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Biochimica et Biophysica Acta 1418 (1999) 320–334

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Enhancing effects of salicylate on tonic and phasic block of Na^+ channels by class 1 antiarrhythmic agents in the ventricular myocytes and the guinea pig papillary muscle

Yasunori Tanaka [a], Ichiro Hisatome [a,*], Jiro Miyamoto [b], Tadashi Urashima [a], Kaoru Ikeda [a], Yumi Yamanouchi [a], Norihito Sasaki [a], Toru Kinugawa [a], Kazuhide Ogino [a], Osamu Igawa [a], Akio Yoshida [a], Chiaki Shigemasa [a], Yasutaka Kurata [c], Ryoichi Sato [d]

[a] *First Department of Medicine, Tottori University Faculty of Medicine, Nishimachi 36-1, Yonago 683, Japan*
[b] *Department of Medicine, Tottori Red Cross Hospital, Tottori, Japan*
[c] *Department of Physiology, Kanazawa Medical University, Ishikawa, Japan*
[d] *Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, Chicago, IL, USA*

Received 4 January 1999; received in revised form 3 March 1999; accepted 3 March 1999

Abstract

Objective: To study the interaction between salicylate and class 1 antiarrhythmic agents. Methods: The effects of salicylate on class 1 antiarrhythmic agent-induced tonic and phasic block of the Na^+ current (I_{Na}) of ventricular myocytes and the upstroke velocity of the action potential (V_{max}) of papillary muscles were examined by both the patch clamp technique and conventional microelectrode techniques. Results: Salicylate enhanced quinidine-induced tonic and phasic block of I_{Na} at a holding potential of −100 mV but not at a holding potential of −140 mV; this enhancement was accompanied by a shift of the h_∞ curve in the presence of quinidine in a further hyperpolarized direction, although salicylate alone did not affect I_{Na}. Salicylate enhanced the tonic and phasic block of V_{max} induced by quinidine, aprindine and disopyramide but had little effect on that induced by procainamide or mexiletine; the enhancing effects were related to the liposolubility of the drugs. Conclusions: Salicylate enhanced tonic and phasic block of Na^+ channels induced by class 1 highly liposoluble antiarrhythmic agents. Based on the modulated receptor hypothesis, it is probable that this enhancement was mediated by an increase in the affinity of Na^+ channel blockers with high lipid solubility to the inactivated state channels.

Keywords: Salicylate; Na^+ channel; Papillary muscle; Ventricular myocyte; Class 1 antiarrhythmic agent; Modulated receptor theory; Liposolubility

1. Introduction

Platelet aggregation plays an important role in the pathogenesis of atherosclerotic complications such as occlusion of coronary arteries leading coronary heart disease. For this reason many studies have investigated the drugs that inhibit platelet aggregation. Acetylsalicylate acid suppresses platelet aggregation by inhibiting the cyclo-oxygenase enzyme and has been the most widely studied antiplatelet drug. Sev-

* Corresponding author. Fax: +81 (859) 348099; E-mail: hisatome@grape.med.tottori-u.ac.jp

0005-2736/99/$ – see front matter
PII: S0005-2736(99)00043-7

eral studies have provided convincing evidence that acetylsalicylate reduces the risk of a second myocardial infarction and the incidence of vascular death in patients with acute myocardial infarction [1]; moreover, it was reported that this drug reduced the risk of non-fatal myocardial infarction in patients who had previously suffered a TIA or minor stroke [2]. On the other hand, class 1 antiarrhythmic agents are not often used to treat the ventricular arrhythmias developing after myocardial infarction, because potent class 1 antiarrhythmic agents have rather dangerous side effects on the normal impulse conduction, reflected as a QRS widening, and increase the probability of converting a single extra beat into terminal ventricular fibrillation [3,4]. In clinical practice it is likely that class 1 antiarrhythmic agents will be administered to patients under treatment with acetylsalicylate, to treat or prevent ventricular arrhythmias. However, at present there are no experimental or clinical data regarding the combined effects of salicylate and class 1 antiarrhythmic agents on the heart. It has been reported that both acetylsalicylate and sodium salicylate prevent arrhythmia during experimentally induced coronary occlusion in dogs and rats [5,6], which may be due to the electrophysiological effects of salicylate on membrane currents [7]. In fact, salicylate decreased potassium conductance and prolonged the inactivation of sodium currents in squid axons [7], it prolonged the refractory period in the cat heart [8], and prolonged the duration of the action potential in sheep Purkinje fibers due to a shift of the activation curve and the reversal potential of the pacemaker current (I_f) to more negative potentials [9]. In contrast, it has been reported that the main effect of salicylate is shortening of the action potential, which is not a typical antiarrhythmic action [10]. Therefore, it is not clear whether salicylate alone has an antiarrhythmic effect or not. It has been demonstrated that salicylate enhances the blocking action of sodium (Na^+) channels induced by procainamide in nerve [11,12]. So, we hypothesized that salicylate in combination with class 1 antiarrhythmic agents may exert a more potent inhibition of the cardiac Na^+ channel, which in turn might modify the pro- or anti-arrhythmic effects of class 1 antiarrhythmic agents. Therefore, we examined the effect of salicylate on the depression of the Na^+ current induced by class 1 antiarrhythmic agents in ventricular myocytes using the whole cell patch clamp technique and conventional microelectrode techniques. Moreover, we discuss the mechanism of the interaction between salicylate and class 1 antiarrhythmic agents.

2. Materials and methods

2.1. Multicellular preparation

Guinea pigs weighing 250–300 g were killed by a blow on the neck. The papillary muscles (5–8 mm long and 0.8–1.0 mm in diameter) from both ventricles were removed, fixed in a tissue bath and superfused with normal Tyrode's solution (mM) (NaCl 137, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 1.8, $NaHCO_3$ 12, NaH_2PO_4 0.4, glucose 5.5, pH 7.4) bubbled with 95% O_2 and 5% CO_2 at a constant flow rate (3 ml/min). The temperature was maintained at 36 ± 0.2°C. The preparation was stimulated using a ring electrode. Using this ring electrode, an homogeneous spread of excitation along the preparation is enhanced [13]. Only cathodal pulses of constant intensity (1.4–1.6 times threshold strength) were used for the stimulation, because the anodal pulses of different intensities sometimes affected the amplitude of V_{max}. The transmembrane potential (E_m) was measured with a conventional glass microelectrode. The V_{max} was obtained using an electronic differentiator which was linear from 0 to 500 V/s. The signal was monitored on an oscilloscope and recorded using a motorized camera.

All procedures met the guidelines stipulated by the Animal Ethics Committee of Tottori University Faculty of Medicine.

2.2. Single cell experiments

Single guinea pig ventricular myocytes were isolated using the enzymatic dissociation technique described previously [14].

The sodium current (I_{Na}) of single ventricular cells was recorded using the whole cell voltage clamp technique. The chamber was continuously perfused at a temperature of 17°C with a low sodium bathing solution having the following composition (mM): NaCl 10, CsCl 5, $CaCl_2$ 1.8, $MgCl_2$ 0.5, $CoCl_2$ 1.0, D-glu-

cose 5.5, HEPES 20, tetramethylammonium chloride 125, and titrated to a pH of 7.4 with 1 mM tetramethylammonium (TMA) hydroxide. The solution inside the suction pipette contained (mM): CsF 145, NaF 10, HEPES 5, and was titrated to a pH of 7.2 with 1 mM CsOH, where fluoride has a sufficient Ca^{2+} buffering effect [15]. Use of this solution allowed an effective isolation of I_{Na} from other ionic currents. The pipette had a tip resistance of less than 0.5 MΩ. Cell capacitance (C_m) was estimated from the current transient produced by a small (10 mV) voltage clamp step, and determined by integrating the current transient; $C_m = 74 \pm 6$ pF ($n = 5$). Series resistance (R_s) was determined by fitting the exponential to the current transient which was well described by a single exponential. R_s was estimated from the time constant (t) of the capacitive transient on the assumption of $t = R_s C_m$. Mean time constant was 98 ± 8 μs ($n = 5$), and R_s was 1.3 ± 0.4 MΩ ($n = 5$). The membrane current signal was filtered at 10 kHz with a two-pore active filter, digitized at a sampling rate of 40 kHz, and recorded on videotapes (video recorder, Mitsubishi HV-F73, Mitsubishi, Tokyo, Japan) through a PCM converter (Shoshin EM, PCM-DP16, Shoshin, Tokyo, Japan) for later computer analysis (NEC PC98XL, NEC, Tokyo, Japan). Several criteria described by Colatsky and Tsien [16] that permit indirect determination of the adequacy of space-clamp control in the cardiac preparation were used.

