British Journal of Pharmacology (2002) 135, 751–763

www.nature.com/bjp

Inhibition of cardiac Na^+ current by primaquine

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1 The electrophysiological effects of the anti-malarial drug primaquine on cardiac Na^+ channels were examined in isolated rat ventricular muscle and myocytes.

2 In isolated ventricular muscle, primaquine produced a dose-dependent and reversible depression of dV/dt during the upstroke of the action potential.

3 In ventricular myocytes, primaquine blocked $I_{Na}{}^+$ in a dose-dependent manner, with a K_d of 8.2 μM.

4 Primaquine (i) increased the time to peak current, (ii) depressed the slow time constant of $I_{Na}{}^+$ inactivation, and (iii) slowed the fast component for recovery of $I_{Na}{}^+$ from inactivation.

5 Primaquine had no effect on: (i) the shape of the I–V curve, (ii) the reversal potential for Na^+, (iii) the steady-state inactivation and $g_{Na}{}^+$ curves, (iv) the fast time constant of inactivation of $I_{Na}{}^+$, and (v) the slow component of recovery from inactivation.

6 Block of $I_{Na}{}^+$ by primaquine was use-dependent. Data obtained using a post-rest stimulation protocol suggested that there was no closed channel block of Na^+ channels by primaquine.

7 These results suggest that primaquine blocks cardiac Na^+ channels by binding to open channels and unbinding either when channels move between inactivated states or from an inactivated state to a closed state.

8 Cardiotoxicity observed in patients undergoing malaria therapy with aminoquinolines may therefore be due to block of Na^+ channels, with subsequent disturbances of impulse conductance and contractility.

British Journal of Pharmacology (2002) **135,** 751–763

Keywords: Primaquine; Na^+ channel; voltage-clamp; cardiac myocytes

Abbreviations: ATP, adenosine triphosphate; δ, electrical distance; dV/dt, rate of change of membrane potential; $g_{Na}{}^+$, sodium inward conductance; $g_{Na}{}^+$-max, maximum inward sodium conductance; GTP, guanosine triphosphate; h_∞, sodium current availability; $I_{Na}{}^+$, whole-cell Na^+ current; k, slope factor; K_d, dissociation constant; PQ, primaquine; τ_f, fast time constant; τ_m membrane time constant; τ_{on}, time constant for block; τ_s, slow time constant; $V_{m_{1/2}}$, half activation or inactivation potential; V_t, test potential

Introduction

Voltage-gated Na^+ channels are responsible for membrane depolarization and action potential conduction in the heart. Indeed, Na^+ channels are the target of many commonly used antiarrhythmic drugs (Grant, 1990; Adelman, 1995; An *et al*., 1996). Class I antiarrhythmic drugs block Na^+ channels with complex voltage, frequency and state-dependent properties that are important for their clinical efficacy (Hille, 1992; Bezanilla & Stefani, 1994; Feng *et al*., 1996; Sakakibara *et al*., 1993). State-dependence of block has been explained by an allosteric model in which a modulated drug receptor has a higher binding affinity when channels are open or inactivated then when channels are resting or closed (Fozzard & Hanck, 1996; Backx *et al*., 1992). While a substantial literature has developed with regards to the biophysical characterization of cardiac Na^+ channels, the toxicological effects of many clinically relevant Na^+ channel antagonists, in particular the aminoquinolines (Harris *et al*., 1988), remains unclear.

Primaquine, an 8-aminoquinoline compound, enjoys wide clinical use for the treatment of relapsing malaria (Greenwood, 1997). In addition, primaquine is also frequently used in the prophylaxis of many forms of latent parasitic infection, including, *Plasmodium vivax*, and *P. ovale* (Cohen *et al*., 1968; De la Pena & Reeves, 1987). While primaquine is very often the first-line drug under such conditions, its use is not without serious adverse effects (Eissen & Ette, 1986). Primaquine and other related aminoquinoline compounds have been reported to have undesirable cardiovascular effects, such as hypotension, depression of myocardial function and disturbances in conduction and rhythm (Tona *et al*., 1990; Iglesias-Cubero *et al*., 1993). While most of these effects have been observed during primaquine overdose, such symptoms are not uncommon during acute or chronic therapeutic use (>60 mg day^{-1}). The most common of these is the depression of myocardial contractility, typically in associa-

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tion with ventricular arrhythmias (Ward *et al*., 1985; De la Pena & Reeves, 1987; Riou *et al*., 1988; Molineaux, 1997). It has been suggested that this negative inotropic effect may be related to the block of cardiac Na^+ channels (Salinas-Stefanon & Morales-Salgado, 1998), but there is as yet no direct information to support this hypothesis.

We have previously reported that the 4-aminoquinoline chloroquine has a non-specific blocking effect on cardiac K^+ channels and blocks Na^+ channels at concentrations greater than 20 μM (Cebada & Salinas, 1994). The present study was therefore undertaken to examine the effect of primaquine on the cardiac Na^+ current ($I_{Na}{}^+$) using the whole-cell voltage-clamp technique.

We observed that primaquine blocks $I_{Na}{}^+$ with a K_d of 8.2 μM. Unlike its structural analogue chloroquine, primaquine had no effect on cardiac K^+ currents (Cebada & Salinas, 1994). Block of Na^+ channels by primaquine occurred approximately 20% into the membrane electrical field and was consistent with an open channel blocking mechanism. Under micro-electrode conditions, primaquine dramatically slowed the rate of change of membrane potential during the action potential, suggesting that pathological perturbations of contractility, and impulse conduction in certain patients taking primaquine may be related to block of $I_{Na}{}^+$.

Methods

Electrophysiological recording in isolated muscles

Thin right ventricular trabeculae or papillary muscles were obtained from adult rat hearts. Preparations were dissected out at room temperature (22°C) in a well-oxygenated Tyrode solution of the following composition (in mM): NaCl 137.0; KCl 2.5; $CaCl_2$ 2.0; $MgCl_2$ 0.5; HEPES 5.0; glucose 20.0; and Na-pyruvate 5.0, pH 7.4. Preparations were mounted in a 1.6 ml perfusion chamber and continuously perfused (10 ml min^{-1}) with the same Tyrode solution at 37°C. After an equilibration period of at least 60 min, transmembrane potentials were recorded by conventional glass microelectrodes filled with a 3M KCl (resistance 10–15 MΩ). The microelectrodes were coupled to the input of a high-impedance preamplifier (KS-700, World Precision Instruments, Sarasota, FL, U.S.A.). The preparations were stimulated at a frequency of 1 Hz with rectangular stimuli (2 ms duration, 1.5 times diastolic threshold intensity), delivered by platinum bipolar electrodes (Grass Instruments, Quincy, MA, U.S.A.). Action potentials signals were digitized at a sampling rate of 10 KHz by use of an analog-to-digital converter (Digidata 1200, Axon Instruments, Foster City, CA, U.S.A.) and stored on a hard disk, Axotape data-acquisition software (Axon Instruments) and a Compaq Presario computer. Data analysis was performed using pClamp software (version 7.01, Axon Instruments).

Resting membrane potential (RMP), the rate of change of membrane potential during the upstroke of the action potential (dV/dt), action potential amplitude (APA) and action potential duration at 30% (APD_{30}) and 90% (APD_{90}) of repolarization from peak APA were measured in 11 different preparations.

Single cell isolation

Isolated rat cardiac myocytes were prepared according to a standard enzymatic perfusion method (Clark *et al*., 1993; Bouchard *et al*., 1993). Briefly, adult Sprague Dawley rats (200–250 g) were heparinized (1000 u kg^{-1}) and anaesthetized with pentobarbitone Na^+ (35 mg kg^{-1}, i.p.). Hearts were excised and perfused through the aorta in a Langendorff system at 37°C, with normal Tyrode solution for 5 min, and thereafter with a nominally zero-calcium Tyrode solution for a further 5 min. The perfusate was then changed to a nominally zero-calcium solution containing collagenase (1 mg ml^{-1}, type IA, Sigma Chemical) and trypsine (0.1 mg ml^{-1}, Boehringer Mannheim) for approximately 20 min. The enzymes were washed out by perfusion with Tyrode solution containing 0.1 mM Ca^{2+} for 5 min. Small pieces of ventricular free wall (1 mm^3) from both left and right ventricles were dissected out and put into separate flasks containing the storage solution. Single cells were obtained by mechanical agitation with a pipette and were stored in Tyrode solution with 0.1 mM Ca^{2+} at room temperature (22°C).

