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The effect of TYB-3823, a new antiarrhythmic drug, on sodium current in isolated cardiac cells

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1 Sodium current (I_{Na}) blockade by TYB-3823, a newly synthesized antiarrhythmic agent, was investigated in isolated single ventricular myocytes by use of the whole cell patch-clamp technique.

2 TYB-3823 blocked I_{Na} under steady-state conditions ($K_{d,rest} = 500\,\mu M$, $K_{d,i} = 4.9\,\mu M$), findings consistent with a shift in the steady state I_{Na} availability curve to more negative potentials.

3 TYB-3823 produced use-dependent block at 2 Hz in conjunction with increase in pulse duration (5–300 ms), that was markedly enhanced at less negative holding potentials.

4 The time course of the onset of block was accelerated and the degree of use-dependent block was decreased at more negative holding potential. The time course of the onset of block was accentuated with enhancing block at more positive holding potentials.

5 The time course of recovery from use-dependent block was accelerated at more negative holding potentials but was accentuated at more positive holding potentials.

6 These results suggest that both tonic block and use-dependent block of sodium channels in cardiac tissue might result from an interaction of TYB-3832 with sodium channels mainly in the inactivated channel states and the kinetics of the interaction between drug and receptor may be modulated by the inactivation gate.

Keywords: Na current; TYB-3823; cardiac myocytes; whole cell voltage-clamp

Introduction

The newly synthesized drug TYB-3823 (1-(2,6,-dimethylphenyl)-4,4,-dimethyl-aminoguanidine hydrochloride) has a potent and long-lasting inhibitory action against supraventricular and ventricular arrhythmias induced by aconitine, ouabain, or coronary ligation-reperfusion in rats, guinea-pig and dogs (Kurthy *et al.*, 1985). The previous experiments in dog isolated ventricular myocytes (Varro *et al.*, 1987) and guinea-pig papillary muscles (Kodama *et al.*, 1988) have shown that TYB-3823 suppresses the maximum upstroke velocity (V_{max}) of action potentials and shortening of action potential duration. These studies were based on measurement of V_{max} of cardiac action potentials. However, V_{max} may not be a linear measurement of I_{Na} in cardiac tissue (Cohen *et al.*, 1984). Therefore it was considered important to elucidate the blocking mechanism of TYB-3823 on I_{Na} in guinea-pig ventricular myocytes, under voltage-clamp conditions.

In the present study, we used the whole cell patch clamp technique to assess the kinetics of I_{Na} blockade by TYB-3823 on single guinea-pig ventricular myocytes. We found that the TYB-3823 could produce resting block only at depolarized holding potentials, suggesting a high affinity of TYB-3823 for the inactivated state of the Na channel. TYB-3823 also shifted the steady state inactivation curve in the negative potential direction and produced use-dependent block markedly at low holding potentials, results consistent with the findings that TYB-3823 slows recovery from inactivation state. It is concluded from these results that TYB-3823 has a high affinity for the inactivated state of the Na channel.

Methods

Single guinea-pig ventricular myocytes were isolated by the enzymatic dissociation technique similar to that previously described by Powell *et al.* (1980) and Ehara *et al.* (1989). Sodium currents of single ventricular cells were recorded by the whole cell clamp technique (Hamil *et al.*, 1981). The chamber was continuously perfused with low sodium Tyrode solution at a temperature of 17°C and of the following composition (mM): NaCl 10, CsCl 5, $CaCl_2$ 1.8, $MgCl_2$ 0.5, D-glucose 11, HEPES 20 and tetramethylammonium chloride 125. The solution was titrated to a pH of 7.35 with 1 M tetramethylammonium hydroxide. The internal solution of the suction pipette was composed of (mM): CsF 145, NaF 10 and HEPES 5 and titrated to a pH of 7.2 with 1 M CsOH. Use of these solutions allowed effective isolation of I_{Na} from other ionic currents. Pipettes had tip resistances less than 0.5 MΩ. Compensation for series resistance was done empirically by applying series resistance compensation to speed the decay of capacitive transient. After compensation, capacitive transients were within 500 μs. The membrane current signal was recorded on video tapes (video recorder, Mitsubishi HV-F73) through a PCM converter (SHOSHIN EM, PCM-PP16) for later computer analysis (NEC PC98XL). Several criteria have been outlined to permit indirect determination of the adequacy of space-clamp control in cardiac preparations. Under our experimental conditions (Hisatome *et al.*, 1987; Miyamoto *et al.*, 1989), current recordings from isolated myocytes satisfied the criteria described by Colatsky & Tsien (1979).