1. The inward current record shows a smooth decay, with no secondary humps or 'abominable notches'.
2. The time course of the current remains unchanged when its magnitude varies due to prepulse inactivation.
3. The measured reversal potential lies close to the theoretically expected value.
4. The activation is a gradual function of the membrane potential of which the current-voltage curve has a wide (> 25 mV) negative slope region, extending over a range of about 50 mV as in other excitable tissue.

Under our experimental conditions [17–19], current recordings from isolated myocytes satisfied these criteria, as shown in the original current traces of control and the I–V curve of the control (Fig. 2).

Calcium inward current (I_{Ca}) and delayed rectifier potassium current (I_K) were also measured using the whole cell patch clamp techniques. For I_{Ca} measurement, KCl in the external and pipette solutions was replaced by CsCl to eliminate the influence of potassium currents. The chamber was continuously perfused at a temperature of 36°C with a bathing solution having the following composition (mM): NaCl 136.9, CsCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, D-glucose 5, HEPES 5, and titrated to a pH of 7.4 with 1 M NaOH. The solution inside the suction pipette contained (mM): CsCl 120, NaH_2PO_4 10, EGTA 1, MgATP 5, HEPES 10, and was titrated to a pH of 7.2 with 1 mM CsOH.

In experiments to measure I_K, the normal Tyrode's external solution was replaced by a sodium-free and potassium-free solution including nisoldipine in order to isolate the current from other current components. The composition of the solution was as follows (mM): *N*-methylglutamine 149, $MgCl_2$ 5, $CaCl_2$ 1.8, HEPES 5, nisoldipine 0.0003. Various depolarization steps up to +50 mV with a duration of 3 s were applied at a frequency of 0.03 Hz from the holding potential at −40 mV.

2.3. Definition of tonic and phasic block of either I_{Na} or V_{max} induced by antiarrhythmic agents

It is known that the block induced by antiarrhythmic agents of either V_{max} of the action potential in the papillary muscle or I_{Na} in a single ventricular cell is composed of a tonic block and a phasic block.

To study the block of either I_{Na} or V_{max} under steady-state conditions (tonic block), the decrease in either I_{Na} or V_{max} during exposure to the drug was defined as tonic block [17,20] at low pulse frequency of stimuli (0.1 Hz) which is sufficient to ensure full recovery from the use-dependent block of either V_{max} or I_{Na}. The extent of the tonic block was calculated as the percent decrease in both V_{max} and I_{Na} after perfusion with the drug with respect to the control.

To study the use-dependent block (phasic block) of either I_{Na} or V_{max}, the decrease of either V_{max} or I_{Na} during a pulse train following a rest of 180 s was defined as the phasic block. The extent of the phasic

block was calculated as the percent decrease in either V_{max} or I_{Na} in the new steady state (20th pulse) with respect to that of the first pulse [17,20].

2.4. Drugs

The following drugs were used: salicylate-sodium (Nakarai Chemical, Tokyo, Japan), quinidine (Nakarai Chemical), aprindine (Mitsui Pharmaceutical, Tokyo, Japan), disopyramide (Chugai Pharmaceutical, Tokyo, Japan), mexiletine (Boehringer Ingerheim, Germany) and procainamide (Daiichi Pharmaceutical, Tokyo, Japan).

Even at the highest concentration used in the study (3 mM) salicylate did not change the pH of the solution (pH = 7.35 ± 0.05 in control, 7.35 ± 0.07 in the presence of salicylate at 1 mM, 7.34 ± 0.02 in the presence of salicylate at 3 mM, respectively; $n = 4$).

2.5. Data analysis

Analysis of the data was performed on a computer (Macintosh LC630) using custom software. All curve fitting was done with a non-linear least squares algorithm using a Marquardt routine. The results were expressed as the mean (S.E.M.). Statistical analysis was done using the one-way repeated measures (ANOVA) test, and the results were considered to be significant when the P value was less than 0.05.

3. Results

3.1. Effects of salicylate on the parameters of the action potential of the ventricular muscle and on the membrane ionic currents of a ventricular myocyte

Table 1 summarizes the values of the various parameters of membrane action potential of guinea pig papillary muscle constantly driven at 0.1 Hz, after exposure to different concentrations of salicylate. Electrophysiological measurements were made before (control) and 30 min after exposure to the test sol-

Table 1
Effects of salicylate on the parameters of the action potential of the ventricular muscles and on the membrane ionic currents of a ventricular myocytes

	V_{max} (V/S)	RP (mV)	APA (mV)	APD_{90} (mV)
Control	257 ± 21	−83 ± 1	123 ± 4	235 ± 33
Salicylate (mM)				
0.03	257 ± 24	−83 ± 1	121 ± 5	223 ± 37
0.1	257 ± 24	−83 ± 1	121 ± 5	223 ± 41
0.3	261 ± 28	−83 ± 1	121 ± 6	200 ± 53*
1.0	257 ± 23	−83 ± 2	115 ± 3*	118 ± 18**
3.0	236 ± 15	−78 ± 4*	106 ± 9**	55 ± 15**
Quinidine 10 μM	233 ± 24*	−83 ± 3	123 ± 2	272 ± 10**
Quinidine 10 μM+salicylate (mM)				
0.03	211 ± 25*	−83 ± 3	123 ± 2	265 ± 30
0.1	197 ± 16*	−83 ± 1	122 ± 3	266 ± 27
0.3	173 ± 15**	−83 ± 2	113 ± 3*	174 ± 40**
1.0	164 ± 15**	−81 ± 6	103 ± 2**	96 ± 14**
3.0	128 ± 24**	−78 ± 2**	93 ± 4**	54 ± 14**
Salicylate (mM)	I_{Na} (%) (HP = −100 mV)	I_{Na} (%) (HP = −140 mV)	I_{Ca} (%)	I_K (%)
0.3	0 ($n = 4$)	0 ($n = 4$)	12.5 ± 7.6 ($n = 5$)	29 ± 0.4 ($n = 4$)
1.0	0 ($n = 4$)	0 ($n = 4$)	22.6 ± 6.8 ($n = 5$)	42 ± 5.6 ($n = 4$)

The upper part of the table indicates the effects of salicylate on the parameters measured after exposure to different concentrations of salicylate ($n = 5$). The middle part indicates the effects of salicylate on the parameters measured after exposure to different concentrations of salicylate in the presence of quinidine ($n = 5$). The lower part indicates the I_{Na}, I_{Ca}, and I_K of a ventricular cardiac myocyte after exposure to salicylate (0.3 and 1 mM).
V_{max}, maximum rate of rise; RP, resting potential; APA, action potential amplitude; APD_{90}, action potential at 90% repolarization.
*$P < 0.05$, **$P < 0.01$, compared with control values.