Electrophysiological recordings of single cells and data analysis

Isolated single cells were placed in a small-volume (0.2 ml) recording chamber on the stage of an inverted microscope (IM35, Zeiss, Germany). Macroscopic current recordings under voltage-clamp conditions were obtained using the whole-cell recording method (Hamill *et al*., 1981) and an Axopatch 1D amplifier (Axon Instruments, Foster City, CA, U.S.A.). Glass pipettes were pulled from borosilicate capillary tubing (TW 150, World Precision Instruments, Sarasota, FL, U.S.A.) using a horizontal puller (Model P-97, Sutter Instruments, Novato, CA, U.S.A.). Membrane potentials were corrected for the liquid junction potential (~4.8 mV). Currents were filtered with an eight pole Bessel filter at 2 kHz, digitized at 5 kHz, and stored on a computer hard disk. Data analysis was performed on leak-subtracted current traces using a P/4 leak subtraction protocol (Bezanilla & Armstrong, 1977).

In a typical voltage-clamp experiment, the time required for the membrane potential to reach 95% of its final value following a step in command potential is equivalent to the product of three times the membrane time constant (τ_m). Prior to the point at which the membrane capacitance is fully charged, membrane currents measured by a voltage-clamp amplifier will be due partly to charging of the membrane capacitance and partly to the flow of ions through channels. Indeed, in previous work we (Bouchard *et al*., 1993) and others (Brown *et al*., 1981; Isenberg & Klockner, 1982) have shown that serious voltage-clamp errors can occur, both at 22°C and 37°C, when large and fast ionic currents, such as $I_{Na}{}^+$ or L-type Ca^{2+} currents ($I_{Ca}{}^{2+}$), are not adequately controlled. Such errors can lead to erroneous measurements of the voltage-dependence of $I_{Na}{}^+$, $I_{Ca}{}^{2+}$, cell shortening and intracellular Ca^{2+} release.

It was therefore necessary during these experiments to carefully control the membrane potential during voltage-clamp of $I_{Na}{}^+$. In the present study, the seal resistance was typically between 2–5 GΩ and the series resistance was

compensated between 80–90% in each cell in order to provide for optimal voltage clamp integrity. In a total of 45 myocytes, the average (±s.e.mean) DC pipette resistance was 3.26±0.02 MΩ, the compensated series resistance was 1.85±0.01 MΩ, and cell capacitance was 82±12 pF, yielding an average τ_m of 148 μs. Compared to the fastest time to peak I_{Na^+} of approximately 1 ms (Figure 2), a τ_m of ~150 μs was sufficient to properly clamp I_{Na^+} under the present experimental conditions.

Solutions

The normal Tyrode solution had the following composition (mM): NaCl 140, KCl 4.5, $CaCl_2$ 1.8, $MgCl_2$ 1.0, HEPES 10, and glucose 10, and pH was adjusted to 7.40 with 0.8 ml of 1 M KOH solution. For experiments in which I_{Na^+} was recorded, cells were superfused with a low-Na^+ external Tyrode solution with the following composition (mM): NaCl 10, CsCl 120, $CaCl_2$ 1.0, $MgCl_2$ 2, $CoCl_2$ 3, HEPES 10, and glucose 10; pH was adjusted to 7.40 with CsOH. Primaquine (diphosphate salt, Sigma Chemical) was dissolved directly in the low-Na^+ external solution. No effects of primaquine on external pH were observed. Pipettes were filled with a Cs^+-rich internal solution of the following composition (mM): CsCl 118, aspartic acid 22, $MgCl_2$ 6.4, ethylene glycol-bis ((-aminoethylether)-N,N,N′,N′-tetra-acetic acid (EGTA) 5, guanosine 5′-triphosphate (GTP) 0.5, ATP-Na 4.2, $CaCl_2$ 2.7, HEPES 5, pH was adjusted to 7.25 using CsOH.

Statistics

Results are given as mean±standard error (s.e.) of the mean. The differences between mean data were analysed with a paired or unpaired Student's *t*-test as appropriate. A P value <0.05 was used to denote statistical difference between the groups.

Drugs

Primaquine (8-(4-amino-1-methylbutylamino)-6-methoxiquinoline,diphosphate salt), ATP-Na (adenosine triphosphate, disodium salt) were purchased from Sigma Chemical (St Louis, MO, U.S.A.). GTP (guanosine 5′-triphosphate) was obtained from Boehringer Mannheim (Germany).

Results

In the first series of experiments, the effect of primaquine (1, 10, and 30 μM) on action potential characteristics was studied in rat ventricular muscle at 37°C. Preparations were bathed in a physiological Tyrode solution and stimulated at 1 Hz using field electrodes. In 11 different preparations, the resting membrane potential, plateau phase of the action potential (APD_{30}) and the terminal phase of repolarization (APD_{90}) were not significantly affected by varying concentrations of primaquine. As shown in Figure 1, primaquine did, however, significantly blunt the rate of rise of the upstroke of the action potential, and this effect occurred in a dose-dependent manner. Differentiation of the upstroke of the action potential (dV/dt) revealed that the depressive effect of primaquine on the action potential is due almost exclusively to a concentration-dependent depression of dV/dt. There was a small change in the early notch of repolarization, but this effect did not significantly affect APD_{30}. Table 1 summarizes the electrophysiological effects of primaquine on action potential amplitude, APD_{30}, APD_{90}, dV/dt, and the resting membrane potential in 11 experiments under control conditions and after the superfusion with incremental concentrations of primaquine. The effect of primaquine after 12 min of washout was partially reversible (~70%), at all concentrations examined.

Figure 2 illustrates whole-cell Na^+ currents obtained from rat ventricular myocytes in the presence of low-Na^+ external solution. This low-Na^+ solution was used as the control solution for the remainder of voltage-clamp experiments. Cells were held at a holding potential of −110 mV. I_{Na^+} was elicited using 20 ms step depolarizing pulses to test potentials between −100 and +40 mV (Figure 2A). Under these experimental conditions, Na^+ channels activated at about −85 mV, peak I_{Na^+} occurred at −40 mV and the reversal potential for I_{Na^+} was +6.7 mV, which is very near to the calculated Nernst potential of +5.8 mV for the solutions used at 18°C. The mean current voltage relationship, time to peak current, and inactivation time constant data for eight cells are given in Figure 2B–D. The results obtained are similar to data reported previously in human (Fozzard & Hanck, 1996) and canine (Hanck & Sheets, 1992) ventricular cells under similar experimental conditions.

Figure 3 shows the effects of primaquine on whole-cell Na^+ currents. I_{Na^+} was elicited by a series of 50 ms step depolarizations between −100 and +40 mV from a holding potential of −110 mV under control conditions (Figure 3A) and after exposure to 10 μM primaquine (Figure 3B). The raw currents show that primaquine blocked I_{Na^+} in both inward and outward directions equally. Figure 3C shows the average current-voltage relationship in seven cells under control conditions and after exposure of the same cells to 10 μM primaquine. The plot in Figure 3C demonstrates that primaquine blocked I_{Na^+} proportionally throughout the entire current-voltage relationship and had no effect on the reversal potential for Na^+. Figure 3D shows the dose-response curve for block of I_{Na^+} by primaquine. The pooled data were fitted a binding model of the form: $I_{Na^+} = 1/(1+([\text{primaquine}]/K_d)n)$, where K_d is the apparent binding constant, and n is the Hill number. The best fit for primaquine block yielded a K_d of 8.2 μM and an n of 1.62.