To study the tonic block of TYB-3823 on I_{Na}, low pulse frequency (0.01 Hz) was used, sufficient to ensure full recovery from rate dependent block of I_{Na}. Thereby, drug-induced decrease in I_{Na} is defined as tonic block. The amount of tonic block is calculated as % decrease in I_{Na} after perfusion with drug as compared to control. To study the use-dependent block, rest periods of 180 s were interposed between the trains of stimuli. I_{Na} decreased during a pulse train and reached a new steady state. The amount of use-dependent block is calculated as % decrease of I_{Na} in the new steady state with respect to that of the first pulse (Hisatome *et al.*, 1990). TYB-3823 was kindly supplied by Toyobo & Co., Ltd., Osaka, Japan; this drug is synthesized by Biogal (Debrecen, Hungary) as G.Y.K.I. 38233 and licensed to Toyobo.

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Results

Voltage-dependency and block of Na current by TYB-3823

Figure 1a shows the families of Na currents under control conditions (upper panel) and after administration of TYB-3823, 30 μM (lower panel). Na current were elicited by depolarizing pulses to selected test potentials from a holding potential (HP) of −90 mV every 90 s. After 8 min exposure to TYB-3823, the Na current was reduced to about 60% of the control value. Figure 1b shows the current-voltage relationship before and 8 min after exposure to 30 μM TYB-3823. TYB-3823 blocked the Na currents without changes in either threshold potential, peak potential or equilibrium potential. Figure 2 shows the relationship between fraction of tonic block and the concentration of TYB-3823 at HP = −90 mV. Each point represents a mean value of the blocked ratio of I_{Na} at each concentration ($n = 4$). The sigmoidal curves drawn through the data points are described according to the following equation:

