Blockade of Na⁺ current by promethazine in guinea-pig ventricular myocytes

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- 1 To elucidate the antiarrhythmic mechanism of promethazine, its effects on the fast Na⁺ current (I_{Na}) were examined in single guinea-pig ventricular myocytes by whole-cell voltage clamp methods.
- 2 Promethazine blocked I_{Na} with a K_D of 42.6 μ M and Hill's coefficient of 1.1 at a holding potential of –140 mV.
- 3 The I_{Na} blockade was enhanced at a less negative holding potential of –80 mV with a change of K_D to 4.4 μ M. Although 10 μ M promethazine did not change the inactivation time constants of I_{Na} , it shifted the steady-state inactivation curve (h_{∞} curve) toward more negative potentials by 19.5 mV with the slope factor unaffected.
- 4 Double pulse experiments revealed that the development of blockade followed two-exponential functions having time constants of 7 and 220 ms at –20 mV.
- 5 Promethazine slowed the repriming of I_{Na} . This was associated with the development of slow phase having a time constant of 1160 ± 59 ms.
- 6 Promethazine produced a profound use-dependent block when the cell was repeatedly stimulated with interpulse intervals shorter than 1 s. However, short pulses of 2 ms duration hardly produced such a use-dependent block. Hence, open channel blockade is considered to play a minor role in the promethazine action on I_{Na} .
- 7 These results suggest that promethazine blocks cardiac I_{Na} in a manner similar to class I antiarrhythmic drugs and that this effect may account for its antiarrhythmic action.

Keywords: Promethazine; voltage-clamp; Na⁺ current; single ventricular myocyte

Introduction

Promethazine, an antihistaminic phenothiazine being widely used for the treatment of allergy, is considered as a competitive antagonist of H₁ histamine receptors (Reinhardt & Borchard, 1982). In addition, this agent has been shown to modify the electrical activity in cardiac tissues. For example, it causes bradycardia and prolongation of the atrioventricular conduction time (Aronson & Hanno, 1979) in perfused rabbit hearts, and exerts a negative chronotropic action on guinea-pig atria (Davis & McNeill, 1973; Hughes & Coret, 1974). Furthermore, promethazine is reported to have anti-tachyarrhythmic effects under certain experimental conditions in atrial (Gupta *et al.*, 1974) and ventricular tissues (Johnson, 1954; Malinow *et al.*, 1955; Rigel & Katona, 1986; Rochette *et al.*, 1988). Some researchers have deduced that these actions are ascribed to a possible 'quinidine-like' local anaesthetic action (Naranjo & de Naranjo, 1958; Giotti & Zilletti, 1981), and not to an H₁ receptor-mediated action (Giotti & Zilletti, 1981; Rigel & Katona, 1986). Indeed, promethazine was reported to suppress the action potential upstroke in skeletal muscle fibres without changing the resting membrane potential, a finding suggesting an inhibitory action on the fast Na⁺ current (I_{Na}) (Buttar & Frank, 1976). In cardiac tissues, however, little is known about its effects on I_{Na} . Hence, in a series of experiments on single guinea-pig ventricular myocytes, we measured I_{Na} directly by the use of whole-cell voltage clamp techniques to clarify promethazine-induced changes in the Na⁺ channel properties.

