczydlowski, E., and R. Latorre (1983) Gating kinetics of Ca²⁺-activated K⁺ channels from rat muscle incorporated into planar lipid bilayers. Evidence for two voltage-dependent Ca binding reactions. *J. Gen. Physiol.* 82: 511–542.
- Momose, Y., G. Szabo, and W. Giles (1983) An inwardly rectifying K⁺ current in bullfrog atrial cells. *Biophys. J.* 41: 311a.
- Neher, E. (1971) Two fast transient current components during voltage clamp on snail neurones. *J. Gen. Physiol.* 58: 36–53.
- Nishi, R., and D. K. Berg (1981) Two components from eye tissue that differentially stimulate the growth and development of ciliary ganglion neurons in cell culture. *J. Neurosci.* 1: 505–513.
- Ohmori, H., S. Yoshida, and S. Hagiwara (1981) Single K⁺ channel currents of anomalous rectification in cultured rat myotubes. *Proc. Natl. Acad. Sci. USA* 78: 4960–4964.

- Sakmann, B., and G. Trube (1984a) Conductance properties of single inwardly rectifying potassium channels in ventricular cells from guinea-pig heart. *J. Physiol. (Lond.)*. 347: 641–657.
- Sakmann, B., and G. Trube (1984b) Voltage-dependent inactivation of inward-rectifying single-channel currents in the guinea-pig heart cell membrane. *J. Physiol. (Lond.)*. 347: 659–683.
- Sakmann, B., A. Noma, and W. Trautwein (1983) Acetylcholine activation of single muscarinic K^+ channels in isolated pacemaker cells of the mammalian heart. *Nature* 303: 250–252.
- Siegelbaum, S. A., J. S. Camardo, and E. R. Kandel (1982) Serotonin and cyclic AMP close single K^+ channels in *Aplysia* sensory neurones. *Nature* 299: 413–417.
- Thompson, S. H. (1977) Three pharmacologically distinct potassium channels in molluscan neurones. *J. Physiol. (Lond.)*. 265: 465–488.
- Trube, G., B. Sakmann, and W. Trautwein (1981) Inward rectifying potassium currents recorded from isolated heart cells by the patch clamp method. *Pfluegers Arch.* 391: R28.
- Vergara, C., and R. Latorre (1983) Kinetics of Ca^{2+} -activated K^+ channels from rabbit muscle incorporated into planar bilayers. Evidence for a Ca^{2+} and Ba^{2+} blockade. *J. Gen. Physiol.* 82: 543–568.