ACTIONS OF RSD1015 AND RSD1000 ON MACROSCOPIC CURRENTS IN RAT VENTRICULAR MYOCYTES

by

RENDING XU

MD, West China University of Medical Sciences, Chengdu, Sichuan, P.R.China, 1992

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

Department of Pharmacology & Therapeutics

Faculty of Medicine

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

November, 1997

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Department of Pharmacology and Therapeutics

The University of British Columbia
Vancouver, Canada

Date Dec. 8, 1997.

ABSTRACT

Two novel agents RSD1015 and RSD1000, compounds with potent antiarrhythmic effects *in vivo,* have been studied in rat ventricular myocytes using the whole-cell patch clamp technique. Both drugs exhibit potent mixed blockade effects on potassium (I_{to}) and sodium current (I_{Na}), with little or no effects on other currents including I_{K1} and I_{Ca}. The effects of these drugs on different kinetic states of potassium and sodium channels were investigated. The ischemic-selectivity of RSD1015 and RSD1000 was also studied using solutions of varying pH. The rationale for this being that physicochemical properties of compounds, especially their pKa, act as one of the important determinants of ischemia-selective antiarrhythmic activity.

At bath solution of pH 7.4, with extracellular concentrations from 1 to 30 μM, both RSD1015 and RSD1000 reduced the inactivation time constant (τ) of the transient outward potassium current (I_{to}) with an EC_{50} values 1.3 ± 0.4 μM and 5.8 ± 0.7 μM respectively. The voltage dependence of both activation and inactivation of I_{to} and the time course of recovery from current inactivation were not significantly changed by the RSD compounds at concentrations up to 10 μM. The inhibition of I_{to} increased with time during depolarizing pulses, indicating that RSD1015 and RSD1000 interacted with the open state of the channel which can be characterized by k_1 (onset) and k_{-1} (offset) rate constants. From the study, the k_1 and k_{-1} for RSD1015 on I_{to} blockade are $16.3 \pm 3.4 \times 10^6$ M^{-1} s^{-1} and 27.6 ± 4.3 s^{-1}, respectively and the k_d ($k_d=k_{-1}/k_1$) is 1.7 μM. The values of the k_1 and k_{-1} of RSD1000 were $8.1 \pm 1.9 \times 10^6$ M^{-1} s^{-1}, 52.3 ± 3.3 s^{-1}

respectively, the k_d is 6.5 μM. The decreased peak amplitudes of I_{to} by agents are also consistent with rapid open channel blockade.

In addition, RSD1015 and RSD1000 decreased the peak amplitudes of the inward sodium current (I_{Na}) with an EC_{50} of 4.1 ± 0.9 μM and 1.5 ± 0.3 μM, respectively. This action was not accompanied by any change in the voltage dependence of activation, but both agents cause hyperpolarizing shifts in the $V_{1/2}$ of I_{Na} inactivation curve. Use-dependence of I_{Na} was evident in the presence of RSD1015 and RSD1000 with a stimulating frequency of 5 Hz or higher; the onset of the use-dependent block is rapid. The blockade of I_{Na} by these agents indicated that they interacted mainly with the inactivated and activated states of sodium channel.

In an acid solution (pH 6.4), the potency of RSD1015 and RSD1000 on I_{to} blockade did not significantly change; however, the potency for RSD1000 on I_{Na} blockade was significantly enhanced from the EC_{50} of 1.5 ± 0.3 μM (pH 7.4) to 0.5 ± 0.1 μM (pH 6.4). Therefore, RSD1000 has higher ischemia-selectivity compared to RSD1015. Use-dependent blockade of I_{Na} by RSD1000 also significantly increased under acidic conditions (pH 6.4) compared with the effects in normal solution of pH 7.4. This ischemia-selectivity of RSD1000 in acidic conditions is consistent with *in vivo* studies and can provide clinical usefulness, especially in the mechanisms of cardiac arrhythmogenesis.

The ischemic-selectivity of RSD1000 appears to be the function of its pKa value, which causing different percentage of molecule protonated in the solution of pH 7.4 or 6.4. The pH-dependent interaction of the tertiary amine sodium channel blockers has been suggested to be a mechanism of pH modulated actions of RSD1000.

Overall, this study showed that RSD1015 and RSD1000 are potent mixed blockers of I_{to} and I_{Na} in rat ventricular myocytes. These results demonstrated the important mechanisms of antiarrhythmic effects of these two agents.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
ANS	autonomic nervous system
AP	action potential
APD	action potential duration
AS	arrhythmia score
BP	blood pressure
CAST	Cardiac Arrhythmia Suppression Trial
°C	degree Celcius
CNS	central nervous system
ECG	electrocardiogram
ED_{50}	dose of drug producing half-maximal response
ERP	effective refractory period
g	gram
hr	hour
Hz	hertz
i.p.	intraperitoneally
i.v.	intravencous
kg	kilogram
µM	micromolar
mg	milligram
MI	myocardial infarction
min	minute
mL	millimitre

mmHg	millimetres of mercury
mM	millimolar
MRH	Modulated Receptor Hypothesis
msec	millisecond
MW	molecular weight
pH	hydrogen ion concentration
PVC	premature ventricular contraction
sem	standard error of the mean
sec	second
VF	ventricular fibrillation
VT	ventricular tachycardia

ACKNOWLEDGEMENTS

I would like to express my gratitude to my research supervisor, Dr. James G. McLarnon for his encouragement and guidance. I am indebted to Drs. Michael Walker, John Church and Ismail Laher for their participation in my thesis committee, and for their valuable discussions and comments for this thesis. I am also want to thank all the faculty and staff members of the Department of Pharmacology & Therapeutics: Drs. Sutter, Pang, van Breemen, Godin for their encouragement and guidance in both pharmacology and life's philosophy; Elaine, Janelle, George, Christian, Wynne and Maureen for their assistance and help. Thank Dr. Wang, Dale, Michael, Wei and everyone in Lab 222 and 413.

I want to express my sincere gratitude to my grandparents, my parents, my brother and his family, Zhaohua Deng and the rest of my family and all my friends for their continuos support, encouragement and understanding through the worst and the best of my life, especially everyday life.

DEDICATION

Dedicated to my father, mother, and brother for their love and support.

1. INTRODUCTION

1.1. The background of antiarrhythmic drugs

1.1.1. The etiology of arrhythmia

An arrhythmia is an irregularity in rhythm of the heart's beating, due to an abnormality of impulse initiation, conduction, or both (Hoffman and Cranefield, 1964; Hoffman and Rosen, 1981). Despite the availability of a number of antiarrhythmic drugs, arrhythmias, especially ventricular tachyarrhythmia or/and ventricular fibrillation remain the main reasons for the mortality (80-90%) due to sudden cardiac death (Cobb, *et al.,* 1980; Panidis, *et al.,* 1983; Bayes, *et al.*, 1989). Recent statistics indicate that sudden cardiac death accounts for more than 300,000 tatalities yearly in the United States (Segal, *et al.,* 1985; Myerburg and Castellanos, 1992; Myerburg, *et al.*, 1992). Because of the strong association between premature ventricular beats (PVBs) and subsequent mortality, it was hypothesized that sudden coronary death resulted from a electrical event which initiates disorganization of cardiac electrical activity and causes fatal arrhythmias. However, at present there is no study demonstrating that agents which inhibit myocaridal PVBs decrease mortality in post- infarction (May, *et al.*, 1982; Furberg, 1983). Some class I antiarrhythmic drugs may even increase sudden death (IMPACT research group, 1984; Hine, *et al.*, 1989). The Cardiac Arrhythmia Suppression Trial (CAST) study showed that patients with asymptomatic ventricular arrhythmias after acute myocardial infarction (MI) receiving encarinide or flecainide had a 3.64 times increase in arrhythmic death/cardiac arrest compared to matched post-infarction patients with placebo (CAST investigators, 1989). This result has prompted

reevaluation of other trials results and the hypothesis underlying the development of antiarrhythmic agents.

There are almost 3 million potential patients who need to be treated for supraventricular and ventricular arrhythmias in the United States (Ratner, 1990). Although electrical (cardioverter implantor, anti-tachycardia pacemakers) and surgical strategies are available for some selected patients, antiarrhythmic drugs would still be the mainstay of the therapy in the majority of patients. Since the current class I antiarrhythmic drugs (e.g., lidocaine) are ineffective for cardiac arrhythmia therapy, it is necessary to develop new agents with less side effects and high therapeutic efficacy.

1.1.2. Mechanisms of Arrhythmogenesis

Common causes of arrhythmias in humans are myocardial ischemia, myocardial infarction, or reperfusion of a previously ischemic myocardium. For some time it was hypothesized that one or several abnormal electrical accidents were the mechanisms of cardiac rhythm disturbances. Those electrical phenomena include abnormal impulse generation (abnormal automaticity, triggered arrhythmias), delayed or blocked impulse propagation and uni-directional conduction block modulated by re-entry.

Many factors could suppress normal sinus node pacemaker function or enhance suppressed pacemaker automaticity and produce an ectopic beat. For example, ischemia causes depolarization of ventricular tissue, inactivation of sodium channels and reduction of repolarizing potassium currents. If there is a decrease in background potassium conductance, the resting transmembrane potential is reduced from -80 mV to -60 mV or less, initiating a spontaneous impulse (Hoffman and Cranefield, 1964). The overdrive suppression that can occur in normal automatic cells is reduced or even lost

in abnormal automatic cells (Rosen, 1988). Some studies show that the sodium/potassium ATPase, which suppresses ectopic pacemaker sites by hyperpolarizing the membrane in normal conditions, was inhibited in ischemic tissue (Binah and Rosen, 1992).

The repetitive impulse formatted by a propagated or automatic action potential is termed triggered activity (Janse and Wit, 1989) and is dependent on oscillations in membrane potential that follow the action potential. The triggered rhythms occur in two forms: early or delay-afterdepolarizations (labelled EAD and DAD). When an oscillation occurs during repolarization, it is called an early afterdepolarization; if early afterdepolarization reaches a threshold, it can produce a triggered response and induce single or multiple extrasystoles or even ventricular tachycardia (VT) (Cranefield, 1977). The increase of slow inward calcium currents (Zeng and Rudy, 1995; Priori and Corr, 1990) or the reduction of outward potassium flow (Coulombe *et al.*, 1984) were proposed as a basis for the early afterdepolarization development; at present the mechanisms underlying the EAD are not totally clear. When such an oscillation occurs after the membrane has repolarized to its maximum diastolic potential, it is called a delayed afterdepolarization. The mechanism of DAD is also not understood, however, the calcium oscillations from the calcium-loaded cytoplasmic reticulum were suggested to contribute to the process (Binah and Rosen, 1992).

Abnormal impulse conduction could be the result of complete failure of propagation or due to unidirectional block and re-entry of an impulse (Rosen, 1988). Re-entry has been suggested to be one of the major causes of ventricular arrhythmias in ischemic tissues. Several factors would contribute to re-entry under ischemic

conditions: scars or fixed barriers, slow conduction, and abnormal refractoriness. Antiarrhythmic drugs can abolish re-entry arrhythmias by either prolonging refractoriness (Class III compounds) or slowing conduction so that uni-directional block converts to bi-directional block within the depressed region (Varro and Surawicz, 1991; El sherif, 1991).

The ineffect and pro-arrhythmic activity induced by class I agents in the CAST study indicated that the pathological consequences of myocardial ischemia also play important roles in arrhythmia besides the abnormal electrical events, since many class I agents can prevent premature ventricular beat (PVB) very well. Ischemia produces changes in a variety of extra- and intra-cellular processes, for example, increased extracellular potassium is associated with acute ischemia (Harris *et al.*, 1954). Changes in pH, O_2 and CO_2 levels and accumulation of lactate were also reported in ischemic tissue (Case *et al.*, 1979). There are also changes occurring inside the cell as a result of ischemia including a decrease of pH from 7.2 to 6.0 (Garlick *et al.*, 1979) and increases of calcium (Steenbergen *et al.*, 1987) and sodium (Wilde and Kleber, 1986). The intracellular anaerobic metabolism causes accumulation of lactic acid and reduction of pH in the procession of glycolysis for making high energy phosphates. The metabolic substances cannot be washed away from the ischemic area by the small blood flow so that the lactate concentration increases and pH decreases. Intracellular potassium can passively move out of the cell with lactate to maintain the electrical neutrality of the membrane. These changes occur to different degrees in different ischemic cells so that this non-equilibrium may contribute to the delayed conduction and block of conduction in acute ischemia (Botting *et al.*, 1986). The increased

extracellular K^+ depolarized the resting membrane potential and this depolarization is enhanced by the pH and CO_2 changes (Kodama *et al.*, 1984). In turn depolarization causes a decrease in action potential amplitude, a slowing of the maximum rate of depolarization and an increase in refractoriness. The effective refractory period (ERP) is prolonged in acutely ischemic cells and the electrophysiological changes can result in heterogeneous conduction and refractoriness in the ischemic tissue.

1.2. Electrophysiology of the myocardium

In order to understand the mechanisms of antiarrhythmic agents, it is important to determine the electrophysiological properties of the cardium and the character of the different ion channels in the myocytes. The normal myocardiac action potential will be reviewed first in this section, and several important channels which contribute to the action potential and the antiarrhythmic drug mechanisms will be considered.

1.2.1. The cardiac action potential

The cardiac action potential consists of 4 phases in atria, ventricular and Purkinje cells. Phase 0 of the action potential is mainly due to the rapid inward movement of sodium ions. Fast activation of the Na^+ channel is responsible for the upstroke of the action potential in most cardiac cells, but not in the SA and AV nodes. Most of the Na^+ channels undergo fast inactivation in normal situations. Recovery from channel inactivation is relatively slow, so that the sodium current is less pronounced at high frequencies, the shortening of the action potential duration at fast heart rate is at least partly due to the smaller inward sodium currents. Phase 1 repolarization is thought to be due to inactivation of I_{Na}, and the activation of the fast transient outward potassium currents (I_{to}) (Coraboeuf and Carmeleit, 1982). The plateau (Phase 2) is the

main determinant of the cardiac action potential duration; and a good balance exists between the inward and outward currents in this phase (The Sicilian Gambit, 1991). The inward currents include the Ca^{2+} current influx though the L-type calcium channels (Bean, 1985) and the Na^{+}-Ca^{2+} exchange current. In particular, a small portion of Na^{+} channels does not inactivate during early repolarization (Phase 1), and can carry inward sodium "window current" for the entire duration of the plateau which contributes to the action potential plateau duration (Coraboeuf *et al*, 1979; Carmeliet, 1987). The outward currents are carried by several K^{+} channels, a Cl^{-} channel and the active Na^{+}-K^{+} pump ATPase. Phase 3 includes the repolarization time of the action potential and consists of a number of ligand- or voltage- activated potassium channels (Carmeliet, 1993), such as the inward rectifier current for the final repolarization and the resting potential in most cardiac cells (Noble, 1975). Some channels have specific functions in different parts of the heart. For example, in atrial and Purkinje tissue, the acetylcholine-activated channel, $I_{K(ACh)}$, plays a very important role. In ventricule, repolarization is mainly due to the opening of delayed rectifier (I_K) potassium channels (McAllister and Noble, 1966).

1.2.2. The sodium channel

Since sodium current comprises the main portion of the upstroke of the action potential and also contributes to the plateau phase by "window sodium" current, mechanisms of antiarrhythmic agents were initially related to the nature and properties of the cardiac sodium channel. In the classic studies on squid axon (Hodgkin and Huxley, 1952), the concept of channel gating was proposed. A mathematical model of the voltage-dependent opening and closing of the sodium channel was derived as the

basis for the generation of the action potential. The activation gate was termed as "m" and the inactivation gate as "h", where the kinetic properties of the gates were highly dependent on the cell membrane potential. Hodgkin and Huxley suggested positive charges (voltage-sensor) were involved in the current activation and the charge movement was called gating current (Armstrong and Bezanilla, 1973; Defelice, 1993). The fast kinetics make the majority of the gating current flow before the "m" gate of sodium opens (Hille, 1976). This gating current is necessary for the macromolecular protein conformation transitions so that the sodium channel gates can be opened by voltage (Hill, 1989).

The molecular characteristics of the sodium channel has been studied (Catterall, 1986; Stuhmer *et al*, 1989; Patton *et al.*, 1992). The sodium channel is comprised of 1820 amino acid residues and contains four homologous internal domains, each of which has six putative transmembrane segments. The α subunit contains a transmembrane helix S4, consists of several arginine or lysine residues and the positively changed residues within the membrane are suggested to be the voltage sensors. The intracellular linkers in segment S4 are involved in the voltage-sensing mechanism for activation of the channel, and the regions between repeats III and IV are responsible for the fast inactivation of channels (Goldin, 1993; Catterall, 1995). The four to six charges within the channel molecule, which move from extra- to intracellular membrane at the channel opening, contributes to the gating current (Noda *et al.*, 1984).

The Na^+ channel was proposed to have three states (section 1.4.1.1). Activation of the sodium channel occurs rapidly and is dependent on depolarization (Hille, 1984). Activation thresholds are normally at -60 to -70 mV and the rate of activation increases

with membrane depolarization (Hodgkin and Huxley, 1952). When the cell membrane depolarizes, the voltage-dependent "m" gate opens and the Na^+ permeability markedly increases (Catterall, 1986; Mitsuiye and Noma, 1992). The activation kinetics are not changed by local anaesthetic or antiarrhythmic agents (Narahashi, 1992), but altered by some plant alkaloids, insecticides and sea anemone toxins, e.g. veratridine promotes activation mechanisms at less depolarized levels.

The Na^+ current permeability through the "m" gate is transient and it decreases to the base-line level after 1 msec (Catterall, 1986). The rate of inactivation increases with an increase in the rate of depolarization. Inactivation also occurs with a time lag which suggested that channels must be open before they could inactivate (Armstrong, 1971). The rate of inactivation was described by a bi-exponential function suggesting that the channels may have two inactivated components: fast and slow (Chiu, 1977; Fozzard *et al.*, 1985). The inactivation " h" gate could be selectively destroyed by the intracellular application of protease or piperazinyl-indole derivative DPI 201-106 (Armstrong *et al.*, 1973; Wang *et al.*, 1990) and the rate of inactivation was slowed by some toxins such as batrachotoxin and aconitine. Inactivation is complete with the return of the membrane potential to its resting level by the repolarizing phase of the action potential.

The interaction of local anaesthetics and antiarrhythmic drugs with the sodium channel in a voltage, time and state-dependent manner was described by the Modulated Receptor Hypothesis (Hille, 1977; Hondeghem and Katzung, 1977), and is discussed in detail below (section 1.4.1.1).

1.2.3. The potassium channels

1.2.3.1. The heterogeneity of potassium channels

Potassium channels participate in establishing the resting membrane potential and contribute to cell repolarization. At least 7 different potassium channels contribute to repolarization and the configuration of phase 3 of the action potential in cardiac tissue (Colatsky and Follmer, 1989; Carmeleit, 1993). Under normal conditions four to five potassium currents participate in action potential repolarization; they include the transient outward current (I_{to}) during the initial phase of repolarization, the delayed potassium current (I_K) in the plateau phase and the inward rectifier potassium current (I_{K1}) during the final repolarization. The acetylcholine-activated channel, $I_{K(ACh)}$, plays an important physiological role in the sinoatrial and atrioventricular nodes and the atrium and the Purkinje system but not in ventricular myocytes in mammals. In pathologic conditions three other K^+ currents can be activated; the ATP-dependent K^+ current, $I_{K(ATP)}$; the Na^+-dependent K^+ current, $I_{K(Na)}$; and the fatty acid-activated K^+ channel, $I_{K(FA)}$. The potassium channels are heterogeneous with regard to gating kinetics, permeability properties and the modulation by neurotransmitter and ions (Hume *et al.*, 1990).

The heterogeneity of potassium channels also exists among different species and regions of the heart. Important electrophysiological distinctions were found in the epicardial and endocardial tissue in many species (Wei *et al.*, 1993). The density of some K^+ channels also differs throughout the heart. For example, I_{K1}, whose density is highest in the Purkinje system and in the ventricule and is practically absent in the

sinoatrial and atrioventricular nodes. Even cells from the same area of the heart may have different kind of currents; for example three different types of action potential duration shape have been established in the rat ventricle cells (Watanabe *et al.*, 1983).

The presence of multiple over-lapping currents in cardiac myocytes complicates the study of individual K^+ channels. Thus the traditional methods using a combination of holding potential modulation, ion substitution, and pharmacological agents to obtain the current of interest have limitations. The rapid advances of molecular cloning and cDNA technologies have allowed the study of a single cloned isoform in the expression system, although this approach has problems in correlating cloned channels with endogenous currents. The identification of the *Shaker* locus has given insights to the molecular structure of potassium channels for the first time (Temple *et al.*, 1987; Pongs *et al.*, 1988). There is strong similarities among the primary amino acid sequences of voltage dependent K^+, Ca^{2+} and Na^+ channels. The voltage-gated K^+ from *Shaker* has a molecular weight of 70,200, this protein is a basic repeat of six or seven hydrophobic membrane-spanning domains of about 20 residues. The fourth domain is an arginine- and lysine-rich region (the S4 domain) and is thought to be the putative voltage sensor (Papazian *et al.*, 1991; Liman *et al.*, 1991). This single repeat was believed to assemble in a tetrameric structure around a central pore (Mackinnon, 1991). Recently, the molecular structures of the family of inward rectifying K^+ channels were described (Nichols *et al.*, 1996), which are similar to that of the outward K^+ channels.

These potassium currents (I_K, I_{to} and I_{K1}) which are very important parts of heterogeneity of potassium channels will be discussed further. In some species, like

guinea pig and rabbit, mainly I_K play major roles in action potential. In rat, I_{to} is the main repolarizing current.

1.2.3.2. Delayed rectifier potassium currents (I_K)

Cardiac delayed rectifier K^+ currents (I_K) play a major role in repolarization following the depolarizing action potential. I_K channels activate in response to a positive shift in membrane potential, but after a distinct and brief delay. These currents do not inactivate appreciable and exhibit a property referred to as "rectification", since the slope conductance either increases or decreases as the membrane is depolarized. At least three distinct types of I_K channels have been identified in isolated cardiac cells based on differences in rate of activation, rectification properties, and pharmacology using whole-cell voltage clamp techniques, I_{Ks}, I_{Kr} and I_{RAK}.

A slowly activating, outwardly rectifying I_{Ks} and a rapidly activating, inwardly rectifying I_{Kr} have been shown in guinea pig atrial and ventricular myocytes (Sanguinetti and Jurkiewicz, 1991). The properties of I_{Ks} and I_{Kr} have been described (Sanguinetti and Jurkiewicz, 1990; Horie *et al.*, 1990; Sanguinetti *et al.*, 1991) and are summarized in Table 1. The expression of the two types of channels is species-dependent, rabbit nodal cells have only a minor I_{Ks} component, but the only measurable I_K in guinea pig nodal cells had similar properties to those of I_{Ks} but not I_{Kr} (Anumonwo *et al.*, 1992). I_K channels with properties consistent with I_{Ks} and I_{Kr} have been cloned and expressed (Sanguinetti and Jurkiewicz, 1990; Honore *et al.*, 1991).