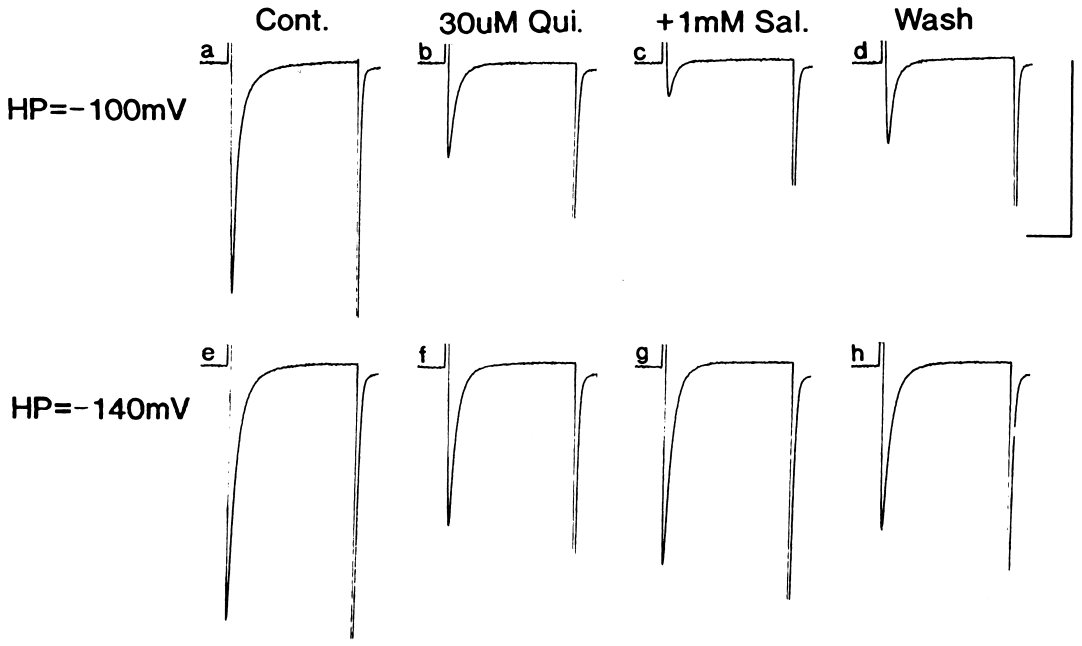


Fig. 1. Enhancing effects of salicylate on the tonic block of I_{Na} induced by quinidine at different holding potentials. (a,e) I_{Na} elicited by the test potential to −30 mV at 0.1 Hz from either an HP = −100 mV or −140 mV. (b,f) Effects of quinidine at 30 μM on I_{Na} at either an HP = −100 mV or −140 mV. (c,g) Effect of salicylate at 1 mM on I_{Na} at either an HP = −100 mV or −140 mV in the presence of quinidine at 30 μM. (d,h) Effect of salicylate washout on I_{Na}. Note that salicylate enhanced the tonic block of I_{Na} induced by quinidine at an HP = −100 mV but not at an HP = −140 mV. The vertical bar indicates 1 nA and the horizontal one 10 ms.

ution containing the various concentrations of salicylate (0.03–3 mM). Although salicylate at 3 mM did not significantly affect the V_{max}, the resting membrane potential (RP) significantly depolarized at the highest concentration of salicylate. Salicylate significantly reduced both the action potential amplitude (APA) and the action potential duration at 90% repolarization (APD_{90}) in a dose-dependent manner. We also studied the action potential parameters in the presence of both quinidine at 10 μM and of different concentrations of salicylate. Quinidine alone significantly prolonged APD_{90} ($P < 0.01$) and reduced V_{max} ($P < 0.05$) without changes in APA and RP. RP significantly depolarized at the highest concentration of salicylate even in the presence of quinidine. Salicylate significantly reduced both APA and APD_{90} in a dose-dependent manner, even in the presence of quinidine. Interestingly, salicylate enhanced the block of V_{max} induced by quinidine in a dose-dependent manner. These results suggested that salicylate alone might change the membrane currents responsible for the plateau phase of the action potential (APD_{90}); and salicylate might enhance the block of I_{Na} induced by quinidine, although salicylate alone did not affect the I_{Na}. To confirm these findings we studied the effects of salicylate alone on I_{Na}, I_{Ca} and I_K of individual cardiomyocytes using the whole cell patch clamp techniques. As shown in Table 1, salicylate at 1 mM did not affect I_{Na} elicited by a test potential of 30 ms to −20 mV either at an HP of −100 mV or of −140 mV, although salicylate significantly blocked I_{Ca} as well as I_K in a dose-dependent manner ($P < 0.01$). These results suggested that salicylate would shorten the action potential duration by reducing I_{Ca} but would not affect I_{Na}.

3.2. Enhancing effect of salicylate on the tonic block of I_{Na} induced by quinidine in single cardiac cells

Quinidine at 30 μM decreased I_{Na} by 58% (from 1.3 to 0.54 nA) elicited by a test potential of 30 ms to −30 mV at an HP of −100 mV (Fig. 1a,b) at 0.1 Hz. Addition of 1 mM salicylate to the quinidine-containing medium resulted in a further decrease of I_{Na} from 0.54 to 0.18 nA (−67%; Fig. 1b,c). After a 15 min washout of quinidine and salicylate, this enhancing effect of salicylate disappeared and the amplitude of I_{Na} returned to the same level as before

the addition of salicylate (Fig. 1d). At an HP of −140 mV, 30 μM quinidine reduced I_{Na} from 1.4 to 0.93 nA (Fig. 1e,f). However, addition of 1 mM salicylate to the quinidine-containing medium did not result in a further reduction of I_{Na} which on the contrary increased to 1.1 nA (Fig. 1g). After the washout of both quinidine and salicylate, the amplitude of I_{Na} returned to the same level as before the addition of salicylate (Fig. 1h).

To avoid the possibility that salicylate shifted the gating kinetics during the experiment, the current–voltage relationship of the Na^+ current was recorded. The I_{Na} under control, in the presence of 30 μM quinidine, in the presence of 30 μM quinidine+1 mM salicylate and after washout of both quinidine and salicylate is shown in Fig. 2. I_{Na} was elicited by 5 mV step depolarizing pulses from an HP of −100 mV at 0.1 Hz. In comparison with the amplitude of I_{Na} under control conditions (a: peak I_{Na} = 3 nA), quinidine at 30 μM produced the tonic block of I_{Na} (b: −42% peak I_{Na} = 1.74 nA).