Methods

The methods for cell isolation and voltage clamp experiments were identical to those previously described by Habuchi *et al.* (1991). Briefly, the excised guinea-pig hearts were perfused on a Langendorff apparatus for 5 min with a nominally Ca²⁺-free Tyrode solution having the following composition (in mM): NaCl 142, MgCl₂ 5, KCl 5.4, NaH₂PO₄ 1, HEPES 5, glucose 20 and 0.2% bovine serum albumin (Sigma, fraction V). The pH was adjusted to 7.4 with NaOH. The perfusate was then changed to an enzyme solution containing 0.3 mg ml^{–1} collagenase, 5 mM MgCl₂ and 1% bovine albumin in the Ca-free Tyrode solution. These perfusates were continuously bubbled with 100% O₂ and the temperature was kept at $36 \pm 1^\circ\text{C}$. Enzymatic treatment for 30–40 min was followed by perfusion with a recovery solution with the following composition (in mM): glutamate 110, oxalate 10, KCl 25, KH₂PO₄ 10, MgSO₄ 2, taurine 20, creatine 5, glucose 20, EGTA 0.5 and HEPES 5 (pH = 7.2 with KOH). The ventricles were subsequently cut into small pieces. The myocytes isolated by gentle agitation were filtered through nylon mesh (200 μ m in diameter) and collected by centrifugation. After incubation for 30–40 min in the recovery solution at 32°C, Ca²⁺-tolerant, rod shape myocytes were obtained, and stocked in a Tyrode solution containing 1.8 mM CaCl₂ and 1% albumin.

Voltage-clamp experiments were carried out by the single-pipette whole-cell clamp technique (Hamill *et al.*, 1981). Suction pipettes were made with a two-stage vertical puller (Narishige, Tokyo, Japan), and electrodes having a tip resistance of 0.8–1.5 M Ω were used for the experiment. The internal solution had the following composition (in mM):

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NaCl 15, CsF 110, CsCl 20, MgCl₂ 2, EGTA-Cs₂ 5 and HEPES 5 (pH = 7.2). I_{Na} was measured during superfusion with low-Na⁺ test solution with the following composition (in mM): NaCl 18, CsCl 30, CoCl₂ 3, MgCl₂ 1, tetramethylammonium chloride 20, 4-aminopyridine 5, sucrose 80, glucose 5 and HEPES 5 (pH = 7.4 with CsOH). The liquid junction potential between internal and external solutions (3 mV) was corrected. The series resistance was compensated to a point just before oscillation. A small bath size of 0.15 ml was used and changing of the perfusate was accomplished within 1 min. All the experiments were carried out at room temperature (20–22°C). Promethazine hydrochloride (Sigma, St. Louis, MO, U.S.A.) was stored at a 10⁻² M concentration.

A voltage-clamp amplifier (ACT ME, 1100T, Japan) designed by M. Yoshii (Narahashi *et al.*, 1987) was used. Current signals were filtered at 10 kHz and stored in a digital cassette recorder (TEAC RD101-T, Japan) or floppy disks attached to a digital oscilloscope (Nicolet 310C). These data were analyzed by a computer (NEC PC 9800, Japan). The linear leakage and capacitive currents were subtracted digitally by P-P/n method ($n = 2-5$; Bezanilla & Armstrong, 1977), and I_{Na} amplitude was measured from 0 current level to the peak inward current. When I_{Na} exceeded 4 nA under these experimental conditions, voltage errors through the clamp circuit was considered to be within acceptable limits (Habuchi *et al.*, 1991). Fit of the experimental data to exponential equations was performed with a non-linear simplex regression algorithm (Nelder & Mead, 1965; Shavers *et al.*, 1979; Turbo Pascal programme, Borland International Inc.). Statistical analyses were made by Student's paired *t* test and the data are expressed as mean $\pm$ s.d.

Figure 1 Effect of promethazine on the current-voltage relationship for I_{Na} : 20 ms depolarizing pulses were applied from a holding potential (HP) of -140 mV to various potentials ranging from -80 mV to $+30$ mV. Test pulses were delivered at a rate of 0.1 Hz. Panel (a) shows families of I_{Na} records before (left) and during (right) superfusion with promethazine $30 \mu\text{M}$. In (b), the peak amplitudes of I_{Na} are plotted against the test potentials. Note that promethazine depressed the current amplitudes without affecting the overall configuration of the current-voltage relationship: (○) control; (●) in the presence of promethazine.

