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Small-Signal Analysis of K^+ Conduction in Squid Axons

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Summary. The maximum potential displacement that gives a linear K conductance response was determined to be 1 mV (rms) from a voltage-clamp analysis of TTX treated axons. For perturbations below this amplitude the K conductance kinetics are indistinguishable from a first-order rate process. Linearity and order of kinetics were assessed by four types of measurements: (i) the shape of the onset of the potassium current (sigmoidal *vs.* exponential); (ii) the symmetry of small hyperpolarizing and depolarizing pulses, (iii) wide band admittance, and (iv) harmonic analysis. The simplest interpretation of the results is that the small-signal linear response arises from a first-order gating mechanism, whereas the large-signal conventional voltage-clamp pulse of tens of millivolts evokes nonlinear phenomena. The small-signal results are consistent with the Hodgkin-Huxley description or any other nonlinear model which fits the large signal data and produces a linear first-order response for small perturbations.

Linear relaxation theory has been used to describe a number of kinetic systems (Eigen, 1968), including axonal conductances (Moore & Jakobsson, 1971); however, no adequate determination of the small-step response of the membrane ionic conductances has been made, although a number of linear analyses have been done (Cole, Baker, 1941; Matsumoto, Inoue, Kishimoto, 1970; Fishman, 1975; Fishman et al., 1977; Poussart, Moore & Fishman, 1977; Fishman, Poussart, Moore, 1979). The purpose of this paper is to compare three kinds of data: small-step currents, admittance, and harmonic generation which relate to a macroscopic description of K conductance kinetics obtained previously by linear analysis (Fishman et al., 1977).

In order to obtain a linear relaxation response, small voltage-clamp steps (< 1 mV) are applied so that the change in the conductance (δg) with respect to its initial value (g_i) is small ($\delta g/g_i < < 1$). The nonlinearity expressed in the Hodgkin-Huxley (1952) formulation (HH) arises from two distinct characteristics of the equations: (i) the well-known voltage dependence of the rate constants and (ii) the multiplicative and power function relationship between the conductance and the voltage-dependent variables (m^3h and n^4). Linearization of the equations replaces the latter with a sum of terms, each of which contains only one time-dependent variable, in contrast with products of the time-dependent variables which occur in the nonlinear equations. Since the linearized equations are reasonably successful in describing impedance or admittance data (Poussart et al., 1977), it is expected that small signal step clamp currents would also show good agreement.

The small perturbation experiments reported in this paper show that the potassium conductance is a linear process, within experimental resolution, for potential displacements less than 0.27 mV. Harmonic analysis also shows that nonlinear effects are 20 dB (10-fold) below the linear response for sinusoidal perturbations of 3 mV (peak to peak) and diminish to about 40 dB (100-fold) at the amplitude level of spontaneous current fluctuations.

The simplest interpretation of the results in this paper is that the small-signal linear response of the potassium conductance near the resting potential arises from a first-order gating mechanism, whereas the large-signal behavior is suggestive of either an additional complexity due to a nonlinear process or a rate process that is linear higher order, but appears

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to be first order for small signals. The linear response is not likely to be higher order at other potentials since relaxations from preceding depolarizations are first order (Cole & Moore, 1960; Moore, Holt & Lindley, 1972).

Materials and Methods

All experiments were done with an internal axial-wire pipette voltage clamp on axons from the squid, *Loligo pealei*, received live at the Marine Biological Laboratory, Woods Hole, Massachusetts. All of the K conductance experiments were done with 10^{-6} M tetrodotoxin (TTX) in the external sea water solution. The procedures used were the same as described previously (Fishman et al., 1977).

In addition, three equal-length (0.4 cm) external platinized platinum plates were placed parallel to the axon to make guarded voltage-clamp measurements. The end plates were connected to ground potential, and a virtual ground amplifier recorded the membrane current in the center region.

Linearity was explored by means of four complementary approaches: (i) analysis of the form of the rise of potassium current (sigmoidal *vs.* exponential) in response to depolarizing steps of voltage, (ii) comparison of the symmetry of response to small hyperpolarizing and depolarizing steps, (iii) wideband admittance in response to pseudorandom perturbations, and (iv) harmonic analysis, in response to sinusoidal excitation.

In the step-clamp experiments the current responses were signal averaged with a 12-bit analog-to-digital converter and signal averager (Nicolet Instruments, Model 535). For measurements (i) above, equal depolarizing and hyperpolarizing responses were added to subtract the leakage conductance for all pulses below 32 mV. Larger pulses were corrected for leakage by assuming a linear leakage current-voltage curve. Averaging, without correction, was used for measurement (ii) above.