The addition of 1 mM salicylate to the quinidine-

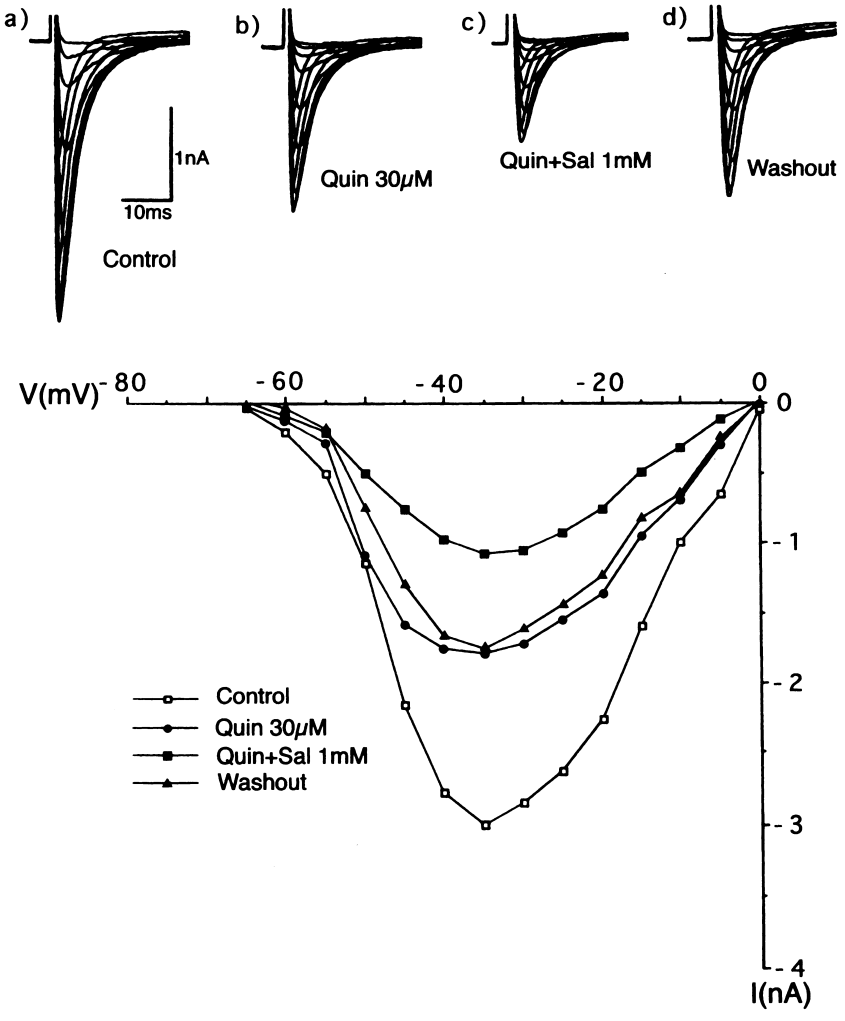


Fig. 2. Effects of quinidine on the current-voltage relationship of Na^+ current in the absence and presence of salicylate. (a–d) The original current traces of Na^+ current under control conditions, in the presence of quinidine (30 μM), in that of quinidine+salicylate (1 mM), and during washout. The holding potential was kept at −100 mV and depolarized by 5 mV step depolarizing pulses of 30 ms duration at 0.1 Hz. (e) The *I-V* curve of Na^+ current under control conditions (□), in the presence of 30 μM quinidine (◆), in that of quinidine+1 mM salicylate (■), and during washout (▲). Note that salicylate could enhance the tonic block of I_{Na} induced by quinidine without changes in either threshold, peak or equilibrium potential.

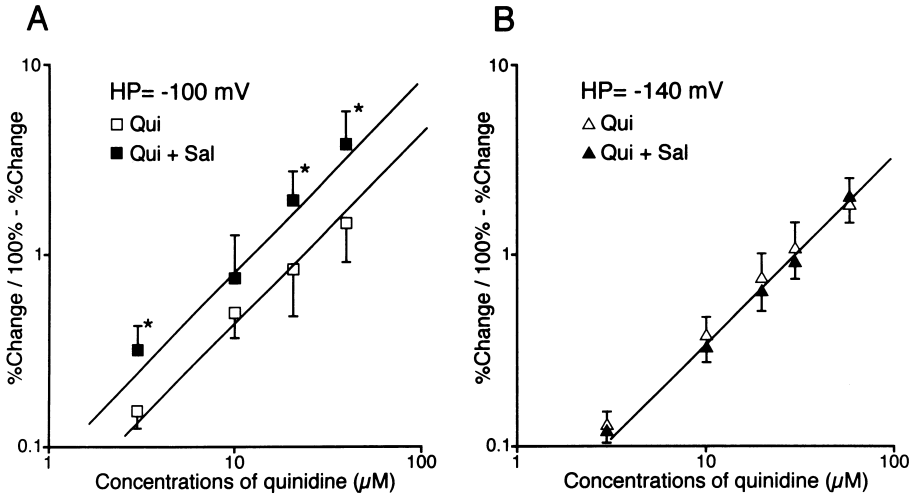


Fig. 3. Hill plot of the concentration-dependent tonic block of I_{Na} induced by quinidine at either an HP = −100 mV or −140 mV. The ordinate shows (% tonic block of I_{Na})/(100%−% tonic block of I_{Na}), and the abscissa indicates the log of salicylate concentration (3–60 μM). (A) Hill plot of the tonic block of I_{Na} induced by quinidine at an HP = −100 mV. □, quinidine alone; ■, quinidine in the presence of 1 mM salicylate. *$P < 0.05$. (B) Hill plot of the tonic block of I_{Na} induced by quinidine at an HP = −140 mV. △, quinidine alone; ▲, quinidine in the presence of 1 mM salicylate. Note that salicylate could shift the dose response of quinidine to the left side at an HP = −100 mV but not at an HP = −140 mV. Each point represents the mean value of five experiments.

containing medium enhanced the tonic block by 67% (c: peak $I_{Na} = 0.99$ nA). After 15 min washout of quinidine and salicylate, this enhancing effect of salicylate was abolished and the amplitude of I_{Na} returned to the same level as before adding salicylate (d: peak $I_{Na} = 1.75$ nA). As shown in the *I–V* curve, there was no shift of either the threshold (−60 mV), peak (−35 mV), or equilibrium potential of I_{Na} (0 mV) under control conditions, in the presence of 30 μM quinidine alone, in the presence of quinidine+1 mM salicylate, or during washout of both quinidine and salicylate.

Fig. 3 shows the Hill plots of the concentration-dependent tonic block of I_{Na} by quinidine (3–60 μM) in the presence and absence of 1 mM salicylate at an HP of −100 mV and of −140 mV. Each point represents the mean (S.E.M.) of five experiments.

We calculated the $K_{d,app}$ and Hill coefficient using the following equation:

$$\% \text{ change} = 1/[1 + [D]^n/K_{d,app}] \quad (1)$$

where D means dose of quinidine, n means Hill coefficient, and $K_{d,app}$ means the dose for 50% block.

At an HP of −100 mV, 1 mM salicylate significantly enhanced the tonic block of I_{Na} by quinidine at various quinidine concentrations, so that the response to quinidine in the presence of 1 mM salicylate ($K_{d,app} = 11.8$ μM; $P < 0.05$) was shifted to a substantially lower concentration of quinidine ($K_{d,app} = 22.8$ μM) without changes in Hill coefficient (= 1.0) as shown in Fig. 3A. In contrast, at an HP of −140 mV salicylate did not cause a shift of the re-

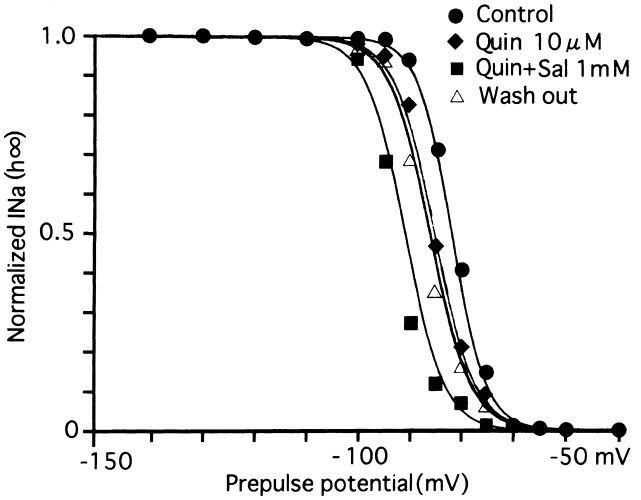


Fig. 4. Effect of salicylate on the h_∞ curve in the presence of quinidine. ●, control conditions; ◆, 10 μM quinidine; ■, 10 μM quinidine plus 1 mM salicylate to the quinidine-containing medium; △, during washout. The ordinate shows the availability of the Na^+ channels and the abscissa the prepulse potentials. Note that salicylate shifted the h_∞ curve in the presence of quinidine further in the hyperpolarized direction.

sponse to quinidine ($K_{d,app}$ = 30 μM) as shown in Fig. 3B.