Figure 2 Concentration-dependent effects of promethazine on I_{Na} : (a) shows I_{Na} records obtained by applying 20 ms depolarizing pulses to -20 mV from a HP of -140 mV (upper traces) and -80 mV (lower traces). The numbers along the current traces indicate the drug concentrations in μM , and C denotes the control record. WO denotes the current after washout of the drug. In (b) are shown the concentration-response curves obtained at -140 mV (○) and -80 mV (Δ). Relative values of I_{Na} normalized by taking the control values as unity are plotted against the drug concentrations. The symbols with vertical bars represent the mean $\pm$ s.d. based on 6 (HP = -140 mV) and 4 (HP = -80 mV) experiments.

Results

Steady-state block of Na⁺ channel by promethazine

Figure 1 shows typical promethazine effects on the current-voltage relationship for I_{Na} obtained with a holding potential (HP) of -140 mV. The cell was depolarized for 20 ms to various potentials ranging from -80 mV to $+30$ mV. Under control conditions, I_{Na} developed at potentials positive to -70 mV, attained the maximum value at -25 mV, and reversed its polarity at $+13$ mV. When promethazine was applied at a concentration of $30 \mu\text{M}$, the I_{Na} was reduced by approximately 30% without significant changes in the shape of current-voltage curve.

The concentration-dependent effects of promethazine on I_{Na} are shown in Figure 2. The left upper panel in Figure 2a shows a family of I_{Na} curves obtained with a HP of -140 mV where Na⁺ channels are free from inactivation. Promethazine ($10-100 \mu\text{M}$) decreased the I_{Na} amplitude in a dose-dependent manner. When the HP was decreased to -80 mV, where a fraction of the channels would be in the inactivated state, the block was enhanced (the left lower panel in Figure 2a). Washing out of the drug restored the I_{Na} almost to the control level at either HP (right panels in Figure 2a). In Figure 2b, I_{Na} values relative to the control are

Figure 3 Steady-state inactivation curves for I_{Na} before (○) and during superfusion with $10 \mu\text{M}$ promethazine (●). As illustrated in the top panel of (a), the application of 5 s conditioning pulses (P_1) to various potentials was followed by a 2 ms interpulse interval and a 20 ms test pulse (P_2) to -20 mV . Families of I_{Na} (I_2) elicited on P_2 are shown in the middle (control) and the lower (promethazine) panels of (a), with the numbers representing the conditioning pulse voltage (P_1). In panel (b), the I_2 amplitudes are plotted against the P_1 potentials. Promethazine at $10 \mu\text{M}$ depressed I_2 at all the potentials tested. In (c), the steady-state inactivation (h_∞) curves for I_{Na} were obtained by normalizing the I_2 amplitudes to the maximal value in each condition.

plotted against the drug concentration. These relationships obtained from 4 (HP = -80 mV) and 6 (HP = -140 mV) experiments were well fitted by the Hill's equation described below:

$$\text{Relative } I_{Na} = 1/[1 + (D/K_D)^n],$$

where D denotes the concentration of promethazine (μM), K_D , the concentration producing the half maximal response, and n , the Hill's coefficient. K_D for the resting state of Na^+

Figure 4 Effect of promethazine on the time course of I_{Na} inactivation in a typical experiment. I_{Na} was elicited by depolarization from the HP of -140 mV to -20 mV .

Figure 5 A typical example of the time course of promethazine binding to Na^+ channels. In (a), P_1 pulses of various durations were applied to -20 mV from the HP of -140 mV . After a 250 ms interval at -140 mV during which drug-unbound channels were allowed to reprimed fully, a 20 ms test pulse (P_2) was applied again to -20 mV . The lower panels show the current (I_1) elicited on P_1 (left) and that (I_2) elicited on P_2 (right) during superfusion with $10 \mu\text{M}$ promethazine. In (b), the relative values of I_{Na} (I_2/I_1) are plotted against the duration of P_1 (in ms): (○) and (●) denote the values before and during the application of promethazine, respectively.

channels was $42.6 \mu\text{M}$ and n , 1.09. At HP of -80 mV , the curve was shifted toward lower concentrations ($K_D = 4.4 \mu\text{M}$, $n = 1.14$).

