Nonstationary fluctuation analysis of the delayed rectifier K channel in cardiac Purkinje fibers

Actions of norepinephrine on single-channel current

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ABSTRACT We have studied the large increase in macroscopic potassium channel current caused by catecholamines in mammalian cardiac cells. An increase in macroscopic K current could result from either an increase in the single-channel current or by an increase in the number of channels that are open. Therefore, we have measured nonstationary potassium current fluctuations under voltage clamp conditions to determine whether norepinephrine increases the current through this channel. The single-channel current (at a potential of −30 mV in 4 mM external [K]) was estimated to be 3.7 pA and was not altered by concentrations of norepinephrine up to 2 μM. The spectral density of the current fluctuations were fitted well by a sum of 2 Lorentzians with corner frequencies that correspond with the measured time constants for deactivation of the macroscopic K current tails. We conclude that the increase in macroscopic K current caused by norepinephrine in these cells is not the result of an increase in single-channel conductance and therefore must involve an increase in the number of open K channels.

INTRODUCTION

We have previously shown that it is possible to quantitatively study the ionic selectivity and kinetics of the delayed rectifier potassium channels in calf cardiac Purkinje fibers (Bennett et al., 1985). This channel is at least 50 times more selective for potassium than sodium ions. The gating kinetics are quantitatively consistent with a three-state Markov model with two closed and one open state. We have also described the effects of norepinephrine (Bennett et al., 1986) on the macroscopic properties of this current. The major effects of norepinephrine on I_K are large (up to fivefold) increases in the current amplitude and a reduction of the slower of the two system time constants. However, the effects on the kinetics are not sufficient to account for the increase in current amplitude. Because the magnitude of the macroscopic K current is equal to the product of single-channel current, i, and the number, N_o, of channels open at a particular time, an increase in the macroscopic current could involve an increase in either i and N_o.

Attempts to directly measure the properties of single delayed rectifier potassium channels in enzymatically dispersed cardiac cells with patch clamp techniques have been hampered by the apparent low density of channels (Clapham and DeFelice, 1984). However, it has been possible to consistently measure the macroscopic properties of this channel and a number of groups have begun to characterize its functional and pharmacological properties (Hume, 1985; Hume and Uehara, 1985; Gintant et al.; Bennett and Begenisich, 1987; Roden et al., 1986, 1987; Balser et al, 1987; Arena and Kass, 1987). While many of the macroscopic properties of this channel appear similar in dissociated and intact preparations, it is nevertheless important to continue to compare as many parameters as possible with those from intact tissues.

Consequently, we have applied nonstationary current fluctuation analysis (Sigworth, 1980) to intact calf cardiac Purkinje fibers. We found that fluctuations of current through the delayed rectifier K channel can be obtained. These fluctuations have a special density predicted by the kinetics of the delayed rectifier K channels. This technique allowed us to estimate a single K channel current of ~3.5 pA. We used this technique to show that the large increase in macroscopic K channel current induced by norepinephrine was not due to an associated increase in single-channel current.

A preliminary presentation of this work has appeared in abstract form (Bennett et al., 1986).

METHODS

The voltage clamp methods for recording potassium currents in calf heart Purkinje fibers have been described in detail (Bennett et al., 1985, 1986). A two micro-electrode arrangement was used to voltage clamp shortened Purkinje fiber segments from calf heart. Bennett et al. (1985) present an analysis of the speed and accuracy of this voltage clamp technique for examining delayed rectifier currents in Purkinje fibers. Additional methods and a study of the actions of norepinephrine on

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macroscopic K channel currents are described in Bennett et al., (1986). Recordings were made in a solution containing (millimolars) 150 NaCl; 4 KCl; 1.8 $CaCl_2$; 0.5 $MgCl_2$; 5 glucose and 10 mM Tris-HCl (pH 7.4). Temperature was maintained at 37 ± 0.5°C. Currents were filtered at 40 Hz by an 8-pole Bessel filter and digitized at 143 Hz by a microcomputer controlled 12 bit analogue to digital converter. The holding potential used in these experiments was −30 mV which inactivated sodium channel currents. Current through calcium channels was also reduced by this relatively positive holding potential. In some experiments remaining calcium channel currents were eliminated by 50–500 nM nisoldipine.

Repetitive voltage pulses of ~1 s duration to membrane potentials between −10 and +30 mV were applied at 0.1 Hz. This pulse served to activate a fraction of the K channels. In these experiments the predominant time dependent current recorded after the return to the holding potential at −30 mV is current through the delayed rectifier K channels (see Bennett et al., 1985, 1986).