TABLE 1 Comparison of I_{Ks} and I_{Kr}

	I_{Ks}	I_{Kr}
τ of activation (at 0 mV)	400 msec	50 msec
τ of deactivation (at -40 mV)	400 msec	170 msec
Midpoint of activation	20 mV	-22 mV
Slope factor of activation (*K*)	12.7 mV	7.5 mV
Inward rectification	slight	marked
Single-channel conductance (150 mM K^+)	< 1-3 pS	10 pS
Activation by β-agonists (1 μM isoproterenol)	Yes	No
Block by dofetilide	No	Yes
Block by 10 μM La^{3+}	No	Yes

Rat atrial cells have an outward rectifier I_K current with very rapid kinetics of activation and very slow inactivation (I_{RAK}) (Boyle and Nerbonne, 1991). I_{RAK} activates with a time constant of 2 msec at +40 mV and has a half-point for activation of -1.5 mV with a slope factor (*K*) of 13.7 mV. The midpoint for steady-state slow inactivation is -41 mV (*K*=9.4 mV), the properties of inactivation distinguish I_{RAK} from other I_{Ks}, since they do not inactivate or only do so at much more positive membrane potentials. I_{RAK} is blocked by 4-aminopyridine, Ba^{2+} and TEA. A similar current has been described in rat ventricular cells and has been expressed in *Xenopus* oocytes (Paulmichl *et al.*, 1991; Aplon and Nerbonne, 1991).

1.2.3.3. The transient outward potassium current (I_{to})

The transient outward potassium current (I_{to}) rapidly activates in a voltage-dependently manner and then inactivates with little or no dependence on potential (Coraboeuf and Carmeleit, 1982). I_{to} starts to activate at membrane potentials more positive than -30 mV, reaches its peak in 3 msec with potential positive to +10 mV, then decays to a non-zero steady-state (Apkon and Nerbonne, 1991; Campbell *et al.*, 1990). The time course of I_{to} inactivation is voltage insensitive and much faster than that of the delayed rectifier current (I_K); has steady-state inactivation at membrane potential positive to -50 mV which is much higher than that of I_K. The reversal potential of I_{to} is -75 mV with 4 mM and 135 mM KCl out- and in-side the cell, respectively. This reversal potential is quite close to the calculated potassium equilibrium potential (E_K) suggesting its high potassium selectivity.

From the kinetics and pharmacological characteristics, the I_{to} current has been suggested as composed of a purely calcium-independent voltage-activated and a small calcium-activated component (Coraboeuf and Carmeleit, 1982, Escande *et al*, 1987), the latter component has been shown to be a Cl^- and not a K^+ current. Only the calcium-independent portion of currents which is sensitive to 4- aminopyridine (4-AP) blockade was considered as I_{to} (Apkon and Nerbonne, 1991) and studied in our experiments.

Because of the fast activation, the I_{to} plays an important role during the early repolarization of spike potential (Katritsis and Camm, 1993). I_{to} is a quite common current which could be found in a wide variety of species, such as ferret, rat and human (Campbell *et al.*, 1995). The differences of channel distribution results in a variable action potential shape in various species or regions of the heart.

1.2.3.4.Inward rectifier potassium current (I_{K1})

The inward rectifier I_{K1} channel is present in high density in cardiac atrial, ventricular and Purkinje cells in the heart (Heidbuchel *et al.*, 1990) but is absent in the pacemaker cells in the sinoatrial and atrioventricular nodes (Irisawa *et al.*, 1987). I_{K1} is the current that sets the resting potential and modulates the final phase of repolarization of the action potential (Shimoni *et al.*, 1992; Ibarra *et al.*, 1991). The current is characterized by a pronounced inward rectification and a time-dependent activation on hyperpolarization (Kurachi, 1985). In myocytes, hyperpolarization rapidly activates I_{K1} and then the current slowly declines; the inactivation of the current was found to be largely due to voltage-dependent block of the channel by external Na^+

(Biermans *et al.*, 1989; Harvey and Ten Eick, 1989). The current-voltage relationship showed a negative slope conductance for potentials more positive than -65 mV and the channels only pass current at membrane potentials negative to -20 mV (Shimoni *et al.*, 1992). The I_{K1} current is potassium-selective and is blocked by external Ba^{2+} and Cs^{+} (Shah *et al.*, 1987; Mitra and Morad, 1991). The external potassium concentration influences the rectification of I_{K1} (Matsuda, 1991; Pennefather *et al.*, 1992) and the conductance of I_{K1} depends approximately on the square root of the external potassium concentration (Sakmann and Trube, 1984). The mechanisms of channel rectification have been studied in detail, it appears that the block of the open channel by intracellular magnesium causes a fast rectification of the channel (Vandenberg, 1987; Matsuda, 1991) and channel gating contributes to a slower process (Kurachi, 1985). Some agents, such as RP58866 and RP62719, possess antiarrhythmic and antifibrillatory potential by blocking the inward rectifying potassium current I_{K1} and causing the prolongation of both the action potential duration and refractory periods (Escande *et al.*, 1992; Rees and Curtis, 1993).

1.2.3.5. The potassium channels in human myocytes

Only a few of potassium channels have been characterized in human atrium: the inward rectifier I_{K1}, the delayed rectifier (I_K), the acetylcholine-activated inwardly rectifying K^{+} current ($I_{K(ACh)}$), the ATP-sensitive potassium current ($I_{K(ATP)}$) and two early transient outward currents (I_{to}), the long-lasting 4-AP sensitive outward current (I_{lo}) and the brief caffeine sensitive current (I_{bo}).

The existence of two transient outward currents in human atrial cells was reported by several studies (Escande *et al.*, 1985; 1987). When isolated myocytes were depolarized from a holding potential, a transient outward current (I_{to}) developed. Pharmacological dissection of this current identified two components: a long-lasting outward current (I_{lo}) inhibited by 4-AP and a brief caffeine-sensitive transient outward current (I_{bo}). An other study demonstrated that the large time- and voltage-dependent I_{to} exhibits a time- and voltage-dependent reactivation that can explain the changes in action potential shapes during different stimulation frequency (Shibata *et al.*, 1989). These results suggest a prominent role for I_{to} in determining the action potential configuration/duration and the frequency-dependent effects on the repolarization in human atrium. I_{to} was also recorded in human ventricular myocytes and its properties were consistent with those in other species.

Although some studies suggested that the delayed rectifier outward current (I_K) does not have an important role in human atrial repolarization (Heidbuchel *et al.*, 1987), recent experiments in physiological temperature (36°C), it has demonstrated the presence of I_K in about two thirds of myocytes isolated from human right atrium (Wang *et al.*, 1993). This current has all the characteristics previously described: time and voltage dependence, kinetics, K^+ selectivity and inward rectification. It was also suggested that the complex kinetics of I_K might be related to the existence of two components similar to I_{Kr} and I_{Ks}. Since I_K is present in human atrium and it is likely to have role in phase 3 and in the refractory period duration, then I_K blockade could increase atrial action potential duration, hinder the deleterious effect of action potential morphology and duration variability, thus prevent re-entrant atrial arrhythmias.

A macroscopic inward rectifying K^+ current has been described in human atrial cells and features similar properties to the I_{K1} previously observed (Escande *et al.*, 1987; Heidbuchel *et al.*, 1987; Shibata *et al.*, 1989). I_{K1} recorded from human atrial cells has a clear inward rectification but no negative slope conductance. Single-channel experiments studied the kinetic and the conductance properties of I_{K1} (Heidbuchel, 1990). It suggested that the contribution of I_{K1} to the basal potassium conductance was quite high, and the resting potassium conductance in human atrium is basically due to I_{K1} channels.

1.3. The classification of current antiarrhythmic drugs

1.3.1. The Vaughan Williams classification

The present classification of antiarrhythmic drugs was first developed by Singh and Vaughan Williams in 1970's (Singh and Vaughan Williams, 1970a) and has become widely accepted. They categorised the antiarrhythmic drugs into four distinct groups according to their electrophysiological actions. Class I agents were local anaesthetics which reduced the maximum rate of rise of action potential depolarisation (V_{max}) by mainly reducing sodium currents in heart cells. Class II agents reduced sympathetic nervous system effects on the heart tissue - the β blockers. Prolongation of action potential duration (APD) and increased refractoriness in cardium by potassium-channel blockade are the mechanisms of the Class III agents. The calcium channel blockers, such as verapamil, comprise the fourth class of antiarrhythmic drugs (Singh and Vaughan Williams, 1972; Vaughan Williams, 1970, 1984a).

The classification of antiarrhythmic drugs is neither complete nor absolute. A fifth class of antiarrhythmic agents which are bradycardic has been suggested,

including alinidine which acts to depress depolarization and increase the threshold potential for spontaneous depolarization in pacemaker cells (Kobinger and Lille, 1987). In addition, a fourth subclass to the Class I category, to describe the electrophysiological properties of the antiarrhythmic agent transcainide, has been suggested (Bennett *et al* 1987). For some drugs, such as amiodarone, the antiarrhythmic properties are not limited to only one group. Amiodarone depresses the fast sodium channel (Mason *et al* 1984), inhibits sympathetic activity (Charlier *et al.*, 1968, 1969), blocks the L-type calcium channel (Nishimura *et al.*, 1989), even though it was initially considered to be a class III antiarrhythmic agent.

Although the cardiac electrophysiological actions of antiarrhythmic agents have been extensively studied and are the basis for the Vaughan Williams classification system, the mechanisms of actions of most compounds are far from clear. An important point is that antiarrhythmic classification was based on experiments on cardiac tissue under normal conditions, so that it would be difficult to extrapolate results to those under ischemic conditions (Brugada, 1990). The choice of an antiarrhythmic agent for patients often remains empirical, and becomes difficult since the same antiarrhythmic agent could have different effects depending on other factors such as the electrolyte disturbances, the degree of ischemia and the rate of heart beat, *etc.* (Brugada, 1990; David *et al* 1986; Szekeres, 1986). The antiarrhythmic therapy may result in not only failure treatment but also aggravating the arrhythmia or generating a new arrhythmia (Zipes, 1987).

1.3.2. Subclassification of class I antiarrhythmic drugs

Because of the important differences among Class I antiarrhythmic drugs, attempts have been made to subclassify them into homogeneous subgroups. Such subclassification would provided additional relevant information for experimental and clinical purposes. Two different subclassifications are discussed here, the Harrison's classification was based on the different effects of drugs on channels and ECG, the Campbell's subclassification emphasized the kinetics of the agents.

1.3.2.1. Harrison's subclassification

In the studies of Hoffman and Bigger (1971), it was proposed that Class I drugs included two groups of agents with distinct properties. The Vaughan Williams classification was modified by subdividing Class I agents into Ia and Ib (Singh and Hauswirth, 1974) and adding a third sodium channel blockade group Class Ic (Harrison, 1981). This Harrison subclassification was primarily based on the different effects of drugs on conduction in the specialised conducting tissue, and on ventricular refractoriness and repolarization. Class Ia agents are moderate to marked sodium-channel blockers in normal cardiac tissue and also block several types of potassium channels. They widen the QRS duration, reduced V_{max}, slow conduction at high drug concentrations, prolong the QT interval and widen the AP; examples included quinidine, procainamide and disopyramide. Class Ib agents are most effective in ischemic or partially depolarized cardiac tissue, they exhibit mild to moderate sodium-channel blockade but have little effect on refractoriness, since there is essentially no blockade of potassium channels, but they shorten the QT interval and APD and

elevated fibrillation threshold; lidocaine, phenytoin, mexiletine and tocainide are the prototypes. Class Ic agents, such as, encainide, flecainide and propafenone, produce small changes in refractoriness by blocking outward-rectifying potassium channels, and they are marked sodium-channel blockers in both normal and ischemic myocardium.

However, this subclassification is not absolute. There are important differences among the members of the same subgroup, and one drug may belong to different subgroups if the situation changes (Vaughan Williams, 1984; Nattel, 1991). Controversy also exists in some classic class I drugs like quinidine and procainamide which prolong refractoriness by mechanisms unrelated to sodium channel blockade at high drug concentration.

1.3.2.2. Campbell's subclassification

According to the rates of onset and offset of the use-dependent block (UDB) of Class I antiarrhythmic drugs, they could also be subclassified as fast, slow and intermediate kinetics in Campbell's subclassification, which corresponded to Harrison's subclassification Ib, Ic and Ia, respectively. Three agents, encainide, flecainide and lorcainide, were studied for the kinetics of onset and rate dependent depression of the maximum rate of depolarization (V_{max} or MRD) *in vitro* (Campbell, 1983a, 1983b). The rates of V_{max} inhibition by these three agents were much slower than those of other drugs (Campbell, 1983b). The speeds of V_{max} decrease to new plateau level were used to separate the class I agents into Ib, Ia and Ic subgroups (Campbell, 1983b).

In the Campbell subgroup, Class Ib agents (e.g. lidocaine, tocainide and mexiletine) have fast kinetics and bind very rapidly to Na^+ channel; a steady-state level

of use-dependent block can be achieved in less than five action potentials. They also unbind quickly during repolarization (τ < 0.5 s), thus at normal heart rates, the rapid offset kinetics permit the complete recovery from block by the time of the next action potential (Hondeghem *et al.*, 1984; Tamargo *et al.*, 1989; Campbell, 1983). The increased refractoriness is determined by the drug's ability to produce an additional inhibition of sodium channels in response to a sudden increase in heart rate (Campbell, 1983) so that the onset kinetics of use-dependent blockade contribute to the effects of antiarrhythmic agents on the atrial and ventricular effective refractory periods (ERP) (Vaughan, 1984; Harrison, 1985). Because of the fast onset and offset, subgroup Ib drugs shorten the action potential duration (APD) but prolong the ERP relative to the APD, they can selectively inhibit conduction and prolong the QRS duration at fast heart rates (during tachycardia) or early premature beats, but have minimal effects at normal heart rates (Valenzuela *et al.*, 1989; Delpon *et al* ., 1991). This confers selectivity of Ib agents for high frequency arrhythmias (Nattel and Zeng, 1984; Varro *et al.*, 1985) but they are less effective than subgroup Ia and Ic agents against slower tachycardias or later PVBs.

Subgroup Ia drugs (quinidine, disopyramide, procainamide) interact with the sodium channel slowly such that it takes about 5-20 action potentials before a steady-state level of use-dependent block could be achieved. The recovery from block appears quite slow (τ=1 to 5 s) (Hondeghem *et al.*, 1984; Tamargo *et al.*, 1989; Campbell, 1983). Because of their slow onset of block, subgroup Ia drugs produce small or moderate increases in the ERP relative to APD. So these agents could have marked effect on the conduction and QRS at high heart rate and could selectively

depress PVBs (Pallandi and Campbell, 1988) even though they could decrease the conduction and prolong the QRS duration at normal sinus rhythm.

Subgroup Ic drugs (encainide, flecainide and lorcainide) bind very slowly to the Na channel (more that 20 beats to reach the use-dependent steady-state level) and unbind from the receptor very slowly ($\tau > 8$ s). These kinetic properties indicates drugs still bind to the channel when the next heart beat starts (Valenzuela *et al.*, 1989; Delpon *et al.* , 1991). Thus, these agents may be more potent than other subgroups since their longer offset kinetics will produce a steadily accumulating depression of V_{max} without significant recovery from diastole (Varro *et al.*, 1985). However, this subgroup are also more toxic since they will decrease the conduction and prolong the QRS at normal heart rates and cause myocardial depression (Schlepper, 1989); such actions could cause proarrhythmia because slow conduction may facilitate or aggravate re-entrant arrhythmias (Hondeghem, 1987).

1.3.3. Subclassification of Class III antiarrhythmic agents

Class III antiarrhythmic agents are defined as antiarrhythmic drugs that act primarily by prolonging the action potential duration. The lengthening of action potential duration can be achieved by reduction in outward currents or increase in inward currents. Thus, Class III agents are not necessarily restricted to potassium channel blockers. Several sodium- and calcium- channels activators can lengthen the action potential duration, for example, DP1201-106, a sodium-channel activator, has been shown to produce class III effects at slow heart rates (Mortensen *et al.*, 1990). However, the typical class III drugs act on potassium channels.

The heterogeneity of potassium channels provides a variety of possibilities for the development of class III antiarrhythmic agents. Besides the normal Class III agents, which demonstrate effects on the physiological important K^+ currents, the agents effects on pathologic K^+ channels could be antiarrhythmic. For example, blockers of ATP-dependent K^+ currents could reduce the incidence of early arrhythmias in ischemic models. ATP-K^+ channel openers (e.g., cromakalim) could also have potential antiarrhythmic effects (Liu *et al.*, 1988), but it is difficult to determine the therapeutic potential of K^+ channels openers in the heart because of drug high selectivity for vascular smooth muscle over heart.

Some class III antiarrhythmic agents tend to be quite non-specific whereas others demonstrate good selectivity in their potassium channel blocking effects and it would be possible to separate them by their specificity in blocking cardiac potassium channels (Colatsky *et al.*, 1990). At present, selectivity of potassium channel blockade provides no particular advantage in antiarrhythmic efficacy or proarrhythmic potential of a drug (Hondeghem and Snyders, 1990). Class III antiarrhythmic agents could also be classified into two subclassifications according to the effects of compounds on action potential duration at different heart rates: Class IIIa, which primarily lengthen action potential duration at excessive heart rate acceleration, and Class IIIb, whose effects on action potential duration length are most prominent during bradycardia (Hondeghem, 1992; 1994). However, this classification has not been widely accepted, since most of class III agents would be defined as class IIIb and be proarrhythmic (e.g., torsade de pointes).

1.4. Mechanism of antiarrhythmic drugs

1.4.1. Class I antiarrhythmic drugs

1.4.1.1. The sodium channel states hypothesis

Sodium channels can be separated into three kinetic states: 1) closed state (R), available to be opened by cell depolarization at potentials near the resting potential; 2) activated-open state (A or O state), permits passage of Na^+ ions though the channel; 3) closed, and not available to be opened, i.e., inactivated state (I) (Fozzard *et al.*, 1985). During diastole, Na^+ channels are in the closed-resting state (R) at negative membrane potentials, but available to be activated-open during a depolarization. During the upstroke, the channels are activated and open then Na permeability rapidly decreases during the plateau. In the plateau phase, the inactivated state (I) predominates. Some factors which depolarize the cardiac tissue will also shift the sodium channel to the I states including ischemia and hyperkalemia. Inactivated channels (I) are not available for opening without first returning to the resting state (R) with repolarization. During the cardiac diastole, a gradual recovery from inactivation occurs and the rate of recovery from the inactivated state is a time- and voltage-dependent process (Chen, *et al.*, 1975). Under normal conditions the reactivation is complete in about 100 ms during diastole so that Na channels are activated and inactivated during each cardiac action potential.

In the presence of antiarrhythmic agents, two types of sodium current inhibition can occur during a train of pulses (Hondeghem and Katzung, 1984; Tamargo *et al*1989). Tonic block indicates the reduction of amplitude of sodium current during the

first depolarization after a long rest period at negative rest potentials. Additional reduction in Na^+ currents that is caused by class I antiarrhythmic agents during repetitive stimulation until a new steady state is obtained is referred to as use-dependent block. The block of class I antiarrhythmic agents was also shown to be voltage-dependent. It was suggested the drug-bound channel complexes favoured the inactived state of the channel by a hyperpolarizing shift of the voltage-dependence of inactivation so that the potency of class I agents, such as lidocaine, was higher at more negative membrane potentials (Werdmann, 1955; Bean *et al.*, 1983).

The voltage- and use-dependent blockade of Na^+ channel by class I agents have been modelled using schemes such as Strichartz-Courtney and Modulated Receptor Hypothesis (MRH). The MRH has been widely tested with many drugs after its development (Hille, 1977; Hondeghem and Katzung, 1977, 1984; Hondeghem, 1989). In this model (Figure 1), a series of equations were developed which defined binding parameters for each state of the channel (R, O, I). This model proposes the following: 1) sodium channels change states (rested, activated-open and inactivated) in a voltage-dependent manner; 2) drug-bound channels could not conduct sodium current; 3) antiarrhythmic agents bind to a specific site on or near the Na channel and can associate or dissociate from each state; 4) the rate of binding and unbinding of agent to their site of action are modulated by the conformational state of the channel. Drug-bound channels exhibit time- and voltage-dependent transitions between the RD, OD and ID states. The affinity of the drug and channel state interactions can be measured from the ratio of association and dissociation rate constants.

Figure 1 Modulated receptor model of the sodium channel. The sodium channel may exist in the resting (R), open (O), and inactivated (I) states. Each state of the channel can bind drug with a characterisitic rate constant to form the drug-associated states RD, OD and ID. Drug-associated channels can make the usual transitions between the various conductance states.

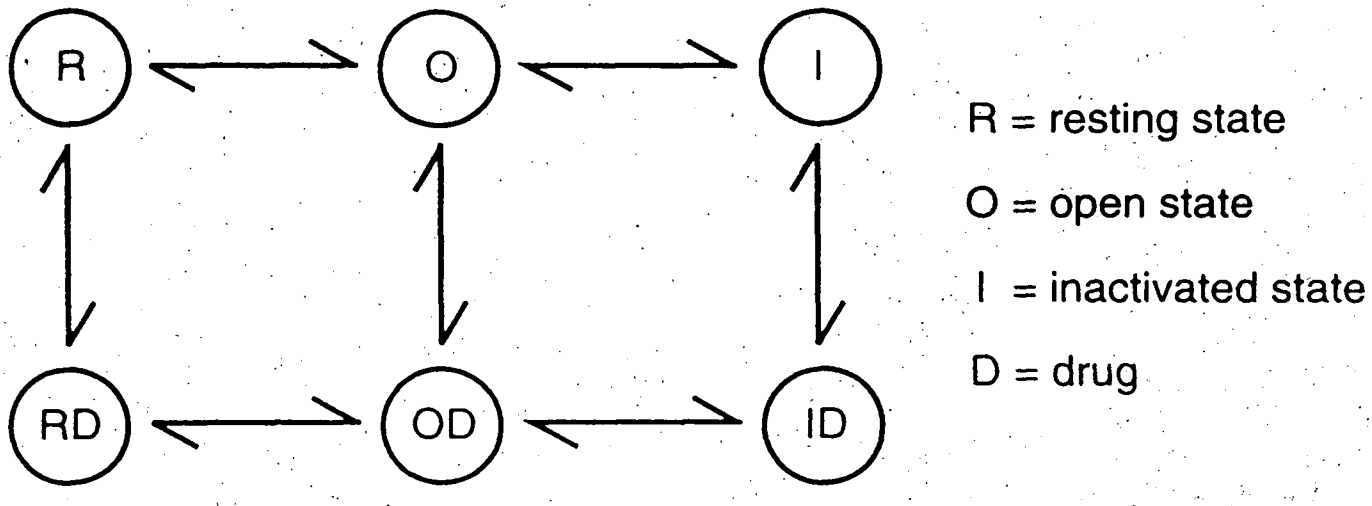

R
O
I
RD
OD
ID
R = resting state
O = open state
I = inactivated state
D = drug

Figure 1

1.4.1.2. State-dependent blockade of class I antiarrhythmic drugs

The channel state selectivity of blockade was studied in guinea-pig cardiac muscles (Kodama *et al*, 1986, 1987). In these studies, class Ia agents block I_{Na} mainly during the upstroke of the action potential (activated channel block), whereas the Ib agents have their main effects during the plateau phase. In general, it has been suggested that class Ia and Ic drugs bind to the activated state of sodium channel and Ib agents exhibit a higher affinity for the inactivated state of the channel (Hondeghem, 1984; Kodama and Toyama, 1988). However, many of the agents are mixed activated and inactivated blockers (Kodama, *et al*, 1987).