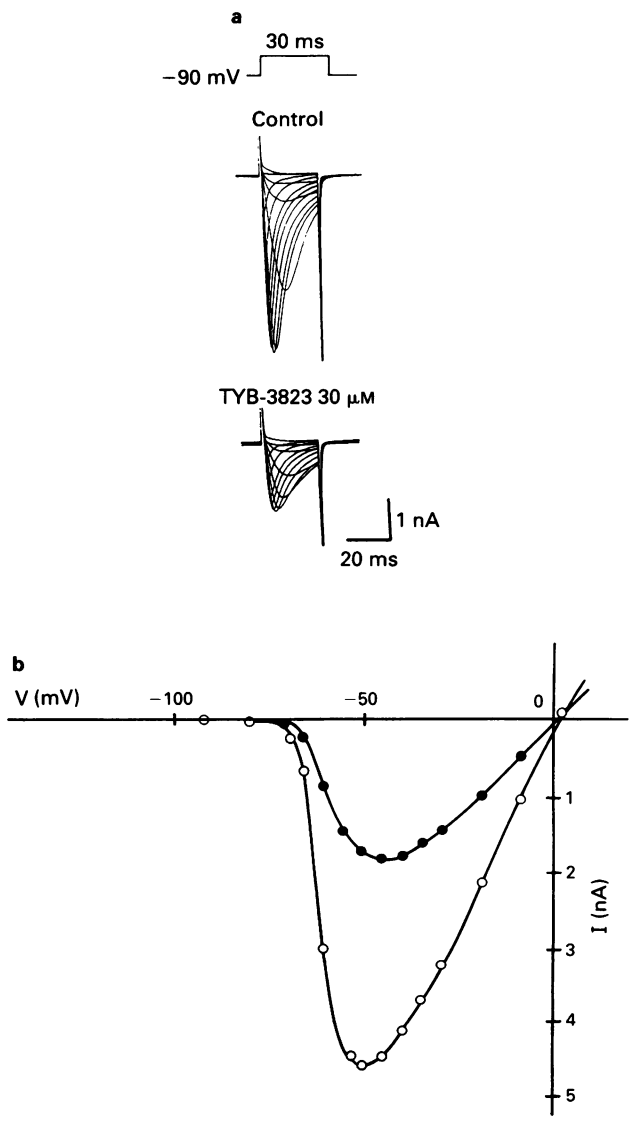


Figure 1 Voltage-dependence of TYB-3823 block of Na current. (a) Families of Na current traces under control conditions and after 30 μM TYB-3823 administration were obtained by applying 5 mV step pulses of 30 ms from a holding potential of −90 mV. Calibration shows 1 nA of current amplitude and 20 ms of time scale. (b) Current-voltage relationship for the peak current under control conditions and in the presence of 30 μM TYB-3823. Na current was blocked at all test potentials without affecting the current-voltage relationship.

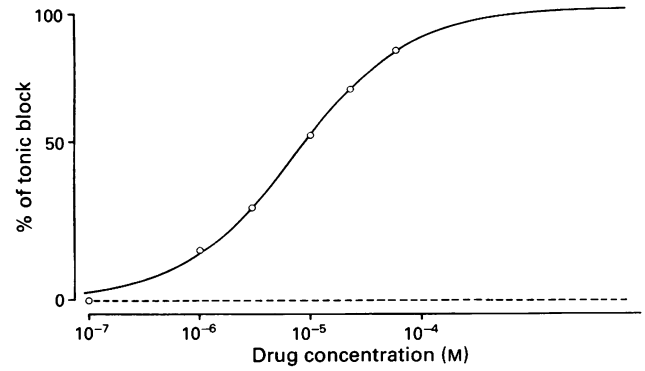


Figure 2 Concentration-dependent TYB-3823 block of Na current. Relationship between blocked reaction of Na current (ordinate scale) and the concentration of TYB-3823 (abscissa scale) are shown. I_{Na} was elicited by a test pulse to −20 mV from HP = −90 mV. The sigmoidal curve was drawn as a best fit to equation (see text). Slope factor was 0.97 and K_d was 15 μM.

$$y = (1 + K_{d,app}/[D])^{-1} \quad (1)$$

where y represents fraction of block, $K_{d,app}$ is apparent dissociation constant at HP = −90 mV and [D] is the drug concentration. $K_{d,app}$ was 15×10^{-6} M at HP = −90 mV.

Resting block by TYB-3823

Figure 3 shows the steady state availability curve for Na current under control conditions and after exposure to TYB-3823 (30 and 200 μM). I_{Na} availability was assessed at selected membrane prepulse potentials by use of a standard two-pulse protocol. A 1 s prepulse to the designated level of membrane potential was followed by a 0.5 ms interval and then by a 30 ms test pulse to −20 mV. This two pulse sequence was applied once every 90 s. The curves drawn through the data points are described by the following equation:

$$h = \{1 + \exp[(V_m - V_h)/k]\}^{-1} \quad (2)$$

where V_m is the prepulse potential, V_h is the prepulse potential at which h = 0.5 and k is a slope factor. At very negative HP (HP = −140 mV), high doses of TYB-3823 could reduce the maximum available I_{Na}. This decrease in I_{Na} at HP = −140 mV and low pulse frequency is defined as resting blockade of I_{Na} by TYB-3823. Although 30 μM TYB-3823 could not produce significant resting block, 200 μM TYB-3823