Analysis of current fluctuations

Current fluctuations were analyzed using techniques appropriate for nonstationary signals (Sigworth, 1980). Macroscopic current, I, from identical, independent channels with one conducting state are determined by the current through a single channel, i, and the number, N_o, of channels open at any particular time:

$$I = i \cdot N_o. \tag{1}$$

N_o is a function of time in the nonstationary case and embodies the channel gating behavior as determined by the rate constants for transitions among the kinetically defined states of the channel. It is equal to the total number of channels in the membrane, N, times the probability, $P(t)$, that one is open. The macroscopic current, I, will fluctuate according to the number of channels open at any particular time. The variance of the macroscopic current is related to the parameters in Eq. 1 in the following way

$$\langle \mathrm{VAR} \rangle = Ii - I^2/N. \tag{2}$$

As described by Eq. 2, the variance of macroscopic current is a parabolic function of the mean current with an initial slope equal to the single-channel current, i. If the number of channels is large (or the probability of opening is small), the second term in Eq. 2 is negligible and there will then be a linear relationship between current variance and mean current. This latter case was the usual situation in our experiments.

Computation of ensemble average current and variance

Deactivating K current tails were measured by applying a depolarizing voltage step into the activation range of the channel (usually 0 to +30 mV) followed by a voltage step to −30 mV. Ensembles of tail currents were recorded were at −30 mV after identical depolarization steps. Individual current tail records were averaged together at each sample point. The averages were then subtracted from each record that contributed to the average. This resulted in differences records that fluctuated about an average of zero. The variance was calculated as

$$\langle \mathrm{VAR} \rangle = [1/(n-1)] \Sigma [I_j(t) - \langle I(t) \rangle]^2, \tag{3}$$

where $\langle\ \rangle$ denotes ensemble averages.

Estimates of single-channel current were obtained either by nonlinear least squares fits of Eq. 2 to experimentally determined $\langle \mathrm{VAR} \rangle$ and $\langle \mathrm{I} \rangle$ or by a fit of the linear form of this equation. In both cases a small zero current variance was included to account for background fluctuations as described by Sigworth (1980).

Computation of spectral density

The discrete Fourier transform (DFT) of digitized difference records was computed using the Fast Fourier transform (FFT) method given by Stearns (1975). The relationship between the Fourier transform, $F(jw)$ and the DFT is given by $F(jw) = T \cdot \mathrm{DFT}$, where T is the sampling interval. The DFT amplitude is found from the quadrature of the real and imaginary parts from the FFT:DFT = $[(F_{real})^2 + (F_{imag})^2]^{1/2}$.

The square of the scaled DFT amplitude, $(T \times \mathrm{DFT})^2$, was used for the estimate of spectral density. Sigworth (1981) has shown that the nonstationary spectral density can be computed from the DFT with a correction term that is small when the probability of channel opening is small or constant. We have employed this correction term in our calculations even though it makes only a small contribution.

Adequacy of nonstationary noise analysis for determining i when P_{open} is small

We tested the ability of the analysis technique to allow accurate determination the single-channel current by modeling the single-channel behavior of I_K. In cases where the probability of channel opening is small, it is difficult or impossible to accurately determine the number of channels using these techniques. However, it is still possible to obtain an estimate of the single-channel current because the initial, linear portion of Eq. 2 is well defined. In our experiments it was not possible to maximally activate I_K. This would have required voltages clamp steps near +100 mV and was not experimentally feasible. As a consequence, the open channel probability, P_{open}, was always <0.5 in our experiments.

The gating behavior of I_K was modeled using the rate constants determined by Bennett et al., 1985. The computations described below allowed us to model the behavior of a single K channel. The noisy macroscopic behavior of 1,000 K channels was calculated by summing together 1,000 episodes of single-channel behavior. Macroscopic currents generated in this way were then subjected to the same analysis as the experimentally measured currents.