Activated channel blockers shown a marked increase of block with each action potential upstroke but little block is shown after the channel is closed (Snyders *et al.*, 1992; Hondeghem, 1990). Open channel blockers (e.g. flecainide, quinidine) decrease single channel mean open time (Kohlhardt, *et al.*, 1989; Valenzuela, *et al.*, 1991). These agents are more effective at high stimulating rates with short action potentials (e.g. atria flutter) but less effective in the depolarized tissues. Since they are not bound to the inactivated state, these drugs are not influenced by the action potential duration for their inhibition effects.

The primary effects of the important class I antiarrhythmic agents like lidocaine are to prolong recovery from inactivation, probably by binding directly to the inactivation gating mechanism, causing a negative potential shift in voltage-dependence and a slow recovery from inactivation (Hondeghem and Katzung, 1977; Courtney, 1980). When the frequency of stimulation increases, the diastolic time for reactivation from inactivation to rested state is shortened, and the inactivated, non-conducting channels

were progressly increased. Thus inactivated-state Na^+ channel blockers will produce only minor effects at normal heart rates but inhibit the sodium current to a great degree during fast heart beat (tachycardia). The effectiveness of these inactivated channel blockers could also be greater in the some situations with more inactivated channels existing (Tamargo *et al.*, 1992), such as in ischemia and hyperkalemia conditions, which cause cardiac tissue to depolarize and more rested channels to be in the inactivated state; in ventricular muscle and Purkinje fibres with long action potentials in which sodium channels are at the inactivated state during most of the action potential duration; under some circumstances (e.g., together with Class III antiarrhythmic agents), which the plateau phase of the action potential duration is prolonged and the inactivated channels increased.

Studies have also been done to investigate the importance of physio-chemical properties in the kinetics of the actions of class I agents (Campbell, 1983c; Courtney, 1980). The properties include molecular weight, lipophilicity and the spatial dimensions of the drug molecule. The proportion of charged-hydrophilic and uncharged-lipophilic moieties varies with pH. The charged drug moiety form will preferentially access the channel receptor through a hydrophilic pathway which is dependent on the activated-open state of channel and cause pronounced use-dependent blockade (Strichartz, 1973; Hille, 1977). Uncharged molecules readily pass through the lipid layer which is not affected by the channel state. Membrane depolarization, acidosis and hyperkalemia favour the interaction of the charged molecular form with its receptor, which may have greater sodium channel blocking effects than that in non-ischemic tissue.

1.4.2. Class III antiarrhythmic agents

1.4.2.1. Pharmacology of different potassium channels

At present, class III antiarrhythmic agents are increasingly favoured to treat patients with serious tachycardias. Since an increase in action potential duration and a prolongation of the effective refractory period without slowing impulse conduction may provide an antiarrhythmic and antifibrillatory action. Many antiarrhythmic drugs showed blockade of multiple potassium channels, such as quinidine blocks I_{to} and IK (Clark *et al.*, 1995), RP62719 blocks I_{K1} and I_{to} (Escande *et al.*, 1992; McLarnon and Xu, 1995). However, it has been suggested that proarrhythmic activity of K channel blockers may be more likely to occur with less selective agents. Thus, new and more selective K channel blocker have been developed, such as E-4031 and dofetilide which effect the rapidly activating component of delayed rectification (I_{Kr}) (Sanguinetti and Jurkiewicz, 1990; Clatsky *et al.*, 1990). Selective inhibition of I_{to} was also suggested to possess specific usefulness for the suppression of supraventricular arrhythmias (Colatsky *et al.*, 1990; Hondeghem, 1992). Table II lists some of the available potassium channel blockers and the reported blocking potencies.

1.4.2.2. The drug interactions with potassium channels

The modulated receptor hypothesis of the interaction of sodium channel blockers with their receptors was time- and voltage-dependent as described in section 1.4.1.1. The same time- and voltage-dependent block of ion channels could also applied to potassium currents. The studies on the mechanism by which TEA^+ blocks the delayed rectifier potassium current indicated that the TEA blocking site was within

the potassium channel pore, accessible only from inside the cell and when the channel is open (Amstrong, 1971). Similar result have been obtained for block of myocardial potassium channels by class III agents, E-4031 (Follmer and Colatsky, 1990).

It has been demonstrated that most calss III antiarrhythmic agents (e.g., sotalol, NAPA and melperone) exhibit reverse use-dependence, which refers to the fact that the drug block effects become less marked with increased use (Strauss, *et al.*, 1970; Dangman and Miura, 1989; Refsum *et al.*, 1981; Roden *et al.*, 1988). The modulated receptor model of potassium channel blockade was used to explain reverse use-dependence (Hondeghem and Snyders, 1990). These drugs primarily reduce the outward current at negative membrane potentials and the block becomes less pronounced during depolarization, thus current blockade increased during diastole (phase 4) and declined during the plateau, resulting in less block with increasing use. This effect is contrasted with the use-dependent block of sodium channels that developed primarily during depolarization (upstroke of the action potential) and diminished during diastole. This phenomenon has been explained by assuming selective binding of the drug to the closed state of the K^+ channel, which was favoured for lipophilic drugs, whereas open-state blockade involves a hydrophilic pathway. Other explanations for reverse use-dependence include the possibility that I_K plays a lesser role in repolarization at faster rates and concomitant increase of other repolarizing currents.

TABLE 2 Different potassium channel blockers

CHANNEL	DRUG	IC_{50}*
I_{K1}	RP58866 and Terikalant	< 3 μM
	Propafenone	7 μM
	LY97241, LY97119	10 μM
	Quinidine	> 10 μM
I_{to}	Clofilium	0.5 μM
	Tedisamil	4.5 μM
	Propafenone	6 μM
	Quinidine	10 μM
	Bupivacaine	23 μM
	Terikalant	17 μM
	Amildarone	10 μM
I_{Ks}	Tedisamil	2.5 μM
	Quinidine	> 10 μM
	Clofilium	50 μM
	Dofetilide	< 0.1 μM
I_{Kr}	Clofilium	1 μM
	Propafenone	0.8 μM
	Quinidine	2 μM
	Flecainide	< 10 μM

* The values of IC_{50} are come from previous studies, and subjected to variations with experimental conditions and voltage clamp protocols.

In order to describe whether compounds are antiarrhythmic by lengthening of action potential duration during tachycardia or proarrhythmic by lengthening action potential duration at normal heart rates, a ratio of lengthening of action potential duration at a fast heart rate to that at a normal heart rate has been defined as the anti- to pro-arrhythmic ratio. The higher the ratio, the greater the antiarrhythmic effects which could be expected (Hondeghem and Snyders, 1990; Hondeghem, 1994). Of the current antiarrhythmic agents, quinidine has the lowest anti- to pro-arrhythmic ratio, amiodarone has the best and sotalol would have an intermediate value between these two (Strauss *et al.*, 1970; Roden and Hoffman *et al.*, 1985; Anderson *et al.*, 1989).

1.5. The new strategies for the development of antiarrhythmic agents

1.5.1. The disadvantage of current antiarrhythmic agents

The most potent antiarrhythmic agents (Ic compounds) can effectively suppress PVBs, however, they do not have high efficacy in clinical trials (The CAST Investigators, 1989). Few clinical trials has ever shown any beneficial effects of class I antiarrhythmic agents on mortality of post-MI patient. The reasons of the inefficacy of antiarrhythmic drugs is not very clear , but it is possible due to many factors, such as the poor discrimination between normal rhythm and tachycardia of encainicde and flecainide and their proarrhythmogenous (CAST Investigators, 1989), lack of pathologically specific effects (Hondeghem, 1991) or excessive toxicity (Botting *et al.*, 1984). Class I antiarrhythmic agents have many common side effects and they are poorly tolerated by patients. One of them is the myocardium depression, which would cause negative inotropic effects (Bigger and Hoffman, 1990, Barrette *et al.*, 1995). Meanwhile decreased left ventricular pressure and vasodilation contribute to

hypotensive effects of these agents (Matsumoto *et al.*, 1993, Barrett *et al.*, 1995). Although lidocaine could provide protection against ischemic-induced arrhythmias by preventing the occurrence of ventricular fibrillation at high concentrations, such doses are rarely used because of the CNS toxicity (Hondeghem, 1991; Roden, 1994). Many class I compounds also had proarrhythmic effects (Furberg, 1983).

Although some studies suggested that the class III agents demonstrate good patient toleration and greater efficacy than conventional class I agents in preventing ventricular arrhythmias occurring during acute ischemia or programmed electrical stimulation (Kou *et al.*, 1987; Anderson 1989), the Survival With Oral d-Sotalol (SWORD) trial was prematurely terminated in 1994 because patients receiving sotalol had high potential for arrhythmic death (Waldo *et al.*, 1995). The reasons for the inefficacy of sotalol in SWORD trial are not very clear, but this is not a surprise since the therapeutic potential of currently available class III antiarrhythmics was limited by several undesirable effects, such as they excessively prolong repolarization at slow heart rates, resulting in pause-dependent new arrhythmia (torsade de pointes) by the development of early afterdepolarizations (Roden, 1993); they have reverse use-dependent block effects, a diminished ability of prolong repolarization at fast heart rates, which reduce the effectiveness in terminating tachycardias (Hondeghem and Snyders, 1990; Roden, 1993; Colatsky, 1995). In several clinical trials, amiodarone had shown the ability to control life-threatening ventricular tachyarrhythmias (Herre *et al.*, 1989; Greene, 1989) and prevent arrhythmias in post-MI patients (Pfisterer *et al.*, 1992; Ceremuzynski *et al.*, 1992), which may partly reflect the high anti- to pro-arrhythmia ratio of amiodarone (Nademanee *et al.*, 1993). But this class III agent

amiodarone was viewed as a "last resort" of antiarrhythmic drug because of its high incidence of toxicity (Nygaard *et al.*, 1986; Mason, 1987), such as pulmonary fibrosis and thyroid dysfunction.

For the development of the new antiarrhythmic agents, many new strategies have been developed. Only some of those strategies related to our study are mentioned here.

1.5.2. Selectivity of antiarrhythmic drugs

The lack of selectivity for tachycardia and ischemic myocardium of antiarrhythmic agents was suggested to be part of the explanations of the ineffect of antiarrhythmics (Tamargo *et al.*, 1992; Barrett *et al.*, 1995). The failure of class I antiarrhythmic drugs in clinical trials indicated the pathological mechanisms play a very important role in the ischemic tissue arrhythmogeny, not only the pure electrical phenomena like re-entry (Nattel *et al.*, 1991). Barrett *et al.* (1995) suggested that class I drugs can provide useful protection against ischemia-induced arrhythmias only if they have high selectivity for ischemic tissue and for high frequencies, such as lidocaine has better antiarrhythmic effects than quinidine and flecainide.

It was mentioned previously that several abnormalities of membrane function may arise during myocardial ischemic situation, e.g., increase of extracellular K^+, intracellular acidosis and depolarization of the resting membrane potential that may lead to partial inactivation of the I_{Na}. Since a higher proportion of sodium channels is inactivated in the ischemic myocardium, agents such as Harrison's class Ib drugs (lidocaine) would inhibit the I_{Na} more selectively in the ischemic than in normal tissues.

The time constant for recovery from block (τ) was also suggested to be important in the drug selectivity. In the depolarized situation, less drug-associated channels move from the inactivated-drug to rested-drug associated state, so that less activation unblocking occurs. Increased inactivation blocking and decreased activation unblocking would result in a selective depression of I_{Na} in depolarized tissue by antiarrhythmic agents (Hondeghem, 1990). The idea window of fast unbind time constant τ from the block was estimated between 300 and 700 ms in normal tissues and less than 1 s in depolarized tissues (Hondeghem 1987; 1990). The τ of lidocaine is around 230 ms in normal tissue, which is much faster than other class I agents and could be part of the reasons that lidocaine showed higher frequency and ischemic dependency blockade than other class I agents. Although in depolarization tissue, the specific selectivity of lidocaine was still about 10 times less than the computed hypothetically ideal agent.

One of the newest studies using some structurally related compounds (e.g., RSD compounds in this study) indicated that the pKa values of agents can modulate the relative partitioning of cation: neutral species in ischemic versus normal myocardium, and cause different ischemic-selective actions (Yong *et al.*, 1997). Although other structural properties of RSD compounds were also suggested as determinants for ischemic selectivity, such as the type/position of certain chemical groups (unpublished data)

The selectivity of class III agents on specific potassium channels was not investigated in details at present. Some assumptions had been made that a specific I_{Kr} blocker would have potential advantages (Colatsky, 1995). Many studies have focused

on use-dependent potassium channel blockade because of the "reverse" use-dependent effects of current class III agents. It has been proposed that the ideal class III drugs should produce a minimal effect on action potential duration at normal sinus rhythms, but a relatively fast, homogeneous and progressive prolongation of the APD and ERP during the tachycardia. However, the hypothetical window for the potassium channel blockade kinetics had not been obtained from the study of automated computer system (Hondeghem, 1994), this may be partly because of the little information available about the different channels which contribute to the repolarization in human ventricular cells, the mechanism of different agent on the channels states, and the chemical structure requirements for a use-dependent prolongation of action potential in cardiac tissues.

1.5.3. Mixed blockade of potassium and sodium channels

A hypothesis suggested that a mixed blocker of potassium and sodium channels would be a new strategy for antiarrhythmic agent development (Hondeghem and Snyders, 1990; Hondeghem, 1990; Tamargo *et al.*, 1992; Colatsky, 1995). As described previously, the ideal Class I agents would have a high selectivity against depolarized-ischemic tissues and use-dependent block against tachycardia, which could be an inactivated Na channel blocker (class Ib), with fast offset kinetics; the ideal class III drugs would have a profile of use-dependent prolongation of action potential duration, which would cause conversion of a tachyarrhythmia in a few beats. An agent with such profile of ideal class I and III agents could have several potential advantages: it can limit the degree of potassium blockade on action potential prolongation by sodium channel inhibition at high drug levels; suppress the possible triggers for early

after-depolarizations, which was proposed as a cellular mechanism for torsade de pointes; and enhance the prolongation of action potential duration at short cycle lengths with positive use-dependence (Colatsky, 1995).

Such mixed K^+ and Na^+ block agents are under investigation, some clinical studies with combinations of the class III and I drugs showed that the drug efficacy enhanced, but the pharmacokinetic drug interactions were extensive (Duff *et al.*, 1991; Marcus, 1992). The efficacy of mixed agents in the treatment of arrhythmia needs detail studies to understand the molecular mechanisms of this hypothesis better, because the drug effects would vary in different types of the tissue studied due to the variance of ionic currents among the different cardiac regions and even within the ventricular muscle, e.g., endocardium or epicardium (Antzelevitch, 1991).

1.6. Experiment approach

1.6.1. Objectives

Desirable characteristics of antiarrhythmic drugs can be measured by analysis the properties of a given agent's, such as the effects of agent on certain cardiac currents, the kinetic parameters of interaction with cardiac channels, the ischemic-selectivity of antiarrhythmic agents.

The experiments conducted in this thesis with two related RSD compounds, RSD1015 and RSD1000, were designed to answer the following questions:

1. The effects of RSD1015 and RSD1000 on several cardiac currents and the mechanic basis of their antiarrhythmic activities.
2. If the interactions of RSD compounds with certain cardiac channel exist, the kinetic mechanisms of the interactions.

3. The ischemia-selectivity of RSD1015 and RSD1000 for ischemic conditions and its relationship to the antiarrhythmic effects.
4. If there is difference between the effects of RSD1015 and RSD1000 on cardiac current, whether it is related to the physicochemical properties of RSD compounds?

1.6.2. Experimental design

The whole-cell patch clamp recording was used to investigate the effects of RSD1015 and RSD1000 on several currents in rat ventricular myocytes, including I_{to}, I_{Na}, I_{k1} and I_{Ca}. Different protocols were used to determine the kinetic mechanisms of drug-channel interactions. The ischemic-selectivities of RSD1015 and RSD1000 were measured using an acidic solution (pH 6.4). Some *in vivo* studies were performed to demonstrate the efficacy of RSD1015 and RSD1000 against ischemia- and electrically-induced arrhythmisa and the ischemic-selectivity in isolated rat heart.

2. MATERIALS AND METHODS

2.1. Isolation of rat ventricular myocytes

Freshly isolated rat ventricular myocytes were used study the ionic effects of RSD antiarrhythmic agents on cardiac channels in this study. Male Sprague-Dawley rats (250-350 g) were used in accordance with the guidelines established by the University of British Columbia Animal Care Committee. The rat ventricular myocytes were dissociated and isolated using procedures which have been described previously (Mitra and Morad, 1985; McLarnon and Xu, 1995). Briefly, a rat was anesthetized by pentobarbitone (ip, 45 mg/kg). The chest was opened, the heart was removed and

immersed in ice-cold, oxygenated, calcium-free Tyrode's solution containing (mM): NaCl (133.5), KCl (4), $MgCl_2$ (1.2), NaH_2PO_4 (1.2), TES (10) and glucose (11), pH at 7.4. The heart was then attached to a constant-flow Langendorff system though a aortic cannula perfused with same Tyrode's solution. The perfusate was oxygenated for 30 minutes and maintained at 37°C. After 4 minutes perfusion which removes most of the blood from ventricular chambers and coronary vasculature, the perfusate was exchanged for another Tyrode's solution containing 0.07% collagenase (Type II, Worthington Biochemical), 25 μM $CaCl_2$ and 200 mg of BSA (Boehringer Mannheim). After 15 to 20 minutes of perfusion, the rat ventricle was removed for three sections. Each section was cut into small pieces carefully in Tyrode's solution with 25 μM calcium, the Tyrode's solution was re-applied with gentle agitation of the digested tissue. The cell suspensions were stored at room temperature between intervals (1-2 hr) of wash with successively increased concentrations of calcium (from 25 to 100 μM). Cells were plated on coverslips precoated with laminin (4 μg per coverslip) and used for electrophysiological study. All cells were prepared and stored at room temperature (20-24C) and were used for the experiments within 10 hr after cell isolation. In terms of morphology the cells had a mean length of 72 ± 6 μm (n=22) and mean diameter of 7.8 ± 0.5 μm (n=22). Only cells which were rod-shaped, clearly striated and quiescent in the 1 mM calcium bath solution were used for study.

2.2. Electrophysiology

2.2.1. Patch-Clamp recording

The development and the advance of patch-clamp recording in the early 1980's makes it possible to record small currents in cell membranes (Hamill *et al.*, 1981; Sakmann and Neher, 1984; Cahalan and Neher, 1992). The use of a high resistance feedback resistor in the recording headstage and the formation of a high-resistance gigohm seal between the membrane and a solution-filled glass recording glass pipette stabilized the recording and reduced background noise and leak currents.

Several configurations of patch-clamp can be used to record membrane currents. These include the basic "cell-attached" and the "inside-out patch" and "outside-out patch" for the single channels and "whole-cell recording" for the macroscopic currents. When the recording electrode is positioned on an intact cell, it forms a "cell-attached" configuration; when the patch of membrane is broken by either air suction or an electrical pulse a whole-cell configuration results. The interior of the cell is homogenous with the contents of the electrical pipette and macro-currents of the cell membrane can be measured. In this study, whole-cell patch clamp recording was used to study the currents in rat myocytes.

2.2.2. whole-cell recording configuration

Patch-clamp electrodes were fabricated using Corning #7052 glass (A-M Systems, Everett, Washington, U.S.A.) with an internal diameter of 1.2 mm and an external diameter of 1.6 mm. The pipette was prepared with a standard two-pull technique with a Narishige PP-83 glass microelectrode puller.

The recording chamber was affixed to the stage of a inverted Nikon TMS microscope and a coverslip containing myocytes was placed in the chamber. The small volume of the chamber allowed for exchange of the experimental solution applied from a gravity-flow system in 30 sec. A suction tube at another end of the recording chamber kept the recording solution at a constant level. An agar-filled bridge was inserted into the bath and a wire connected to ground was inserted in the bridge to complete the recording circuit.

Pipettes filled with solution (see below) were inserted into a holder on the head-stage (Axon model CV 201A, gain = 1), which was mounted on a Newport 360-90 lab jack fitted with a Newport 423 series actuator powered by a Newport model 860 controller for manipulation of the pipette; the microscope and lab jack were place on a nitrogen-suspended floating table (Newport, USA) to achieve a vibration-free environment.

An Axopatch amplifier (200A, Axon Instruments, Foster City, CA) was used to record whole-cell currents with the low-pass filter set at 1 or 2 kHz. The sampling frequency of the A/D converter was 10 kHz. Capacitive currents and series resistance were compensated by use of analogue circuitry and leak subtraction was also used in some experiments. The mean value of seal resistance was 36 $\pm$ 5 GΩ (n=12) with series resistance compensation set at 80-85%, the mean series resistance was 4.2 $\pm$ 1 MΩ (n=10) and the mean cell capacitance was 92 $\pm$ 6 pF (n=6). P/N subtraction was not routinely done and there was no evident effect on macroscopic currents when it was employed. The protocols used were generated by computer loaded with pClamp 6.0.2 (Axon Instruments Inc.) and data were recorded on disk for subsequent analysis;

unless otherwise noted the holding potentials were -70 mV. A detailed description of the specific protocols which were used in the work is included in the results section below. Complete sets of data (control, drug, recovery) were obtained from each cell studied. All experiments were performed at room temperature (20-24 °C).

2.3. Data analysis

The data analysis used pClamp 6.0.2 software (Axon Instruments Inc.). The inactivation time courses of I_{to} were best fitted with single exponential functions with time constants denoted by τ. To determine the potency of agents on I_{to}, dose-response curves were plotted for the effects of the compounds to alter τ. In these plots the concentrations of drug were represented linearly and the dose-response data were fitted with a single exponential function to obtain a value for the EC_{50}. The potency of inhibition of I_{Na} (EC_{50}) was determined from a single exponential function fit of dose-response plot where the response was measured as the effect of drug to reduce peak current. The results in this study are given as mean value $\pm$ SEM and statistical significance was determined using Student's t-test or two-way ANOVA. Statistical analysis was performed by the use of the NCSS statistical package. A difference at $P < 0.05$ was considered significant.

2.4. Experimental drug and solutions

The agents used in this study, RSD1015 and RSD1000, were obtained from Nortran Pharmaceutical Inc. (Vancouver, BC, Canada). The chemical structure of the compounds are shown in Figure 2. RSD1015 (FW=439.42) contains an N-methyl piperazine ring which gives it two charged tertiary amine centres. The N-morpholino

containing RSD1000 (FW = 390) could have two possible conformations of the charged forms (Yong *et al* 1997). The drug pKa values were determined by the equivalence point method by adding HEPES (pKa = 7.35) at a temperature of 25°C. The respective pKa for RSD1015 and RSD1000 were 8.1 and 6.1. A fresh stock solution of drug was prepared for each experiment (dissolved in distilled water).