Effect of promethazine on the kinetics of Na⁺ channel inactivation

The effect of promethazine on the steady-state inactivation (h_∞) curve for I_{Na} was studied by use of the double-pulse protocol. Results from a typical experiment are shown in Figure 3. The h_∞ curves were obtained by normalizing the test current amplitudes (I_2) by taking the maximum value under each condition as unity. Curves were fitted by the equation:

$$h_\infty = 1/[1 + \exp((E - V_h)/s)],$$

where E denotes the P_1 potential, V_h , the potential showing $h_\infty = 0.5$, and s the slope factor. Promethazine $10 \mu\text{M}$ shifted the curve to more negative potentials by 18.1 mV without affecting the slope factor. On average, $V_h = -80.5 \pm 3.6 \text{ mV}$ and $s = -6.2 \pm 0.37 \text{ mV}$ under control conditions, and $V_h = -100.0 \pm 5.7 \text{ mV}$ and $s = -5.8 \pm 0.48 \text{ mV}$ in the presence of $10 \mu\text{M}$ of promethazine, respectively ($n = 5$, $P < 0.01$ for V_h and $P > 0.1$ for s).

Promethazine did not significantly modify inactivation kinetics (Figure 4). The I_{Na} elicited on depolarization to -20 mV was fitted by double exponential functions by means of a non-linear simplex regression algorithm (Nelder & Mead, 1965; Shavers *et al.*, 1979) according to the following equation:

$$I_{Na} = A \times \exp(-t/\tau_{\text{fast}}) + B \times \exp(-t/\tau_{\text{slow}}) + C,$$

The I_{Na} decay under the control condition ($n = 5$) showed two time constants of $\tau_{\text{fast}} = 1.00 \pm 0.12 \text{ ms}$ and $\tau_{\text{slow}} = 3.33 \pm 0.33 \text{ ms}$. Comparative values in the presence of $100 \mu\text{M}$ promethazine were $0.98 \pm 0.06 \text{ ms}$ and $3.13 \pm 0.27 \text{ ms}$, respectively.

Time course of the development of I_{Na} block by promethazine

The time course of the development of I_{Na} block by promethazine was studied by double-pulse methods (Figure 5a). P_1 pulses of various durations were applied to -20 mV . After a 250 ms interval at the HP of -140 mV , which allowed the drug-free channels to reprimed fully, a 20 ms test pulse (P_2) was applied to -20 mV . The amplitude of I_{Na} produced by the P_2 pulse (I_2) was normalized to that elicited on P_1 (I_1). In Figure 5b, the relative I_{Na} at P_2 is plotted against the P_1 duration. Under control conditions, I_2 was hardly decreased even when the P_1 duration was prolonged to 1 s . Conversely, in the presence of $10 \mu\text{M}$ promethazine, lengthening of the P_1 duration progressively decreased I_{Na} in two phases. The line connecting the closed circles represents the least squares fit to the following equation:

$$\text{Relative } I_{Na} = 1 - A_{\text{fast}} \times \exp(-t/\tau_{\text{fast}}) - A_{\text{slow}} \times \exp(-t/\tau_{\text{slow}}),$$

where A_{fast} and A_{slow} represent the fraction of the fast and slow components, respectively. From 5 experiments, A_{fast} , A_{slow} , τ_{fast} , and τ_{slow} were calculated to be 0.09 ± 0.05 , 0.61 ± 0.83 , $7.0 \pm 5.0 \text{ ms}$, and $220 \pm 70 \text{ ms}$, respectively.