Knowledge of the rate constants allows computation of the exponential lifetimes of each of the three channel states (Bennett et al., 1985). The single-channel gating behavior after a voltage transition was determined by calculating the steady state probabilities of occupying each state at the voltage before the transition (see Eqs. A9, A10, and A11 of Bennett et al., 1985). These probabilities were used to weight the random placement of the channel in one of the three states just before the step to a new voltage (and a new set of rate constants). The channel was considered to be in the open state just before the voltage step if P_{open} exceeded a uniformly distributed random number between 0 and 1, otherwise the channel was closed. If not in the open state, one of the two closed states was chosen based on the conditional probability of being in either closed state given that it was not in the open state. Because $P_1 + P_2 + P_3 = 1$, the conditional probabilities of occupying the closed states were calculated as follows:

$$\mathrm{Prob}\{P_2|1 - P_3\} = P_{200}/(1 - P_{300}) \tag{4}$$

$$\mathrm{Prob}\{P_1|1 - P_3\} = 1 - \mathrm{Prob}\{P_2|1 - P_3\}. \tag{5}$$

If the conditional probability of occupying P_2 given that P_3 was not occupied (Eq. 4) exceeded a number between 0 and 1, chosen at random, then the channel was in state 2 otherwise it was in the other closed state.

After the voltage transition the rate constants at the new voltage were used to calculate the exponential distributions of lifetimes for the new conditions. A time was selected at random from the distribution and the channel remained in the state for that period of time. At the end of that time a transition into an adjacent state occurred. The channel remained in the new state for a time chosen at random from its distribution of life times. If the channel was in state 2 it could go into state 1 or 3.

The probability of going from state 2 into state 1 is $P_{21} = k_{21}/(k_{21} + k_{23})$ and the probability of going from state 2 into state 3 is $P_{23} = k_{23}/(k_{21} + k_{23})$.

These probabilities were used to weight the random placement of the channel in either state 1 or 3 after it was in state 2. In this way the behavior of a single channel was generated. The process was repeated N times and the results were summed together to simulate the macroscopic behavior of N channels.

The results of these simulations are illustrated in Fig. 1. Panel A shows the summation of 8, 16, 32, and 64 channels. The simulation was carried out using rate constants appropriate for a voltage clamp step from +10 to −30 mV. As the number of channels in the simulation is increased, the currents increasingly resemble a deactivating K current tail. Panel B shows deactivating current tails generated by summing the behavior of 1,000 channels. At the top are four (*a–d*) different tails generated in this way. The calculations were made using rate constants appropriate for a voltage step from +30 mV to a potential of −30 mV. The tails relax with two time constants of 89.5 and 566 ms. Beneath the individual records is the ensemble average (*e*) of ten such records. The ensemble average was subtracted from the individual records to give difference records (*g*) and these were used to calculate the variance (*f*) of the current fluctuations about the average current.

Panel C illustrates the relationship between the ensemble average current ⟨I⟩ and the ensemble variance ⟨VAR⟩ for two different conditions. The variance was calculated from ten difference records as described above. In part *a* the channel behavior was simulated for a voltage step from +60 mV ($P_{open} \gg 0.5$) to −30 mV ($P_{open} < 0.1$). The solid curve represents the best fit of Eq. 2 to the data. The best fit estimates of were i = 4.4 pA and N = 860. The true values of i and N were 4 pA and 1,000 channels (errors of 9.5 and 14%, respectively). Part *b* shows the results of using current tails at −30 mV after an activating voltage step to +10 mV ($P_{open} < 0.5$). In this case, the least-squares estimate of the single-channel current was 3.6 pA (a 10% error).

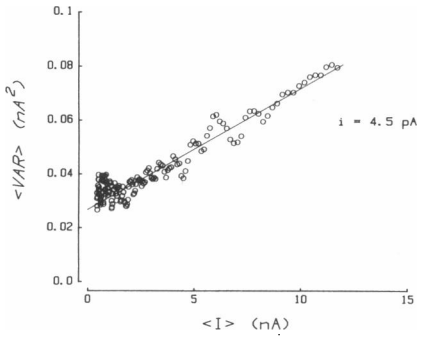

FIGURE 1 Computed behavior of K channel current fluctuations and a test for the fidelity of extracting the single-channel current from macroscopic fluctuations. (*A*) Summation of 8, 16, 32, and 64 K channels whose kinetic behavior was calculated from the rate constants derived by Bennett et al (1985). Channels were allowed to reach steady state appropriate for a membrane potential of +10 mV and then the rate constants were changed to those appropriate for −30 mV (see Methods). The process was repeated N times to simulate N channels. (*B*) Simulated deactivating K-tail currents (*a–d*) calculated from 1,000 channels, the ensemble average of ten such records (*e*), the ensemble variance calculated from ten records (*f*), and difference records (*g*) obtained by subtracting the ensemble average current tail from the individual records. (*C*) The relationship between the ensemble current and variance when P_{open} was >0.5 (*a*) and (*b*) when P_{open} was small (<0.5). The fitted curves (see Eq. 2) give estimates of the single-channel current and the number of channels. In part *b* the P_{open} was too small to allow a reliable estimate of the number of channels, but the single-channel current could still be estimated.

