CONDUCTION in normal myocardium and the His-Purkinje system is sustained by a transient inward sodium current (Fozzard, 1979). The transmembrane movement of sodium ions is believed to occur through specific permselective channels. Measurement of action potential maximum upstroke velocity and macroscopic currents under voltage clamp have provided important information about the average behavior of the aggregate of sodium channels in multicellular and isolated myocyte preparations (Carmeliet and Vereecke, 1979; Colatsky and Tsien, 1979; Colatsky, 1980; Ebihara et al., 1980; Brown et al., 1981). However, little is known about the properties of individual ionic channels. Two recent developments now make it possible to study the behavior of individual ionic channels in adult cardiac myocyte preparations. Powell et al. (1980) have refined a technique for preparation of calcium-tolerant myocytes from adult hearts. Hamill et al. (1981) have described an extracellular patch clamp technique which permits the resolution of the unitary membrane current through individual ionic channels. We have combined both techniques to study single sodium channel currents in response to step changes in membrane potential. Our initial results indicate that many of the kinetic properties of sodium channels suggested by macroscopic currents are borne out by single channel measurements. We observed other kinetic properties which could not have been directly inferred from macroscopic current measurements.

Methods
Myocytes were isolated from the heart of adult rabbits by a modification of the technique described by Isenberg and Klockner (1982). Rabbits (1.4–2.5 kg) were anesthetized with ketamine (5–10 mg/kg, im) and pentobarbital (40–70 mg/kg, iv). Each heart was rapidly excised and transferred to ice-cold Ca++-free Krebs-Henseleit (K-H) solution. The aorta was cannulated and perfused by the Langendorff technique with Ca++-free Krebs-Henseleit solution at 36 ± 1°C. The composition of all salt solutions are listed below. After the heart had been cleared of blood, the perfusate was switched to enzyme medium for 45 minutes. The perfusate was finally changed to Kraftbruhe (KB) medium for a 5-minute period. The ventricles were cut into small segments and gently agitated in KB medium. The isolated myocytes were separated from chunks by filtration through a 200-µm mesh. The suspension of isolated myocytes was centrifuged for 1 minute and the pellet resuspended in fresh KB medium. The cells were stored at 4°C for at least 1 hour before use. All solutions passed through a 0.2-µm filter prior to use.

The composition of salt solutions (in mM) were: Krebs-Henseleit solution NaCl, 118.2; KCl, 4.7; MgSO4, 7H2O, 1.2; CaCl2, 2.7; NaHCO3, 25; NaH2PO4, 1.2, glucose, 11. "Ca++-free" Krebs-Henseleit solution: as above with CaCl2 omitted. K-H solution was gassed with 95% O2, 5% CO2. Enzyme medium: Ca++-free K-H solution containing 69.4 U/ml collagenase and 50 U/ml hyaluronidase. 25 mM Ca++ was added to the nominally Ca++-free solution. K-B medium (mm): KCl, 85; K2HPO4, 5; Na2ATP, 5; pyruvic acid, 5; β-OH butyric acid, 5; creatine, 5; taurine, 20; glucose, 20; polyvinyl povidone, 50 g/liter, EGTA, 0.04. The pH was adjusted to 7.2 with KOH. Patch-pipette solution (mm): NaCl, 120; KCl, 4; MgSO4, 1.2; CaCl2, 2.7; Hepes, 5; NaH2PO4, 1.2. The pH was adjusted to 7.4 with NaOH (± 5 mm). Cadmium-pipette solution contained 1 mM CdSO4 and PO4− was omitted. Hepes solution (mm): NaCl, 138.2; KCl, 4; CaCl2, 2.7; MgSO4, 7, H2O, 1.2; glucose, 11; Hepes, 5. The pH was adjusted to 7.4 with NaOH.

Batrachotoxin was employed at a concentration of 1 µM and was kindly provided by Dr. John Daly, National Institutes of Health, Bethesda, Maryland.
Recording Techniques

The base of the recording chamber consisted of a glass coverslip (22 × 22 mm) coated with Alcian blue (Sommer, 1977). The recording chamber was mounted on the stage of an inverted microscope (American Optical Co. Biotar). After introduction of 200 µl of cell suspension into the chamber, perfusion was started at a rate of 1 ml/min with K-H solutions. One to 50% of the cells remained rod-like with definite cross-striations. Experiments were performed on rod-like cells only at room temperature (20–22°C). A fresh sample of cells was used every 30–60 minutes. Patch micropipettes were prepared according to the method of Hamill et al. (1981). The micropipettes were filled with "pipette" solution and coupled to a current-to-voltage (I-V) converter via an Ag/AgCl wire coated with Teflon up to its tip.