The effect of primaquine on the voltage dependence of steady state-inactivation of Na^+ channels (h_∞) and Na^+ conductance (g_{Na^+}) was examined in 12 cells. We employed a conventional double-pulse protocol to study h_∞, as shown in the inset to Figure 4A. A 1 s conditioning pulse was followed by a 2 ms return to a holding potential of −110 mV, followed by a 25 ms test pulse to −20 mV. Figure 4A illustrates a family of current traces obtained using this protocol under control conditions (centre) and following 10 μM primaquine (right). The pooled data in Figure 4B demonstrate that significant inactivation occurred at −50 mV under control conditions, with complete inactivation of channels at −90 mV. Despite blocking I_{Na^+} by over 50%, exposure of cells to 10 μM primaquine effected very little change in the level of steady-state inactivation of Na^+ channels. Exposure of myocytes to primaquine produced a small leftward shift of the steady-state inactivation curve, but

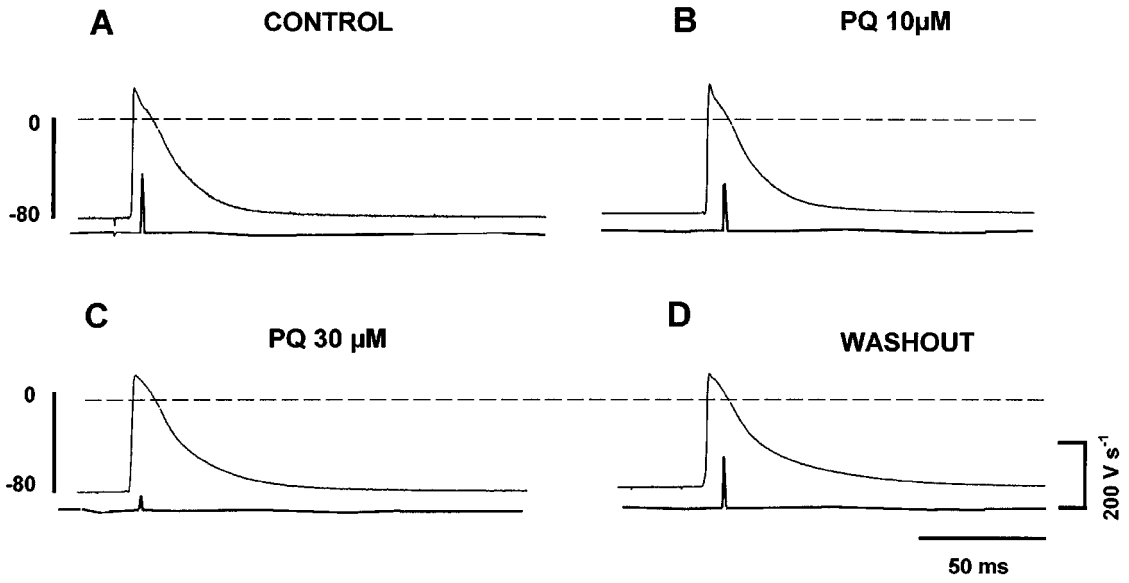

Figure 1 Effect of primaquine (PQ) on transmembrane action potentials recorded from a single papillary muscle. Action potentials are shown under control conditions (A), following superfusion with 10 μM (B) and 30 μM (C) primaquine and after washout of drug (D). The bottom trace in each panel is the derivative of the upstroke of the action potential (dV/dt). Dashed line in each recording represents 0 mV. Stimulation frequency was 1 Hz. Temperature was 37°C.

Table 1 Effect of primaquine on rat ventricular papillary muscle action potentials

	APA (mV)	*dV/dt%*	APD_{30}(ms)	APD_{90}(ms)	*RPM* (mV)
Control	110±4.0	100	8.1±2	45±8.0	−82.5±1
PQ 10 μM	104.1±3.0	62.9±1.0**	7.7±0.5	46±6.1	−84±2.2
PQ 30 μM	107.1±4.0	25.8±5.5***	7.9±1.0	48.5±6	−84±2.3
Washout	105.2±3.5	73.9±6.2	7.2±1.2	46±5.3	−84±3.2

APA, action potential amplitude; dV/dt, rate of change of membrane potential expressed as per cent of control; APD_{30}, action potential at 30% of total repolarization; APD_{90}, action potential duration at 90% of total repolarization; RPM, resting membrane potential; PQ, primaquine concentration. $^{**}P<0.01$, $^{***}P<0.001$; (n = 11).

this shift was not statistically significant. The solid lines in the h_∞ curves were calculated by normalizing the current recorded during the test pulse to their maximum value and plotting these data against the pre-pulse current levels. Normalized currents were well fitted by a Boltzmann function, as shown by the curves in Figure 4B. The averaged data indicate that control cells were half-inactivated ($V_{m_{1/2}}$ at −59.3±1.9 mV, with a slope factor (k) of 7.4±0.1 mV (n=12). The $V_{m_{1/2}}$ and k in the presence of primaquine were 65.9±0.5 and 8.5±0.1 mV (n=12).

Figure 4C shows the effect of primaquine on membrane Na^+ conductance. Data for g_{Na^+} were calculated from current-voltage relationship data obtained from the pre-pulses in Figure 4B. Data points were calculated using the equation: $g_{Na^+} = I_{Na^+}/(V_{m_{1/2}} - V_{rev})$, where g_{Na^+} is membrane Na^+ conductance, $V_{m_{1/2}}$ the half activation potential of the current, and V_{rev} the equilibrium potential for I_{Na^+}. For both controls and after primaquine treatment, the ratio was fitted to a Boltzmann function of the form: $g_{Na^+} = g_{Na^+\text{-max}}/1+\exp[(V_{m_{1/2}} - V_m/k]$, where $g_{Na^+\text{-max}}$ is the maximum inward conductance, $V_{m_{1/2}}$ is the half activation potential and k is the slope factor. Using this procedure, the mean (±s.e.mean) $V_{m_{1/2}}$ was −65.7±1.2 mV and k was 4.7±0.3 under control conditions and −61.2±5.6 and 6.2±1.3 mV following exposure to primaquine (n=12). The data from control cells and primaquine treated cells were not statistically different.

Figure 5 shows the effects of primaquine on the time course of activation and inactivation of I_{Na^+}. Time to peak current was measured from the deflection at zero current level to the point of maximum inward current. The raw data from a representative cell before and after exposure to 10 μM primaquine is given in Figure 5A,B. Cells were held at −110 mV and depolarized for 50 ms to test potentials between −50 and +30 mV. Stimulation frequency was 0.1 Hz. The dependence of time to peak I_{Na^+} on membrane potential under control conditions was curvilinear, similar to previous data reported for dog cardiac myocytes (Hanck & Sheets, 1992), and for sinoatrial node cells (Muramatsu *et al*., 1996). As shown in Figure 5C, superfusion of the same myocytes with primaquine resulted in a marked slowing of time to peak current at all test potentials negative to 0 mV (n=7).

The effect of primaquine on inactivation of Na^+ current is shown in Figure 5B,D. Inactivation of I_{Na^+} was measured by calculating the best fit to the decay phase of I_{Na^+} elicited by 50 ms test pulses from −50 to +10 mV. The decay phase of I_{Na^+} at each test pulse was well fitted to the sum of two