The conditions used in the computations for part *b* of Fig. 1 *C* were designed to simulate those of the experiments of this report. That is, we used activating voltages such that P_{open} was <0.5. These conditions preclude an accurate determination of the number of channels, N. But still permit a determination of the single-channel current, i. These simulations can also be taken as a worst case analysis in that only 10 sweeps were used to estimate i. In the experiments presented below estimates were always made using 20 or more individual records. In addition it should be pointed out that none of the results depends on the validity of the kinetic model used. Eq. 2 is valid regardless of the kinetic model provided there is a single open state. The model was used simply to provide a reasonable time dependent behavior of the macroscopic noise by which to test the adequacy of the estimation of the single-channel current.

RESULTS

Implicit in the analysis described above is the assumption that the measured current variance arise from K channel current fluctuations and not from other sources such as instrumentation noise, noise from other channel types. It is also necessary that the properties of the K channel be relatively stable over the period over which the data were obtained. We have performed several experiments designed to validate these assumptions.

A requirement for successful analysis of the variance of current fluctuations is a minimal systematic drift or run-down of the current being measured. Fig. 2 shows the results of the test for slow changes in the current amplitude that would alter the computed variance. Current amplitude was measured at two different times during deactivation of a current record since this also tests for stability of the current kinetics. There is no systematic trend as a function of pulse number indicating the absence of slow drifts that would contaminate the estimated variance. Similar tests were done for all experiments and only experimental results that showed a similar lack of systematic variation with pulse number are reported.

As described in Methods, several means were employed to eliminate currents through channels other than the delayed rectifier K channel. Consequently, the time dependence of the current variance should resemble the time course of the K channel tail current. The data presented in Fig. 3 show that this is, indeed, the case.

Fig. 3 *A* shows individual I_K currents recorded at −30 mV after an activation step to +15 mV and the ensemble

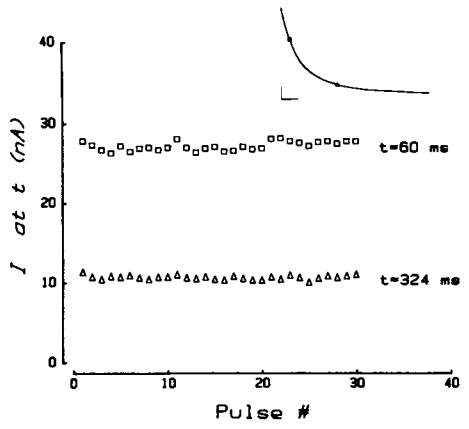

FIGURE 2 Pulse to pulse variation in currents used for ensemble averages. Current amplitude was measured at two times during the deactivation phase of I_K. Inset shows cursors on a deactivation I_K tail at 56 and 882 ms after a step from +13 to −30 mV.

average of 30 individual I_K records is shown in Fig. 3 *B*. Subtraction of a local average ot 3–10 sweeps from the individual records gives the difference records in Fig. 3 *C*. Ensemble variance as a function of time is illustrated in Fig. 3 *D*. The similarity between the time course of both the current and the current fluctuations is apparent.