The wideband admittance measurements (Poussart & Ganguly, 1977; Poussart, et al., 1977) were done after the K conductance had reached a steady state and within a 1–2 sec period after the onset of the depolarization. The time required for the measurement itself was the theoretical minimum determined by the frequency resolution and band used. Typically, for a 1-kHz band the acquisition time was 250 msec.

The harmonic analysis was done by successive application of various amplitude 10 or 100 Hz low distortion sinusoids to the voltage-clamp system and a simultaneous Fourier analysis of the harmonics generated in the current response in a steady state. The clamp system had sufficient loop gain (> 60 dB) at frequencies below 1 kHz to ensure that harmonics produced in this frequency range were due entirely to the membrane and not generated by the measurement system.

Results

The Sigmoidal Response of K Conductance

The sigmoid shape of the voltage clamped potassium currents is clearly demonstrated for step changes in the membrane potential from rest, as shown in Fig. 1. In Fig. 2 the responses of the small amplitude steps were signal averaged and normalized with respect to their maximal values, to show that the "delay" of the current response diminishes as the step amplitude is reduced to low levels. In Fig. 3 the disappearance

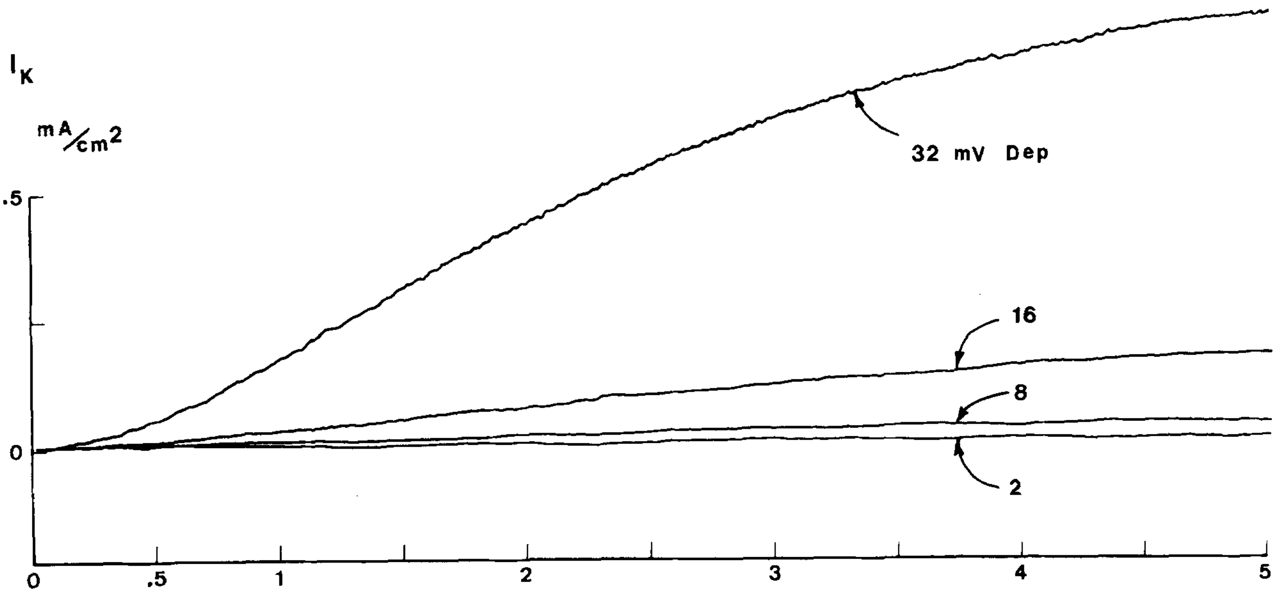

Fig. 1. Delayed potassium currents. The numbers refer to the level of depolarization for each voltage-clamp step. All currents were leakage corrected with hyperpolarizing pulses of equal magnitude referred to the corresponding depolarizing pulse. The membrane was hyperpolarized by 10 mV for 170 msec before applying the equal magnitude additional hyperpolarizing pulse used for leakage correction. This procedure removed relaxations of g_K due to hyperpolarization pulses which occur at rest potentials. The rest potential was -60 mV. 1 μM TTX was present in the external solution. In this and subsequent figures all currents were scaled according to an estimated membrane area in the central guarded region of 0.1 cm^2. The temperature in this and subsequent figures was 10 °C.