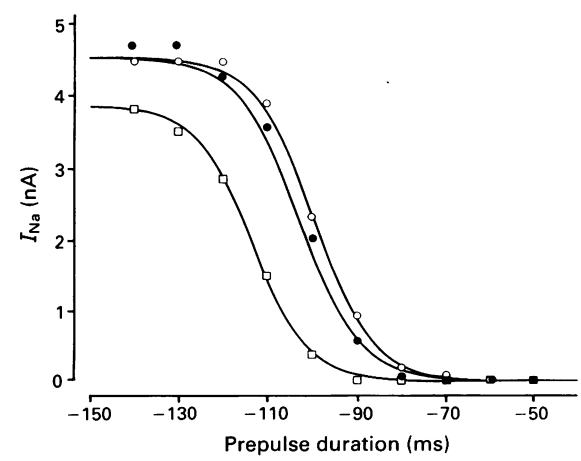


Figure 3 Dose-dependent effects of TYB-3823 on Na availability curve. Prepulse potential where I_{Na} is one-half maximum (V_h) and the slope factor (k) was calculated using Boltzmann distribution: $h = 1/1 + \exp[(V_m - V_h)/k]$ where V_m = prepulse potential. V_h was −95.3 mV and k was 6.3 (○, control), −102.8 mV and 6.3 in 30 μM TYB-3823 (●) and −113.3 mV and 6.2 in 200 μM TYB-3823 (□), respectively.

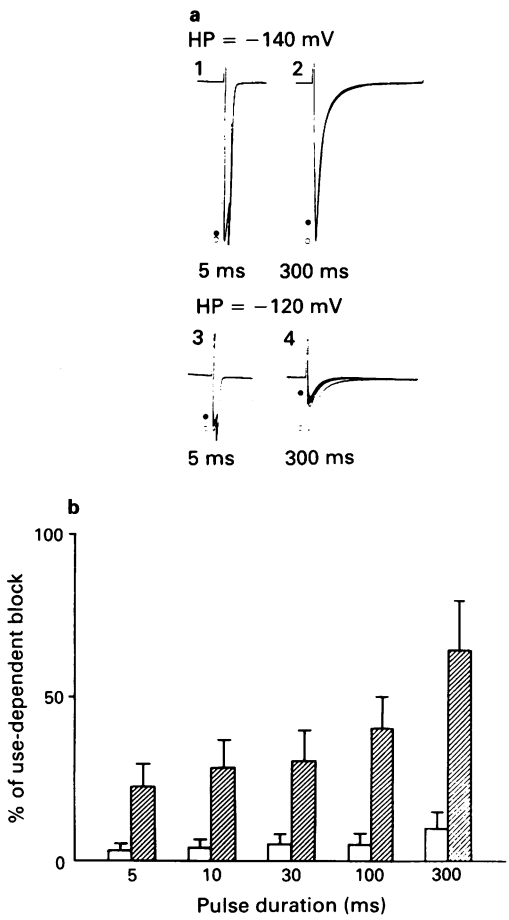


Figure 4 The relationship between pulse duration and use-dependent block at 2 Hz in the presence of 100 μM TYB-3823: effects of holding potential. (a) (1–4): I_{Na} elicited during a train of pulse to −20 mV from HP = −140 mV or −120 mV at 2 Hz. Open circle shows I_{Na} at 1st pulse and closed circle I_{Na} at 10th pulse, duration of which is 5 ms (1) and 300 ms (2) at HP = −140 mV, 5 ms (3) and 300 ms (4) at HP = −120. For calibration, see Figure 1a. (b) The relationship between pulse duration and phasic block at 2 Hz in the presence of 100 μM TYB-3823. Vertical bars show s.e. (n = 4) of use-dependent block at various pulse durations at HP = −140 (open columns) and −120 mV (hatched columns).