Fig. 4 shows the steady-state inactivation curve of I_{Na} under control conditions, in the presence of 10 μM quinidine and in the presence of both 10 μM quinidine and 1 mM salicylate. The inactivation curve of I_{Na} was assessed at selected membrane (prepulse) potential using a standard two-pulse protocol from an HP of −140 mV. A 1 s prepulse to the designated level of membrane potential was followed by a 0.5 ms interpulse interval then by a 30 ms test pulse of −20 mV. The protocol sequence was applied once every 40 s. The curve drawn through the data points is described by the following equation:

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

where V_m is the prepulse potential, V_h is the prepulse potential at $h = 0.5$ and k is a slope factor. Under control conditions, V_h was −81.8 mV and k was 8.6, while in the presence of 10 μM quinidine, V_h was −85.3 mV and the slope factor was 8.6, and in the presence of both 10 μM quinidine and 1 mM salicylate, V_h was −91.0 mV and the slope factor was 8.7. After the washout, V_h was −86.0 mV and the slope factor was 8.8. From the data obtained from four experiments, 10 μM quinidine significantly shifted the I_{Na} inactivation curve by −3.2 ± 1.7 mV ($P < 0.05$) and treatment with both 10 μM quinidine and 1 mM salicylate further produced a significant shift of I_{Na} inactivation by −9.9 ± 3.4 mV ($P < 0.05$) in the hyperpolarized direction along the voltage axis while the slope factor remained unchanged at 8.6 ± 2.2.

3.3. Enhancing effect of salicylate on the phasic block of I_{Na} induced by quinidine in single cardiac cells

Fig. 5a–d shows the enhancing effect of salicylate

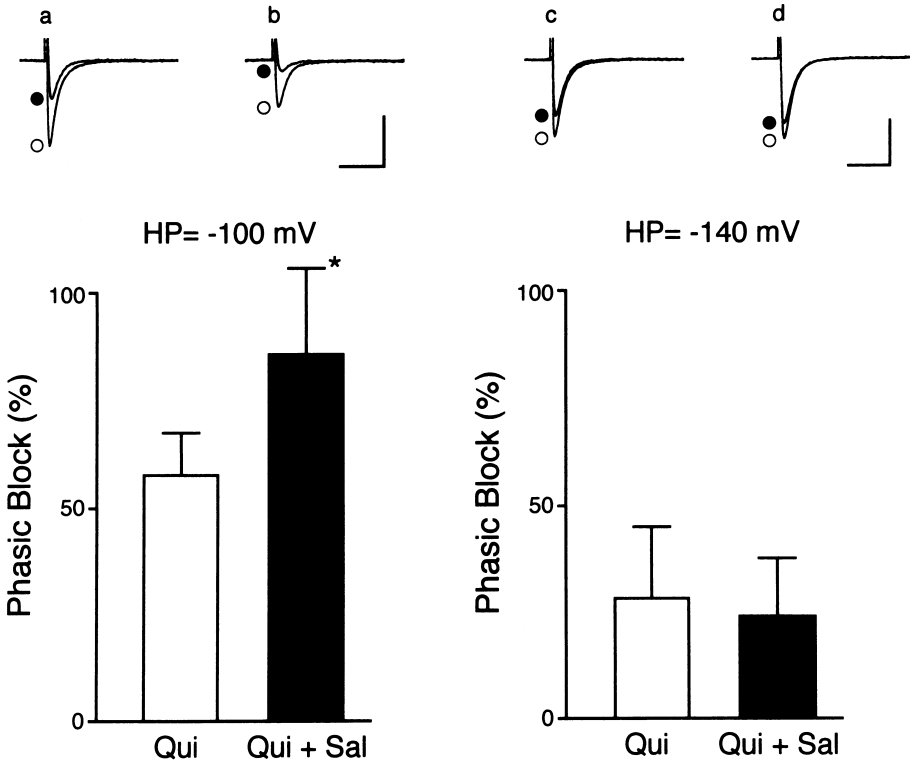


Fig. 5. Effect of salicylate on the phasic block of I_{Na} induced by quinidine at different holding potentials. ○, I_{Na} elicited by the first depolarizing pulse to −20 mV; ●, I_{Na} elicited by the 20th pulse at either an HP = −100 mV or −140 mV. (a,c) In the presence of 30 μM quinidine; (b,d) in the presence of both 30 μM quinidine and 1 mM salicylate. (Lower panels) Phasic block of I_{Na} at either an HP = −100 mV (left panel: $n = 4$) or HP = −140 mV (right panel: $n = 4$). Qui, in the presence of 30 μM quinidine; Qui+Sal, in the presence of both 30 μM quinidine and 1 mM salicylate. *$P < 0.05$. Note that salicylate enhanced the phasic block of I_{Na} induced by quinidine at an HP = −100 mV but not at an HP = −140 mV.

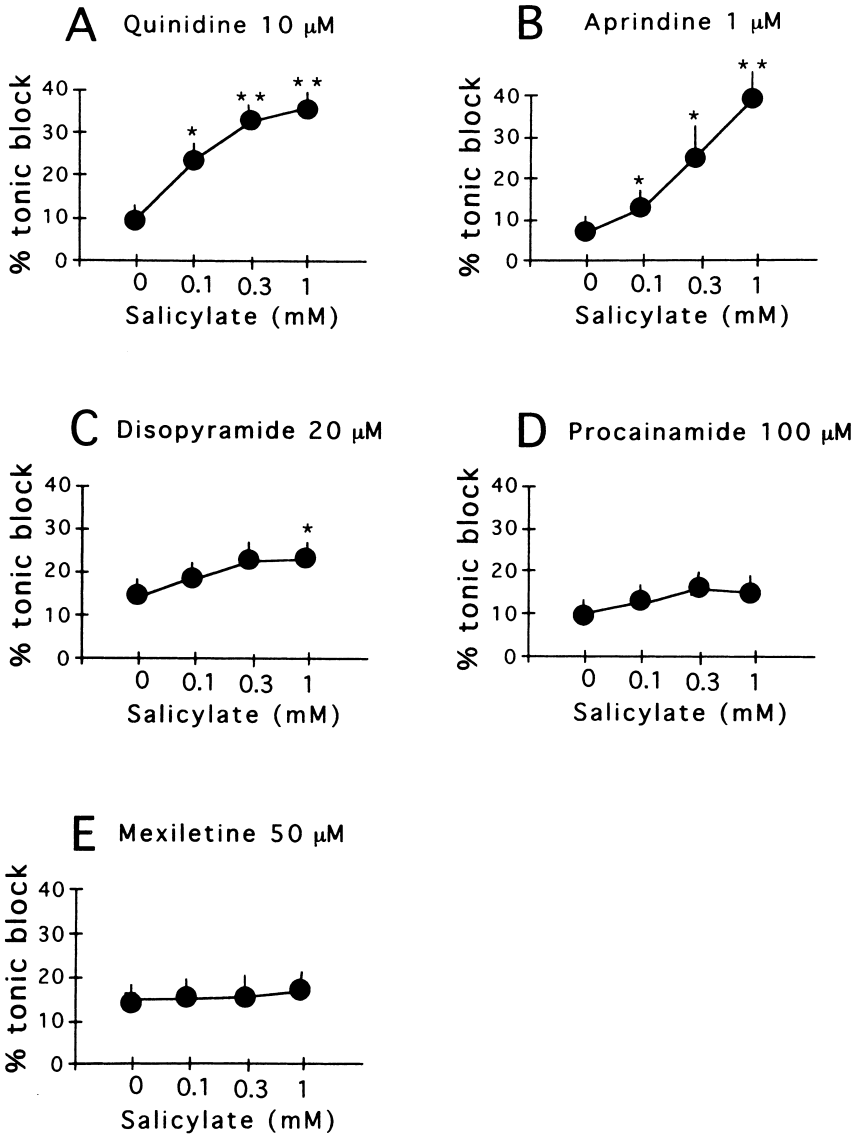


Fig. 6. Concentration-dependent enhancing effects of salicylate on the tonic block of V_{max} by class 1 antiarrhythmic drugs. The ordinate indicates the % tonic block of V_{max} and the abscissa the salicylate concentration. (A) In the presence of 10 μM quinidine; (B) in the presence of 1 μM aprindine; (C) in the presence of 20 μM disopyramide; (D) in the presence of 100 μM procainamide; (E) in the presence of 50 μM mexiletine. Note that salicylate significantly enhanced the tonic block of V_{max} induced by aprindine or disopyramide but not that induced by procainamide or mexiletine. Each point represents the mean value of five experiments. $^{*}P<0.05$, $^{**}P<0.01$.