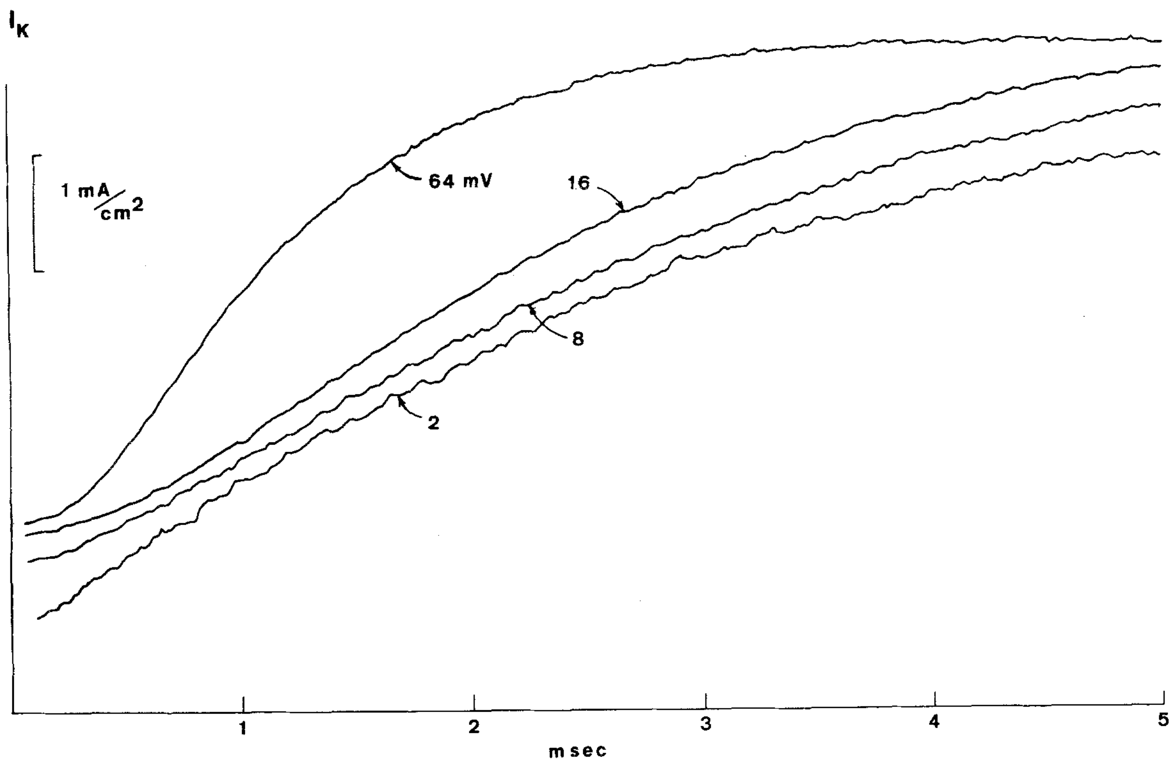

Fig. 2. Decreasing delay of the potassium currents. The vertical calibration refers only to the 64-mV depolarization. The currents at the other depolarizations are arbitrarily scaled and vertically offset to illustrate the decrease in sigmoidal character as the pulse amplitude is reduced. The leakage correction was the same as in Fig. 1. The currents were averaged 64, 4, and 2 times for the 2, 8, and 16 mV pulses, respectively.