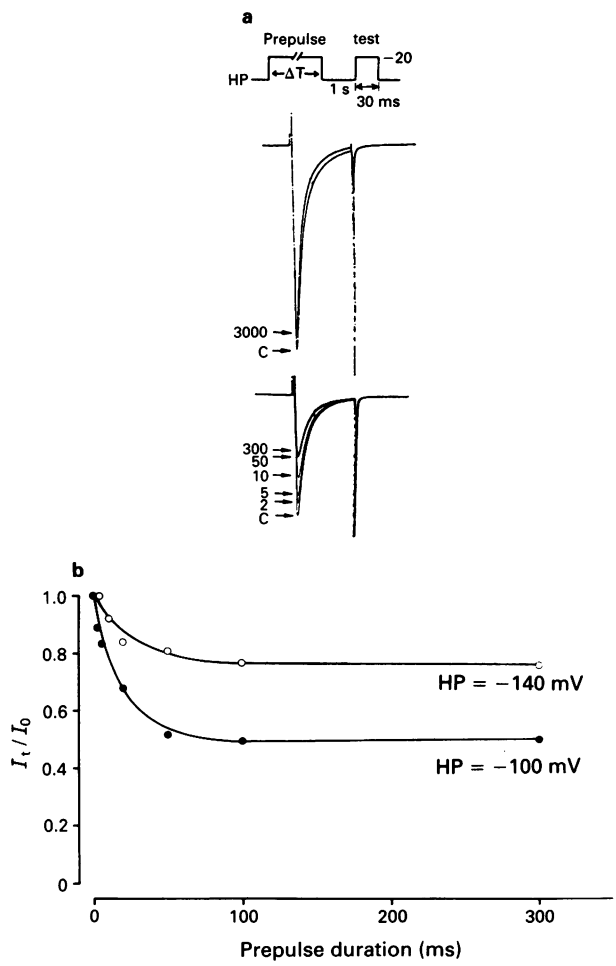


Figure 5 The time course of onset block in the presence of 100 μM TYB-3823. (a) The two-pulse protocol was used to assess the onset of use-dependent block. I_{Na} elicited by control (bottom arrow) and the test pulse following each conditioning pulse, the duration of which was 2, 5, 10, 50, 300, or 3000 ms (upper arrow) is shown in the presence of 100 μM TYB-3823. HP was −140 and −100 mV and test potential was −20 mV. For calibration, see Figure 1a. (b) The time constant of onset of block were 29 ms and 135 ms at HP = −140 (○) and −100 mV (●), respectively.

reduced maximum I_{Na} by 20% (n = 4) on average. TYB-3823 also shifted the Na availability curve toward more negative potentials in a dose-dependent manner without changes in slope factor. Therefore, resting block was observed only at a high dose of TYB-3823, and the I_{Na} blocking action of TYB-3823 was markedly dependent on membrane potential. According to equation (1), $K_{d,rest}$, i.e., dissociation constant in rested state, was 500 × 10^{-6} M.

Use-dependent block by TYB-3823

In addition to tonic block, TYB-3823 produced use-dependent block of I_{Na}. The magnitude of the use-dependent block of I_{Na} was dependent on the pulse duration and holding potential. Figure 4a(1) shows I_{Na} elicited by the first depolarizing pulse from HP = −140 to −20 mV (open circle) and 10th pulse (closed circle) in the presence of 100 μM TYB-3823 at 2 Hz, of which pulse duration was short (5 ms). Figure 4a(2) shows I_{Na} elicited by the long pulse duration (300 ms) from HP = −140 mV. Even long train pulses could produce only a small amount of use-dependent block at HP = −140 mV. Figure 4a(3) shows I_{Na} elicited by a first depolarizing pulse (open circle) from HP = −120 to −20 mV, and 10th pulse (closed circle) in the presence of 100 μM TYB-3823 at 2 Hz, with a short pulse duration (5 ms). Figure 4a(4) shows I_{Na} elicited by a long duration pulse (300 ms) from HP = −120 mV at 2 Hz. Even short duration pulses could reveal TYB-3823-induced use-dependent block of I_{Na}. Figure 4b shows the % of use-dependent block at 2 Hz by train pulses of 5, 10, 30, 100 and 300 ms in the presence of 100 μM TYB-3823 at HP = −140 and −120 mV (n = 4). The % of use-dependent block is calculated by the peak current for the 10th pulse normalized relative to that of the first pulse. The degree of use-dependent block at each HP increased with increase in the pulse duration. In addition, more cumulative block was observed at more depolarized HP. These results suggest that the magnitude of use-dependent block by TYB-3823 was dependent on both the HP and the pulse duration. It is worth noticing that even short duration pulses such as 5 or 10 ms produced use-dependent block, and the proportion of use-dependent block induced by 5 and 10 ms duration pulses relative to that by 300 ms was about 30% at either HP = −120 or −140 mV.