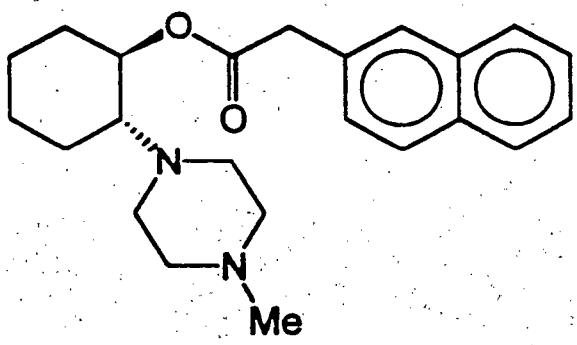
In the experiments on I_{to} and I_{K1}, the bath solution was a modified Tyrode's solution and contained (mM): NaCl (133.5), KCl (4), $CaCl_2$ (1), $MgCl_2$ (1.2), TES (10), NaH_2PO_4 (1.2) and glucose (11); pH was adjusted to 7.35 with NaOH in normal solution, and titrated to 6.4 with HCl in acid buffer solutions. The patch pipette solution contained (mM): NaCl (10), KCl (140), $MgCl_2$ (1), EGTA (10), Mg-ATP (5) and HEPES (10); pH adjusted to 7.35 with KOH.

For studies of I_{Na}, the bath solution contained 4 mM CsCl (to replace the KCl) and 50 mM NaCl with 87 mM TRIS (to replace 133.5 mM NaCl), pH was adjusted to 7.35 with NaOH in normal solution, and titrated to 6.4 with HCl in acid buffer solutions. The pipette solution contained (in mM): NaCl (10), CsCl (120), EGTA (12), TES (10), $MgCl_2$ (1), Na-ATP (5); pH adjusted to 7.4 with CsOH.

In several experiments studies on I_{Ca} were undertaken and in these cases the bath solution contained (in mM): TRIS (137), $CaCl_2$ (5.5), $MgCl_2$ (1), CsCl (20) and glucose (5.5); pH adjusted to 7.2 with CsOH. The pipette solution used (in mM): CsCl (125), Mg-ATP (5), EGTA (15), TEACl (20) and HEPES (10); pH adjusted to 7.2 with CsOH.

Figure 2 Chemical structure of RSD1015 (A) and RSD1000 (B).

A



.2HCl

B

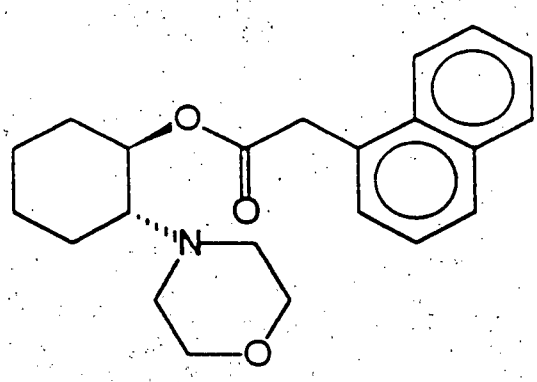


Figure 2

3. RESULTS

Two putative antiarrhythmic agents RSD1015 and RSD1000 (Nortran Pharmaceutical Inc., Canada) have been studied in this work. These two agents have shown dose-dependent antiarrhythmic activities against ischemic- or electrical-induced arrhythmias *in vivo* (Yong, *et al*, 1997; unpublished data). Since similar experimental strategies and protocols have been applied to characterize the drug properties, the results are organized on the basis of the drug interactions with specific cardiac currents. The results with RSD1015 are illustrated in figures; some results where RSD1000 had actions different from RSD1015 are also shown.

3.1. The interactions of RSD1015 and RSD1000 with transient outward potassium currents (I_{to})

3.1.1. The effects of RSD1015 and RSD1000 on transient outward potassium currents (I_{to})

A single depolarization pulse to +60 mV from a holding potential of -70 mV for 400 ms was used to activate the I_{to} in rat ventricular myocytes. Typical I_{to} are shown in Figure 3A and Figure 4A. I_{to} exhibited rapid activation followed by inactivation to a non-zero steady-state level as previous described (Josephson *et al*, 1984; Apkon and Nerbonne, 1991). Based on pharmacological actions and kinetic behaviour, the inactivating and steady state macroscopic currents are considered as separate entities (Apkon and Nerbonne, 1991; Castle and Slawsky, 1992; McLarnon and Xu, 1995) and only the transient inactivating component, sensitive to 4-AP, is termed I_{to}. The effects

of RSD1015 and RSD1000 on the residual steady state current which may represent a delayed rectifier type of K^+ current were not investigated in this work (see discussion).

Figure 3 is a recording from one cell. In the presence of 2 μM RSD1015 in the extracellular solution (Figure 3B), the time course of the inactivation (τ) was decreased; in addition the peak amplitude was also diminished. The effects of RSD1015 on the time course of I_{to} was dose-dependent as shown in Figure 3C with the concentration of the agent increased to 30 μM. Partial recovery of peak amplitudes and the inactivation time course was found after wash-off of RSD1015 (Figure 3D).

RSD1000 had similar effects on the time course of inactivation and peak amplitudes of I_{to}, which were also dose-dependent, as illustrated in a single cell in Figure 4B and 4C with 5 μM and 30 μM RSD1000. Partial recovery was evident after wash-off of the compound (Figure 4D).

The time course (τ) of I_{to} inactivation is independent of voltage (Apkon and Nerbonne, 1991; McLarnon and Xu, 1995). The inactivation time course (τ) at +60 mV was used to illustrate the dose-response effects of the two compounds on I_{to}. The τ was determined by computer fits using single exponential function.

The dose-response effects of RSD1015 on the decay time course (τ) of I_{to} is shown in Figure 3E. Each point on the graph represents a value (normalized to control) with application of 5 concentrations of RSD1015 to each of the 7 cells. In control, the value of τ was 51.7 ± 4.1 ms (n=7). The resultant dose-response curve was fit by a logistic function and the concentration of RSD1015 which reduced τ to 50% of control value (i.e., EC_{50}) was 1.3 ± 0.4 μM (n=7).

Figure 3 Effects of RSD1015 on I_{to}. (A) A single I_{to} in control solution with a depolarizing step from -70 mV to + 60 mV; (B) Effects of 2 μM and (C) 30 μM RSD1015 on I_{to}; (D) Recovery of I_{to} after prolonged wash-off of RSD1015; (E) Dose-response data for effects of RSD1015 on time constant τ of I_{to} inactivation. The data points are means ± sem from 7 cells and the solid line is a fit to the normalized data using a logistic function. In control, the value of τ was 51.7 ± 4.1 ms (n=7).

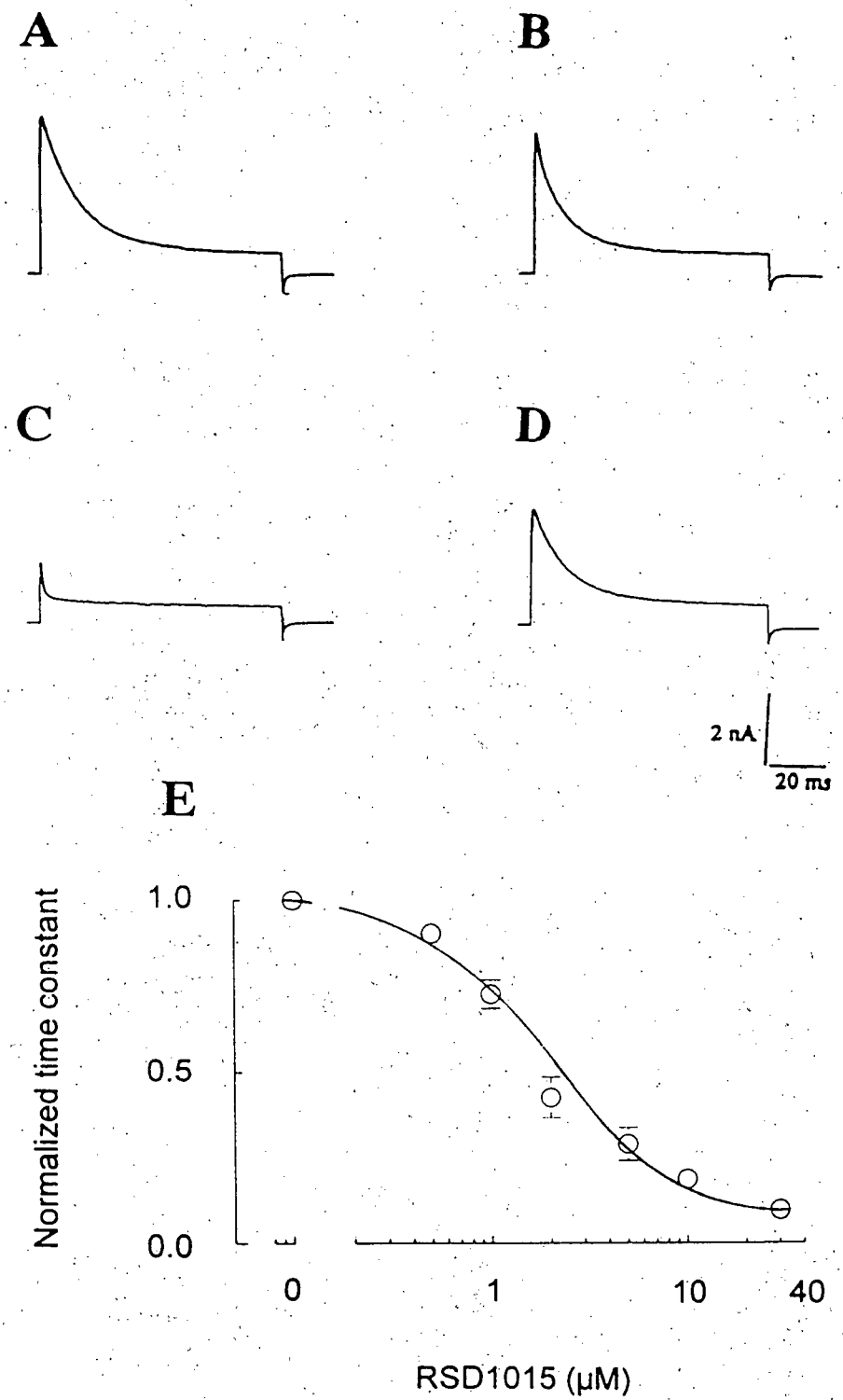

A
B
C
D
2 nA
20 ms
E
Normalized time constant
1.0
0.5
0.0
0
1
10
40
RSD1015 (μM)

Figure 3

Figure 4 Effects of RSD1000 on I_{to}. (A) A single control I_{to} recorded with a depolarizing step from holding voltage of -70 mV to + 60 mV; (B) Effects of 5 μM and (C) 30 μM RSD1000 on I_{to}; (D) Recovery of I_{to} after prolonged wash-off of RSD1000; (E) Dose-response curve for effects of RSD1000 on time constant τ of I_{to} inactivation. The normalized values are means ± sem from 5 cells and the solid line is a fit to the normalized data using a logistic function. In control, the value of τ was 48.2 ± 3.9 ms (n=5).

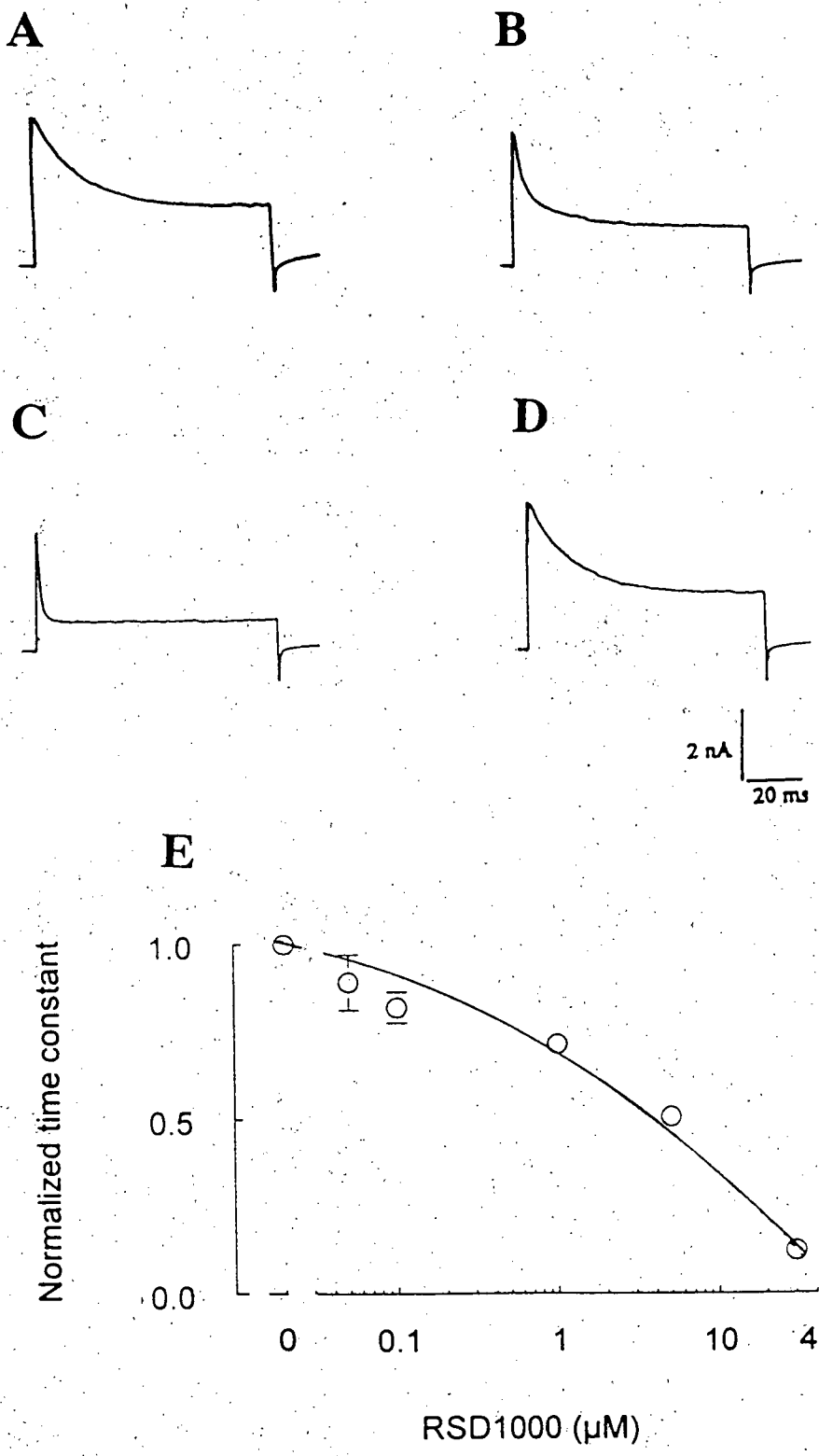

A
B
C
D
2 nA
20 ms
E
Normalized time constant
1.0
0.5
0.0
0
0.1
1
10
40
RSD1000 (μM)

Figure 4

Figure 4E shows a dose-response plot for RSD1000 using the same procedures noted above. The value of τ was 48.2 $\pm$ 3.9 ms in control solution (n=5). The EC_{50} for RSD1000 to diminish the time constant of I_{to} inactivation was 5.8 $\pm$ 0.7 μM (n=5). The difference in potency between RSD1015 and RSD1000 blockade of I_{to} was statistically significant ($P < 0.05$).

3.1.2. The interactions of RSD1015 and RSD1000 on voltage-dependence for I_{to} activation

A family of currents, evoked by depolarizing steps to potentials up to +60 mV from holding potential of -70 mV, was used to study the effects of RSD compounds on the voltage-dependence of activation. The procedure was to measure the peak currents at different test depolarizing potentials and compare the activation thresholds in control and in the presence of RSD1015 and RSD1000.

A plot of the peak current *vs.* membrane potential is presented from one cell in Figure 5A with control (open circles) and 2 μM RSD1015 (closed circles). It is clear from Figure 5A that the amplitudes of I_{to} were decreased by 2 μM RSD1015 compared to control values after the current was activated. However, the thresholds for activation of I_{to} were -34.5 mV in the absence and -36 mV in the presence of the compound. Thus the voltage threshold for I_{to} activation was not changed with RSD1015; the same result was also obtained in another 4 cells at the same concentration of RSD1015. The partial recovery of peak I_{to}, after wash-off of the agent, can also be noted in Figure 5b (upward open triangles).

Figure 5 Effects on voltage-dependence of activation of I_{to} with 2 μM RSD1015 in a cell. Activation of I_{to} was illustrated by peak currents shown as a function of depolarizing potentials from a holding level of -70 mV. Control currents were presented as open circles, amplitudes in the presence of 2 μM RSD1015 as closed circles and after wash-off of drugs as open triangles.

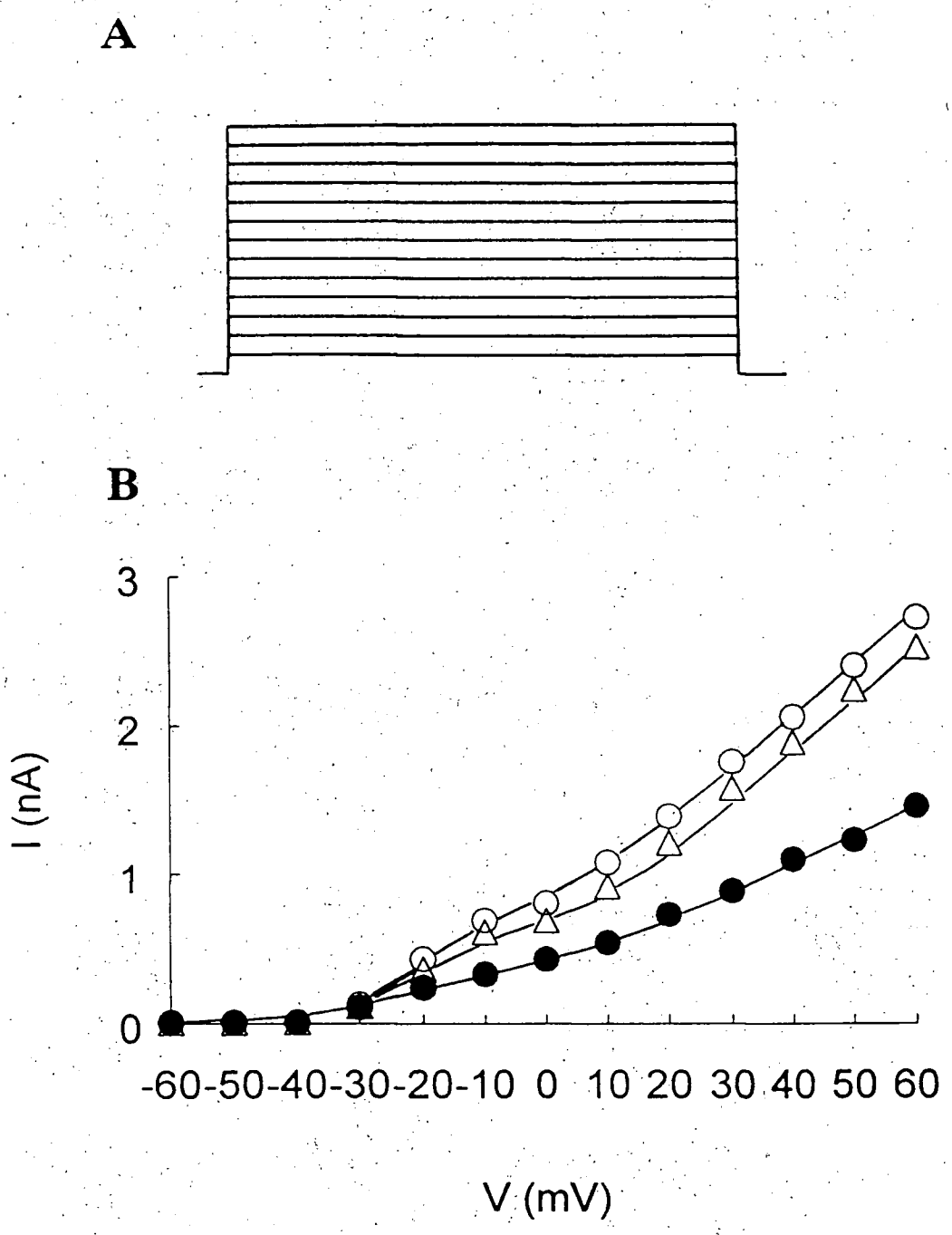


Figure 5

The effects of RSD1000 on the activation of I_{to} was also studied (data not shown). In five cells, the thresholds for activation of I_{to} were -36 ± 1.5 mV in control (n=5), and -35 ± 2 mV in the presence of 5 μM RSD1000 (n=5), the difference of the threshold of I_{to} activation was not significant ($P > 0.05$). The same result was also obtained using a higher concentration of RSD1000 (30 μM) (n=3).

3.1.3. The effects of RSD1015 and RSD1000 on voltage-dependence of steady-state inactivation of I_{to}

As noted above, the generalised modulated receptor model can be applied in the analysis of I_{to}. A number of protocols were applied in order to determine mechanisms of RSD compounds interaction with different states of the I_{to} system. The interactions of compounds with the inactivated state of the I_{to} channels were studied using a two-pulse protocol (Castle, 1990, 1991; McLarnon and Xu, 1995; McLarnon and Xu, 1997). In order to determine the drug effects on the steady state inactivation of I_{to}, conditioning pulses from -90 mV to -30 mV (duration 300 ms) were followed with a single test pulse to +60 mV for 200 ms with the holding voltage of -70 mV (Figure 6A). The peak amplitudes of the I_{to} associated with the test pulse were normalized to the I_{to} amplitude at a conditioning pulse of -90 mV, then the normalized data were plotted *vs.* the conditioning voltages. The resulting curves were fit by the Boltzmann equation:

$$Normalized\ I_{to} = 1/\{1+exp[(V-V_{1/2}\}/k]\}$$

where V is the condition pulse potential, $V_{1/2}$ is the potential at which the normalized I_{to} equals 0.5 and k is the slope factor. The results of RSD1015 is shown in

Figure 6 Actions of agents on steady-state inactivation of I_{to}. The protocols used in this study is shown in (A), where 300 ms conditioning pulses from -90 mV to -30 mV was followed with a single test pulse to +60 mV for 200 ms, the holding voltage is -70 mV. The results in control (open circles) and the effects of 2 μM RSD1015 (closed circles) from one cell are shown in (B). Normalized peak I_{to} have been plotted *vs.* conditioning potentials. The solid line is a fit using the Boltzmann equation (see section 3.1.3). Only the fit to control curves is shown.