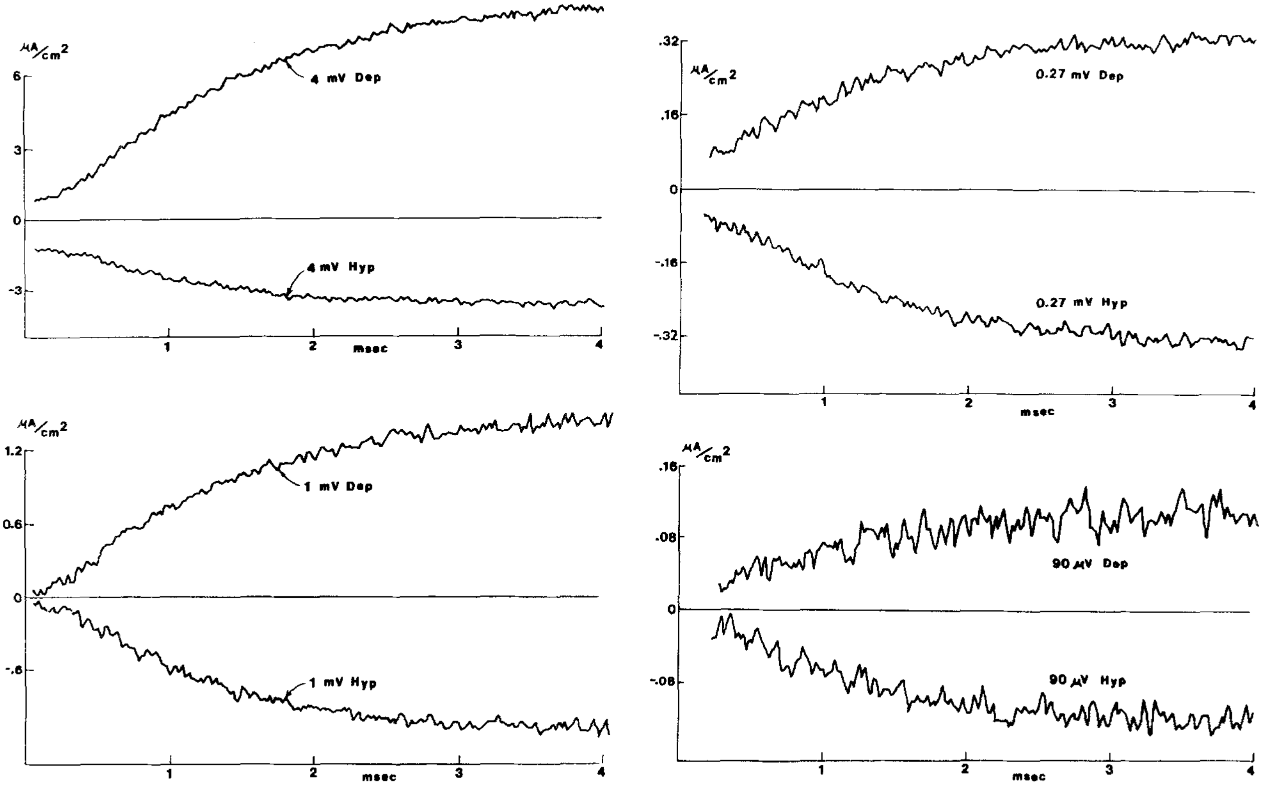

Fig. 3. Relaxation of K conductance to equal amplitude small-signal depolarizations and hyperpolarizations. The zero current is arbitrary. The 4-mV pulses were averaged 16 times and the 1-mV pulses, 128 times. The 0.27- and 0.09-mV pulses were averaged 1024 times. These records are not corrected for leakage.

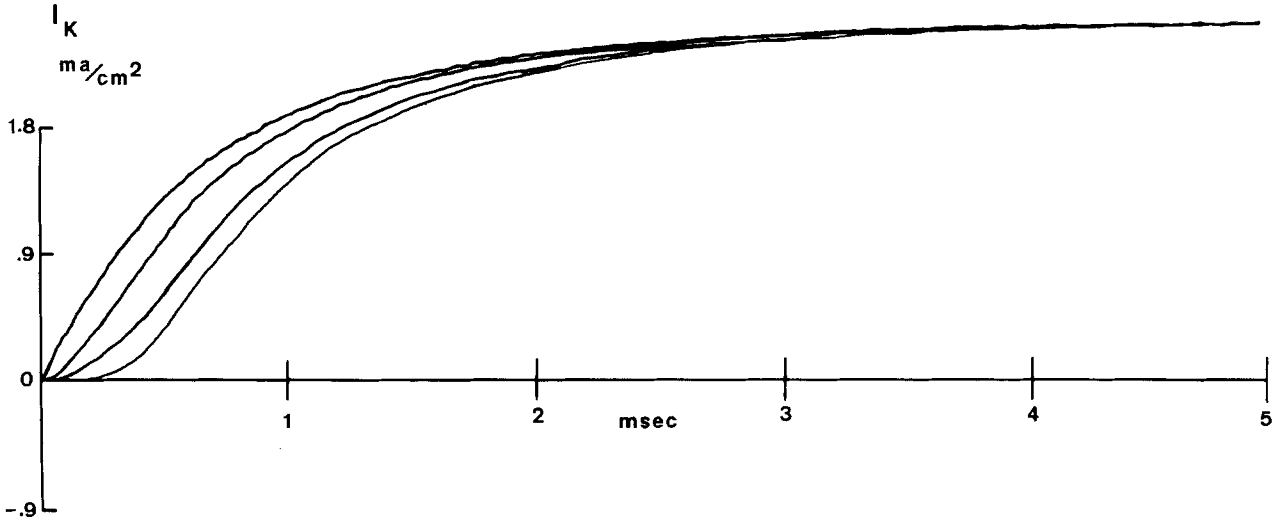

Fig. 4. Potassium currents after different prepulse levels. The calibration of the ordinate refers only to the lower curve. The other currents were scaled. The currents were recorded at a membrane potential of +35 mV. The prepulse duration was 15 msec, ending at time 0 msec. The prepulse levels were −40, −55, −105, and −135 mV for the upper through lower curves, respectively. The resting level was −55 mV. All currents were leakage corrected as in Fig. 1 except that the hyperpolarizing pulse used for the leakage current was $^1/_4$ the magnitude of the depolarizing pulse.

of the sigmoidal response is demonstrated as the step amplitude is decreased from 4 mV to the amplitude level of spontaneous voltage fluctuations (100 µV peak-to-peak). A plot of the data for a 0.27 mV step on semi-logarithmic graph paper is a straight line, thus indicating that a single exponential function is sufficient to describe the time-course of the K conductance response at this perturbation amplitude.