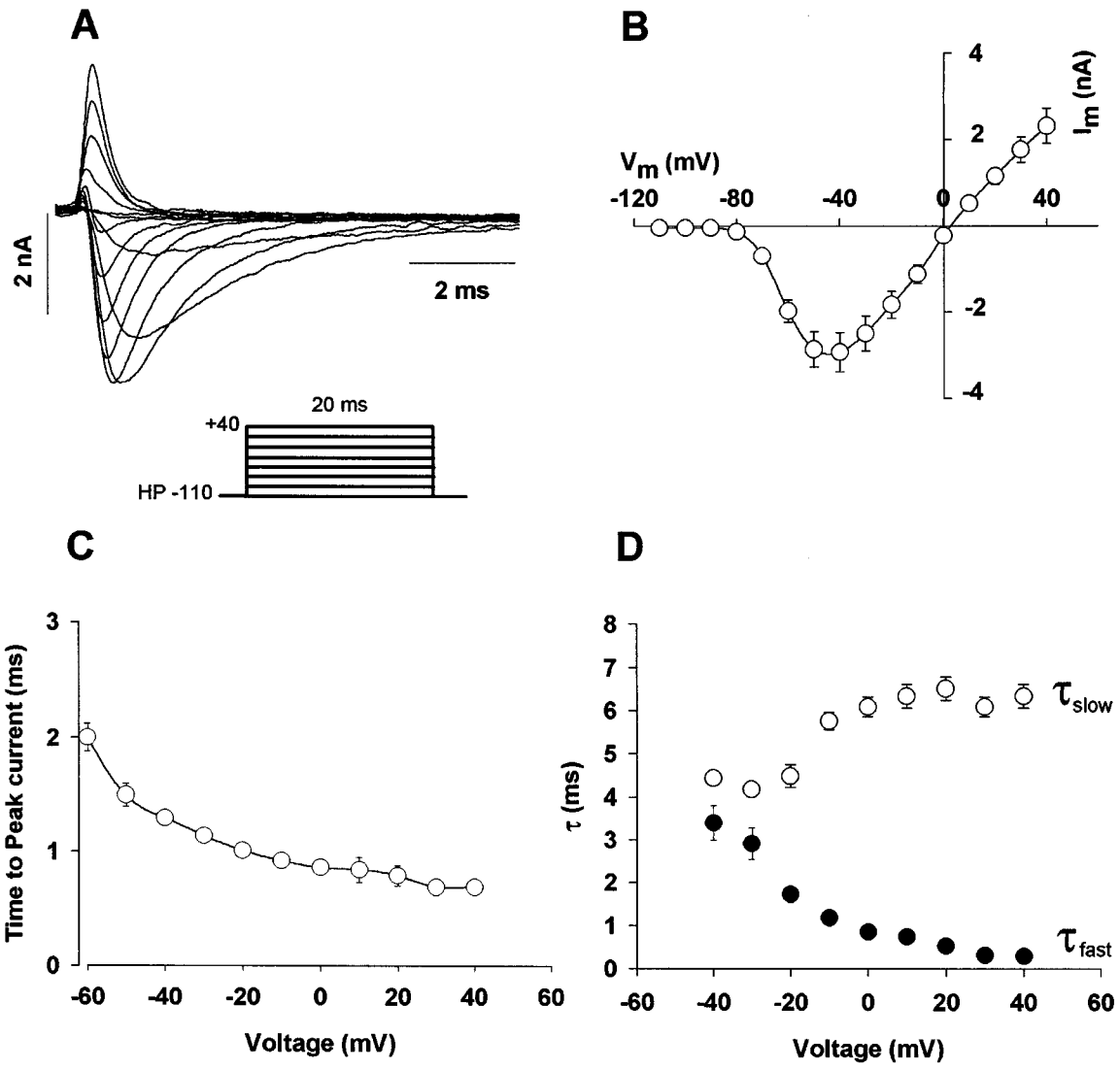

Figure 2 Whole-cell Na^+ currents (I_{Na^+}) from rat ventricular myocytes recorded in low-Na^+ Tyrode solution. (A) A family of currents elicited in a representative myocyte by step depolarizations from a holding potential of −110 to +40 mV in 10 mV increments. Data shown are for test potentials between −80 and +40 mV. Depolarizing test pulses were 20 ms duration and applied at a frequency of 0.1 Hz. Extracellular and intracellular Na^+ concentrations were 10 and 8.4 mM, respectively. Rs and Cm were 0.66 MΩ and 66 pF, yielding a membrane time constant (τ_m) of 44 μs. (B) Average current–voltage relationship under control conditions. Open circles represent mean (±s.e.) data from eight cells. (C,D) Average time to peak current and time constants for inactivation of I_{Na^+} during depolarizing pulses to the test voltages indicated. Data were obtained from the same cells as in (B). Mean (±s.e.) R_s, C_m, and τ_m were 1.2±0.1 MΩ, 63.3±4.2 pF, and 75.6±0.09 μs ($n=8$), respectively.

exponentials for all potentials tested. The decay phase of I_{Na}^+ at each test potential from −40 to +10 mV was well fitted to the sum of two exponentials (τ_f and τ_s). Our results indicate that inactivation of I_{Na}^+ was significantly slowed by primaquine, particularly the slow component of inactivation. Mean values of τ_f and τ_s at +10 mV were 1.6±0.3 and 5.5±0.9 ms under control conditions ($n=7$), and 1.8±1.3 and 10.6±1.9 ms, following exposure of myocytes to primaquine ($n=7$). Only the slowing of τ_s with primaquine treatment was statistically significant.

Figure 6 illustrates the time course of recovery from I_{Na}^+ inactivation in the absence and presence of 10 μM primaquine. In these experiments, I_{Na}^+ (I_c) was inactivated by a 500 ms conditioning pulse to −20 mV, and the membrane was returned to a holding potential of −110 mV for a variable time period (Δt) before eliciting a second current (I_t) with a test pulse to −20 mV in order to assay recovery of channels from inactivation after a 10 s pulse-free period at −110 mV. The time course of recovery from inactivation was determined by plotting the ratio of the amplitude of the I_{Na}^+ current during the second pulse (I_t) over the amplitude of control I_{Na}^+ during the first pulse (I_c) as function of Δt and fitting the resulting trajectories with double exponential functions.

Shown in Figure 6A is a family of current traces elicited by the double-pulse protocol under control conditions (left) and after exposure to 10 μM primaquine in the same cell (right). As demonstrated above, primaquine blocks I_{Na}^+ and rather slows the late stage of current inactivation. Figure 6B,C illustrate the mean values of the I_t/I_c ratio (fraction of the

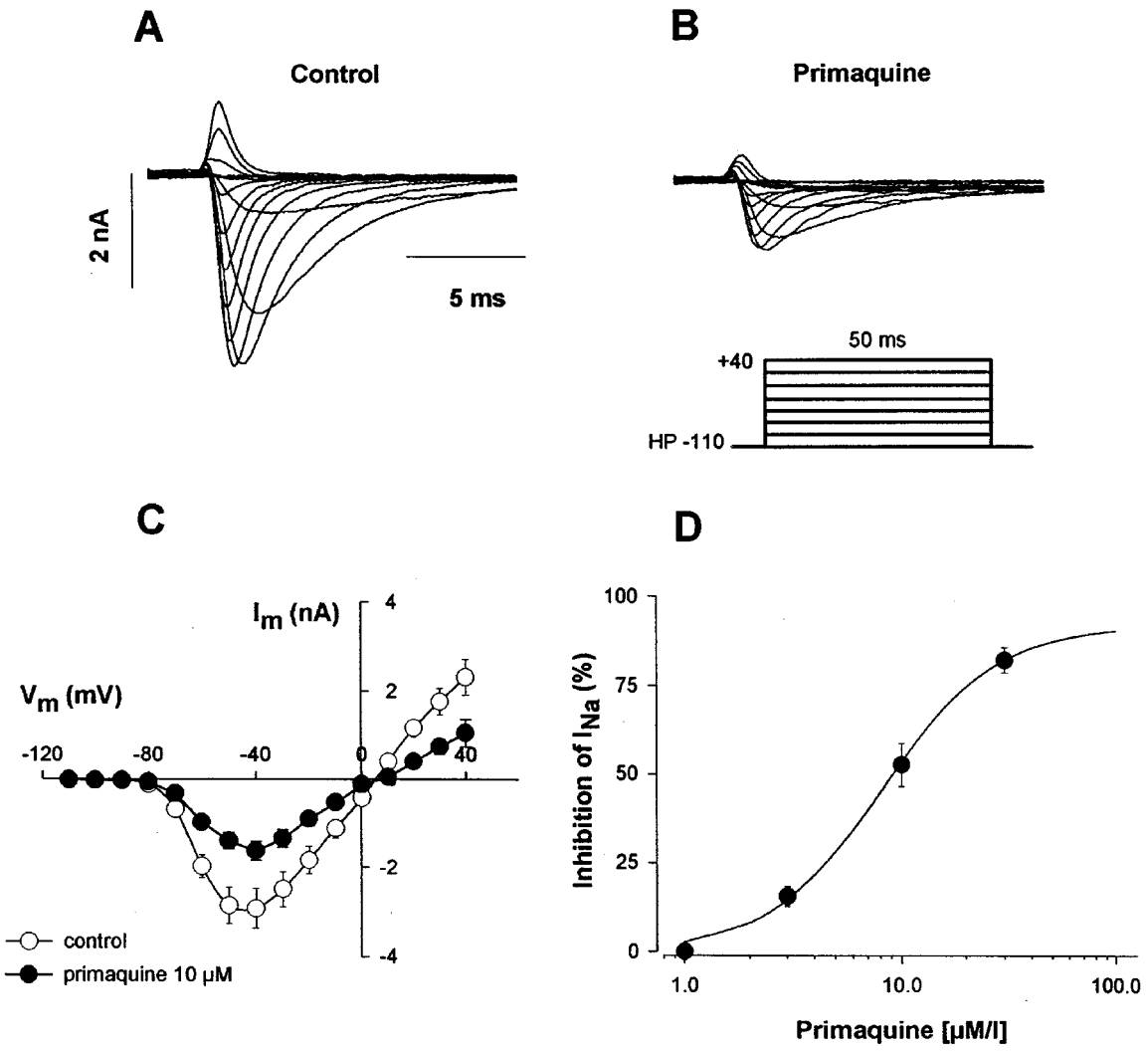

Figure 3 Effect of primaquine on I_{Na^+}. Current traces for 10 mV steps depolarizations from a holding potential of −110 mV under control conditions (A) and after the exposure of the same cell to 10 μM primaquine (B). (C) Mean (±s.e.) current–voltage relationships from seven myocytes before and after exposure to 10 μM primaquine ($n=7$). (D) Concentration-response curve shows the blocking effect of varying concentrations of primaquine on I_{Na^+}. I_{Na^+} was elicited by a series of 50 ms test pulses to −20 mV, from a holding potential of −110 mV. Stimulation frequency was 0.1 Hz.

channels recovered) in the absence and presence of primaquine during the recovery process. Both sets of data were well fit by double exponential functions, and the best fit functions are plotted as smooth curves. The parameters of the fits were $\tau_1=5.9$ ms and $\tau_2=122.4$ ms under control conditions and $\tau_1=12.8$ ms and $\tau_2=129.3$ ms in the presence of primaquine. The fast phase of recovery (τ_1) of I_{Na^+} after the conditioning depolarization is preferentially slowed by primaquine. These results suggest that drug-bound channels require more time to unblock after depolarization.