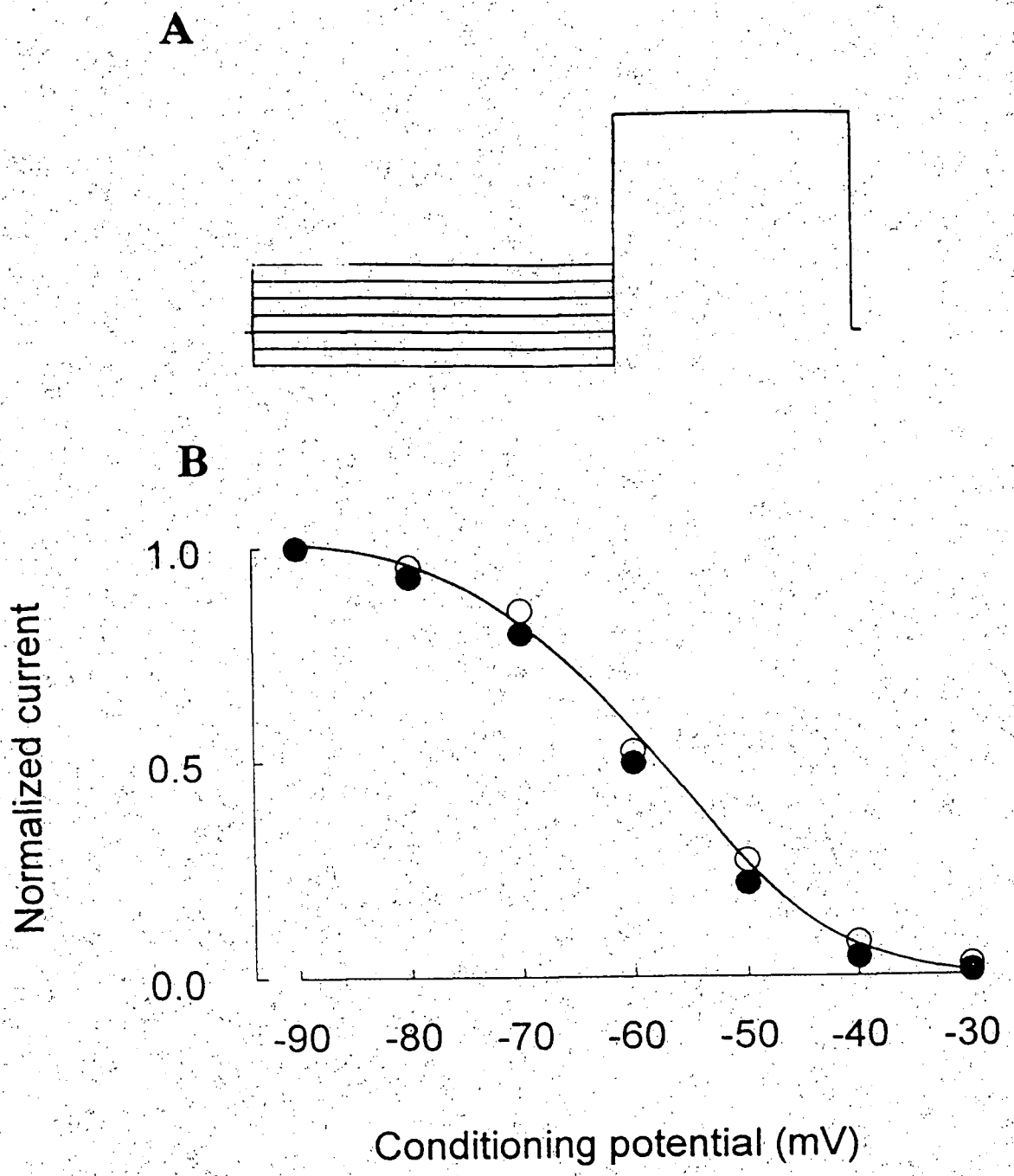

A
B
1.0
0.5
0.0
Normalized current
-90
-80
-70
-60
-50
-40
-30
Conditioning potential (mV)

Figure 6

Figure 6B, control data are represented by open circles and the effects of the compound by closed circles.

The $V_{1/2}$ and k in the absence and presence of compounds were determined from the Boltzmann equation and tabulated in Table 3.

Overall, RSD1015 changed $V_{1/2}$ by 3.5 ± 1.6 mV (hyperpolarizing shift, n=4) and k by 1.6 ± 0.6 mV (n=4); for RSD1000, the changes of $V_{1/2}$ and k are 2.8 ± 1.4 mV and 0.7 ± 0.7 mV. There are no significant difference between $V_{1/2}$ or k in control and in the presence of RSD1015 or RSD1000 ($P > 0.05$).

3.1.4. The effects of RSD1015 and RSD1000 on recovery from steady-state inactivation of I_{to}.

Another procedure was used to determine possible effects of agents with the inactivated state of I_{to}. The protocols to study the kinetics of recovery from steady-state inactivation of I_{to} initially used a depolarizing conditioning pulse to +60 mV for 200 ms to ensure full inactivation of I_{to}. This conditioning pulse was followed by a variable recovery time at -70 mV holding potential and a single test pulse of +60 mV to assess the extent of recovery (Figure 7A). The interval time between the conditioning and test pulse was from 5 ms to 1000 ms. The recovery from inactivation was determined by the fractional change of I_{to} (test pulse peak amplitude divided by conditioning pulse peak amplitude at each episode). The normalized amplitudes were then plotted against the interpulse recovery time and the time courses of recovery from inactivation were obtained by an exponential fitting of the data.

The effects of RSD1015 and RSD1000 on recovery from steady-state inactivation of I_{to} are summarized in Table 4.

TABLE 3 The effects of RSD compounds on voltage-dependence of steady-state inactivation of I_{to}

	$V_{1/2}$ (mV)	k (mV)
Control (n=4)	-57.8 ± 3.1	8.5 ± 2.3
2 μM RSD1015 (n=4)	-54.3 ± 4.8	10.1 ± 1.9
Control (n=5)	-57.1 ± 5.9	8.3 ± 2.7
5 μM RSD1000 (n=5)	-54.3 ± 4.6	9.4 ± 3.3

The results are expressed as means ± SEM.

TABLE 4 The effects of RSD compounds on the time course of inactivation recovery

	Time course of inactivation recovery (ms)
Control (n=4)	56.7 ± 4.3
2 μM RSD1015 (n=4)	61.1 ± 3.7
Control (n=5)	58.1 ± 3.3
5 μM RSD1000 (n=5)	60.8 ± 4.5

The results are expressed as means ± SEM.

Figure 7 Effects on drugs on recovery from steady-state inactivation of I_{to}. A two-pulse protocol was used in this study (A), a 100 ms initial depolarizing condition pulse was followed by a different recovery time at -70 mV holding potential and a single test pulse of +60 mV for 100 ms. This pulse protocol measured the extent of recovery of peak I_{to} after variable recovery times at -70 mV subsequent to complete inactivation induced by the conditioning pulse. The recovery from inactivation was determined as the fractional change of I_{to} (test pulse peak current divided by conditioning pulse peak current). The results in control (open circles) and the effects of 2 μM RSD1015 (closed circles) from one cell are shown in (B). The solid line is an exponential fit to the data points with normalized I_{to} value plotted *vs.* the interpulse recovery time, only the fit to control curve is shown.

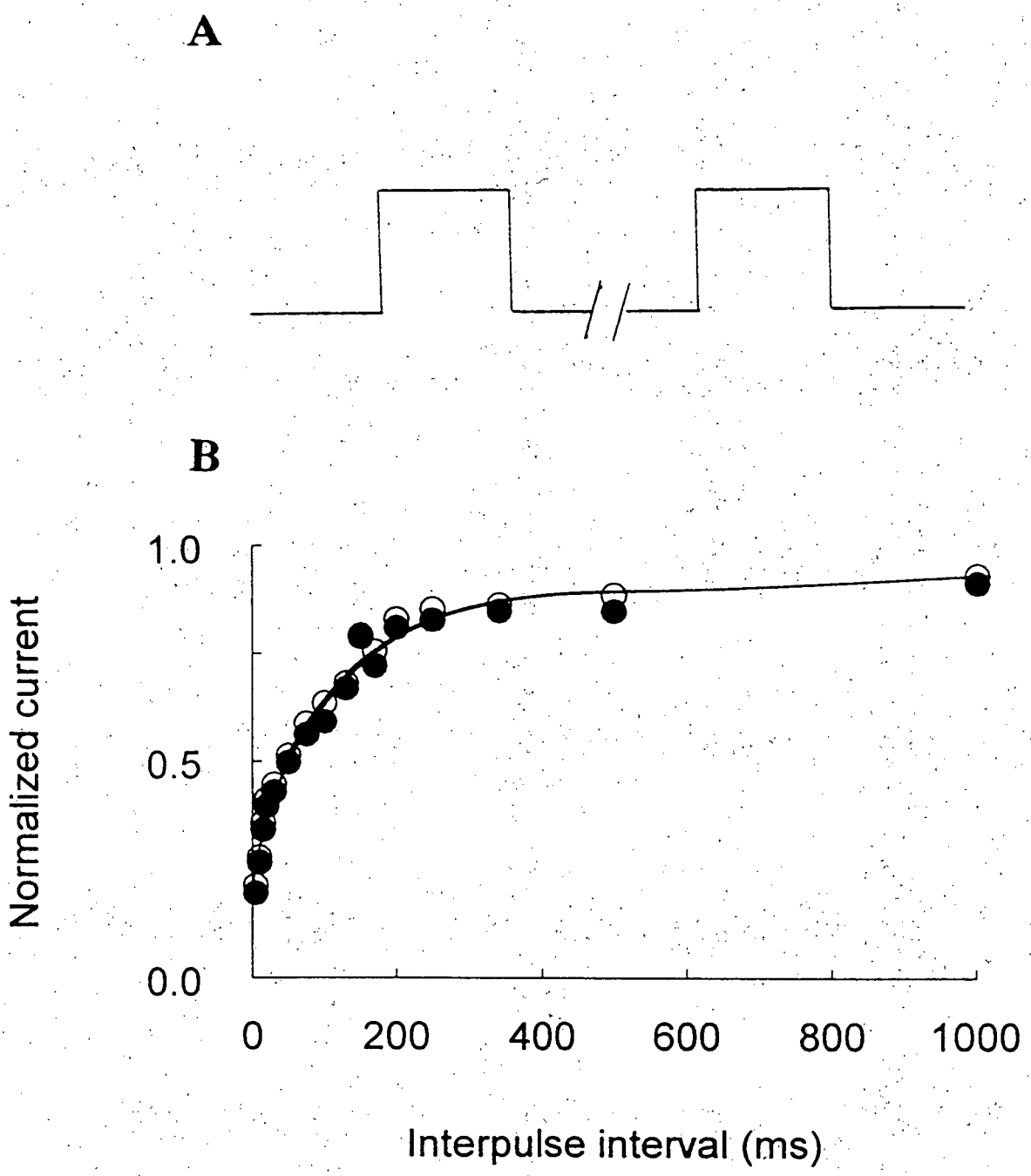

A
B
1.0
0.5
0.0
Normalized current
0
200
400
600
800
1000
Interpulse interval (ms)

Figure 7

Thus the results described above show both agents, in a dose-dependent manner, reduce the amplitudes of I_{to} and the time constant (τ) of I_{to} inactivation. However, there was no evident interactions of the agents with either the inactivated state or the activation of the current. The possibility that RSD1015 and RSD1000 acted on the open state of the I_{to} system was then investigated.

3.1.5. The open state blockade of the I_{to} system of RSD1015 and RSD1000

As mentioned in section 1.4.1.1, the modulated receptor model can be applied to potassium channels. In the simplest form, kinetic states of the channel can be written as:

$$C \rightleftharpoons O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B$$
$$\downarrow$$
$$I$$

Where C, O, I and B indicated closed, open, inactivated and drug-blocked state of the channel, respectively. The k_1 is the rate constant of open state transition to an open-blocked state and the k_{-1} is the unblocking rate constant. The effective onward rate constant is k_1[D], where D is the drug concentration. The on- and off-rates of the drug-channel interactions are very important in determining the clinical application of drugs and the use-dependent block of agents. Several protocols were used to determine these rate constants of RSD1015 and RSD1000 in this study.

3.1.5.1. Voltage dependence of decay time course and the relation to channel block

The voltage-dependence of the inactivation time constant (τ) has been plotted in Figure 8. The magnitudes of the time constants in control solution (open circles of Figure 8) were not significantly altered ($P > 0.05$) by the different levels of voltage in the range of +10 to +60 mV. Also shown are the effects of RSD1015, at concentrations of 2 and 10 μM (upward and downward triangles of Figure 8, respectively). The compound caused dose-dependent decreases in τ, however, for a given concentration there was no significant dependence of τ on potential ($P > 0.05$). Thus drug actions on the time course of decay were independent of voltage. These results would be consistent with a simple channel block model where the open state undergoes a transition to an open-blocked state with a voltage-independent rate constant k_1. In this case the inverse of the decay time constant in the presence of drug can be expressed as $k_1[D] + k_{-1}$ where D is concentration of RSD1015 and k_{-1} is the off or unblocking rate constant (McLarnon and Xu, 1995; McLarnon and Xu, 1997). An estimate for the blocking rate constant can be found by assuming that the rate constant $k_{-1} << k_1[D]$ and using the data from Figure 8 to determine τ. For example, with RSD1015 at a concentration of 10 μM, the k_1 was estimated to be $19.4 \pm 3.6 \times 10^6$ M^{-1} s^{-1} at +50 mV (n=4). Since the values of τ were relatively constant over the voltage range from +10 to +60 mV, the derived values of k_1 would not differ markedly from the value obtained at +50 mV. Using the same analysis procedure, the k_1 of RSD1000 was estimated to be $9.1 \pm 2.1 \times 10^6$ M^{-1} s^{-1} at voltage of +60 mV (n=7). A more quantitative analysis for the channel block rate constants is considered below.

Figure 8 The actions on voltage dependence of time constant (τ) of I_{to}. The time constant (τ) values are means $\pm$ sem values and plotted against the membrane potentials. (A) Effects of RSD1015 with control (open circles), 2 μM RSD1015 (upward closed triangles) and 10 μM (downward closed triangles) (n=4) are shown.

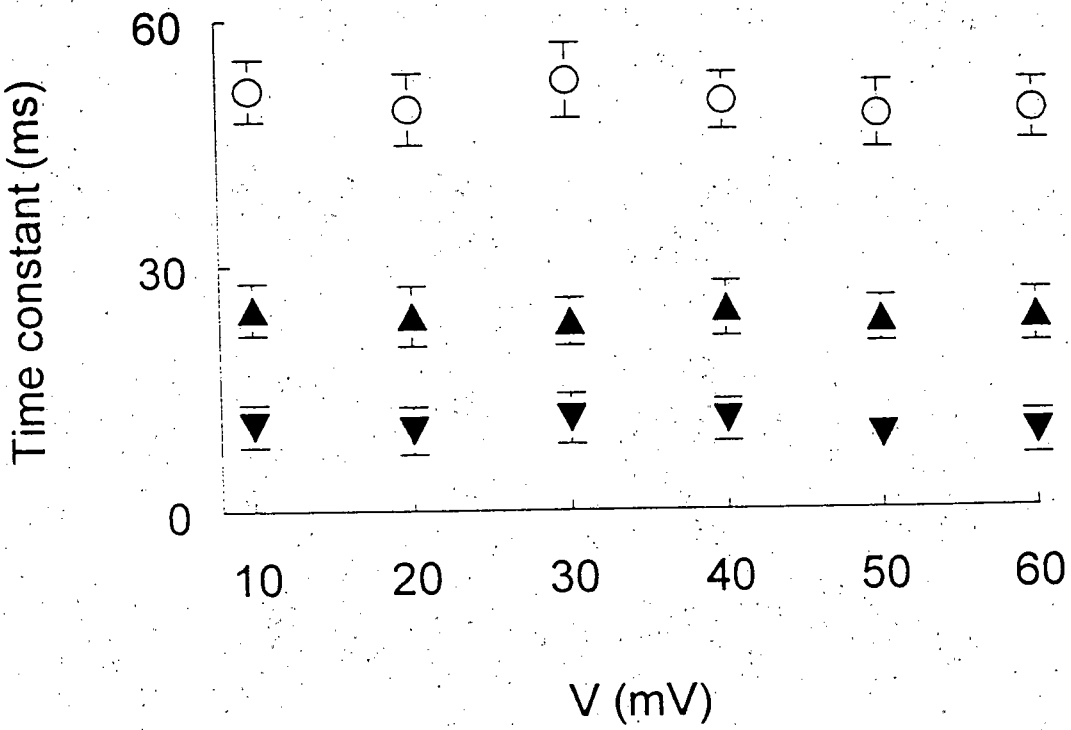


Figure 8

The estimated values of the onset rate (k_1) of RSD1015 and RSD1000 are summarized in Table 5.

3.1.5.2. Rate constants for channel blockade using time-dependent inhibition of I_{to}

In order to determine values for the magnitude of the unblocking rate constant k_{-1} and obtain more accurate values of k_1, measurements of the effects of two concentrations of agents on the decay of I_{to} were carried out (Castle, 1990; McLarnon and Xu, 1995; McLarnon and Xu, 1997). In essence, the time course of agents actions could then be determined by subtraction of the decay time course in the presence of the compound from that of control. Thus the development of enhanced inhibition of I_{to} with time was assumed to reflect a time-dependent block of open channels with rate constants k_1 (onward) and k_{-1} (off). This procedure was carried out at two different concentrations of drug and the magnitudes of k_1 and k_{-1} were determined using a solution of two simultaneous equations.

Typical I_{to} in control and with two concentrations of RSD1015 (at 2 μM and 30 μM) are shown in Figure 9A. The results of subtracting the two time-courses measured with drug from control data are presented in Figure 9B where the results have been normalized using the expression $(1-I_{drug}/I_{control})$. It can be noted that the increase of inhibition was both time and concentration-dependent. Secondly, the inhibition approached a maximum value which depended on the drug concentration. The following equation has been used to fit the curves shown in Figure 9B (McLarnon and Xu, 1995; McLarnon and Xu, 1997):

$$I(t)=I_{max}\,(1-e^{-(k1[D]+k\text{-}1)t})$$

where I_{max} equals maximum block at drug concentration [D]; $I(t)$ refers to the amount of inhibition at any time t; and k_1 and k_{-1} are defined above. The single exponential fits, extrapolated to zero at the beginning of the depolarization steps, indicate that there was little block of I_{to} before activation. This result implies little or no tonic block by the RSD compounds. The analysis of the data shown in Figure 9B gave $k_1 = 15.8 \times 10^6\ M^{-1}\ s^{-1}$ and $k_{-1} = 24.5\ s^{-1}$. A similar analysis was also carried out on 3 other cells and the mean values of rate constants were: $k_1 = 16.3 \pm 3.4 \times 10^6\ M^{-1}\ s^{-1}$, $k_{-1} = 27.6 \pm 4.3\ s^{-1}$ (n=4). The dissociation constant associated with channel block k_d ($k_d=k_{-1}/k_1$) was then found to be 1.7 μM, a value close to the EC_{50} for RSD1015 effects on τ.

The same analytic procedure was used to determine open channel blockade of RSD1000. The values of k_1 and k_{-1} were $8.1 \pm 1.9 \times 10^6\ M^{-1}\ s^{-1}$, $52.3 \pm 3.3\ s^{-1}$ (n=4) respectively, the k_d is 6.5 μM.

The results of RSD1015 and RSD1000 open channel blockade are summarized in Table 6.

The use-dependent effects of RSD1015 and RSD1000 on I_{to} were also investigated. In these experiments, a series of depolarizing test pulses to +60 mV (for 100 ms from a holding potential of -70 mV) were used. At a frequency of 1 Hz, the peak I_{to} current produced by each pulse remained constant during the series of stimulating. In the presence of 2 μM RSD1015, there was no decline of the current

TABLE 5 The estimated onset rates of RSD1015 and RSD1000 on I_{to}

	RSD1015 (n=4)	RSD1000 (n=7)
k_1 (M^{-1} s^{-1})	$17.3 \pm 3.6 \times 10^6$	$9.1 \pm 2.1 \times 10^6$

The results are expressed as means ± SEM.

TABLE 6 The values of I_{to} open channel blockade of RSD1015 and RSD1000

	RSD1015 (n=4)	RSD1000 (n=4)
k_1 (M^{-1} s^{-1})	16.3 ± 3.4	$8.1 \pm 1.9 \times 10^6$
k_{-1} (s^{-1})	27.6 ± 4.3	52.3 ± 3.3
k_d (μM)	1.7	6.5

The results are expressed as means ± SEM.

Figure 9 Enhancement of drug channel blockade with time. (A) The traces shown are I_{to} in control (con) and after addition of RSD1015 at either 2 or 30 μM for a step depolarization to +60 mV. (B) Time course of the block of I_{to} with the ordinate a measure of the fraction of current blockade, $(I_{control} - I_{drug})/I_{control}$. The fits shown were according to the equation in the text.

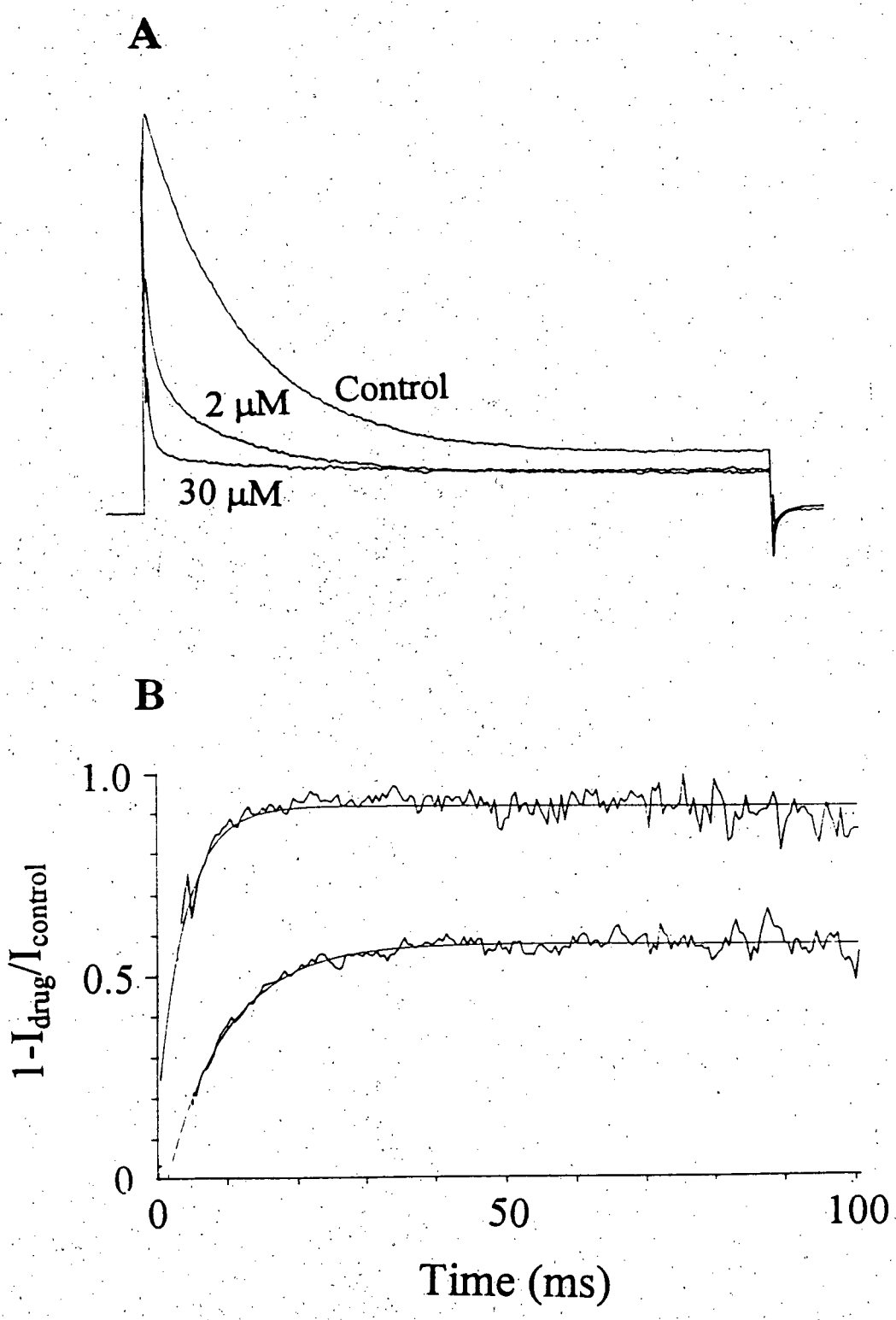


Figure 9

amplitudes with successive pulses. At different concentrations of RSD1015 (5 and 10 μM), and different stimulating frequency of 2 Hz, no evident use-dependent effect was observed. Similar results were obtained with RSD1000 at concentrations of 5 and 10 μM with stimulating frequency of 1 or 2 Hz.