This behavior is remarkably similar to the previously observed shift in the delay that occurs for large test pulses as the prepulse amplitude is varied (Frankenhaeuser & Hodgkin, 1956; Cole & Moore, 1960). The data of Fig. 4 show similar results in the present series of experiments for a prepulse of 15 msec followed by a large step to +35 mV. As before, the delay vanishes, although at a pulse amplitude of 75 rather than 1 mV.

Linearity and Definition of Small Perturbation

An important consideration, independent of the order of the rate process, is whether the system is linearizable. The nonlinearity of the voltage-clamp response can be determined by comparing the current responses to equal amplitude steps of opposite polarity. In principle, an arbitrary nonlinear system should show a symmetrical relaxation response for perturbations of opposite sign, if the system is linearizable and the perturbation is small enough to allow the linear component of the response to dominate. This definition of a linear system applies irrespective of the nature of the nonlinearities produced by any model and, in particular, those in the HH description introduced by the voltage dependence of the rate constants or by the delay in the onset of a change in conductance.

The experiments illustrated in Fig. 3 show that all current responses were asymmetrical above a step of 270 µV. Thus, as with consideration of the (kinetics) delay, a linear (amplitude) response is obtained only in the limiting case of step perturbations below 1 mV. Since all depolarizing pulses above 270 µV resulted in asymmetrical relaxations, the membrane response is significantly nonlinear for all perturbations above this level, although the nonlinearity does not become pronounced until the applied potential becomes greater than 1 mV.

Wideband Admittance

An alternative way of obtaining a linear characterization of membrane conduction is by low-frequency measurements of the complex admittance (Fishman et al., 1977, 1979; Poussart et al., 1977). A deterministic broadband signal is used to make a 1-mV peak-to-peak (*p-p*) perturbation of the membrane potential in a specific manner under voltage-clamp conditions. The current response to the perturbation signal is then processed together with the input perturbation function by discrete Fourier transform techniques to obtain the complex admittance in terms of a pair of 256-point functions of frequency, the admittance magnitude, $|Y|$ and phase, ϕ. Figure 5 shows a com-