Previous work has demonstrated that the effectiveness of lidocaine in blocking voltage-dependent Na^+ channels is enhanced if the channels are repetitively activated by membrane depolarization (An *et al*., 1996; Salazar *et al*., 1996). Therefore, it was of interest to see if primaquine produce a similar use-dependent block of Na^+ channels in rat ventricular cells.

Use-dependent block by primaquine was addressed using trains of 10 ms pulses from a holding potential of −110 mV to a test potential of −20 mV at different frequencies. In each case stimulation was resumed after a short rest period of 1 min in the continued presence of 10 μM primaquine. Frequency-dependent block was examined by measuring the ratio of the last test pulse (P_{20}) to the first test pulse (P_1) as function of stimulation frequency. The current traces in Figure 7A, show representative use-dependent effects of 10 μM primaquine at 0.1 Hz (left), 1 Hz (centre) and 2 Hz (right) stimulation. The raw currents show that primaquine clearly blocks cardiac Na^+ channels in a use-dependent fashion. Figure 7B is a plot of the peak current values at various frequencies from the cell in Figure 7A.

Figure 7C shows pooled data from nine cells obtained under control conditions and after superfusion of 10 μM primaquine at a maximum blocking frequency of 2 Hz. Cells were stimulated continuously following a 1 min rest period with depolarizing pulses from a holding potential of −110 to −20 mV. The time course of block was well fitted by a

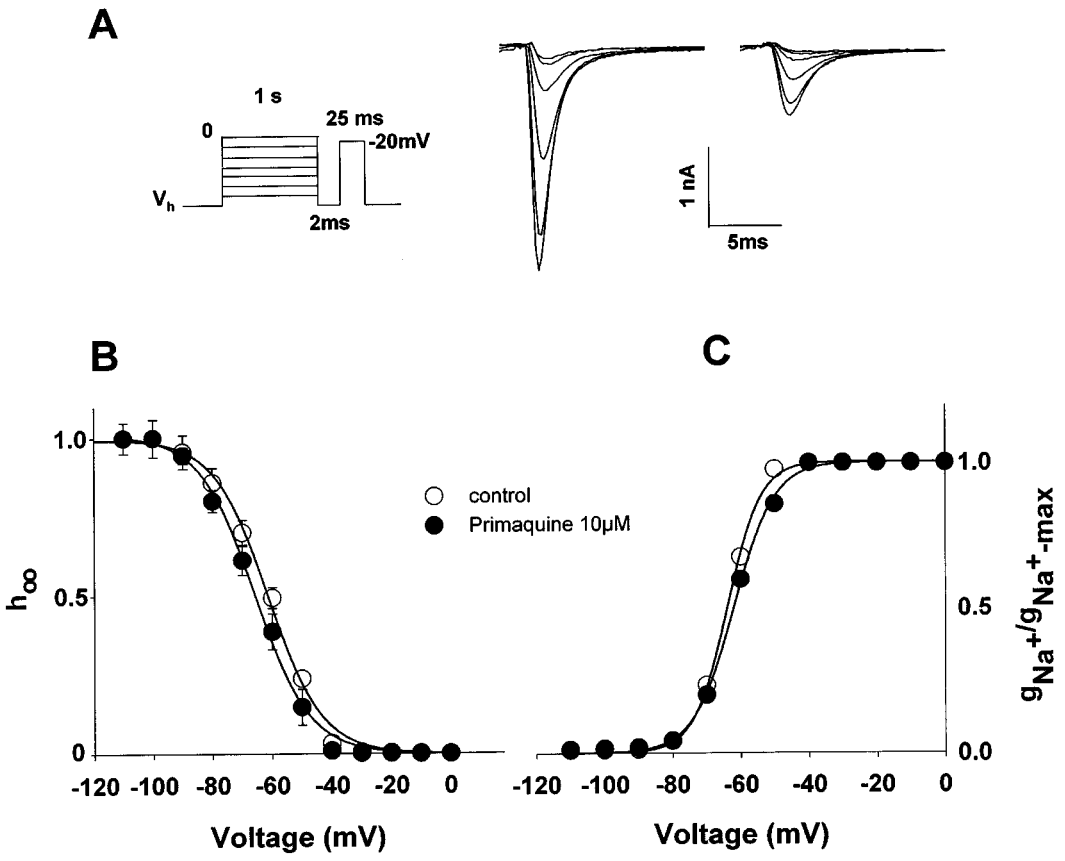

Figure 4 Effect of primaquine on the voltage dependence of steady-state inactivation (h_∞) and Na^+ conductance (g_{Na^+}). The h_∞ curve was obtained using the double pulse protocol shown in the inset to (A). A 1 s conditioning pulse to various test potentials was applied from a holding potential of −110 mV at a frequency of 0.1 Hz. The raw currents in the right of (A) are original current recordings obtained in the same myocyte under control conditions and after exposure to 10 μM primaquine. Normalized data are plotted in (B). Data under control conditions and following primaquine were plotted against membrane potential. Lines through the data are the best fit using a Boltzmann equation. (C) Normalized g_{Na^+}-voltage relationship expressed as a function of maximal of Na^+ conductance ($g_{Na^+\text{-max}}$) under control conditions ($n=12$) and in the presence of 10 μM primaquine ($n=6$). The fitted curves were calculated using a conventional Boltzmann equation.

monoexponential function, with a relatively fast time constant of block (τ_{on}) of 66 ± 0.1 ms.

The results reported thus far illustrate that primaquine blocks cardiac Na^+ channels with relatively high affinity and that the blocking effects of primaquine are enhanced with increased stimulation frequency, suggesting an open channel blocking mechanism. We also tested for the possibility of closed channel block using two stimulus trains at 1 Hz separated by a rest period during which cells were superfused with 10 μM primaquine (Choquet & Korn, 1992; Bouchard & Fedida, 1995). The voltage-clamp protocol consisted of a pair of depolarizing trains to −20 mV from a holding potential of −110 mV. The first was a 'control' train and the second was a 'test' train. The control and test trains were separated by a rest period of 2 min during which the cells were held at a holding potential of −110 mV and the superfusate was switched from the control solution to the primaquine solution. This allowed sufficient time for the channels to recover from inactivation and allowed the opportunity for primaquine binding to closed channels. During the rest period input resistance was monitored continually. Cells that evidenced a change in seal resistance or compensated series resistance of >10% from the final control pulse (P_{20}) to the first test pulse (P_1) in primaquine were discarded.

In the absence of primaquine, the amplitude of peak I_{Na^+} after the 2 min rest period remained unchanged compared to the final I_{Na^+} prior to termination of stimulation. However, when stimulation was resumed after a 2 min rest period in the presence of primaquine, all, or substantially all, of the blocking effect occurred with successive pulses in a use-dependent fashion, consistent with the results in Figure 7. In addition, there was a very small but measurable decrease in the current amplitude of the first I_{Na^+} elicited after rest (4.8%). It was not possible to determine if this was a small component of closed channel block, or resulted from small and difficult to measure changes in series resistance during the 2 min rest period. These results demonstrate that the majority, if not all, of the blocking effect of primaquine on I_{Na^+} is a result of open channel block.