The patch microelectrodes had resistances of 3–10 MΩ. Scanning electronmicrographs of three patch microelectrodes showed a mean tip diameter of 0.8 µm. The patch microelectrode mounted on the head stage of the I-V converter was advanced at an angle of 60–80° to a cell surface with a Leitz micropositioner, until dimpling was just perceptible. Gentle suction was applied while the shunt resistance to ground was monitored with a 100-µV pulse. Gigaseal formation was an abrupt event, and associated with a corresponding decrease in the noise level of at least one order of magnitude. For resolution of single sodium channels, we used membrane patches with seal resistances to 10 GΩ or greater. Inside-out excised patches were formed by withdrawing the pipette from the cell surface following gigaseal formation. The pipette then was passed very briefly (approximately 1 second) across the air-water interface.

The output of the I-V converter was filtered (corner frequency, 1 KHz, Bessel response). Voltage command pulses were provided by an analog stimulator (WPI model 830) or a custom-built microprocessor-based stimulator. Membrane patch current and voltage clamp command pulses were displayed on a Tektronix RM565 oscilloscope and photographed with a kymographic camera (Grass Instrument Company C4).

The pipette voltage was under experimental control. Following gigaseal formation, the membrane patch potential was the sum of the cell resting potential and the patch microelectrode potential. The patch microelectrode potential was held at 10–20 mV positive to the ground (10–20 mV hyperpolarized from the resting potential of the cell) and changed in 5–10 mV depolarizing steps of 40 msec duration. Ten to 60 traces were obtained at each test potential level. Capacitative transients were reduced by analog electronic compensation with a two-time-constant system (Hamill et al., 1981).

Single channel currents were digitized from film. Initially, we measured all deviations from the noise of the current baseline. For an event to be included in the analysis, the amplitude of the current fluctuation had to exceed 50% of the amplitude of a clearly resolved event.

Single channel current amplitudes and open times at various membrane potentials were compared by an analysis of variance. Values of variables are quoted as mean ± sem. Results were obtained in 16 patches.

Results

Figure 1 shows the response of a membrane patch to a step change in patch membrane potential, \( V_m \). \( V_m \) was held 20 mV hyperpolarized from the resting potential and stepped 55 mV for 40 msec. 5.6 msec after the onset of the clamp step, there was a 1.3 pA inward rectifying current change, 3.6 msec in duration. The step change in current represents a unitary conductance change in a membrane channel. The occurrence of such events in response to a step change in patch potential was highly variable, with characteristics dependent on the level of the voltage clamp step. It was not possible to obtain measurements of the transmembrane potential and gigaseals with a sodium channel in the same cell. Like Reuter et al. (1982), we have quoted the patch membrane potential as the sum of the cell resting potential and the applied potential in the electrode. We measured the transmembrane potential in 10 representative cells by rupturing the membrane patch following gigaseal formation and immediately recording the transmembrane potential. Mean transmembrane potential was \(-46 ± 3\) mV in these cells. However, the membrane potentials grouped around a low range of \(-6.6\) mV (\( n = 3 \)) and a high range of \(-63 ± 1.3\) mV (\( n = 7 \)). If more than 40 mV steady hyperpolarization were required to elicit unitary events, further experimentation on that cell was terminated.

To illustrate some of the variability, we have presented current traces in response to 10 consecutive voltage clamp steps to each of three test potentials in Figure 2. No events were observed in one of the 10 traces for voltage clamp steps of 40 mV (\( R_p + 20 \) mV). Events tended to occur throughout the 40 msec of the clamp pulse. When the clamp step was increased to 50 mV (patch potential \( R_p + 30 \) mV), no events occurred in three of the 10 clamp steps, and all channel openings occurred within 10 msec of the onset of the clamp. In response to a 70-mV step (patch potential \( R_p + 50 \) mV), no events were evident in four of 10 clamp steps, and all openings started within the first 1 msec of the clamp.