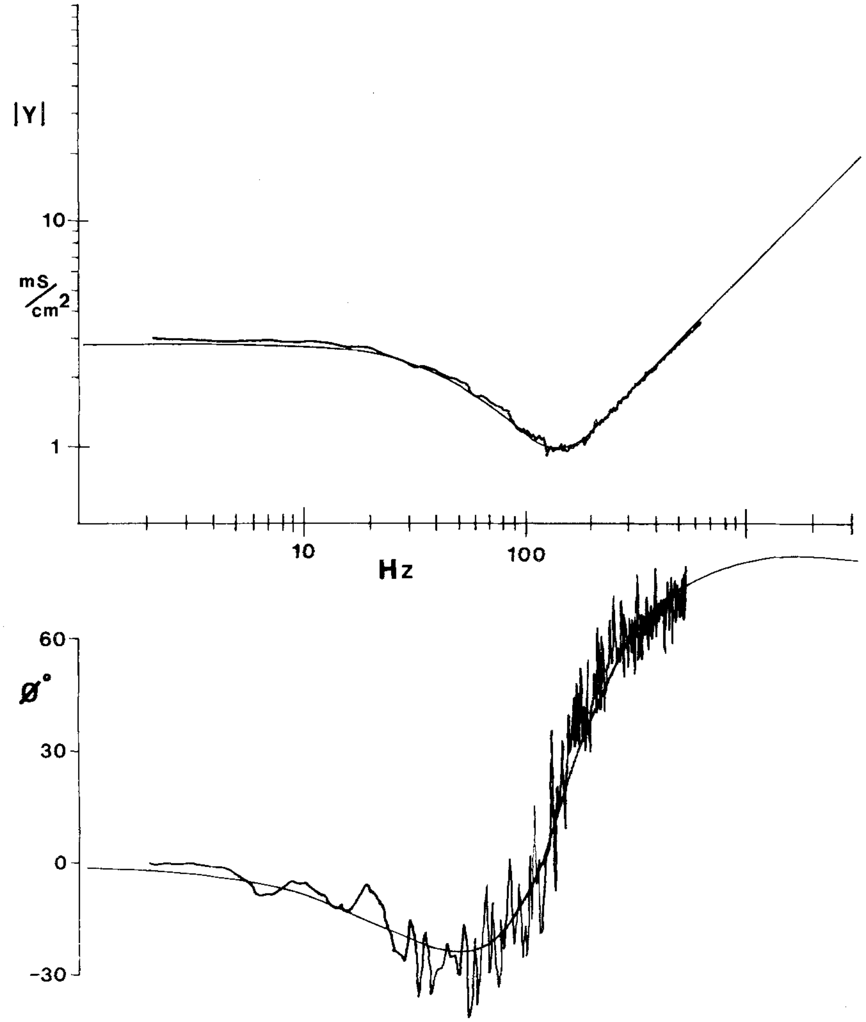

Fig. 5. Magnitude and phase of the membrane admittance with no sodium conduction. The amplitude of the perturbation was 1 mV peak to peak. The smooth curves drawn through the data points were calculated from the Hodgkin-Huxley equations with $\bar{g}_K = 50$ mS/cm^2, $\bar{g}_{Na} = 0$, and $V_K = -22$ mV. The temperature was 10 °C.

parison between the $|Y|$ and ϕ functions obtained on a squid axon, clamped at the rest potential with TTX applied externally, and the computed functions from the linearized HH equations with $\bar{g}_{Na} = 0$. It was shown previously (Fishman et al., 1977) that the linear properties of K ion conduction dominate the admittance at low frequencies (< 100 Hz). Thus, the admittance shows an anti-resonance at about 100 Hz that reflects the interaction of membrane capacitance (assumed to be ideal) with a parallel resistance-inductance like branch (the frequency domain equivalent of the first-order kinetics of K conduction as expressed in the linearized HH equations). These data confirm the fact that for small signals, irrespective of shape of the stimulus signal, K conduction kinetics are indistinguishable from a first-order rate process.

In order to explore the range of linearity the amplitude of the perturbation signal was raised. For signals exceeding 5 mV (*p-p*), a clear indication of nonlinearity was observed in the admittance spectrum. The relatively smooth $|Y|$ function became very "noisy" with some fine structure in the curve probably reflecting harmonic generation due to the nonlinearity (Poussart et al., 1977).

Harmonic Analysis

Additional observations of the transition from linear to nonlinear responses was made by harmonic analysis. A sinusoid ($< 1\%$ distortion) was applied as the command to the voltage-clamp system and a spectral analysis was performed on the current response to determine its harmonic content. Since the response of a linear system to a sinusoid is always another sinusoid at the same frequency differing only in amplitude and phase, harmonic analysis provides a sensitive means of assessing linearity. The solid circles in the graph of Fig. 6 show the responses for increasing amplitude sinusoidal voltages applied as a steady-state (10 Hz) voltage clamp command about the normal resting potential. The second harmonic component (20 Hz), indicated by the X's in Fig. 6, dominates the nonlinear (total harmonic content) response for amplitudes up to 10 mV *p-p*. However,