Recovery of I_{Na} from inactivation

The speed of the dissociation of promethazine from inactivated Na⁺ channels was then assessed. The pulse protocol used is shown in Figure 6a. The Na⁺ channels were adequately inactivated by a 5 s pulse to -20 mV (P_1). Following various recovery intervals ranging from 2 to 8000 ms at -140 mV , a test pulse (P_2) was again applied to -20 mV . The unrecovered fraction of I_{Na} at P_2 ($1 - I_2/I_1$) was semilogarithmically plotted against the recovery time (Figure 6b). Under control conditions, I_{Na} rapidly recovered from inactivation within 200 ms . In the presence of $3 \mu\text{M}$ promethazine, on the other hand, I_{Na} recovery was markedly retarded. The semilogarithmic plots obtained from 5 experiments revealed that the retardation could be ascribed to the development of

Figure 6 Effect of promethazine on the recovery of I_{Na} from inactivation in a typical experiment. The pulse protocol is shown in (a). The P_1 pulse to -20 mV for 5 s was followed by a 20 ms pulses (P_2) to -20 mV , with an intervening recovery period of various durations. The current (I_2) elicited by P_2 was compared to the current I_1 . (b) The unrecovered fraction of I_{Na} ($1 - I_2/I_1$) was semilogarithmically plotted as a function of the recovery intervals (in ms): (O) under control conditions; (●) in the presence of $3 \mu\text{M}$ promethazine. Note that a slow recovery phase (time constant = 1230 ms) developed on superfusion with $3 \mu\text{M}$ promethazine.

a much slower phase having a mean time constant of $1160 \pm 159 \text{ ms}$.

Use-dependent block of I_{Na} by promethazine

The apparent use-dependent effects of promethazine were tested by applying 15 consecutive depolarizing pulses. As shown in Figure 7a, 200 ms pulses to -20 mV were applied in the presence of a HP of either -140 mV or -90 mV . Two interpulse intervals of 500 and 1000 ms were used. Values of I_{Na} , normalized to that on the first pulse in each train, are plotted against the pulse number in Figure 7c and d. It is evident that $3 \mu\text{M}$ promethazine blocks I_{Na} in a frequency-dependent manner. Such a blocking action was exaggerated when the HP was set at -90 mV . In a subsequent experiment illustrated in Figure 8, the pulse duration was shortened to 2 ms as it is considered that, during such a brief depolarization, I_{Na} inactivation would not develop sufficiently to allow promethazine binding to the inactivated channels. Use-dependent I_{Na} block was hardly observed under these conditions.

Discussion

The present experimental study demonstrated that promethazine would cause a reversible blockade of I_{Na} in guinea-pig ventricular myocytes with a 1 to 1 stoichiometry and a K_D of $42.6 \mu\text{M}$ in the resting state. Since the therapeutic plasma concentration of this drug ranges from 3 to 23 ng ml^{-1} (less than $0.3 \mu\text{M}$; DiGregorio & Ruch, 1980), the drug would be expected barely to bind to the resting Na⁺ channels in the clinical situation. However, marked voltage-dependent block of I_{Na} by promethazine, indicated by a shift of the dose-response curve toward lower promethazine concentrations at the HP of -80 mV and a shift of the I_{Na} availability (h_∞)

Figure 7 Use-dependent block of I_{Na} by promethazine. In (a), the pulse protocol is shown: 200 ms pulses to -20 mV were given in the presence of the HP of either -140 mV or -90 mV. The interpulse intervals (t) were set at 500 ms or 1000 ms. Panel (b) illustrates the actual current traces obtained at the HP of -140 mV (upper section) and -90 mV (lower section). In (c) and (d), sequential I_{Na} values were normalized to the I_{Na} elicited on the first of the 15 consecutive pulses: (○), under control condition; (●), and (▲), in the presence of $3 \mu\text{M}$ promethazine. It is evident that promethazine exerts use-dependent block, which is enhanced at a less negative HP.

curve to more negative potentials indicate that promethazine molecules have a higher affinity for inactivated Na^+ channels than for resting channels. We then estimated the K_D of the drug for the inactivated Na^+ channels according to the following equation (Bean *et al.*, 1983):

$$\Delta V_h = s \times \ln((1 + D/K_r)/(1 + D/K_i)),$$

in which ΔV_h is the midpoint shift of the I_{Na} availability curve, s , the slope factor, D , the concentration of pro-