![Figure 1](http://circres.ahajournals.org/July 11, 2017
http://circres.ahajournals.org/Downloaded from)

**Figure 1.** Unitary sodium current in response to step change in membrane potential. The upper trace shows patch membrane current recorded from an isolated myocyte. Membrane current was filtered (1 kHz, Bessel response), and capacitative transient was reduced by analog compensation. Inward current is downward. The lower trace shows the 40-msec depolarizing step change in pipette voltage. Pipette holding potential was +20 mV (i.e., 20 mV hyperpolarizing from the cell resting potential).
FIGURE 2. Unitary membrane currents in response to step changes in membrane potential. Ten consecutive current records obtained at each of three potentials are shown. The pipette holding potential was +20 mV, and the pipette potential was stepped by 40, 50, and 70 mV, in the records shown (potential during the clamp was +20, +30, and +50 mV). The sample of records at the 40-mV clamp (Rp + 20) was chosen to illustrate multiple channel openings during a single clamp. A 2.5-pA calibration is shown on the right. The clamp step was 40 msec in duration. The records or data in Figures 2, 3A, and 5 were obtained in the same patch.

The overall frequency of clamp steps without events was 41, 5, 15, and 23% with steps of 40, 50, 60, and 70 mV, respectively. Opening probability was lowest with voltage clamp steps to the highest (most negative intracellular) potential at which events were first observed. With even greater depolarization, channel activation overlapped the capacitative transient. Because of the increase in duration of the events with increasing depolarization and the short time frame over which both the first and all subsequent events occurred, simultaneous events were much more likely to occur at depolarized command potentials. The occurrence of multiple levels, e.g., trace number 9 at Rp + 30, suggested the presence of two channels in this patch. At the more depolarized command potential, e.g., Rp + 50 mV, rarely were more than two events observed per voltage clamp step (1 in 56 steps). In contrast, the frequency of more than two openings was much greater with small depolarizations. The data suggest that, with small depolarizations, a channel may open more than once during the same step. With greater depolarizations, the channel rarely undergoes a second opening during a given step. Fukushima (1981) observed current bursts that were interpreted as multiple openings of sodium channels in tunicate eggs. Fenwick et al. (1982) made similar observations in chromaffin cells. For small depolarizations, channel closure probably occurs by deactivation (reversal of activation) and may quickly reopen. For larger depolarizations, channel closure occurs by inactivation and does not reopen during the clamp step.

Figure 3A shows the cumulative probability to first opening after onset of the clamp step. With increasing membrane depolarization, time to first opening decreases and is steeply dependent on transmembrane voltage. This corresponds to the decrease in the time constant of activation of the macroscopic currents with increasing depolarization. We performed a more detailed analysis in two patches in which overall opening probability was high. Histograms of the probability density of first opening and latency were obtained at each of several potentials. At most potentials, histograms had maxima at times of latency greater than zero msec (Fig. 3B). With strong depolarizations the maxima occurred within the width of the first bin (0–0.05 msec), and the possibility that events were lost in

FIGURE 3. Probability of first opening as a function of time from onset of the voltage clamp step. In Figure 3A, the clamp duration is divided into 0.2-msec bins, and all events occurring at a time interval less than or equal to each limit are summed. The summed events are then divided by the total number of events to give cumulative probabilities for each interval. The symbols represent records obtained at Rp + 50 (○), Rp + 30 (●), and Rp + 20 (□). Figure 3B was obtained from a different patch. Events occurring within equal time intervals are divided by the total number of events during the clamp to give a probability density histogram.
FIGURE 4. Summing of the unitary currents of multiple channels. Panel A shows the membrane current in response to a 40-mV voltage clamp step. The holding pipette voltage was $R_p + 20$ mV. It consists of an initial large current which is thought to represent the summing of the current of individual sodium channels. Three unitary events are also evident. Current calibrations are 5.0 pA and 10 msec. In panel B, the peak amplitude of the summed currents are plotted against voltage clamp step size. The capacitative transient was more prominent than in the usual preparations, e.g., compared with Figure 2.

The capacitative transient could not be excluded. The delayed maximum is consistent with at least two closed states of the sodium channels (French and Horn, 1983).

Over a voltage range of 40 mV, mean channel open time increased. In the experiment illustrated in Figure 2, mean open time was $1.20 \pm 0.5$ msec ($n = 61$) at $R_p + 20$ mV, $1.43 \pm 0.07$ msec ($n = 110$) at $R_p + 30$ mV, and $2.11 \pm 0.12$ msec ($n = 39$) at $R_p + 50$ mV. The increase in mean open time between $R_p + 20$ and $R_p + 50$ mV were statistically significant. There was little difference in the mean current amplitude at each of the three clamp potentials, $1.20 \pm 0.03$ pA, $1.3 \pm 0.03$ pA, and $1.20 \pm 0.03$ pA at $R_p + 50$, $R_p + 30$, and $R_p + 20$ mV. Fenwick et al. (1982) observed a flat single channel I-V curve between $-70$ and $-40$ mV, followed by a positive slope at more depolarized potentials. This raised the possibility that if the I-V curves were determined over a sufficiently wide potential range, decreases of the single channel current amplitude may be observed at depolarized potentials.