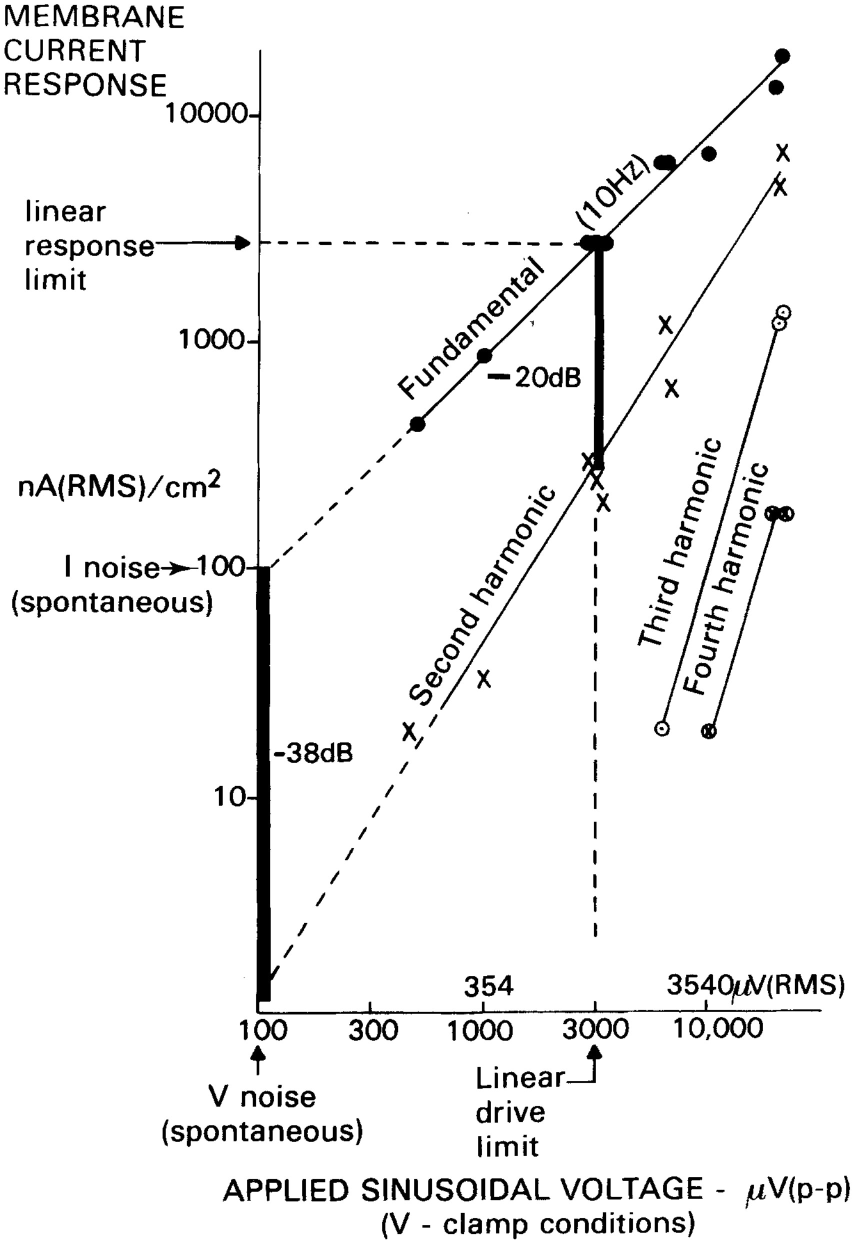

Fig. 6. Harmonic analysis of the potasium conductance. The root mean square (rms) current responses to a voltage-clamped 10-Hz command sinusoid stimulus are shown for narrow frequency bands (Hanning filter) at 10 Hz (fundamental), 20 Hz (second harmonic), 30 Hz (third harmonic), and 40 Hz (fourth harmonic). The peak-to-peak amplitudes of the sine waves are shown on the abscissa. The solid bar, labeled '*−20 dB*', indicates that the amplitude of the second harmonic response to a 3 mV peak-to-peak stimulus is 10-fold smaller than the fundamental response. The solid bar, labeled *−38 dB*, is based on an extrapolation of the measured data to the spontaneous fluctuation level and shows that the total harmonic response would be nearly 100-fold below the fundamental at the amplitude level of the ion conduction noise.

the second harmonic response is not significant with respect to the fundamental response for amplitudes below 3 mV *p-p*. At 3 mV *p-p* the second harmonic is one tenth that of the fundamental linear response and represents a 10% distortion in the current response waveform. Thus, these data suggest that the limit of the linear response occurs for a stimulus of about 1 mV (rms) and are consistent with the linear range determined by the previous methods.

The straight lines drawn through the data points in Fig. 6 for each type of response empirically indicate a power-law relationship between stimulus and spectral response. The dashed line extrapolation of the fundamental and second harmonic responses allow an estimation of the linearity at the amplitude level of the spontaneous current or voltage noise, since the third and fourth harmonics are negligible compared to the second harmonic. It is apparent from the extrapolation that the nonlinear components of the response for a driving voltage comparable to spontaneous membrane potential fluctuations are below the linear fundamental response by a factor of about 100 (1% distortion in the response waveform). Data similar to these in Fig. 6 have been obtained for a sinusoidal voltage at a fundamental of 100 Hz as well as for different holding potentials. It was