3.2. The interactions of RSD1015 and RSD1000 with inward sodium current (I_{Na})

The applications of RSD1015 or RSD1000 *in vivo* showed evidence for inhibition of sodium currents (Yong *et al*, 1997). Such actions were suggested from the increased magnitudes of the P-R interval after doses of RSD1000 or RSD1015 were applied.

3.2.1. The effects of RSD1015 and RSD1000 on I_{Na}

Inward sodium current is the main component of the spike phase of the cardiac action potential. It can be activated by a protocol which consists of a prepulse to -130 mV, to remove resting inactivation of sodium current, followed by a depolarizing test pulse to -10 mV. Typical I_{Na} in control solution are illustrated in Figure 10A and 11A. Tonic block, defined as the decrease in I_{Na} at a low pulse frequency (0.3 HZ) sufficient to ensure full recovery from the use-dependent block of I_{Na}, was studied initially. The effects of RSD1015 on I_{Na} are shown in Figure 10. This compound, in a dose-dependent manner at 1 μM (Figure 10B) and 10 μM (Figure 10C), diminished the amplitude of the sodium current with no evident effects to alter the time course of inactivation. The blockade was reversible as shown after wash-off of the drug (Figure 10D).

Figure 10 Actions of RSD1015 on I_{Na}. (A) A typical trace of I_{Na} in control; (B) Effects of 1 μM and (C) 10 μM RSD1015 on I_{Na}; (D) Recovery of I_{Na} after wash-off of RSD1015. The protocol for recording I_{Na} consisted of a prepulse to -130 mV for 30 ms then depolarizing to -10 mV for 20 ms. (E) Dose-response curve for normalized I_{Na}, with the fit shown using a logistic function, the data shown are means ± sem (n=6). The peak value of sodium current is 6.7 ± 0.8 nA in control (n=6).

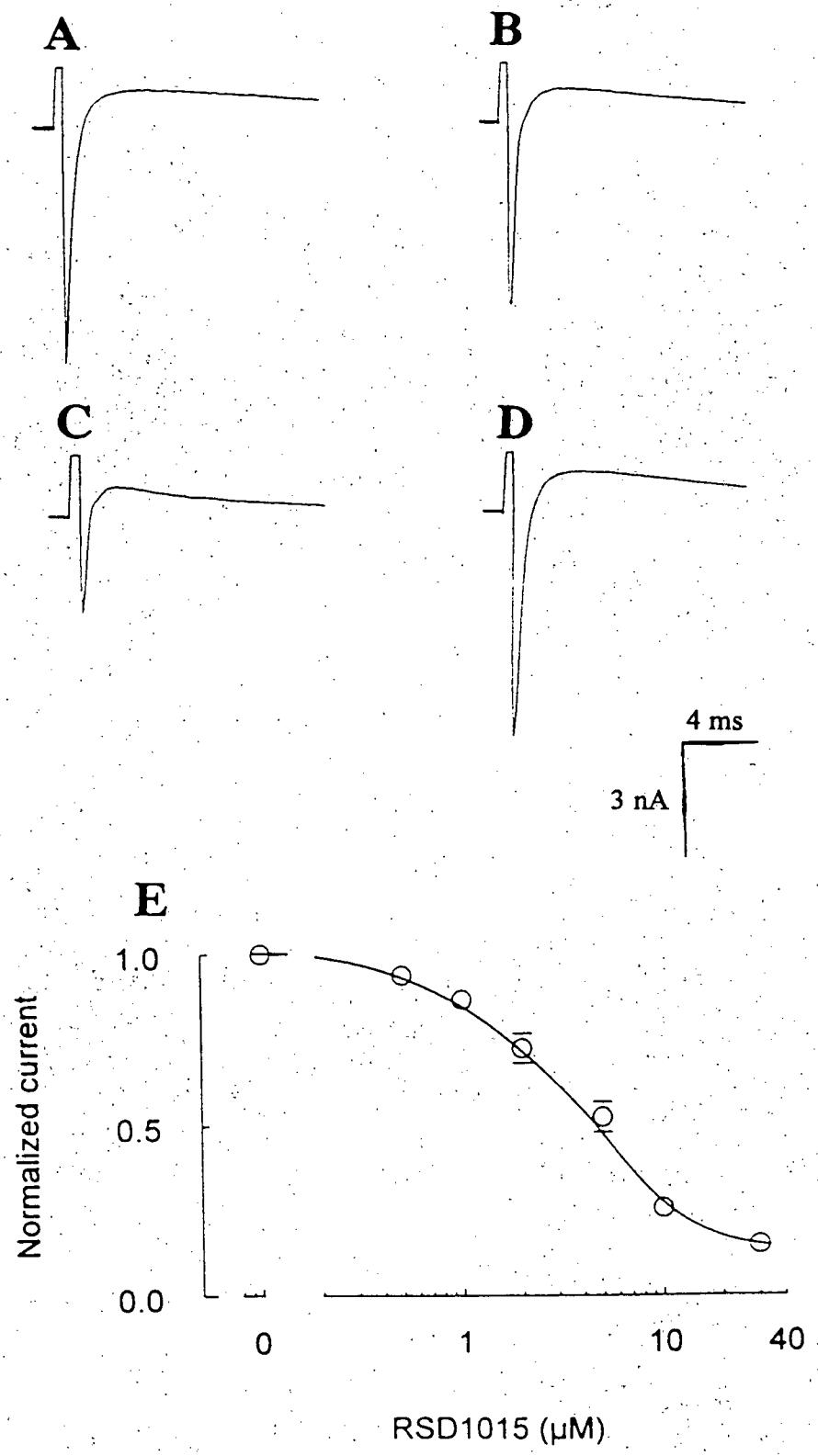


Figure 10

Figure 11 Actions of RSD1000 on I_{Na}. (A) A typical trace of I_{Na} in control; (B) Effects of 0.5 μM and (C) 10 μM RSD1000 on I_{Na}; (D) Recovery of I_{Na} after wash-off of RSD1015. The protocol for I_{Na} recording was the same as for Figure 10. (E) Dose-response curve for normalized I_{Na}, with the fit shown using a logistic function, the data shown are means ± sem (n=9). The peak value of sodium current is 6.3 ± 0.6 nA in control (n=9).

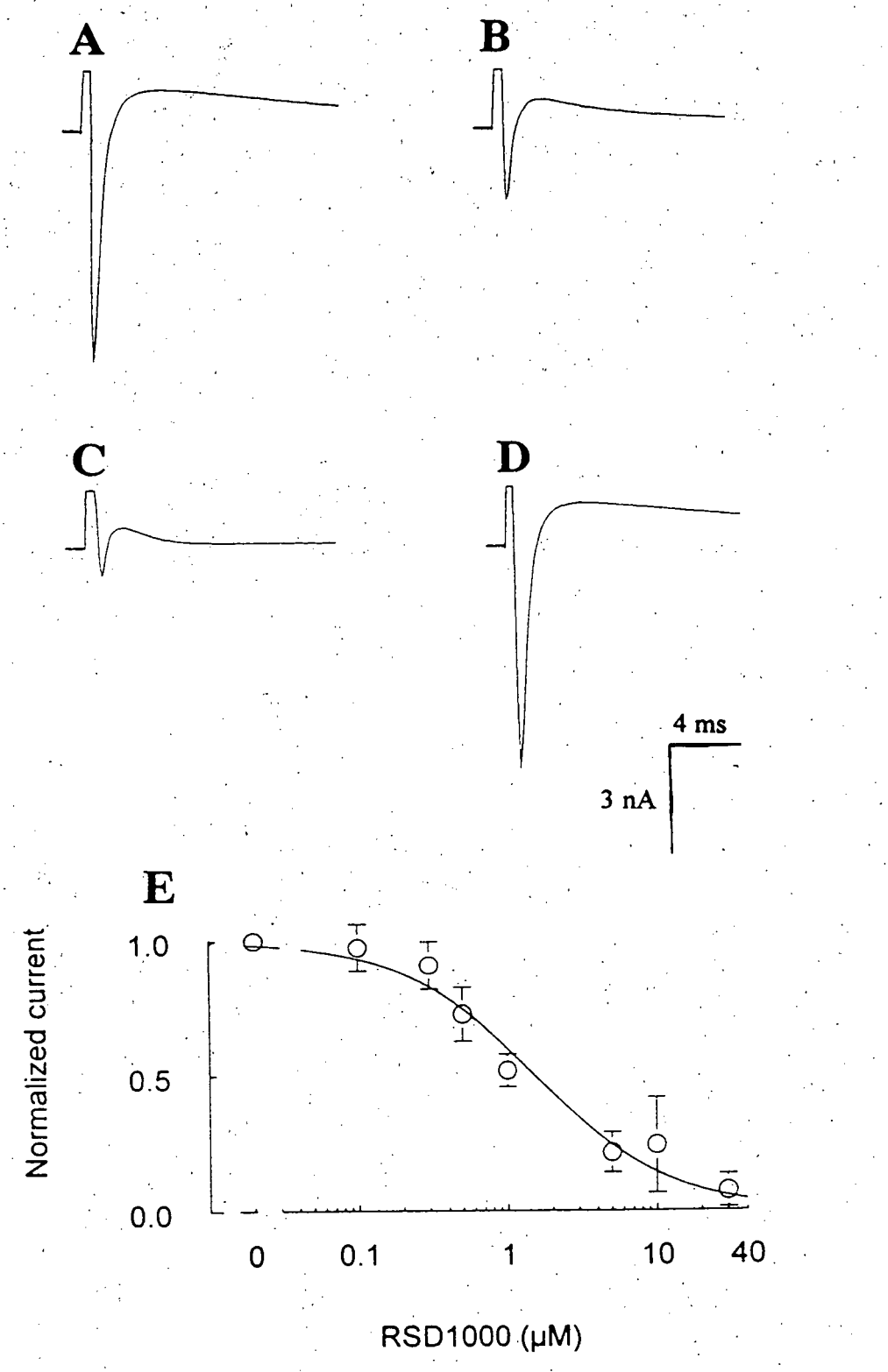

A
B
C
D
4 ms
3 nA
E
Normalized current
1.0
0.5
0.0
0
0.1
1
10
40
RSD1000 (μM)

Figure 11

RSD1000 also caused amplitude decrements of sodium current in a dose-dependent manner. These actions are illustrated at 0.5 μM (Figure 11B) and 10 μM (Figure 11C), recovery of the sodium current after re-application of control solution is shown in Figure 11D.

The peak amplitudes of sodium currents were used as a measurement of drug effects. Two modes of sodium current inhibition can be observed following a train of pulses. The reduction of I_{Na} during the first depolarization after a long rest period at sufficiently negative potentials is referred to as tonic block. In contrast, during repetitive stimulation, the I_{Na} falls beat to beat until a new steady state is obtained. This additional reduction in sodium amplitudes produced during repetitive depolarizations in the presence of antiarrhythmic agents is termed use-dependent block. Because RSD1015 and RSD1000 showed use-dependent effects on sodium (see section 3.2.4), the steady-state level of block, obtained from the 20th pulse at the stimulation frequency of 10 Hz, was used for the analysis of drug dose-response curve, which evaluated both the tonic and the use-dependent block effects. The amount of block was calculated as the percentage decrease in I_{Na} after perfusion with the compound in comparison with the value of the 20th pulse in control.

Figure 10E presents the dose-response plot of RSD1015 effects on I_{Na}. The data were fit with a logistic function and the EC_{50} value derived from the dose-response plot was 4.1 ± 0.9 μM (n=6). The dose-response curve of RSD1000 is shown in Figure 11E and the EC_{50} was 1.5 ± 0.3 μM (n=9).

3.2.2. The effects of RSD1015 and RSD1000 on voltage dependence of activation of I_{Na}

The protocol utilized a series of depolarizing steps (from -70 mV to +20 mV) to activate the sodium current with each step following an initial hyperpolarizing step to -130 mV to remove inactivation at the rest potential of -60 mV. The amplitudes of current were plotted against the corresponding voltage to analyze for the voltage dependence of activation.

A typical I/V plot of the voltage-dependent of I_{Na} activation is shown in Figure 12B. The voltage corresponding to peak sodium current was -30 mV for control, -32 mV for 10 μM RSD1015 and -30.5 mV following wash-off of the agent. There was no significant shift by 10 μM RSD1015 in the voltage-dependent activation of I_{Na} in 7 cells ($P > 0.05$).

A similar I/V relationship of I_{Na} activation in control, 1 μM RSD1000, and recovery was obtained. There was no shift of the voltage-dependent activation by 1 μM RSD1000. The same result on voltage-dependent activation of I_{Na} were obtained in other 4 cells with 1 μM RSD1000.

3.2.3. The effects of RSD1015 and RSD1000 on voltage-dependent inactivation of I_{Na}

The I_{Na} inactivation was assessed at selected membrane (prepulse) potentials using the standard two-pulse protocol. A series of 600 ms pre-pulses (range -150 mV to -50 mV) from the rest potential of -60 mV was followed by a test potential to -20 mV. This protocol was applied once every 3 s. The amplitude of I_{Na} was normalized to that

of I_{Na} at -150 mV. The curve was fit by the same Boltzmann relation described above in the analysis of I_{to} inactivation (section 3.1.3).

The results from one cell, in the presence of 5 μM RSD1015, are presented in Figure 13B. The value for $V_{1/2}$ was -86.3 mV in control and was -94.3 mV with 5 μM RSD1015. Inactivation relations were plotted for 5 cells and 5 μM RSD1015 shifted the inactivation curve -8.3 ± 2.1 mV in a hyperpolarizing direction which is significant ($P < 0.05$). There is no significant change in the slope factor k ($P > 0.05$), which was 4.8 ± 1.6 mV in control and 5.1 ± 1.8 mV with 5 μM RSD1015 (n=5).

The same analytic procedure was used to determine the effect of RSD1000 on voltage-dependent inactivation of I_{Na}. There is significantly hyperpolarization shift in the $V_{1/2}$ of I_{Na} inactivation curve in the presence of 5 μM RSD1000 ($P < 0.05$). The change of the slope factor k is not significant ($P > 0.05$). The results of RSD1015 and RSD1000 on voltage-dependence of inactivation of I_{Na} are described in Table 7.

3.2.4. Use-dependent actions of RSD1015 and RSD1000

3.2.4.1. Use-dependent block of RSD1015 and RSD1000

In addition to tonic blockade, the use-dependent block of I_{Na} was investigated in the experiments. A series of 20 depolarization pulses (to -20 mV with 30 ms duration) was applied from the holding voltage of -100 mV. The frequency of stimulation was 10 Hz. I_{Na} decreased during the pulse train and reached a steady state at the 20 pulse in the presence of drugs. The amount of use-dependent block was calculated as the percentage decrease in I_{Na} in the steady state (at 20th pulse) with respect to the value for the first pulse (Hisatome *et al*, 1990).

Figure 12 Effects of agents on voltage dependence of I_{Na} activation. (A) The protocol consisted of an initial hyperpolarizing step from the rest potential of -60 mV to -130 mV for 50 ms followed by a series of deplarizing steps from -70 mV to +20 mV for 20 ms. (B) I/V relationship showing activation of sodium currents in control (open circles), 10 μM RSD1015 (closed circles) and recovery of I_{Na} (open triangles).

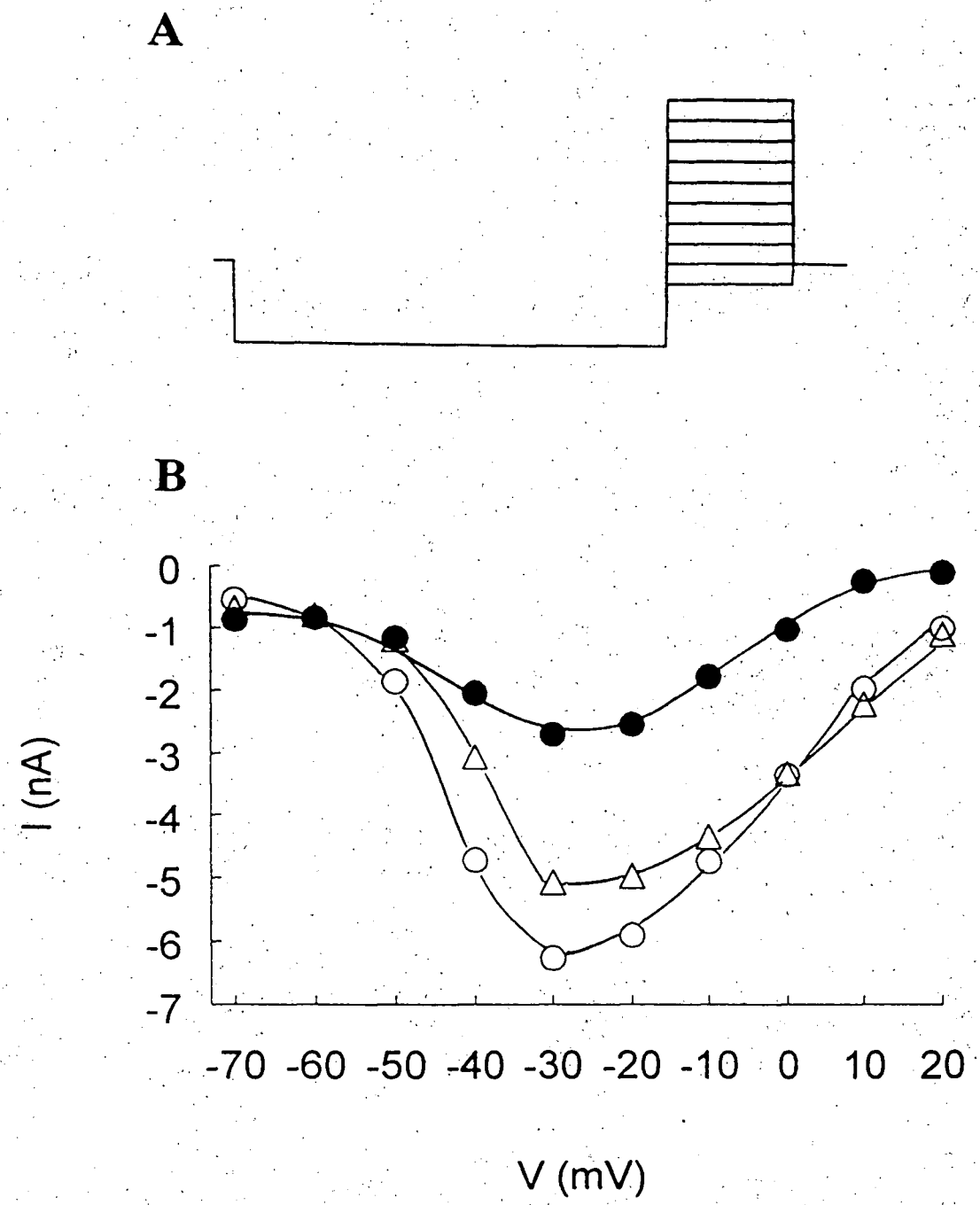


Figure 12

Figure 13 The effects on voltage dependence of the inactivation of I_{Na}. (A) The protocol utilized a series of prepulses from -150 mV to -50 mV (duration of 600 ms) followed by a test potential to -20 mV for 20 ms, the rest potential is -60 mV. (B) The normalized I_{Na} results from one cell plotted *vs.* prepulse potentials in control (open circles), 5 μM RSD1015 (closed circles). The peak values of sodium current at the prepulse of -150 mV are 8.3 nA in control and 4.5 nA in the presence of 5 μM RSD1015 in this cell. The solid line is a fit by the Boltzmann equation to the curves.

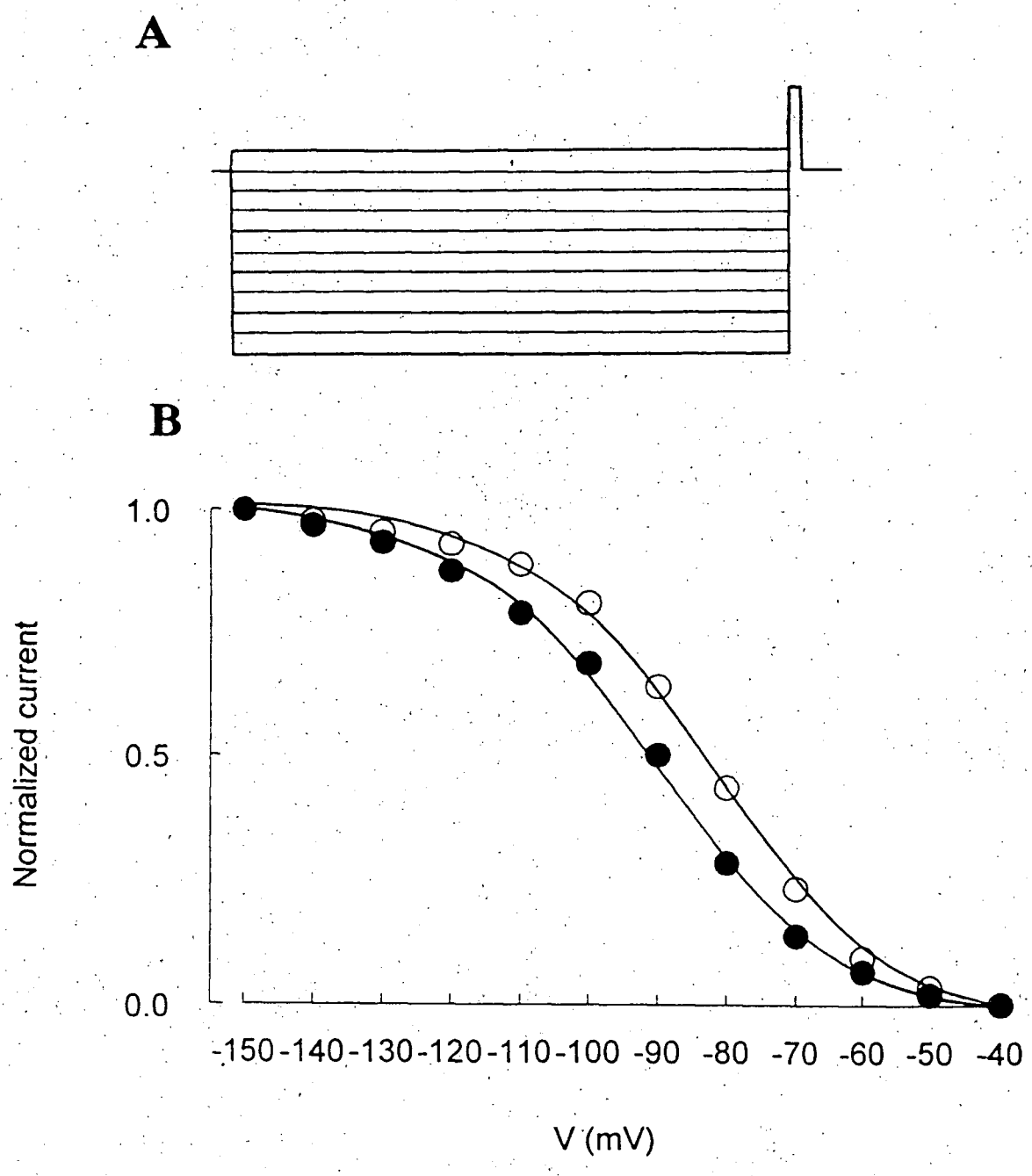

A
B
1.0
0.5
0.0
Normalized current
-150 -140 -130 -120 -110 -100 -90 -80 -70 -60 -50 -40
V (mV)

Figure 13

TABLE 7 The effects of RSD compounds on voltage-dependence of inactivation of I_{Na}

	$V_{1/2}$ (mV)	k (mV)
Control (n=5)	-85.8 ± 4.1	4.8 ± 1.6
5 μM RSD1015 (n=5)	-94.1 ± 4.3 *	5.1 ± 1.8
Control (n=5)	-84.1 ± 3.4	4.3 ± 1.2
5 μM RSD1000 (n=5)	-96.6 ± 2.6 *	4.5 ± 1.3

The results are expressed as means ± SEM. The symbol * indicates statistical significance at $P < 0.05$ for difference from control.