In a single patch with at least five channels, we were able to perform clamps over a large voltage range (five increments of 10 mV). In many traces, we observed both summed and single channel activity in the same voltage clamp step (Fig. 4A). Figure 4B shows the mean of 13–20 summed currents plotted against voltage clamp step size. The increase in current for clamp steps of 30–50 mV probably results from an increase in the number of open channels; the subsequent decline probably resulted from a decrease in driving force. Some overlap of the capacitative transient could have contributed to the decline of current at strong depolarizations, but it is not likely that the overlap was responsible for the 50% decrease of the summed current at a $\Delta V$ of 80 mV.

A histogram of the duration of the single channel events provides information about the closing process of the channel (Horn and Patlak, 1982). We selected the responses to the 50-mV voltage clamp step ($R_p + 50$ mV) for illustration (Fig. 5) because the largest number of events were seen at this potential level. Openings were most frequently 1–1.2 msec in duration. The frequency of opening of greater duration decreased progressively. How-

FIGURE 5. Frequency distribution of channel open times. The duration of channel open times at a clamp potential of $R_p + 50$ mV are grouped in 0.2-msec bins. The frequency of events in each time range is plotted on the ordinate.
ever, the bandwidth limitation may have made the first bin artificially small. Correlation coefficient for a single exponential distribution of open times was $-0.92$, with a mean closing rate of $1.42/\text{msec}^{-1}$ at $R_p +30 \text{ mV}$. The single exponential suggests transitions from a single conducting state.

Channel activity was observed in excised inside out patches with the same solution on either side of the membrane patch. However, the transmembrane membrane potential had to be hyperpolarized to $-110$ to $-140 \text{ mV}$ before voltage clamp steps elicited channel activity. We are not sure of the reason for this apparent shift of voltage dependence of channel gating.

We examined the effect of 1 $\mu\text{M}$ batrachotoxin on the unitary sodium channels in two patches. Sodium channels opened spontaneously in the absence of voltage clamp steps. Channel opening tended to occur in bursts interspersed by quiescent intervals. Open times ranged from $2.5$ to $860 \text{ msec}$ and showed the same pattern of increase in open times with depolarization as the normal channels. Open channel amplitude was uniform at each potential, and we did not observe subconductance levels despite the prolonged channel open times.

We have used a number of approaches to validate that the inward unitary channel currents result from opening of sodium channels. When the pipette holding potential is varied and the voltage clamp stepped to a fixed value above the resting potential, inactivation changed from zero to one over a 40-mV range. The macroscopic sodium currents in whole cell or multicellular preparations inactivate over a 30 to 40-mV range. We have obtained records similar to that in Figure 2 with patch pipettes containing 1 $\text{mM}$ cadmium. Kostyuk et al. (1977) have shown that 1 $\text{mM}$ cadmium blocks the slow or secondary inward current carried by calcium ions without shifting the $h_{\text{oc}}$ curve of the $I_{\text{Na}}$ along the voltage axis. With pipettes filled with isotonic barium and the cells superfused with phosphate-free Hepes-buffered solution, we have briefly observed unitary channel current events through the slow channels. To date, we have not been able to make an analysis of these channels, as the rabbit myocytes do not survive for long periods in nonbicarbonate buffers and the slow channel current fatigues rapidly (Fenwick et al., 1982).

When these studies were initiated, we were interested first in the feasibility of the approach. We therefore performed pilot experiments in a number of other preparations. Similar unitary sodium current events were observed in isolated ventricular myocytes of toad and guinea pig and primary culture of rat neonatal myocytes. We elected to do these initial studies in the rabbit, as we plan comparative studies with pacemaker cells of the SA node, and they are best characterized in the rabbit. Potassium channels with open times of tens to hundreds of milliseconds have also been identified in rabbit ventricular myocytes.