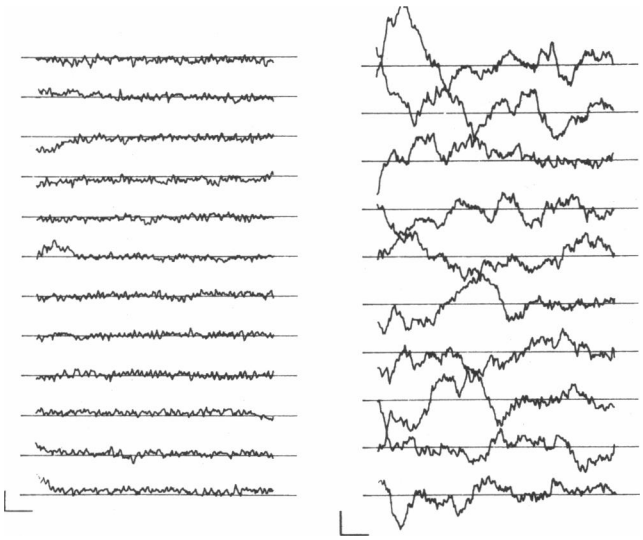

FIGURE 3 Time course of ensemble current and ensemble variance. (*A*) Time Course of individual I_K tails at −30 mV after an activation step to +15 mV. (*B*) Ensemble average of 30 individual I_K tails. (*C*) Difference records obtained by subtracting local averages of 3–10 sweeps from the individual sweeps that were used to compute the averages. (*D*) Ensemble variance as a function of time. Calibration bars: 5 nA for *A* and *B* and 0.029 nA^2 for *D*, 200 ms. The small horizontal bars in the lower right of *B* and *D* are the respective zero levels.

If the measured fluctuations arise from K channels, then conditions that reduce K channel current should also reduce the measured current fluctuations. A test of this hypothesis is illustrated in Fig. 4. In this experiment the external concentration of potassium was raised to 12 mM to shift the K ion equilibrium potential to near −60 mV. If the fluctuations are from the gating of K channels, then the current fluctuations should disappear near the K ion equilibrium potential. A voltage clamp step to +13 mV activated I_K and repolarization to −30 mV resulted in a deactivating current tail. The time course of the ensemble variance was similar to the deactivation time-course. When the pulse protocol was repeated with a repolarization to −55 mV, near the K^+ equilibrium potential, the current tail was reduced because of the reduced driving force for potassium. Similarly, the variance associated with the current tail deactivation was diminished.

Fig. 5 demonstrates the form of the spectral density of I_K fluctuations. Because we have already shown that the K channel deactivation currents are composed of two exponential components, a spectral density composed of two Lorenztian components is expected with the corner frequencies predicted by the current time constants. The deactivating tail current in this fiber was fit with a biexponential function with time constants of 89 and 418 ms. The solid line in the figure represents the sum of two

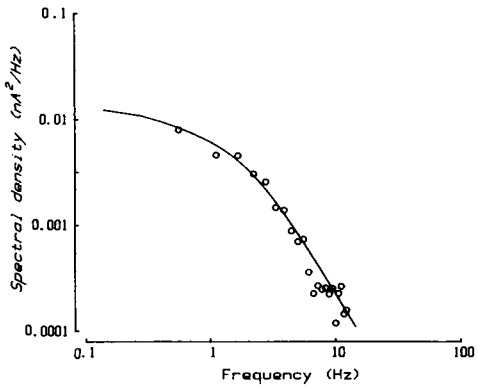

FIGURE 4 Time course of ensemble current and variance at two different membrane potentials. The top row shows the voltage protocol used. The middle row illustrates the ensemble average currents at the potentials indicated in the top panel. On the left, I_K deactivates at −30 mV after activating steps to +13 mV. Beneath the ensemble average current is the ensemble variance during deactivation which has a time course similar to the deactivating current. The middle column of traces show the voltage, ensemble current, and ensemble variance at −30 mV when there is no activating step. The records in third panel are similar to those of the left panel but with a holding potential of −55 mV near the channel current reversal potential of −57 mV. The preparation was bathed in an $[K]_o$ of 12 mM. The small horizontal bar in the lower right corner is the zero variance level.