Figure 8 Use-dependent block of I_{Na} caused by shorter depolarizing pulses. As shown in inset at the left, 15 consecutive pulses of 2 ms duration were applied at the rate of 5 Hz. The current traces are shown in the insets at the right. Relative values of I_{Na} are plotted against the pulse number: (○), during control condition; (●), in the presence of $3 \mu\text{M}$ promethazine. Promethazine produced little use-dependent block with this protocol.

methazine, and K_r and K_i , the dissociation constants for resting and inactivated Na^+ channels, respectively. Using the values of ΔV_h , D , s , and K_r obtained in the present study, the K_i was calculated as $0.36 \mu\text{M}$, a value close to the plasma concentration of the drug encountered clinically. In view of such a high affinity of promethazine for inactivated Na^+ channels, the drug may well depress excitability of and conduction in Purkinje fibres and ventricular muscle, especially under pathological conditions associated with rapid heart actions and partially depolarized membranes.

According to the modulated receptor hypothesis, an antiarrhythmic drug binds either to the activated or inactivated state during membrane depolarization (Hille, 1977; Hondeghem & Katzung, 1977). The binding characteristics of promethazine were described by a double exponential function with time constants of 7 ± 5 ms and 220 ± 70 ms at -20 mV. Under these experimental conditions, most of the Na^+ channels were inactivated during the initial 10 ms-depolarization to -20 mV. If a considerable fraction of Na^+ channels is blocked in the open state during the brief activation, the binding process should be faster than development of Na^+ channel inactivation as was demonstrated by Clarkson *et al.* (1988) with lignocaine. Although the rapid phase of promethazine binding may be contaminated with the drug binding to the open channels, this possibility appears remote because (1) promethazine did not affect the I_{Na} decay time course, and (2) repetitive activation of Na^+ channels for 2 ms in the presence of promethazine caused no significant use-dependent block. Therefore, both binding phases would predominantly reflect the binding of the drug to the inactivated channels. Compared to the slow kinetics of lignocaine binding to the inactivated Na^+ channels with a time constant of 350 ms (Clarkson *et al.*, 1988), the time course of promethazine binding to the Na^+ channels is relatively fast.

Another striking finding, which may contribute to the electrophysiological effects of promethazine, is the slow recovery of the Na^+ channel from inactivation. Class I antiarrhythmic drugs are generally characterized by recovery kinetics, which probably represent the process of drug dissociation from inactivated Na^+ channels (Hille, 1977; Hondeghem & Katzung, 1977). In this regard, the time constant of 1230 ms for promethazine to dissociate from Na^+ channels classifies it as an intermediate type Na^+ channel blocker. This relatively slow repriming of I_{Na} leads to an incomplete recovery of Na^+ channels from inactivation during short

diastolic intervals and would contribute to use-dependent block by this agent. Since use-dependent block of I_{Na} was voltage-dependent, being enhanced at a less negative HP of -90 mV than at -140 mV, the drug molecules may dissociate from Na⁺ channel receptors after the drug-bound inactivated state is converted to the drug-bound resting state. Hence, promethazine would exert a potent use-dependent blocking action in depolarized and possibly injured myocardium.

Based on the results of the present study, it is concluded that promethazine blocks Na⁺ channels in a channel state-

dependent manner, much like other class I antiarrhythmic drugs. These features of the Na⁺ channel blockade would probably explain previously reported experimental findings such as electrophysiological alterations in the myocardium induced by promethazine (Hughes & Coret, 1972; Davis & McNeill, 1973; Aronson & Hanno, 1979) and the antiarrhythmic properties of this agent (Winbury & Alworth, 1959; Rigel & Katona, 1986; Rochette *et al.*, 1988). Since there is no clinical evidence available to support these experimental findings, clinical study is necessary to elucidate fully the electrophysiological actions of promethazine in man.

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(Received December 24, 1991)

Revised March 10, 1992

Accepted April 6, 1992)