on the quinidine-induced phasic block of I_{Na} elicited by the first 30 ms depolarizing pulse (open circle) at 2 Hz from either an HP of −100 mV or an HP of −140 mV to −20 mV and by the 20th pulse (closed circle) in the absence or presence of 1 mM salicylate. At an HP of −100 mV, 30 μM quinidine produced a phasic block (to 58%, Fig. 5a). The addition of 1 mM salicylate enhanced the phasic block of I_{Na} induced by 30 μM quinidine by 85% (Fig. 5b). In contrast, at an HP of −140 mV, 30 μM quinidine produced a phasic block of I_{Na} (28%, Fig. 5c), but the addition of 1 mM salicylate did not enhance the phasic block of I_{Na} induced by quinidine (23%, Fig. 5d). The lower panels of Fig. 5 show the summary of the

enhancing effects of salicylate on the phasic block obtained from each of the four experiments. At an HP of −100 mV, 1 mM salicylate could significantly enhance the phasic block induced by 30 μM quinidine (54 ± 12% without salicylate vs. 87 ± 15% with 1 mM salicylate; $P < 0.05$). On the contrary, at an HP of −140 mV 1 mM salicylate did not enhance the phasic block of I_{Na} induced by 30 μM quinidine (28 ± 19% without salicylate vs. 24 ± 14% with 1 mM salicylate).

3.4. *Modification of the tonic and phasic block of V_{max} of the action potential induced by the class 1 antiarrhythmic drugs by salicylate*

Since salicylate specifically enhanced the tonic and phasic blocks of I_{Na} induced by quinidine at a partially depolarized membrane potential (at an HP of −100 mV) but not at a well-polarized membrane potential (at an HP of −140 mV), we thought that salicylate might enhance the tonic and phasic block

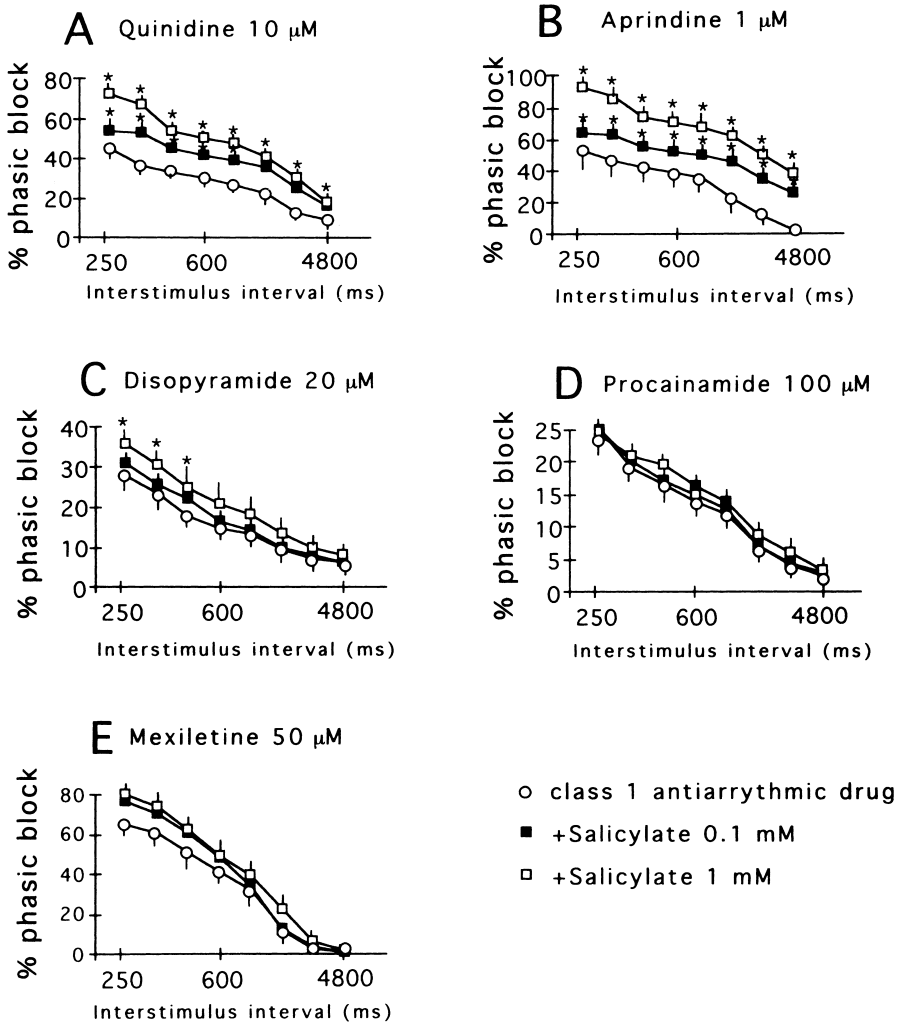


Fig. 7. Concentration-dependent effects of salicylate on the phasic block of V_{max} induced by class 1 antiarrhythmic drugs. The ordinate indicates the % phasic block of V_{max} and the abscissa the interstimulus interval. (A) In the presence of 10 μM quinidine; (B) in the presence of 1 μM aprindine; (C) in the presence of 20 μM disopyramide; (D) in the presence of 100 μM procainamide; (E) in the presence of 50 μM mexiletine. ○, class 1 antiarrhythmic drug alone; ■, class 1 antiarrhythmic drug combined with 0.1 mM salicylate; □, class 1 antiarrhythmic drug combined with 1 mM salicylate. Note that salicylate significantly enhanced the phasic block of V_{max} induced by aprindine or disopyramide but not that induced by procainamide or mexiletine. Each point represents the mean value of five experiments. *$P < 0.05$.

of V_{max} of the action potential at the resting membrane potential (RP = around −80 mV) induced by class 1 antiarrhythmic agents. Fig. 6 shows the effects of salicylate on the tonic block of V_{max} induced by quinidine, aprindine, disopyramide, procainamide and mexiletine in each of the five experiments. Salicylate significantly enhanced the tonic block of V_{max} induced by 10 μM quinidine (Fig. 6A), 1 μM aprindine (Fig. 6B), or by 20 μM disopyramide (Fig. 6C) in a dose-dependent manner (0.1–1 mM). However, even at the highest concentration of 1 mM, salicylate failed to enhance the tonic block of V_{max} induced by 100 μM procainamide (Fig. 6D) or 50 μM mexiletine (Fig. 6E).

Fig. 7 shows the effects of salicylate on the phasic block of V_{max} induced by quinidine, aprindine, disopyramide, procainamide and mexiletine in each of the five experiments. Salicylate significantly enhanced the tonic block of V_{max} induced by 10 μM quinidine (Fig. 7A), 1 μM aprindine (Fig. 7B), or 20 μM disopyramide (Fig. 7C) in a dose-dependent manner (0.1–1 mM). However, even at the highest concentration of 1 mM, salicylate failed to enhance the tonic block of V_{max} induced by 100 μM procainamide (Fig. 7D) or 50 μM mexiletine (Fig. 7E).