Onset of use-dependent block of I_{Na} by TYB-3823

The above data suggest that onset of use-dependent block by TYB-3823 might be fast. Therefore, this possibility was tested by use of a two-pulse protocol as follows. In the presence of 100 μM TYB-3823, a prepulse (2 to 300 ms) to −20 mV from

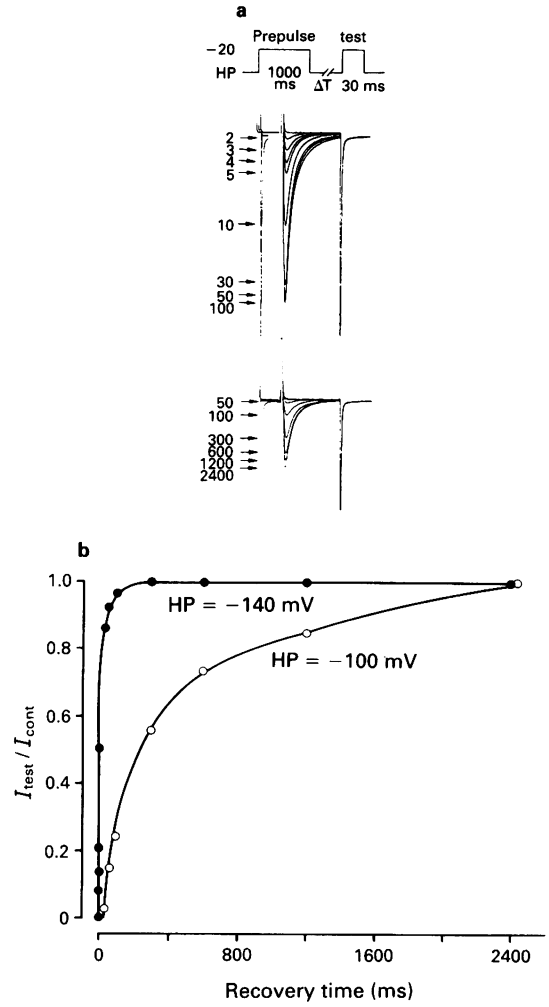


Figure 6 Effects of TYB-3823 on the recovery from inactivation of I_{Na}. Recovery of I_{Na} from inactivation was assessed by two-pulse protocol shown in the inset in upper part of (a). (a) I_{Na} elicited by the test pulse to −20 mV following 1000 ms prepulse is shown after selected recovery times (ms) as shown by arrows from HP = −140 and −100 mV in the presence of 100 μM TYB-3823. For calibration, see Figure 1a. (b) I_{Na} elicited during test pulse (I_{test}) was normalized to that elicited by the control pulse (I_{cont}). At HP = −140 mV (●), I_{Na} recovered within 300 ms, whereas at HP = −100 mV (○) full recovery of I_{Na} required more than 3 s.