Discussion

In the present study, we used both a conventional microelectrode technique in multicellular preparations and

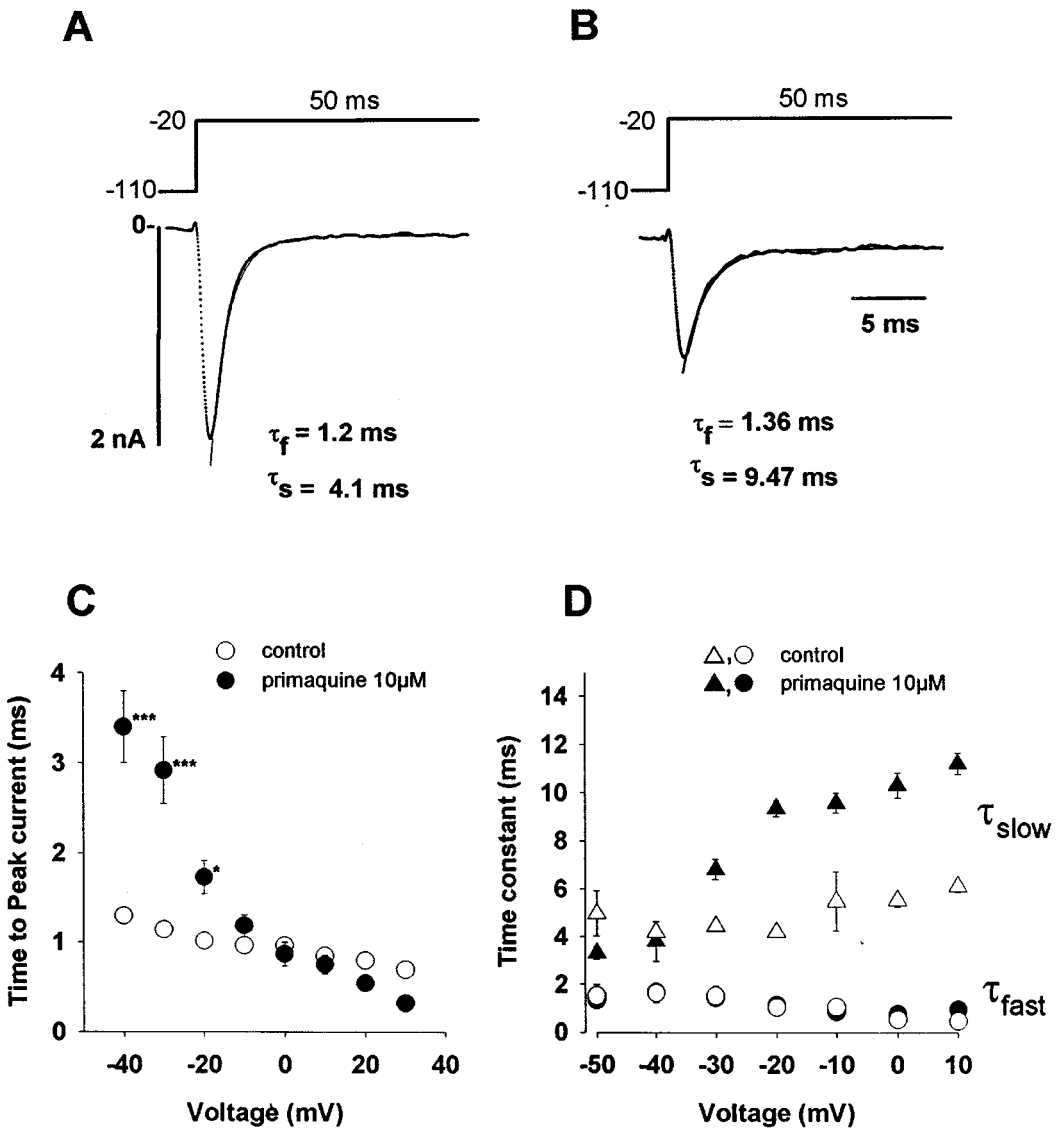

Figure 5 Effect of primaquine on I_{Na}^+ kinetics. (A,B) Representative I_{Na}^+ elicited by a depolarizing pulse to −20 mV from a holding potential of −110 mV under control conditions (A) and after exposure to 10 μM primaquine (B). Stimulation frequency was 0.1 Hz. Inactivation of I_{Na}^+ was well fitted by a double exponential function. The solid lines through the decay phase of I_{Na}^+ represent the fitted curves. (C) Dependence of time to peak I_{Na}^+ under control conditions and following superfusion with 10 μM primaquine (n=7). (D) Plot showing the mean values (±s.e.) for time constants of inactivation of I_{Na}^+ as a function of test potential. Each point represents the mean of seven experiments. Standard error bars are not visible when the amplitude of the standard error is smaller than the symbol size.

the whole-cell configuration of the patch clamp technique to characterize the electrophysiological effects of primaquine, an 8-aminoquinoline drug, on the Na^+ current in rat ventricular myocytes. This is the first biophysical report which provides evidence that antimalarial drugs, like primaquine, may produce a deleterious effect on cardiac excitability by blocking cardiac Na^+ channels.

The present results show that: (1) primaquine blocks whole-cell I_{Na}^+ with a K_d of 8.2 μM (Figure 3), and a relatively fast time constant of block of 66 ms (Figure 7). This is reflected by a dose-dependent decrease in dV/dt in whole muscle studies (Figure 1); (2) primaquine slowed the time-to-peak current and preferentially depressed the slow component of I_{Na}^+ inactivation (Figure 5); (3) primaquine produced very little change in the steady-state activation and inactivation curves (Figure 4); (4) the fast time constant for recovery from inactivation was depressed by primaquine, with less change in the slow time constant of recovery (Figure 6); (5) primaquine blocks I_{Na}^+ in a use- and frequency-dependent manner (Figure 7); and (6) there was no appreciable closed channel block of I_{Na}^+ by primaquine (Figure 8).

Block of I_{Na}^+ by primaquine

Figure 3 shows that primaquine blocks cardiac Na^+ channels, and this block is reflected in whole muscle studies as a concentration-dependent decrease of dV/dt during the upstroke of the action potential (Figure 1, Table 1). The observed blocking effects of primaquine share some of the

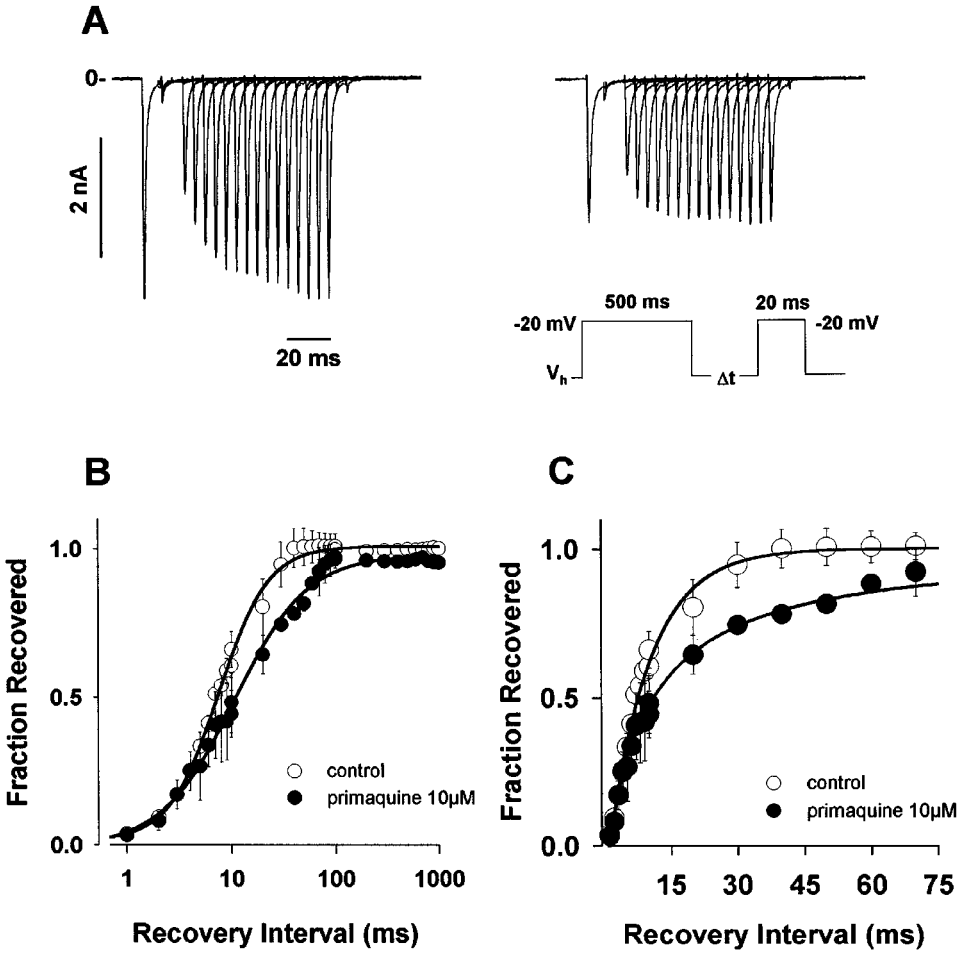

Figure 6 Effects of primaquine on the time course of recovery from inactivation of I_{Na}^+. Recovery from inactivation was measured using a conventional double UU pulse protocol. The raw currents in (A) show superimposed currents recorded under control conditions (left) and following exposure of the cell to 10 μM primaquine (right). Paired pulses were applied from a holding potential of -110 mV. Stimulation frequency was 0.1 Hz. (B) Mean ($\pm$s.e.mean) peak current levels during the test pulse normalized to peak current during conditioning pulse plotted against the recovery interval. The best fits of the data to a double exponential function are also shown. The time constants under control conditions were $\tau_1 = 5.9$ ms and $\tau_2 = 129.37$ ms ($n = 7$) and following exposure to primaquine were $\tau_1 = 12.85$ ms and $\tau_2 = 224$ ms ($n = 7$). (C) Expanded time scale showing the first 75 ms of recovery on a semilogarithmic scale.