Discussion

We have used the extracellular patch clamp technique to demonstrate unitary changes in membrane current in response to step changes in pipette potential. Membrane patches were studied in the cell-attached and inside-out configuration with pipettes filled with Na⁺, Ca²⁺, K⁺, and Cl⁻ as potential permeable ions. The unitary events showed the potential dependence and insensitivity to cadmium characteristic of sodium channels. Events occurred in response to depolarization of 10 or more millivolts from the resting potential of the cell. In the study of Brown et al. (1981), macroscopic sodium currents were activated between $-60$ and $-70 \text{ mV}$. A change in holding potential of 40 mV causes steady state inactivation to vary from zero to one. This is similar to the observation of Colatsky (1980) in a multicellular preparation, but is narrower than the range reported by Brown et al. (1981). Time to first event decreased rapidly over a very narrow voltage range. It corresponds to the rapid decrease in activation time constant for depolarization to potentials of $-55 \text{ mV}$ or less in the study of Brown et al. (1981). The 1 kHz corner frequency used to filter current records, and the duration of the capacitative current, limited the resolution of unitary events with strong depolarization, as events overlapped the capacitative transient. In particular, we were not able to demonstrate reversal of the unitary membrane currents.

The persistence of the unitary events when pipettes contained 1 $\text{mM}$ Cd²⁺ support our conclusion that the events resulted from conductance changes in sodium channels. Calcium channels are activated over a much wider range of membrane voltage (Reuter et al., 1982). Estimates of unitary currents through the slow channel with calcium ions as the charge carrier are less than 0.2 pA (Fenwick et al., 1982). We have recorded unitary events with micropipettes filled with isotonic KCl (three patches) or K aspartate (one patch). The latter was less satisfactory as K aspartate led to polarization of the electrode. We were able to record unitary events of tens to hundreds of milliseconds in duration. It is unlikely that chloride ions are the charge carriers for the currents reported here. Chloride ions should produce a net outward current over the potential range studied. There is no convincing evidence for a gated chloride current in rabbit ventricular myocardium.

The relative constancy of the amplitude of unitary sodium channel currents with increasing depolarizations was a surprising result. The conductance of most unitary channels recorded to date have been ohmic. The fact that events were of shorter duration at higher membrane potential means that the restricted bandwidth of the recording system could lead to an error in the estimate of the amplitude of events at high potentials [see Fig. 3 of Hamill et al. (1981)]. However, the duration of events at high membrane potentials was well within the range that
could be recorded with a corner frequency of 1 kHz. The observation of a relative flat I-V curve of single channels is still true if events of duration greater than 1 msec, only, are considered. Fukushima (1981) observed a flat I-V curve negative to −56 mV for the sodium channels of tunicate egg. Similarly, Fenwick et al. reported a flat I-V curve for the sodium channels in isolated bovine chromaffin cells over the range of voltage reported in our study. Our results also provide some insight into the mechanism(s) of an increase in the macroscopic Iₜ, with membrane depolarization. The channels remain open longer in response to increasing membrane depolarization.

There was evidence for only two primary conductance states of the sodium channel, open and closed. We did not observe subconductance states. The delay in the maximum of the latency-probability density histogram is consistent with multiple closed states. The bursts with quiescent intervals during batrachotoxin exposure is also consistent with at least two closed states. Occasionally, we have observed openings interrupted by multiple closures in the absence of batrachotoxin. Fukushima (1981) observed bursting of the sodium channel in the absence of batrachotoxin exposure in the tunicate egg. We did not have sufficient events to plot closed-time histogram (two events are required to obtain a single point).

Two abstracts have recently appeared describing single sodium channels in myocytes of rat and chick embryonic heart (Kunze and Brown, 1982; Ten Eick et al., 1982). Kunze and Brown reported that events were confined to the first several milliseconds of the depolarizing voltage steps, and were 1.1 pA in amplitude at −50 mV. Ten Eick et al. noted a mean current amplitude of 1.27 pA, duration of 2.54 ± 0.28 msec, and slope conductance was 10 pS between −40 and −20 mV. The distribution of open times was exponential with a mean closing rate of 685/sec. The amplitude of unitary events in our study is similar to that in the two previous reports. Further, we have shown that the probability of first opening of a channel occurs over a decreasing interval as membrane depolarization is increased. This suggests depolarization increases the rate of the conformational change of the channel macromolecules from the nonconducting to the conducting state. Our study also shows that an increase in the duration of channel opening is one of the mechanisms by which the macroscopic sodium current increases with depolarization. The recent study by Cachelin et al. (1983) demonstrates similar properties of the Na⁺ channels in neonatal rat myocyte. The only significant difference is the presence of a definite slope to the I-V curve with conductances of 15 pS. However, they clearly state that, in some experiments, single channel amplitude was "little voltage dependent" or may even decrease at hyperpolarized potentials. The properties of cardiac sod-

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