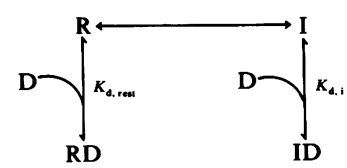
HP = −140 and HP = −100 mV was followed by a 1 s recovery period and a test pulse to −20 mV from each HP to assess current recovery as shown in the pulse protocol of Figure 5a. This sequence was applied at 90 s interval to avoid build-up of use-dependent block. The upper panel of Figure 5a shows the original current traces followed by 10 μs prepulse (control) and by 3000 ms prepulse at HP = −140 mV. The lower panel of Figure 5a shows the original current traces followed by 10 μs, 2, 5, 10, 50, and 300 ms prepulse at HP = −100 mV. Figure 5b shows % of onset of use-dependent block following prepulse duration varying from 2 to 300 ms in the presence of 100 μM TYB-3823 at HP = −140 (open circles) and −100 mV (closed circles). More positive HP accelerated the degree of onset of block, which was already revealed from prepulse duration = 2 ms at HP = −100 mV. This experiment indicates that, at less negative HP, even short pulse durations such as 2 ms could induce use-dependent block of I_{Na}, and block was also enhanced at longer prepulse durations such as 100 and 300 ms. By contrast, the more negative holding potential accentuated the degree of onset block. The onset of block was fitted by a single exponential curve. The time constants of onset of block were 39 and 175 ms at HP = −140 and −100 mV, respectively, which suggested that more negative HP accelerated the time constant of onset of block.

Recovery from inactivation of I_{Na} in the presence of TYB-3823

Recovery from inactivation was assessed by the protocol shown in the inset of Figure 6a. A 1000 ms prepulse was followed by a variable recovery period and a test pulse to assess the amount of current recovered at HP = −140 and −100 mV. Each two-pulse sequence was applied at 90 s interval. The upper panel of Figure 6a shows the original current traces at HP = −140 mV and the lower panel of Figure 6a shows the original current traces at HP = −100 mV. Figure 6b shows that the peak current for each test pulse was normalized to that for the preceding control pulse and plotted as a function of recovery time. At HP = −140 mV (closed circles), the Na current recovered rapidly, being described by single exponential with a time constant of 27 ms in the presence of 100 μM TYB-3823. The time course of recovery was slowed markedly at HP = −100 mV, and was characterized by the time constant of 587 ms. Thus, the more positive HP slowed recovery from inactivation markedly, a finding consistent with its effects in inducing use-dependent block.

Discussion

The present experiments have shown that the TYB-3823 decreased I_{Na} of guinea-pig ventricular myocytes, in both a tonic and use-dependent fashion, and also shifted the Na channel availability curve in the hyperpolarizing direction. The time constant for the decay of capacitive transients did not change before and after administration of TYB-3823, so that the shift of the inactivation curve was not due to a voltage drop across the residual series resistance. We tried to reconcile the present results with the modulated receptor hypothesis (Hille, 1977; Hondeghem & Katzung, 1977). According to the modulated receptor theory, (1) drugs bind to the receptor site or very close to the sodium channel, (2) the affinity of the receptor of the drug is modulated by the channel state: rested, inactivated and activated state, (3) drug-associated channels differ from the drug-free channels in that they do not conduct and their ability to be activated is shifted to more negative potentials. It is known that the tonic block is composed of rested and inactivated state block. A simplified scheme showing the relation between TYB-3823 and inactivation and the resting state is reproduced, which was shown by Bean *et al.* (1983).



where D is the drug molecule, R is the rested state, RD is the resting state with the neutral form of drug bound; I and ID are the corresponding forms of inactivation. Therefore, the apparent affinity for TYB-3823 ($1/K_{d,app}$) will depend largely on the apportionment of channels between rested and inactivated state (comprising fraction h and 1 − h, respectively) as following equation:

$$1/K_{d,app} = h/K_{d,rest} + (1 - h)/K_{d,i} \quad (3)$$

where h is the fraction of channels in the rested state in the absence of drug and $K_{d,i}$ is the dissociation constant for

binding to the inactivated state. $K_{d,rest}$ is the dissociation constant in the rested state. $K_{d,app}$ at HP = −90 mV was 15×10^{-6} M. $K_{d,rest}$ was 500×10^{-6} M ($n = 3$) and $K_{d,i}$ was 4.9×10^{-6} M.

Mean shift of the inactivation curve induced by the drug is obtained from the following equation:

$$dV_h = k \ln\{(1 + [D]/K_{d,rest})/(1 + [D]/K_{d,i})\} \quad (4)$$

where dV_h is the shift of inactivation curve, k is the slope factor of inactivation curve and [D] is drug concentration.