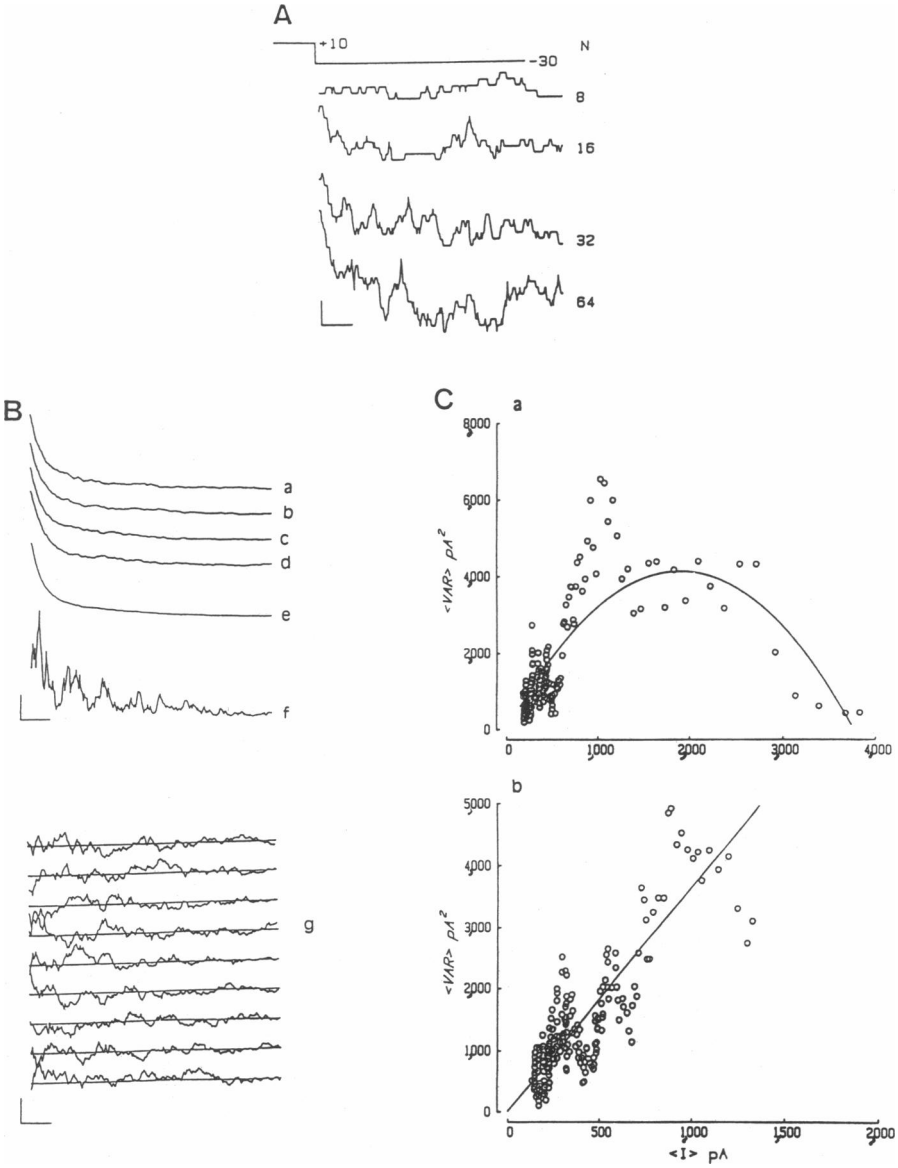

FIGURE 5 Spectral density of current fluctuations. Local averages of 3 to 10 records were computed as described in Methods and the averages were subtracted from the individual records. The resulting difference records of current fluctuations about a mean of zero were used to calculate the spectral density as a function of frequency. The circles are the average of spectra calculated from 13 difference records. The ensemble averaged current tail was fit by a 2-exponential function with time constants of 89 and 418 ms (f_c: 1.78 and 0.38 Hz). The solid curve through the data represents the sum of two Lorenzians with corner frequencies of 1.78 and 0.38 Hz. Data were filtered at 20 Hz by an 8-pole Bessel filter and digitized at 143 Hz.

Lorentzian terms with corner frequencies that correspond to these time constants (f_c = 1.78 and 0.38 Hz). Spectra obtained in the presence of norepinephrine was similarly described by a sum of Lorentzians.

Potassium channel current variance as a function of mean K channel current, obtained from the same fiber of Fig. 3, is shown in Fig. 6. The K channel current was measured at the holding potential of −30 mV after a voltage pulse to +15 mV. We have previously shown that only a small fraction (~0.2) of the maximum K conductance is activated by pulses to +15 mV. That is, the probability of channel opening is small and the linear relationship between these current and variance is expected to be a special case of Eq. 2, as described in