same actions on cardiac Na^+ channels as those reported for other structural aminoquinolines. For example, quinidine, an open channel blocker of the transient outward K^+ current (I_{to}) at low concentrations ($K_d = 19.4$ μM; Clark *et al*., 1995), has also been demonstrated to block I_{Na}^+ in guinea-pig ventricular cells with a K_d of 50 μM (Snyders & Hondeghem, 1990). In our experiments (data not shown), we did not find any significant effect of primaquine at concentrations up to 30 μM on I_{to} or the inward rectifying K^+ current (I_{K1}). In earlier work (Cebada & Salinas, 1994) we found, however, that chloroquine (10 μM), another aminoquinoline, blocks a number of cardiac K^+ currents, including I_{to}, I_{K1}, and the delayed rectifier K^+ data current (I_K), as well as blocking I_{Na}^+. Therefore, unlike reported for other aminoquinolines, the blocking effects of primaquine in cardiac muscle appear to be relatively specific to Na^+ channels.

In six cells (data not shown) we found that no block of I_{Na}^+ occurred when 10 μM primaquine was included in the pipette solution. Block of I_{Na}^+ occurred only when primaquine was added to the extracellular solution, suggesting that primaquine blocks cardiac Na^+ channels from the extracellular side of the membrane. The fractional block by primaquine of cardiac Na^+ channels can be described by the Woodhull equation (Woodhull, 1973): $f = [D]/([D] + K_d^{-\delta FVm/RT})$, where [D] is the drug concentration, K_d is the apparent binding constant (at -20 mV), and δ is the equivalent electrical distance, i.e., the fraction of the transmembrane potential sensed by a single charge at its binding site in the channel. This equation assumes that a single primaquine molecule is required to block the channel, and these molecule is charged.

The fractional electrical distance for primaquine block of Na^+ channels under our experimental conditions derived from the ratios of the blocked and unblocked current over a wide voltage range, was 0.22, suggesting that one primaquine molecule enters the channel from a extracellular side and travels approximately 22% of the transmembrane field to reach its binding site. This result is similar to previous work demonstrating that quinidine and lidocaine, other cardiac Na^+ channel blockers that share similar blocking

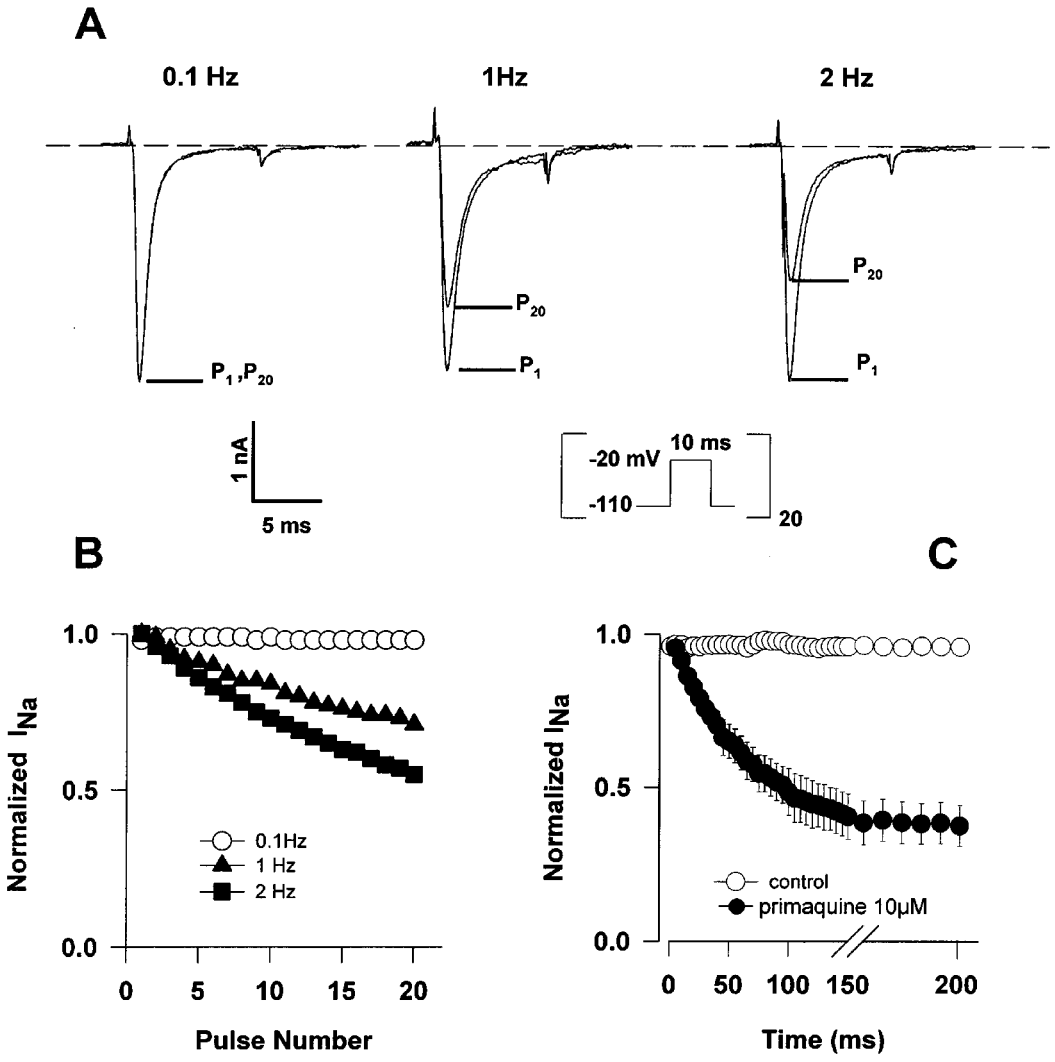

Figure 7 Use-dependent block of I_{Na}^+ by primaquine. (A) Superimposed I_{Na}^+ traces elicited by stimulus train of 20 pulses to -20 mV from a holding potential of -110 mV in the presence of 10 μM primaquine at different frequencies (0.1, 1, and 2 Hz). Only the first (P_1) and the last (P_{20}) currents elicited are shown to preserve clarity of the traces. (B) Time course and degree of block of I_{Na}^+ achieved during resumption of stimulation after a rest of 1 min in the continued presence of drug at stimulation frequencies of 0.1, 1 and 2 Hz. The average ratio of currents (taken from the same cell as in (A)), during the first 20 pulses is plotted as a function of pulse number. (C) Mean ($\pm$s.e.) time course of block of I_{Na}^+ in nine cells at a maximal blocking frequency of 2 Hz. The decay phase was well fitted by a single exponential, with a time constant for block of I_{Na}^+ of 66 ± 0.01 ms.

characteristics to primaquine, bind 19% (Yeola *et al*., 1996) and 22% (Ragsdale *et al*., 1994) of the way into the transmembrane field from the extracellular side of the membrane. Further experiments will therefore be needed to determine the precise blocking site of primaquine and how many primaquine molecules are necessary to block cardiac Na^+ channels.

Figure 4C illustrates that primaquine has no effect on the voltage-dependence of Na^+ conductance when compared to the conductance of unblocked channels. These results are consistent with the lack of effect of primaquine on the shape of the current-voltage relationship (Figure 3C) or the steady state activation (Figure 4B) and g_{Na}^+ (Figure 4C) curves suggesting that there is no or very little voltage-dependence of block of cardiac Na^+ channels by primaquine.