4. Discussion

4.1. Mechanism of the enhancing effects of salicylate based on the modulated receptor hypothesis

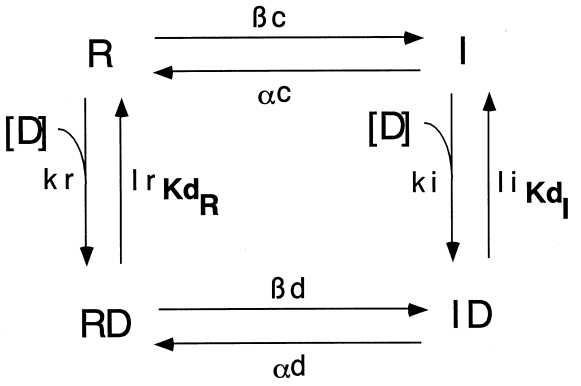
From the single cell experiments, we concluded that salicylate enhanced the quinidine-induced tonic block and phasic block of Na^+ channels at a depolarized membrane potential but not at a hyperpolarized membrane potential, enhancing the hyperpolarizing shift in the h_∞ curve induced by quinidine.

According to the modulated receptor theory [21], (1) drugs bind to a receptor site on or very close to the sodium channel, (2) the affinity of the receptor for the drug is modulated by the channel state: the resting, the inactivated, and the activated state, (3) drug-associated channels differ from drug-free channels in that they do not conduct, and their ability to be activated is shifted to a more negative potential. It is well known that under steady-state conditions the tonic block is composed of the resting state block and the inactivated state block [21].

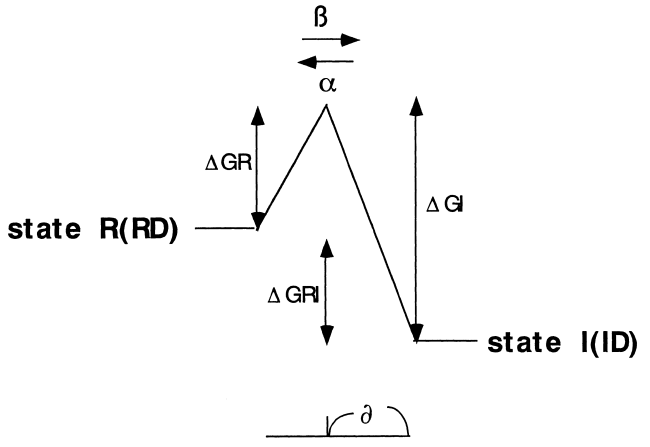
Scheme 1 is a simplified scheme showing the relationship between the quinidine molecule and both the resting and inactivated states of the channel. In this scheme D is the drug molecule, R is the resting state, RD is the resting state with the neutral form of drug bound, I is the inactivated state, and ID is the inactivated state with the neutral form of the drug bound. kr and ki are the drug-binding rate constants. lr and li are the corresponding dissociation rate constants. α is the forward rate constant from I to R (αc) or from ID to RD (αd). β is the backward rate constant from R to I (βc) or from RD to ID (βd). Kd_R and Kd_I are the equilibrium dissociation constants for the resting and inactivated states, respectively ($K_{dR} = l_r/k_r$, $K_{dI} = l_i/k_i$). Based on the modulated receptor hypothesis, administration of salicylate could further reduce the quinidine-induced reduction of the number of available channels under steady-state conditions. There are two possible explanations regarding the salicylate-induced reduction of the available channels in the presence of quinidine.

1. Salicylate could increase the number of drug-bound non-conducting channels by decreasing the K_{dI}.
2. Salicylate could increase the number of drug-bound inactivated channels (ID) through the acceleration of the transition from RD to ID by shifting the h_∞ curve toward the hyperpolarized direction.

To examine the above possibilities, we applied the



Scheme 1.



Scheme 2.

experimental values of K_{dR}, slope factor, and V_h to the equations below and calculated the probability of Na^+ channels dwelling in R (drug-free resting state) in the presence of 10 μM quinidine before and after adding the 1 mM salicylate according to the Erying rate theory. We assumed that Na^+ channels transited between R (RD) and I (ID) over the energy barrier as shown in Scheme 2.

In this scheme α and β represent the rate constants for the state transitions, ΔGR, ΔGI and ΔGRI denote the energy difference in the Gibbs free energy among state (in RT unit). The forward and backward rate constants were expressed as functions of energy differences.

$$\alpha = kT/h\{\exp(-[\Delta G_I + \partial zFV/RT])\} \quad (3)$$

$$\beta = kT/\{\exp(-[\Delta G_R + (\partial - 1)zFV/RT])\} \quad (4)$$

$$\Delta G_I = \Delta G_R + \Delta G_{RI} \quad (5)$$

$$\Delta G_{RI} = -V_h/S \quad (6)$$

$$S = RT/zF \quad (7)$$

where S is the slope factor, z the number of gating charges, δ the electrical distance and V_h the midpoint potential of the h_∞ curve. h, k, R, F and T have their usual meanings.

A drug could bind to or dissociate from the receptor over the energy barrier as shown in Scheme 3.

In this scheme k and l are the rate constants for drug-binding and drug dissociation, and ΔGk, ΔGkl, and ΔGl are the energy differences among the states. Association and dissociation rate constants are given by the following equations:

$$k = kT/h\{\exp(-\Delta G_k)\} \quad (8)$$

$$l = kT/h\{\exp(-\Delta G_l)\} \quad (9)$$

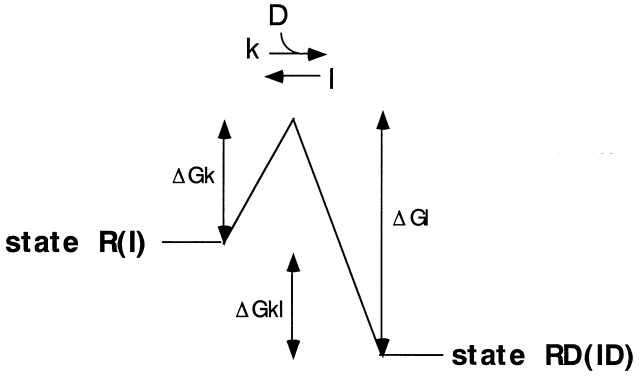
$$\Delta G_l = \Delta G_k + \Delta G_{kl} \quad (10)$$

$$\Delta G_{kl} = \ln(K_d) = \ln(l/k) \quad (11)$$

Since the experimental data obtained by Koumi et al. [22] showed that the recovery time constant of quinidine from the resting state block was 8.4 ± 1.9 s, we assumed that the rate of transition between R and RD was similar to that of the transition between I and ID. Using the equations described above, we calculated the probability of state R (h_∞ curve) in the presence of 10 μM quinidine before and after adding 1 mM salicylate. As illustrated in Fig. 8A,B, the reduction of the K_{dI} value resulted in a shift of the midpoint of the h_∞ curve to the hyperpolarized direction and was accompanied by a reduction of the $K_{d,app}$ values.

Under a K_{dI} of 9.8 μM and a K_{dR} of 30 μM (as depicted by K_{dI} = 9.8 in Fig. 8C,D) the V_h was −89.1 mV, S was 8.6 and $K_{d,app}$ was 22.7 μM, which was very close to the experimental data obtained in the presence of 10 μM quinidine only. Under a K_{dI} of 2.8 μM and a K_{dR} of 30 μM (as depicted by K_{dI} = 2.8 in Fig. 8C,D) the V_h was −96.1 mV, S was 8.6 and $K_{d,app}$ was 11.9 μM, which was very close to the experimental data obtained in the presence of 10 μM quinidine+1 mM salicylate.