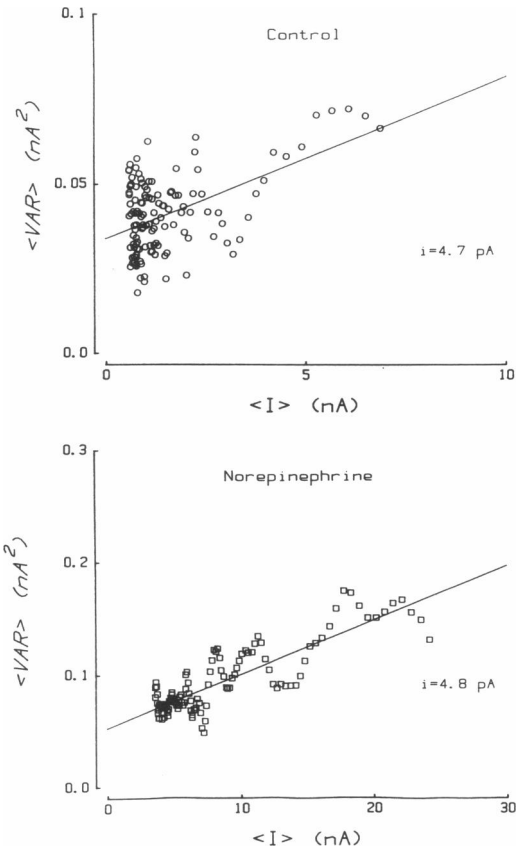

FIGURE 6 Potassium channel current variance as a function of mean K channel current, obtained from the same fiber of Fig. 3. The K channel current was measured at the holding potential of −30 mV after a voltage pulse to +15 mV. The solid line is the best fit linear relationship and the slope is the single-channel current value of 4.5 pA.

Methods. The variance at zero current represents (mostly) instrumentation noise as has been described by Sigworth (1980). The solid line in the figure is the best fit linear relationship and the slope of this line is equal to the single-channel current. In this experiment it was 4.5 pA.

Single-channel current values were determined in this manner from a total of five similar experiments (in 4 mM external K). The average single-channel K current at −30 mV from these five experiments was 3.7 ± 0.36 pA (SEM).

Fig. 7 shows current fluctuations in a fiber (bathed in 12 mM K_o) both before and during exposure to norepinephrine. Difference records were obtained by subtracting the ensemble average from the individual records as described in Methods. Macroscopic K channel currents increase markedly in the presence of norepinephrine and, as shown here, the magnitude of the current fluctuations also increased. To determine if the increase in the current and the current fluctuations was produced by an increase in the single-channel current or the number of open

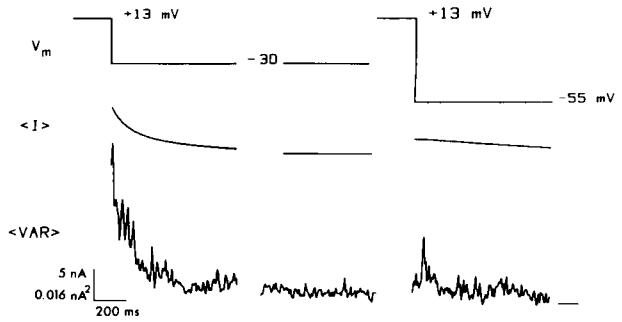

FIGURE 7 Difference records obtained by subtracting local averages from individual currents tails. Individual difference records are plotted as a function of time. Local averages were subtracted from each record so the fluctuations are around a mean of zero (reference line drawn). The left panel shows fluctuations under control conditions. In the right panel fluctuations in 2 μM norepinephrine are shown. The external potassium concentration, $[K]_o$, was 12 mM. The calibration bars at the lower left of each panel represent 0.5 nA (*vertical*) and 200 ms (*horizontal*).

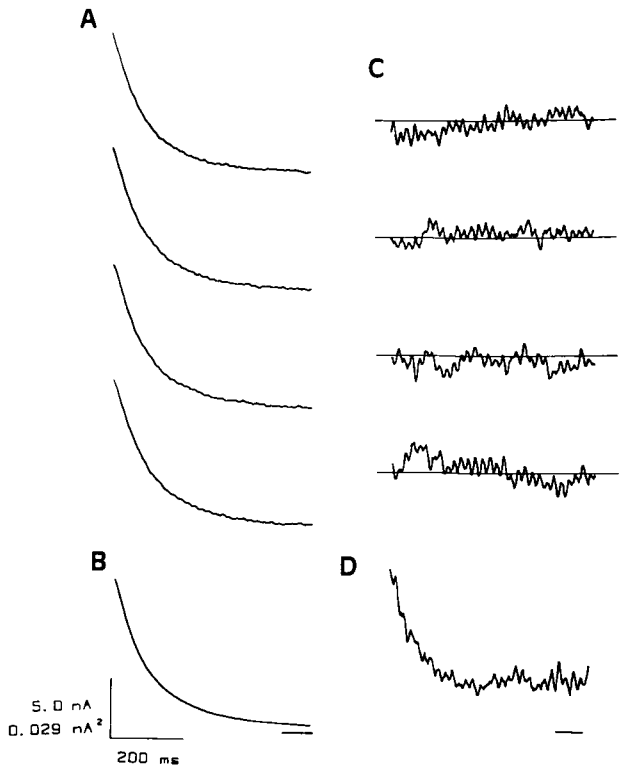

FIGURE 8 Ensemble variance as a function of ensemble current in the absence (*upper panel*) and presence (*lower panel*) of norepinephrine. Ensemble average variance, ⟨VAR⟩, and current, ⟨I⟩, were calculated as described in Fig. 3 and in Methods. The slope of the relationship between ⟨I⟩ and ⟨VAR⟩ was the same in control and in the presence of 2 μM norepinephrine indicating that the single-channel current did not change even through the macroscopic current was increased in this experiment by a factor of 3.3