Effect of primaquine on I_{Na}^+ kinetics

It is known that cardiac Na^+ channels pass through several inactivated states during repolarization (Stühmer *et al*., 1989; Nuss & Marban, 1999), and it is possible that primaquine could influence each of these state transitions. Figure 5 shows that primaquine has a selective effect on the late phase of I_{Na}^+ inactivation slowing τ_s to almost twice the control values while τ_f remained essentially unchanged. In addition, the difference in τ_s values following exposure of myocytes to primaquine was slightly voltage-dependent (Figure 5D) while the effect of primaquine on τ_f was comparatively small. These results are consistent with the effect of primaquine on the recovery of Na^+ channels from inactivation (Figure 6), where primaquine slowed the fast component of recovery from inactivation to twice the

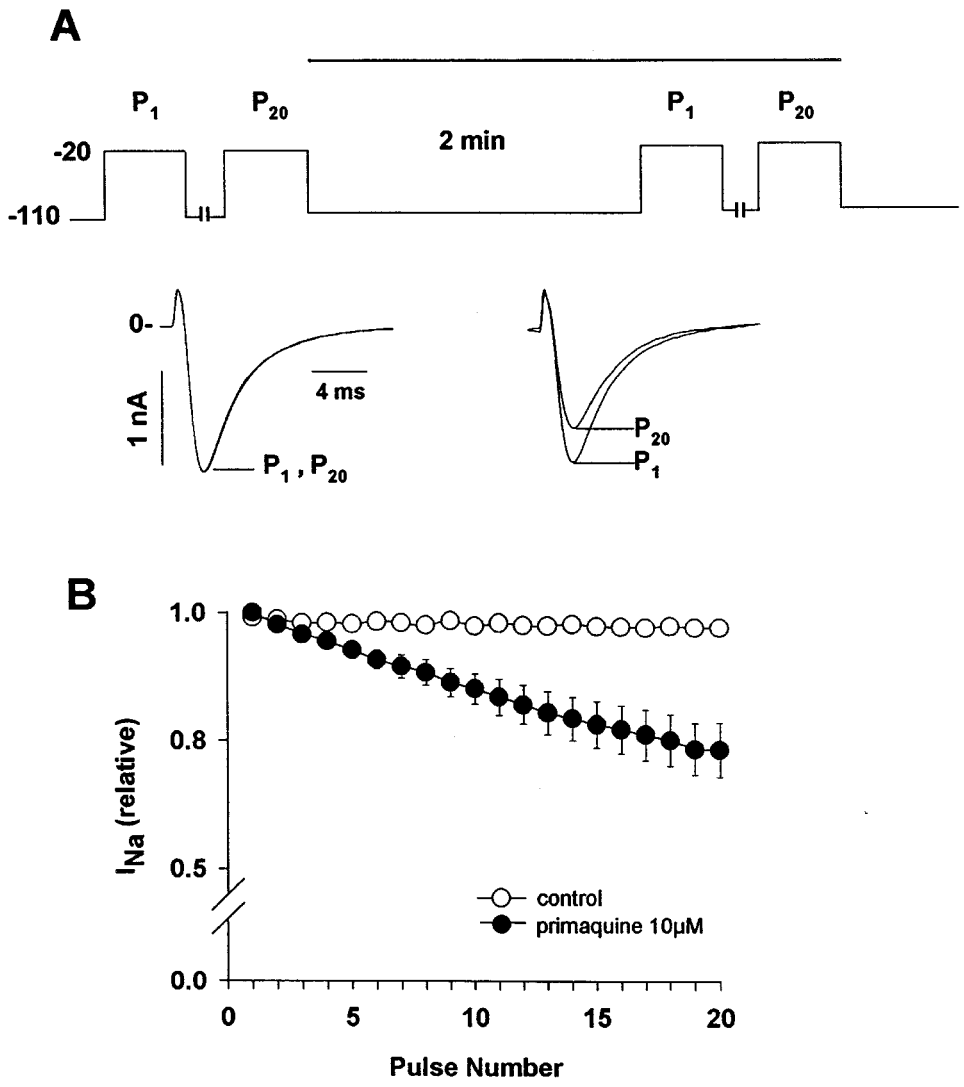

Figure 8 Test for closed channel block of I_{Na}^+ by primaquine. (A) Superimposition of currents recorded during a train of 20 pulses from a holding potential of −110 mV to a test potential of −20 mV in the absence and presence of primaquine (10 μM, bar). Cells were stimulated at 1 Hz under control conditions, following which stimulation was temporarily halted and the superfusate was immediately changed to a primaquine-containing solution. After a 2 min rest period stimulation was resumed in the absence (left) or presence (right) of primaquine. For simplicity, only the 1st and 20th currents are shown. (B) Mean (±s.e.) data from five cells. The current amplitudes for I_{Na}^+ in each pulse in the train have been normalized to peak I_{Na}^+ from first pulse after rest. No significant change in current amplitude from P_1 to P_{20} was observed under on resumption of stimulation in the presence of 10 μM primaquine. The maximum current decreased in a use-dependent fashion during the stimulus train.

control value while having substantially less effect on the slow component of recovery.

The data in Figures 5 and 6 suggest that primaquine slows the movement of cardiac Na^+ channels between inactivated states, most likely inactivated states subsequent to the initial inactivated state. In Figure 5, the depolarizing pulse was short (50 ms) compared to the interpulse interval of 10 s allowing for full recovery from inactivation (Figure 6), with a very fast activation and inactivation process, i.e., a mean time to peak current of 1 ms and a fast time constant of inactivation of 1 ms at test potentials positive to −20 mV. That the depolarizing pulse was relatively short in comparison to the interpulse interval, also allowed a slow time constant of inactivation of 4–6 ms to develop. This can be compared to the recovery protocol in Figure 6, where channels were inactivated using a relatively long depolarizing pulse of 500 ms. This longer pulse allowed the development of a further inactivated state, with a time constant of 122 ms under control conditions (129 ms with primaquine). The preferential suppression of the slow phase of inactivation in Figure 5 combined with the preferential decrease of the fast phase of recovery from inactivation of I_{Na}^+ in Figure 6 may reflect a comparatively slow transition of Na^+ channels between early and late inactivating states (i.e. I_2 and I_5) in the presence of primaquine compared to under control conditions.

Finally, superfusion of myocytes with primaquine resulted in a voltage-dependent increase in time to peak I_{Na}^+ at test potentials negative to 0 mV. This result could be due to a delay in (i) latency to first channel opening, (ii) development

of inactivation or (iii) movement of channels between open and closed states. In the absence of single channel data, it is not possible to offer a reasonable explanation for this result other than to say that the movement of Na^+ channels from the open state through multiple inactivating states is slowed by primaquine.

Mechanism of block of $I_{Na}{}^+$ by primaquine

Figure 7 demonstrates that block of cardiac Na^+ channels by primaquine occurs in a strongly use-dependent fashion. Resumption of stimulation in the continued presence of primaquine at 0.1 Hz stimulation produces a stable level of block during the entire stimulus train. This reflects a steady-state level of block due to recovery of channels during the long 10 s interpulse interval. However, when the interpulse interval is shortened such that there is insufficient time for recovery from inactivation, the degree of block is strongly use-dependent (Figure 7B). These results are consistent with previous data obtained using other blockers of cardiac Na^+ channels (Ragsdale *et al*., 1994; Snyders & Yeola, 1995; Yeola *et al*., 1996; Dumaine & Hartmann, 1996).

We also conducted experiments in order to determine whether primaquine was able to block closed Na^+ channels. This was examined using a post-rest stimulation protocol previously described for 4-aminopyridine block of K^+ channels in lymphocytes (Choquet & Korn, 1992) and human Kv1.5 channels (Bouchard & Fedida, 1995). Figure 8 illustrates that resumption of stimulation after a 2 min rest under control conditions, results in no change in peak $I_{Na}{}^+$ from the last pulse before rest to the first pulse after rest. The peak current level remains constant throughout both stimulus strains. The result was different when myocytes were superfused with primaquine during the 2 min rest period. In the presence of primaquine, peak $I_{Na}{}^+$ on first pulse after rest was unchanged from the final control pulse before termination of stimulation, suggesting there was no development of closed channel block during the rest period. Block of $I_{Na}{}^+$ developed only with continued stimulation (Figure 8B), consistent with the use-dependent block $I_{Na}{}^+$ data in Figure 7. Together these results imply that block of cardiac Na^+ channels by primaquine is due almost entirely to binding of primaquine to open channels, similar to previous results obtained with Class I antiarrhythmic drugs (Salata & Wasserstrom, 1988; Yeola *et al*., 1996; Sanchez-Chapula, 1996).

In summary, the results of this study indicate that primaquine blocks cardiac Na^+ channels with relatively high affinity and is selective for Na^+ channel block over cardiac K^+ channels. Most, if not all, of the blocking effects of primaquine are due to open channel block. It is possible that the depression of dV/dt during the cardiac action potential may give rise to some of the electrical and mechanical disturbances observed in patients undergoing malaria therapy with aminoquinalines.

The authors thank Dr Robert Clark and Dr Julio Alvarez for helpful comments. This work was supported by a grant from CONACyT 26538/N.

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(Received July 6, 2001
Revised September 19, 2001
Accepted October 26, 2001)