Therefore we concluded that salicylate enhanced the quinidine-induced tonic block of Na^+ channels



Scheme 3.

by decreasing the K_{dI} for quinidine from 9.8 μM to 2.8 μM without changing the K_{dR} (30 μM) for quinidine, and resulting in the reduction of $K_{d,app}$ for quinidine. In other words, salicylate could increase the number of drug-bound non-conducting state Na^+ channels through the acceleration of the transition from I to ID by increasing the affinity of quinidine to the inactivated state channel.

This would explain the enhancement of the quinidine-induced phasic block of Na^+ channels by salicylate as well. As phasic block is composed of the activated and/or the inactivated state block, the salicylate-induced increase in the affinity of quinidine to the inactivated state channels would lead to an accumulation of the quinidine-bound non-conducting inactivated channels during the train of stimuli, resulting in an increased phasic block of quinidine.

4.2. *Mechanisms of the enhancing effects of salicylate based on the structure of class 1 antiarrhythmic agents*

The fact that salicylate enhanced quinidine-, aprindine-, and disopyramide-induced tonic and phasic blocks of V_{max}, but not procainamide- or mexiletine-induced tonic and phasic blocks is very interesting. These differences could be related either to their action potential prolonging effect [23,24] or to their chemical structure.

In the present study, salicylate always shortened the action potential duration mainly via inhibition of Ca channels even in the presence of quinidine (as shown in Table 1) as well as in that of the other class 1 antiarrhythmic agents (data not shown). Therefore, changes in the action potential duration

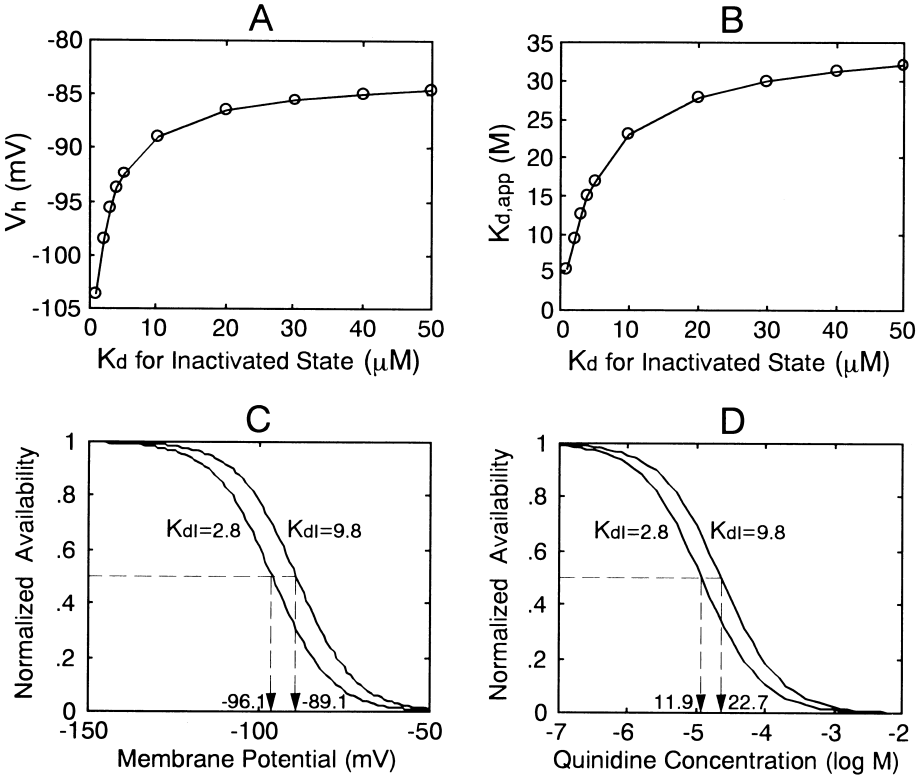


Fig. 8. Prediction of the voltage-dependent availability curve of I_{Na} or concentration dependence of quinidine block of I_{Na} in the presence and absence of salicylate. (A,B) Dependence of V_h (A) and $K_{d,app}$ (B) on K_{dI}. Steady-state probability of state R in Scheme 1 was calculated from Eqs. 2–10 to construct the voltage-dependent availability curve or concentration dependence of quinidine block, and then the V_h or $K_{d,app}$ was determined by a fit with Eq. 1. K_{dI} = 1, 2, 3, 4, 5, 10, 20, 30, 40, 50 μM. $\Delta G_{lc} = \Delta G_{lb} = 32RT$, $\Delta G_{lr} = \Delta G_{li} = 32RT$. (C,D) Voltage-dependent availability (C) and concentration dependence of quinidine block (D) in the absence (K_{dI} = 9.8 μM) or presence (K_{dI} = 2.8 μM) of salicylate. The steady-state probabilities of state R were normalized to the maximum values determined by the fits with Eq. 1. The numbers in the figure represent the mid-potentials in mV (C) or the IC_{50} values in μM (D).

would not explain the heterogeneity of the enhancing effect of salicylate on the phasic block of V_{max} induced by class 1 antiarrhythmic agents.

The receptor site for local anesthetics in nerve Na channels has been suggested to lie within the channel lumen [25], and a drug would thus reach it from the pore or through the lipid bilayer of the cell membrane, i.e., the former is the hydrophilic pathway and the latter is the hydrophobic pathway. The hydrophilic pathway is only used by the charged form of the drug, which is favored by a low molecular weight, while the hydrophobic pathway is only used by the neutral form of the drug, which is favored by its lipid solubility estimated by log P (log of the n-octanol:water partition coefficient) [26–29]. Although the ratio of neutral to charged form of each drug is known to depend on its pK_a, quinidine, aprindine, disopyramide, procainamide, and mexiletine have almost the same pK_a values, that is, 8.6–9.2. Therefore, there is no difference in the proportion of the neutral forms among these antiarrhythmic agents. As the values of log P of quinidine (3.6), aprindine (5.0) and disopyramide (1.8) are higher than those of procainamide (0.8) and mexiletine (1.3) [30], quinidine, aprindine and disopyramide have stronger lipophilicity than procainamide and mexiletine. Consequently, salicylate might specifically enhance the blocking effect of a drug which mainly uses the hydrophobic pathway. It has been reported in nerve that organic acid salts like salicylate or butyrate enhanced the action of local anesthetics, and this was attributed to an acceleration of the adsorption of the local anesthetics to the lipid bilayer and a decreased adsorption ratio without changes in the desorption rate [12]. This information would support the hypothesis that salicylate increased the intracellular concentration of the drug by accelerating the absorption of drugs with stronger lipid solubility to the lipid bilayer, resulting in an enhancement of the tonic and phasic blocks of V_{max} induced by class 1 antiarrhythmic agents.

4.3. Limitations and clinical relevance of the study

Since class 1 antiarrhythmic agents were utilized in the present experiment at concentrations slightly higher than the therapeutic range for patients with arrhythmia (quinidine <9 μM, aprindine <6.7 μM, disopyramide <17.7 μM, procainamide <73.6 μM, and mexiletine <9.3 μM) and higher than the dose commonly prescribed for post-myocardial infarction patients, the clinical relevance of the present work is limited. The dose of salicylate used here was far in excess of the effective levels in cardiac patients as well, although the therapeutic plasma concentration of salicylate for rheumatic disease is approx. 0.625–1.87 mM [31] and these concentrations are large enough to attain the enhancing effect of salicylate on the class 1 antiarrhythmic agent-induced block of V_{max}. However, the salicylate-induced enhancing effects on the blocking action of the class 1 antiarrhythmic agents with high lipid solubility could produce a new area of unidirectional block in the depolarized (diseased) cardiac cells.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (C) of the Ministry of Eduction, Science, Sports and Culture (I.H.) and Health Sciences Research Grants (Research on Pharmaceutical and Medical Safety) of the Ministry of Health and Welfare (I.H.).

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