channels, the single-channel current was obtained as in Fig. 6 and illustrated in Fig. 8.

Mean current and current variance data from the experiment of Fig. 7 are shown in Fig. 8. From the data in the absence of norepinephrine (*upper panel*) we obtained a single-channel current value of 4.7 pA. In the presence of norepinephrine (*lower panel*) the value was 4.8 pA. That is, the single current was not changed during exposure to norepinephrine. In a second, similar experiment (in 12 mM external K) a single-channel current value of 3.7 pA was obtained in the absence and 2.9 pA in the presence of norepinephrine. The results of these two experiments in which it was possible to determine in the same fiber the single-channel current in both the absence and presence of norepinephrine show that the increase in K current and K current fluctuations do not arise from an increase in the single-channel current.

We also obtained estimates of the single-K-channel current in the presence of norepinephrine in two additional experiments with 4 mM external K. The average single channel value in the presence of norepinephrine was 3.1 ± 0.86 pA which is not significantly different from the average value of 3.7 pA in the absence of norepinephrine. This supports the results from the two experiments in which both control and norepinephrine data were obtained on the same fiber. Norepinephrine certainly does not increase the single-channel current, yet, in these experiments, the mean macroscopic K channel current increased by a factor of 2 with 0.5 μM norepinephrine and by an average of 5.0 ± 0.6 (4) with 2 μM.

DISCUSSION

We have investigated the mechanism underlying the catecholamine induced increase in the delayed, voltage activated potassium channel current. Macroscopic K current was doubled by 0.5 μM norepinephrine and was increased by a factor of 5 by 2 μM norepinephrine, but the single-channel current at −30 mV was not increased ($p < 0.05$). Because the macroscopic current is the product of the single-channel current and the number of open channels, we conclude from these results that norepinephrine increased the number of open channels.

Clapham and DeFelice (1984) used single-channel recording techniques (cell attached patch and outside-out patch) and identified a channel in embryonic chick heart as the delayed rectifying K channel. The properties of this channel are quite similar to those of the channel that is the subject of this report. For example, Clapham and DeFelice found a highly K^+ selective channel with inward rectification and with a distribution of open channel lifetimes described by a single-characteristic time constant but with a biexponential closed time distribution. We have previously reported (Bennett et al., 1985, 1986) a P_{Na}/P_K of 0.02 for the delayed rectifier channel in cardiac Purkinje fibers. This channel exhibits inward rectification and has gating behavior consistent with one open and two closed states.

The single-channel conductance for the *K* channel described by Clapham and DeFelice (1984) is 68 pS with an extrapolated reversal potential of −77 mV in 4 mM $[K]_o$. We computed a single-channel conductance at −30 mV in our experiments of 68.9 ± 6.1 pS.

At present there are few single channel data on this type of potassium channel in cardiac cells, probably because of the very low density of these channels in the cell membrane. Clapham and DeFelice estimated the channel density to be 2/100 μm^2 which is more than a 1,000 times less than in nerve. Thus, if the channels are uniformly distributed, a 1-μm diam patch pipette would be placed over a channel only once in 50 attempts; and successful measurements could be made only when those attempts resulted in the formation of a high quality giga-Ω seal. Thus, successful measurements of single delayed rectifier K channels in heart will require an substantial effort, and the estimation of drug effects may be quite difficult. Nevertheless, it will be important to compare our results obtained by fluctuation analysis of macroscopic currents of intact tissue with the measurement of single-channel events in dissociated mammalian cardiac cells.

This work has demonstrated the utility of nonstationary fluctuation analysis in determining single channel properties based on macroscopic measurements. Further experiments will be required to determine the exact biochemical nature of the increase in the number of open K channels by catecholamines. Experiments similar to the elegant work of Bean et al. (1983) and Kameyama et al. (1985, 1986) will be needed to fully unravel the detailed workings of this channel system.

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