The use-dependent blockade of I_{Na} by RSD1015 is illustrated in Figure 14. The data shown are control (Figure 14A) and after application of 5 μM RSD1015 (Figure 14B). As evident from Figure 14A, no attenuation of currents was found in control. The first trace of Figure 14B was attenuated from that recorded in control, which indicates some tonic block as was discussed above (in section of 3.2.1). However, the subsequent I_{Na} showed a progressive decrease to a steady level (traces shown correspond to stimulating pulses 1, 2, 3, 6 and 20) where the amplitude of the 20th pulse was 78% that of the 1st pulse. Following a level of steady block, a period of another two minutes was allowed whereby no stimulation was applied. Despite the continued presence of the compound, the initial I_{Na}, evoked by another series of depolarizing pulses, showed substantial recovery from the steady state use-dependent block. Thus RSD1015 exhibits both tonic and use-dependent blockade of I_{Na}. Similar results were obtained from 5 other cells at a single concentration of 5 μM RSD1015, the amount of use-dependent block of I_{Na} was 72 ± 3.2 % of the first pulse (n=6) at frequency of stimulation at 10 Hz.

Use-dependent block of 5 μM RSD1000 on I_{Na} is shown in Figure 15B and normalized in Figure 15C. The amount of use-dependent block of I_{Na} was 62 ± 4.6 % of the first pulse (n=7) with a stimulation frequency of 10 Hz. Thus the use-dependent block of I_{Na} was more pronounced with RSD1000 compared with that observed with RSD1015.

Figure 14 I_{Na} use-dependent inhibition of RSD1015. (A) The traces shown are the I_{Na} for the pulses 1, 2, 3, 6 and 20 of a 20 pulse sequence of depolarizing steps to -10 mV for 30 ms; frequency of 10 Hz and holding voltage of -100 mV in control (A) and 5 μM RSD1015 (B). (C) The I_{Na} peak amplitudes were normalized to the first pulse in control (open circles) and 5 μM RSD1015 (closed circles) in one cell. The peak amplitudes of sodium current of first pulse are 7.9 nA in control and 5.1 nA in the presence of 5 μM RSD1015 in this cell.

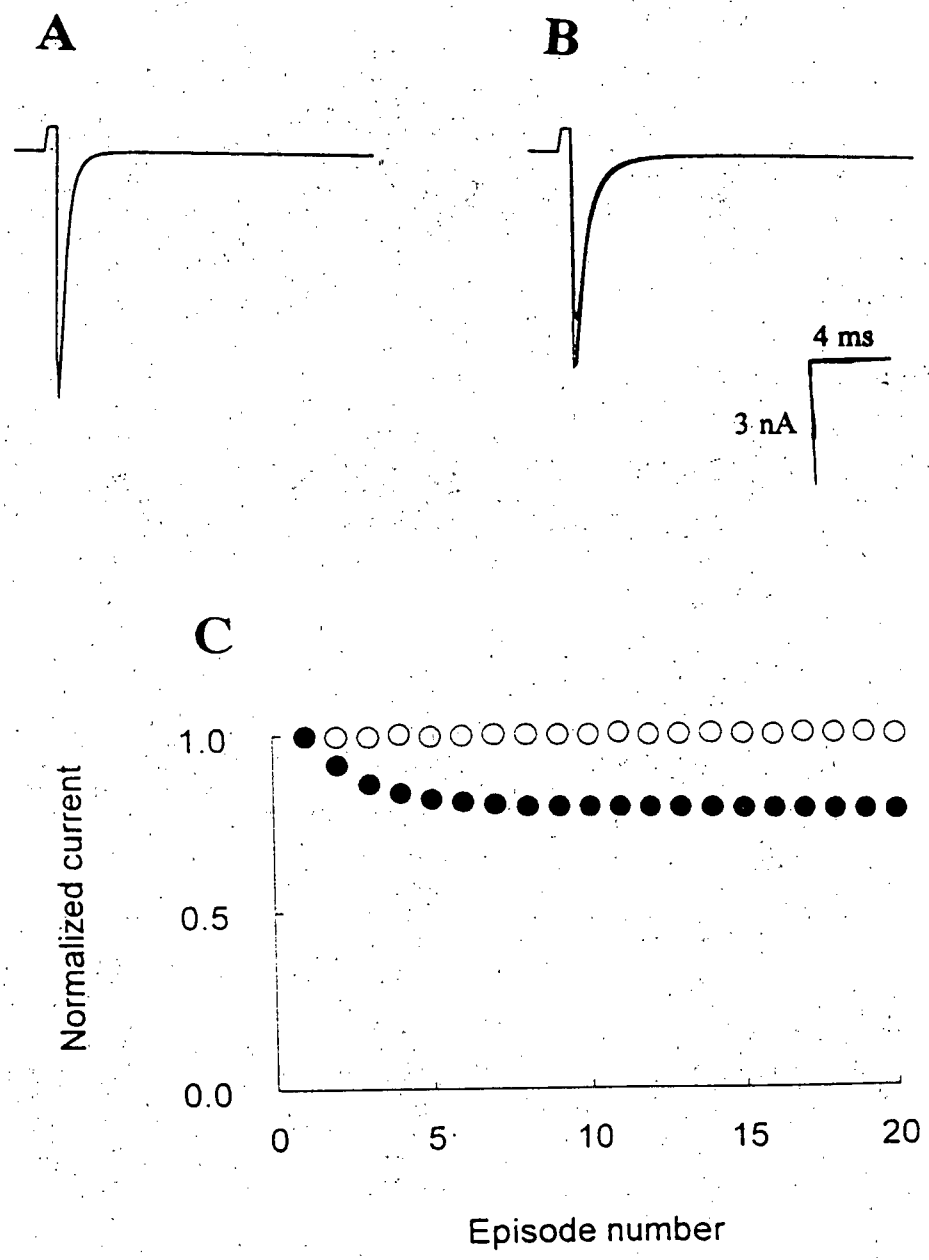

A
B
4 ms
3 nA
C
1.0
0.5
0.0
Normalized current
0
5
10
15
20
Episode number

Figure 14

Figure. 15 I_{Na} use-dependent blockade of RSD1000. (A) The traces shown are the I_{Na} for the pulses 1, 2, 3, 6 and 20 of a 20 pulse sequence of depolarizing steps to -10 mV for 30 ms; frequency of 10 Hz and holding voltage of -100 mV in control (A) and 5 μM RSD1000 (B). (C) The I_{Na} peak amplitudes were normalized to the first pulse in control (open circles) and 5 μM RSD1000. (closed circles) in one cell. The peak amplitudes of first pulse of sodium current are 8.2 nA in control and 4.9 nA in the presence of 5 μM RSD1000 in this cell.

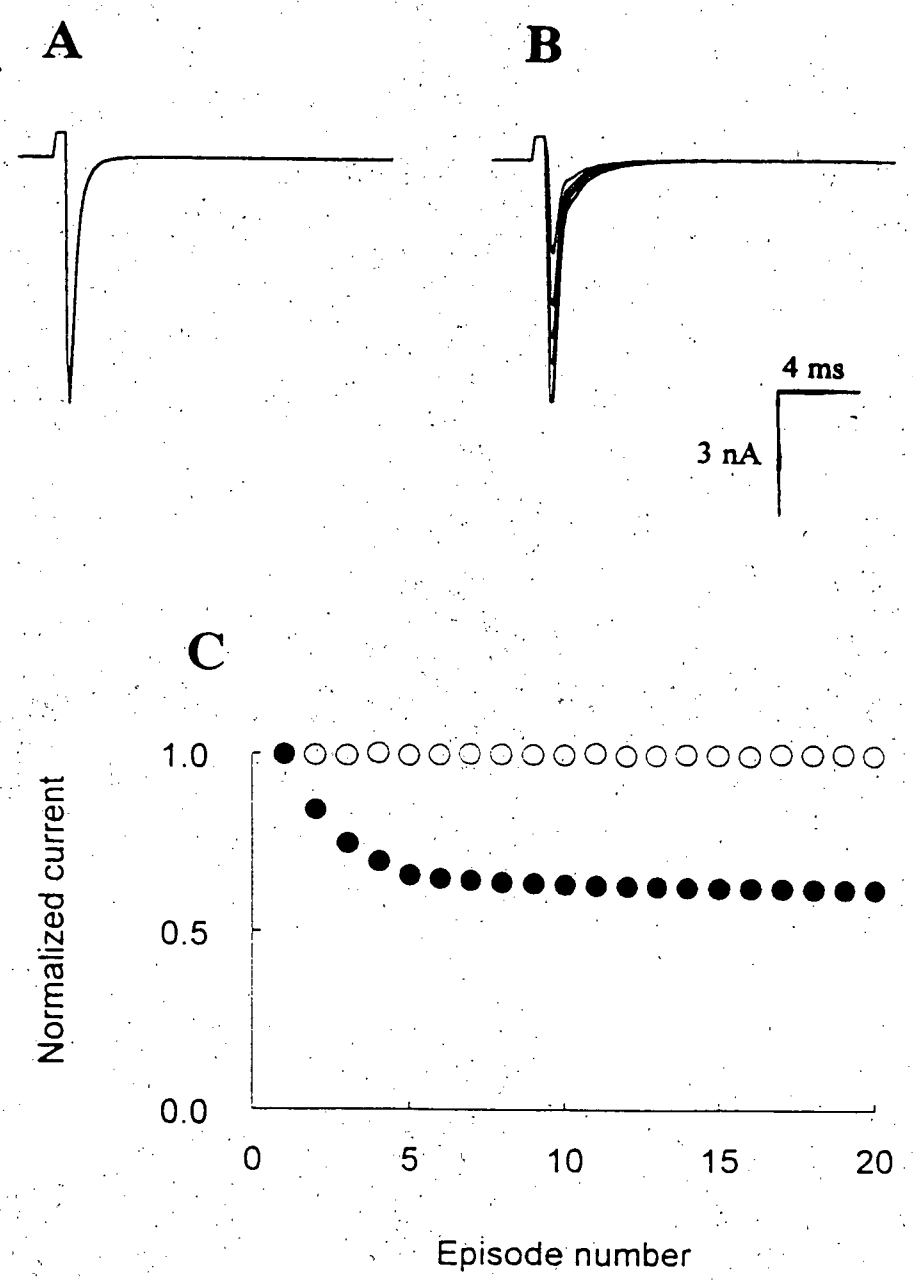

A
B
4 ms
3 nA
C
Normalized current
1.0
0.5
0.0
0
5
10
15
20
Episode number

Figure 15

3.2.4.2. Use-dependent blockade of RSD1015 and RSD1000 at different stimulation frequencies

The I_{Na} use-dependent blockade of RSD compounds at different stimulation frequencies of 1, 5, 10 and 20 Hz were studied. The same test protocols and concentration as described in section 3.2.4.1 were used.

As illustrated in Figure 16A, 5 μM RSD1015 caused only a small reduction in I_{Na} at the lowest frequency of 1 Hz. With higher frequency stimulation of 5 and 10 Hz, the currents exhibited more use-dependent blockade. It was evident that the levels of inhibition of I_{Na} reached steady level at each frequency. The fastest rate to obtain the steady-state use-dependent blockade and the largest degree of inhibition was obtained at 20 Hz. The same procedures were applied to 4 other cells with similar results to those presented in Figure 15A. The use-dependent block effects of 5 μM RSD1000 at different stimulation frequencies are presented in Figure 16B. The amount of use-dependent blockade of 5 μM RSD1015 and 5 μM RSD1000 at frequencies of 1 Hz and 20 Hz are summarized in Table 8.

3.2.4.3. The use-dependent blockade of RSD1015 and RSD1000 with different pulse durations

To determine the onset of use-dependent block, a protocol with variable depolarization durations (10 or 30 ms) was used at a stimulation frequency of 10 Hz. The holding potential was -100 mV and the depolarization voltage was -20 mV.

The I_{Na} use-dependent blockade of RSD1015 and RSD1000, with different durations of 10 and 100 ms, is shown in Figure 17. The amount of use-dependent

blockade of these two RSD compounds with different depolarization durations are listed in Table 9. There are no significant difference in the use-dependence of I_{Na} with two duration pulses ($P > 0.05$).

TABLE 8 Use-dependent blockade of RSD1015 and RSD1000 at different stimulation frequencies

	5 μM RSD1015 (n=4)	5 μM RSD1000 (n=4)
1 Hz	90 ± 2.4 %	89 ± 3.8 %
20 Hz	66 ± 3.6 % *	54 ± 4.7 % *

The results are expressed as means ± SEM. The symbol * indicates statistical significance at $P < 0.05$ for difference from the amount of use-dependent blockade at frequency of 1 Hz.

TABLE 9 The use-dependent blockade of RSD1015 and RSD1000 with different pulse durations

	5 μM RSD1015 (n=5)	5 μM RSD1000 (n=4)
10 ms	78 ± 5.2 %	65 ± 3.1 %
100 ms	71 ± 4.6 %	61 ± 5.5 %

The results are expressed as means ± SEM.

Figure. 16 The effects of stimulation frequency on the use-dependent block. (A) and (B) shown the effects of 5 μM RSD1015 and 5 μM RSD1000 in one cell, respectively. The peak amplitudes of I_{Na} were normalized to the first pulse. The effects of different stimulation frequencies are shown as control (open circles), 1 Hz (closed circles), 5 Hz (triangles) and 20 Hz (crosses). The peak amplitudes of first pulse of sodium current are 8.1 nA in control, 7.2 nA (5 μM RSD1015 + 1 Hz), 6.4 nA (5 μM RSD1015 + 5 Hz) and 4.3 nA (5 μM RSD1015 + 20 Hz) in this cell. The peak amplitudes of first pulse of sodium current are 8.3 nA in control, 7.1 nA (5 μM RSD1000 + 1 Hz), 6.1 nA (5 μM RSD1000 + 5 Hz) and 4.1 nA (5 μM RSD1000 + 20 Hz) in this cell.

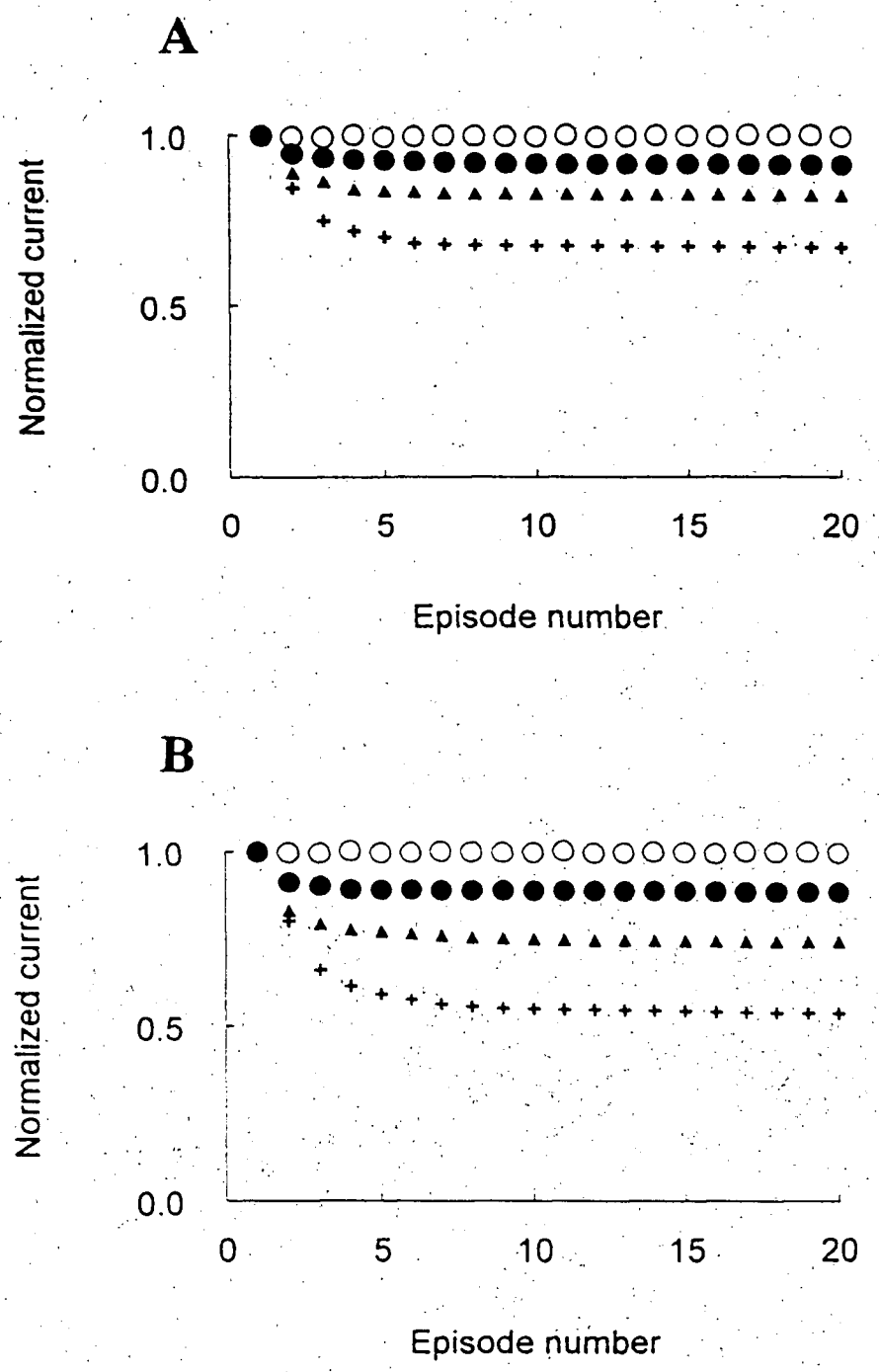

A
1.0
0.5
0.0
Normalized current
0
5
10
15
20
Episode number
B
1.0
0.5
0.0
Normalized current
0
5
10
15
20
Episode number

Figure 16

Figure 17 The effects of depolarizing pulse duration on use-dependent block. In the presence of 5 μM RSD1015 (A) or 5μM RSD1000 (B), normalized I_{Na} presented the results with different depolarization duration of 10 ms (closed circles) or 30 ms (triangles) at the same frequency of 10 Hz, control is shown by open circles. The peak amplitudes of first pulse of sodium current are 8.0 nA in control, 7.3 nA (5 μM RSD1015 + 10 ms), 6.9 nA (5 μM RSD1015 + 30 ms) in this cell. The peak amplitudes of first pulse of sodium current are 8.4 nA in control, 7.2 nA (5 μM RSD1000 + 10 ms), 6.7 nA (5 μM RSD1000 + 30 ms) in this cell.

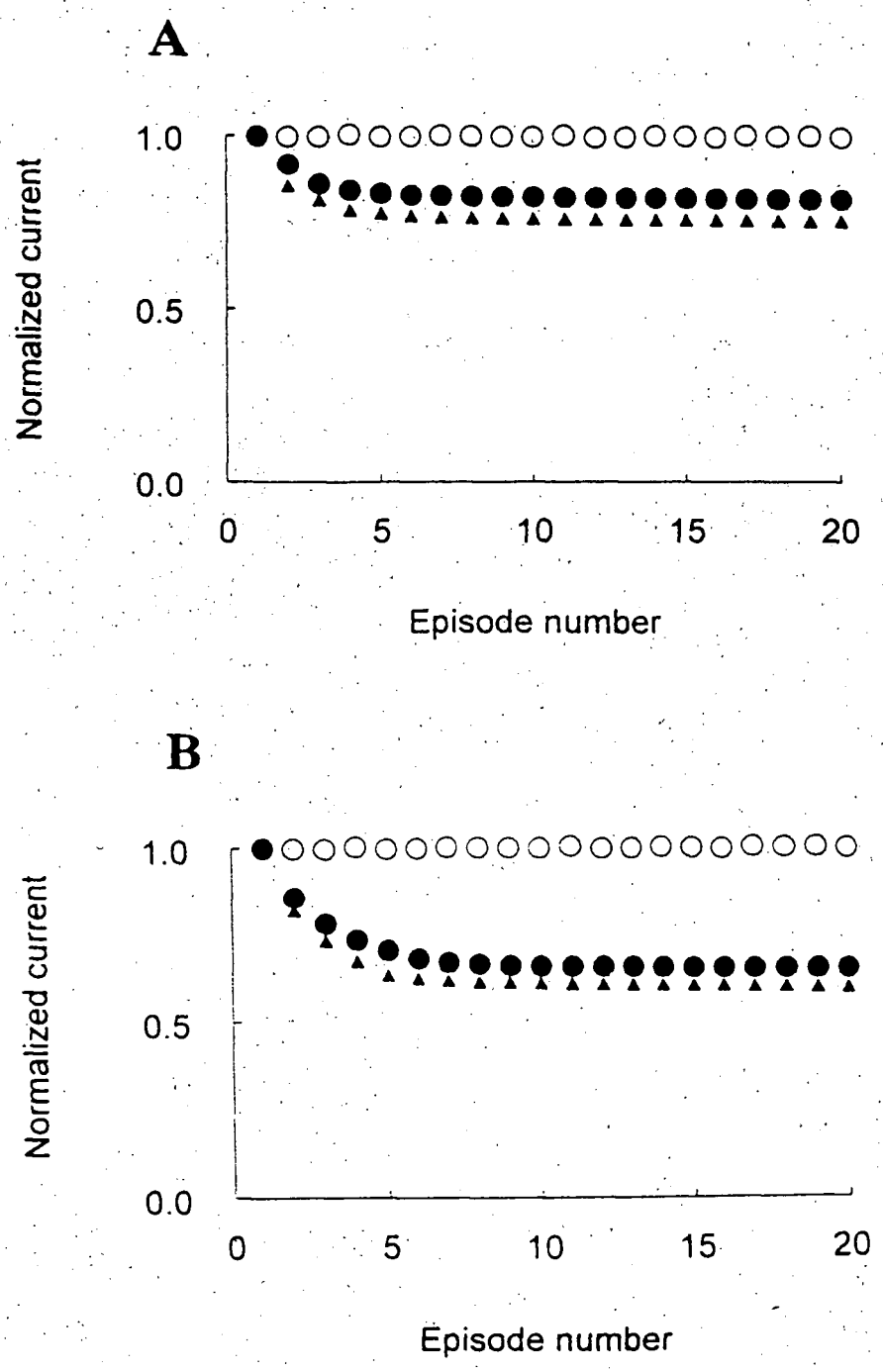

A
Normalized current
1.0
0.5
0.0
0
5
10
15
20
Episode number
B
Normalized current
1.0
0.5
0.0
0
5
10
15
20
Episode number

Figure 17

3.3. The effects of RSD1015 and RSD1000 in acid solution

In this study, the effects of these two compounds in acid solution were studied to obtain drug selectivity in the ischemic tissue. The solution used in this study was adjusted to pH 6.4; the protocols and analytic procedures were the same as those used in the corresponding result section above with normal bath solution of pH 7.3.