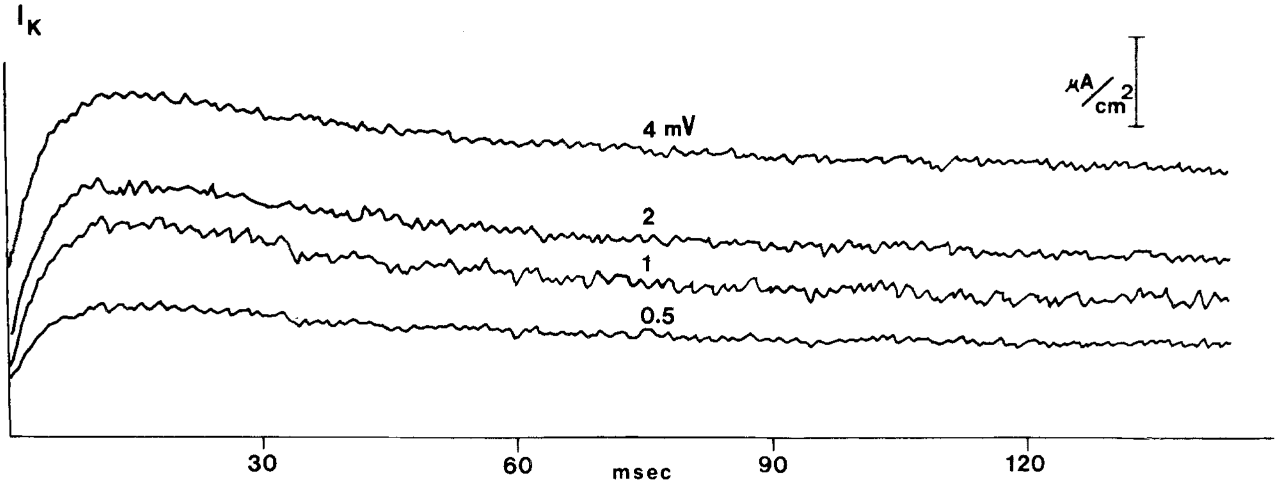

Fig. 7. Steady-state potassium currents. The currents were scaled and vertically offset for clarity. The vertical scales for the 4, 2, 1, and 0.5 mV pulses were 6, 3, 1.5, and 0.4 nA/cm^2, respectively.

found that the relative magnitude of the nonlinear second harmonic response decreased significantly when the membrane was depolarized by 25 mV.

Previous measurements of the impedance of the K$^+$ conduction process indicated a low frequency feature below 50 Hz not present in the linearized HH model (Fishman et al., 1977). Similar results using small-step analysis confirm the higher order complexity of the total axon system. Responses to long step pulses in Fig. 7 showed a slight reduction in the outward current at long times even for small steps. This feature may be related to potassium accumulation in the Schwann cell space or an axonal membrane conductance process similar to the *h* process of the sodium conductance system (Grisell & Fishman, 1979).

Discussion

The data presented in this paper show that the response of the potassium conductance system in the squid axon is indistinguishable from that of a first-order kinetics system that behaves linearly for diminishing small-step changes from rest potential. Since the small-step behavior of the K-conduction system is *both* linear and first order, the higher order kinetics that are apparent for large steps may reflect either nonlinearity induced in the gating process and/or additional non linearities elicited by other membrane phenomena. The finding that the delay seen with large pulses is reduced or abolished by a depolarizing prepulse (Cole & Moore, 1960) suggests that the usual sigmoidal time course of K current is a consequence of a nonlinear process. If the gating process were linear and of higher order, then the small-step response should not be first order. The finding that the small signal response is first order is consistent with any nonlinear process which, when linearized, gives first-order behavior. The problem that remains is to correctly describe the large amplitude response of the gating mechanism coupled to the ionic conductance of the membrane. The use of more complicated linear kinetic models (Roy, 1975; Palti, Ganot & Stämpfli, 1976; Armstrong & Bezanilla, 1977) to explain the large-signal behavior appears to be an alternative to a nonlinear formulation.

Although quantitatively different, the sodium conductance of the frog node is remarkably analogous to the K conductance; namely, the delay is increased by either a hyperpolarizing prepulse or an increased pulse amplitude at a fixed holding potential (Neumcke, Nonner & Stämpfli, 1976). In the node experiments an arbitrary measure of the delay vanished for pulses below 20 mV. Increases in the Na-current delay with hyperpolarizing prepulses has also been seen in squid axon (data of Fishman in Offner, 1972; Keynes & Rojas, 1976). Similarly, the Na delay in squid axon is dependent on pulse amplitude, progressively increasing with potential steps from 4 to 32 mV (*unpublished experiments*).

In conclusion, these results suggest that the conventional voltage-clamp experiment may evoke nonlinear phenomena; however, small-signal experiments provide linear responses that are consistent with the linearized Hodgkin-Huxley description of the membrane conductances. Although the complete Hodgkin-Huxley description of the large-signal data is sufficient to predict action potentials and other experimental results, its interpretation at the molecular level remains uncertain. Since the linear step response occurs for pulses evoking currents less than 3 nA/cm^2 (Fig. 6), it would seem that the response to spontaneous perturbations, which are well below this level, could yield to a linear description. However, this may not be the case since a nonlinear system may show fluctuations not described by a linear approximation (Van Kampen, 1965). In this respect, it appears that the only present means of determining whether the microscopic process associated with ion conduction is linear or nonlinear is to compare the kinetics derived from fluctuation analysis directly with those obtained from linear analysis on the same axon (Stevens, 1972; Fishman, 1973).

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