In order to confirm our calculation, we applied our data to the above equation. In the presence of 200 μM TYB-3823, the hyperpolarizing shift is calculated to be 20 mV, which was very close to the experimental data (18.2 mV). These results suggest that under steady state conditions, TYB-3823 had a very low affinity for the rested state, and blocked the Na channel mainly by binding to the inactivated state, resulting in a decrease in number of the sodium channels available.

Use-dependent block is produced when the drug-channel interaction is too slow to reach equilibrium within a single cycle of Na channel activation and inactivation. Drug binding to open channels and resting channels is also attributed to the accessibility of the receptor, which is controlled by the Na channel gate (Stimers *et al.*, 1985; Tanguy & Yeh, 1985). That is, the use-dependent block is attributed to a specific interaction between the sodium channel and the charged form of the drug molecules. This use-dependent blocking action is explained by two different theories, the modulated receptor hypothesis as already mentioned in the previous section and the guarded receptor hypothesis (Starmer *et al.*, 1984). In the latter, the affinity of the binding site of the drug is constant, but access to the binding site is guarded by activation and/or inactivation gates, such that the forward binding rate is faster when the channel is open or inactivated than under resting conditions. The present experiment showed that TYB-3823 exerted pronounced use-dependent block dependent on the longer duration of the depolarizing pulse and the more depolarized level of the holding potential. Thus, a train of long pulses (300 ms) could produce a small amount of Na channel block at HP = −140 mV, because at this holding potential, Na channels are predominantly in the rested state conformation but not in the inactivated state conformation. By contrast, at HP = −120 mV, even a train of brief pulses (5 ms) produced a large degree of use-dependent block and this degree of block was enhanced by a train of longer pulses (300 ms), because at this holding potential, Na channels are partially in the inactivated state conformation. These results suggest that TYB-3823 has little effect on the Na channel in its activated state, but binds to the inactivated state, i.e., inactivated gate (state)-dependent block, resulting in block of I_{Na} due to a decrease in the number of channels available. Since TYB-3823 can be supposed to block inactivated channels with an affinity that is larger compared to that for the rested state channels, at depolarized holding potentials (where tonic block is marked and most channels are distributed between drug bound and unbound inactivated state) rapid train pulses can increase the fraction of inactivated state.

It is worthwhile to notice that the time course of both onset block and recovery from block was accelerated at more negative holding potentials while decreasing the degree of use-dependent block, and was accentuated at more positive holding potentials while increasing the degree of use-dependent block. Based on the guarded receptor hypothesis, the receptor site should lie within the channel lumen and the charged form of the drug would reach it from the pore. At more negative holding potentials, the access of drug molecules to the binding site could not be guarded by the inactivation gate. Under these conditions, the drug molecules could easily gain access to the binding site and could also easily leave the site, which might reflect on the fast kinetics of drug-receptor interaction, resulting in a decrease in the degree of use-dependent block due to a reduction in the time that the drug was present on the receptor. By contrast, at more positive holding potentials, the access of drug molecules to the binding site would be guarded by the inactivation gate. In this condition, the drug not only could hardly gain access to the binding site but also would have difficulty leaving the site, which might reflect on the slow (intermediate) kinetics of drug-receptor interaction, resulting in an enhanced degree of use-dependent block because of the longer time the drug was present on the receptor. Kodama *et al.* (1989) recently demonstrated that at a resting membrane potential of −90 mV, TYB-3823 should be regarded as an intermediate kinetic class 1 drug according to the scheme of Campbell (1988) using V_{max} of the action potential in multicellular preparations, which is in favour of our data at a more positive holding potential. However, at more negative holding potentials, this substance has been proposed as a fast kinetic Class 1 drug and assumed to have the characteristics of inactivated channel blockers rather than activated channel blockers. In summary, our results show that TYB-3823 is a potent blocker of Na current in guinea-pig ventricular myocytes. The magnitude of tonic and use-dependent block may be a major factor in the anti-arrhythmic efficiency in depolarized tissue in such conditions as ischaemia.

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