3.3.1. The effects of RSD1015 and RSD1000 on I_{to} in acid solution

As found in the normal solution of pH 7.3, RSD1015 also decreased the time course of the inactivation of I_{to} currents in the acid solution of pH 6.4. The inactivation time course (τ) at +60 mV was used to illustrate the dose-response effects of drug. The blockade effect was recoverable after wash-off of the drug with bath solution of pH 6.4.

A dose-response figure for the RSD1015 on the decay time course (τ) of I_{to} is showed in Figure 18A. As for the experiments in solution of pH 7.3, τ was determined by using a single decreasing exponential function to fit the current. Each point on the dose-response graph represents a normalized value to the control value of τ at pH 6.4 in the absence of drug. The dose-response curve was fit by a logistic function and the concentration of RSD1015 which reduced τ to 50% of control value (EC_{50}) was 1.8 ± 0.7 μM (n=5). There was no statistically significantly difference between the EC_{50} of I_{to} blockade with solution at pH 7.3 and pH 6.4 ($P > 0.05$).

Similarly, RSD1000 dose-dependently diminished the time course of the inactivation of I_{to} currents. The dose-response curve of RSD1000 on I_{to} in the acid solution is shown in Figure 18B, the EC_{50} was 6.2 ± 0.8 μM (n=5) (Table 10). The EC_{50}

Figure 18 Effects on I_{to} in acid solution of pH 6.4. The dose-response curve of RSD1015 or RSD1000 on the time constant of I_{to} inactivation τ was shown in (A) and (B), respectively. Open circles presented the data in normal bath solution of pH 7.3, closed circles was the results in acid solution of pH 6.4. The data were fitted by a logistic function. In Figure 18A, the value of τ was 51.7 ± 4.1 ms (n=7) in control solution (pH 7.3), and 56.8 ± 3.7 ms (n=5) in acidic solution (pH 6.4). In Figure 18B, the value of τ was 48.2 ± 3.9 ms (n=5) in control solution (pH 7.3), and 54.7 ±5.1 ms (n=5) in acidic solution (pH 6.4).

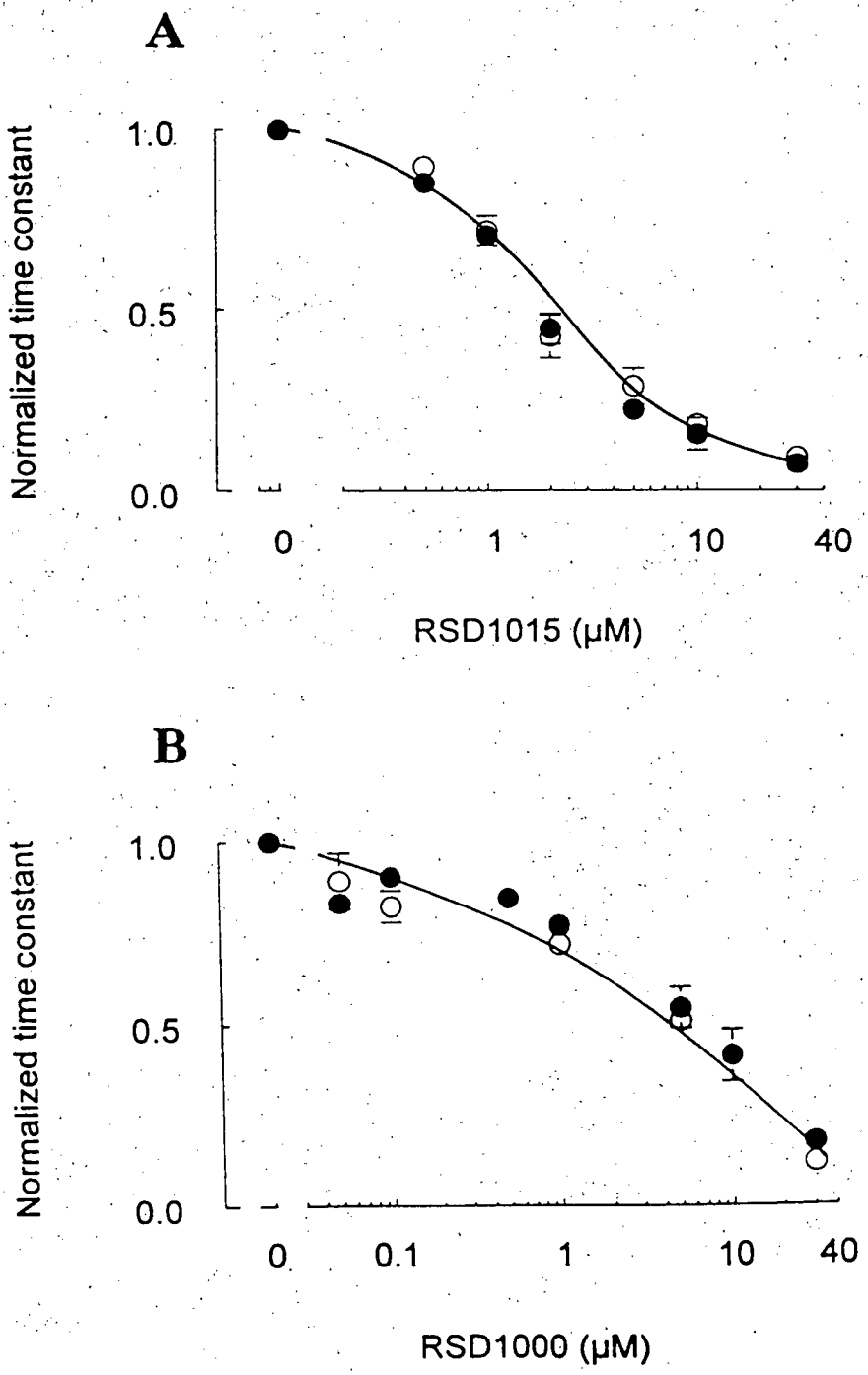

A
Normalized time constant
1.0
0.5
0.0
0
1
10
40
RSD1015 (μM)
B
Normalized time constant
1.0
0.5
0.0
0
0.1
1
10
40
RSD1000 (μM)

Figure 18

of RSD1000 at pH 6.4 was not significantly different from that of normal solution at pH 7.3 ($p > 0.05$).

3.3.2. The effects of RSD1015 and RSD1000 on I_{Na} in acid solution

RSD1015, in a dose-dependent manner, diminished the amplitude of the sodium current with no evident effect to alter the time course of inactivation. From one cell, the peak amplitude was decreased to 76% of that of control by 1 μM RSD1015. The blockade effect was reversible after wash-off of the drug. A dose-response figure for the RSD1015 on steady-state blockade of sodium current amplitude is shown in Figure 19A. The resultant dose-response curve was fit by a logistic function and the EC_{50} of RSD1015 was 3.3 ± 0.8 μM (n=5) (Table 10), which is not statistically significant from the EC_{50} value found in normal solution of pH 7.3 ($P > 0.05$). RSD1000 also decreased the amplitude of the sodium current with an EC_{50} of 0.5 ± 0.1 μM (n=4) (Figure 19B, Table 10), which is significantly different from the EC_{50} value of RSD1000 obtained from bath solution of pH 7.4 ($P < 0.05$).

Table 10 listed the EC_{50} of RSD1015 and RSD1000 on I_{to} and I_{Na} in normal solution of pH 7.3 and acid solution of pH 6.4.

3.3.3. The effects of RSD1015 and RSD1000 on sodium use-dependent blockade in acid solution.

The I_{Na} use-dependence blockade of these two agents was studied in acid solution (pH of 6.4). The protocols used were the same as in the normal bath solution of pH 7.4 (section 3.2.5.1). The amount of use-dependent block was described as the percentage decrease in I_{Na} in the steady state at the 20th pulse with respect to the value for the first pulse.

Figure 19 Effects on I_{Na} in acid solution of pH 6.4. The dose-response curve of RSD1015 or RSD1000 on the amplitudes of I_{Na} was shown in (A) and (B), respectively. Open circles presented the data in normal bath solution of pH 7.3, closed circles was the results in acid solution of pH 6.4. The data were fitted by a logistic function. In Figure 19A, the peak value of sodium current is 6.7 ± 0.8 nA in control solution (pH 7.3)(n=6), and 6.6 ± 0.7 ms (n=5) in acidic solution (pH 6.4). In Figure 19b, the peak value of sodium current is 6.3 ± 0.6 nA in control solution (pH 7.3) (n=9), and 6.1 ± 0.5 ms (n=4) in acidic solution (pH 6.4).

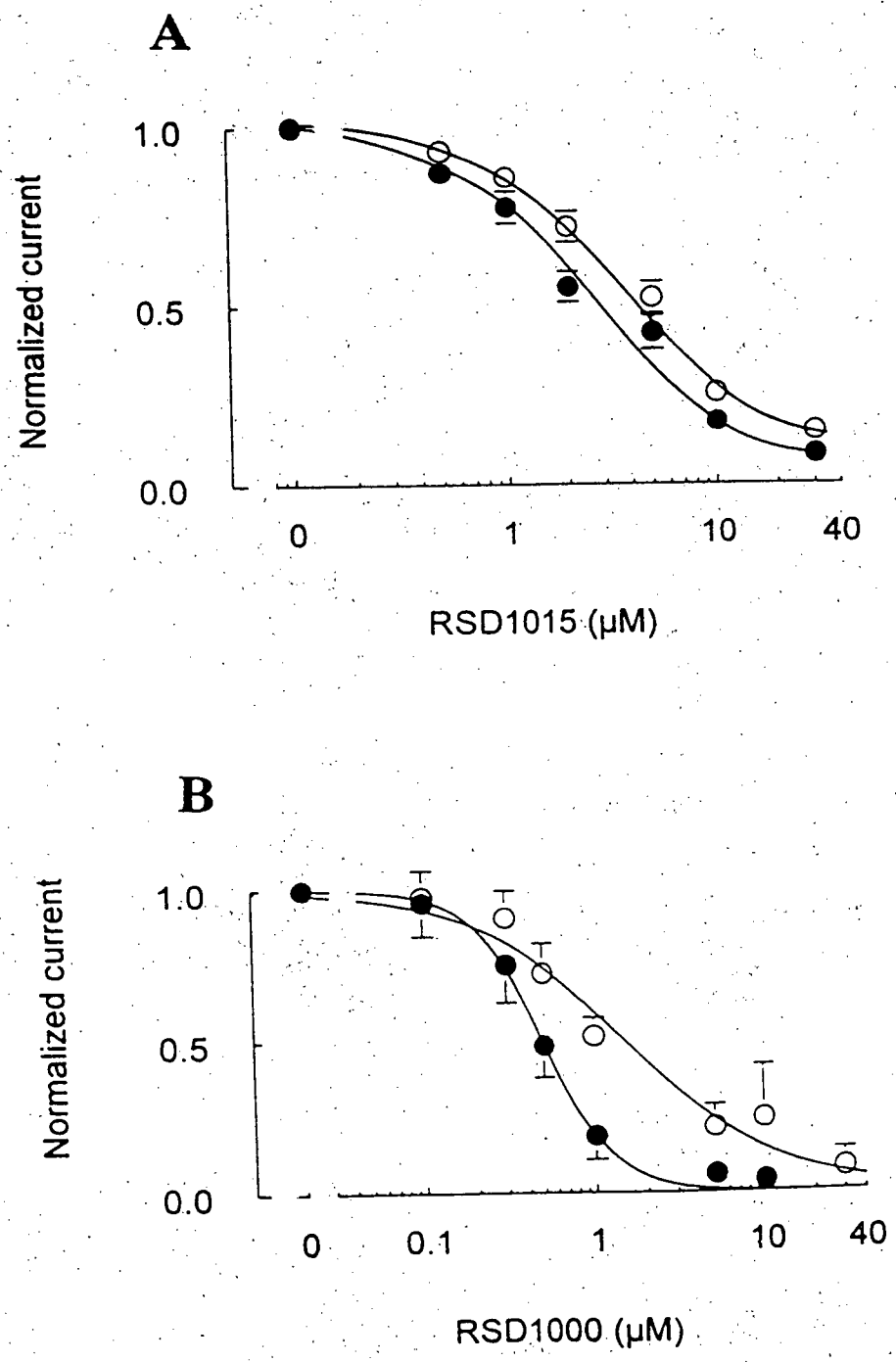

A
1.0
0.5
0.0
Normalized current
0
1
10
40
RSD1015 (μM)
B
1.0
0.5
0.0
Normalized current
0
0.1
1
10
40
RSD1000 (μM)

Figure 19

Figure 20 Effects on I_{Na} use-dependent in acid solution. In the presence of 5 μM RSD1015 (A) or 5 μM RSD1000 (B), normalized I_{Na} presented the results of use-dependence with depolarization duration of 30 ms at the frequency of 10 HZ. Data in control are shown by open circles, the results of drugs are by closed circles. The I_{Na} peak amplitudes were normalized to the first pulse in one cell. In Figure 20A, the peak amplitudes of first pulse of sodium current are 7.9 nA in control and 5.7 nA in the presence of 5 μM RSD1015 in this cell. In Figure 20B, the peak amplitudes of first pulse of sodium current are 8.2 nA in control and 5.3 nA in the presence of 5 μM RSD1000 in this cell.

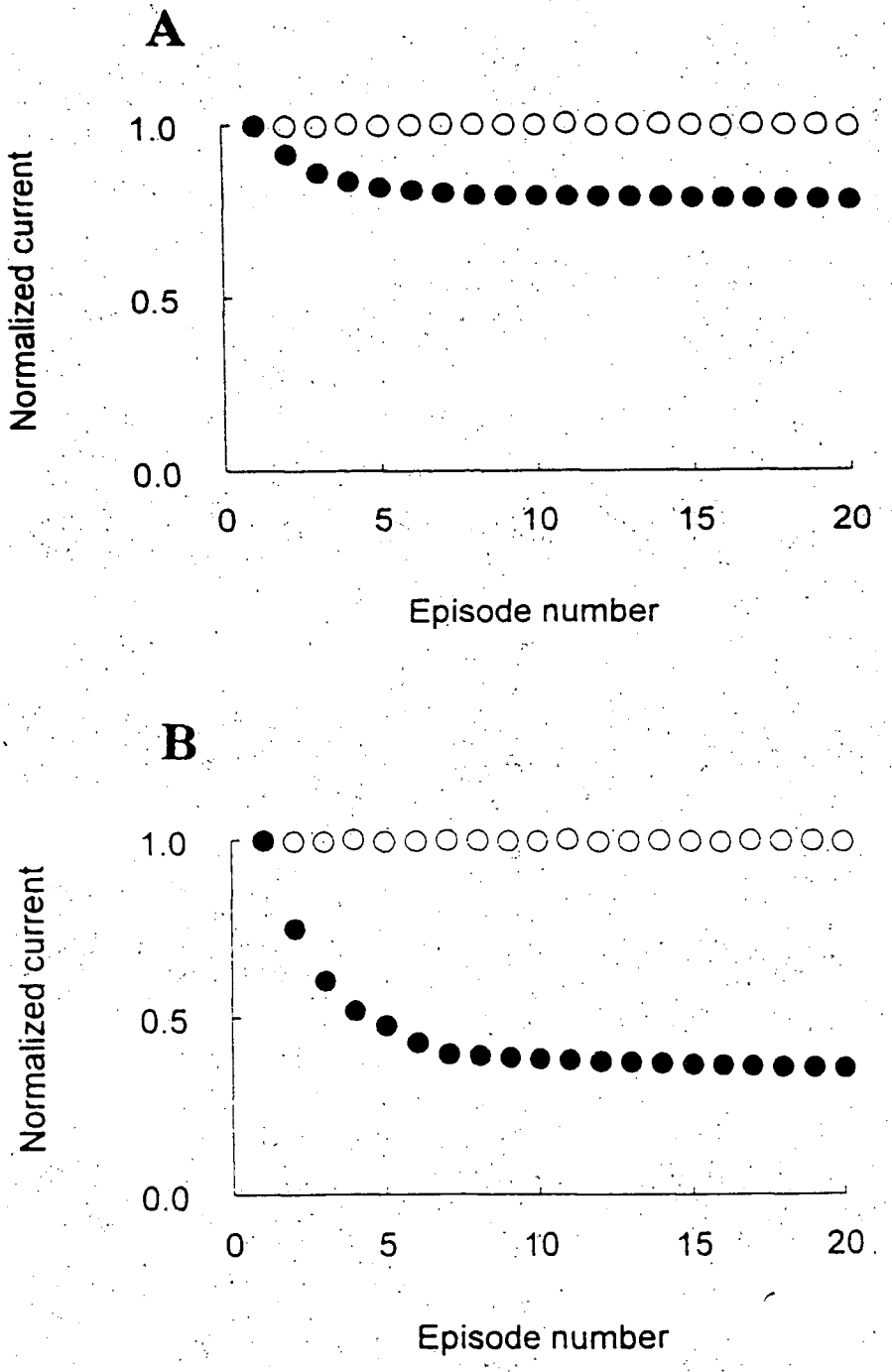

A
1.0
0.5
0.0
Normalized current
0
5
10
15
20
Episode number
B
1.0
0.5
0.0
Normalized current
0
5
10
15
20
Episode number

Figure 20

TABLE 10 The EC_{50} of RSD1015 and RSD1000 on I_{to} and I_{Na} in normal solution of pH 7.3 and acid solution of pH 6.4.

	Normal solution (pH 7.3)		Acid solution (pH 6.4)	
	EC_{50} of I_{to} (μM)	EC_{50} of I_{Na} (μM)	EC_{50} of I_{to} (μM)	EC_{50} of I_{Na} (μM)
RSD1015	1.3 ± 0.4 (n=7)	4.1 ± 0.9 (n=6)	1.8 ± 0.7 (n=5)	3.3 ± 0.8*(n=5)
RSD1000	5.8 ± 0.7 (n=5)	1.5 ± 0.3 (n=9)	6.2 ± 0.8 (n=5)	0.5 ± 0.1*(n=4)

The results are expressed as means ± SEM. The symbol * indicates statistical significance at $P < 0.05$ for difference from the EC_{50} value in normal solution of pH 7.3.

TABLE 11 Use-dependent blockade of RSD1015 and RSD1000 in normal solution of pH 7.3 and acid solution of pH 6.4

	Normal solution (pH 7.3)	Acid solution (pH 6.4)
RSD1015	72 ± 3.2 % (n=6)	78 ± 5.6 % (n=5)
RSD1000	62 ± 4.6 % (n=7)	38 ± 6.4 % * (n=5)

The results are expressed as means ± SEM. The symbol * indicates statistical significance at $P < 0.05$ for difference from the amount of use-dependent blockade in normal solution of pH 7.3.

The use-dependent blockade of I_{Na} by 5 μM RSD1015 in acid solution from one cell was normalized and illustrated in Figure 20A. The I_{Na} current showed a progressive decrease to a steady level, where the amplitude of the 20th pulse was 74% of the 1st pulse. Overall, at a stimulation frequency of 10 Hz, the amount of use-dependent block of I_{Na} by 5 μM RSD1015 was 78 ± 5.6 % (n=5), which is not significantly different from the use-dependent effect in the normal solution of pH 7.4.

The use-dependent effects by 5 μM RSD1000 were enhanced in acid solution, as presented in Figure 20B. Overall, the amount of use-dependent blockade of 5 μM RSD1000 was 38 ± 6.4 % (n=5).

The use-dependent blockade of 5 μM RSD1015 and 5 μM RSD1000 in normal solution of pH 7.3 and acid solution of pH 6.4 are summarized in Table 11.

3.4. The effects of RSD1015 and RSD1000 on other cardiac currents

In rat ventricle both inward rectifier K^+ currents and inward Ca^{2+} current contribute to the genesis of the action potential. To test for selectivity of the RSD compounds, both RSD1015 and RSD1000 were applied to investigate the effects on these two currents. In these experiments a single concentration of 20 μM was used to simply determine whether there was any effect to alter I_{K1} or I_{Ca}. This concentration was considerably higher than the EC_{50} for blockade of I_{to} and I_{Na}.

3.4.1. The effects of RSD1015 and RSD1000 on I_{K1}

The I_{K1} was activated with hyperpolarization steps from a resting potential of -70 mV to levels from -50 mV to -140 mV. No significant change in amplitudes or time

courses of I_{K1} at -140 mV were found with either 20 μM RSD1015 or RSD1000 (n=6) (data not shown).

To investigate the effects of RSD1015 in I_{K1} in the physiological range of potential and the voltage dependence of drug actions, another protocol was used in the study (McLarnon and Xu, 1995). A ramp potential was applied over the range from -140 mV to -40 mV at the speed of 0.25 mV/sec with holding level of -70 mV. The current/voltage (I/V) relations could be obtained from computer analysis. The I/V plot in control (trace a) and in the presence of 20 μM RSD1015 (trace b) are shown in Figure 21, there was no significant blockade over the range of potentials studied. Similar results were obtained from 20μM RSD1000 (data not shown).

3.4.2. The effects of RSD1015 and RSD1000 on I_{Ca}

The recording of I_{Ca} required use of a specific solution (see methods section) to block contributions from other currents. Inward I_{Ca} were evoked with depolarizing steps to -10, 0 and +10 mV from a holding potential of -60 mV. The calcium currents, under our experimental conditions, could be recorded for up to 6 min before a slow run-down of amplitude occurred in the normal bath solution. This slow rundown of current (due to flow through L-type channel) has been documented in other work (Bean, 1985; Nilius *et al* 1985). Measurements were thus obtained within two minutes after attaining the whole-cell clamp. As presented in Figure 22A, there is no change in the amplitudes or time courses of currents in the presence of 20 μM RSD1015. The amplitudes of I_{Ca} current were plotted against the depolarized potential (Figure 22B), which shows no change in control and in presence of 20 μM RSD1015. Similarly, 20 μM RSD1000 causes no effects on I_{Ca}.

Figure 21 Effects of RSD1015 on I_{K1}. I_{K1} recorded from a single myocyte with application of a voltage ramp from -140 mV to -40 mV. The current/voltage relations are with control solution (a) and after addition of 20 μM RSD1015 to the solution (b).

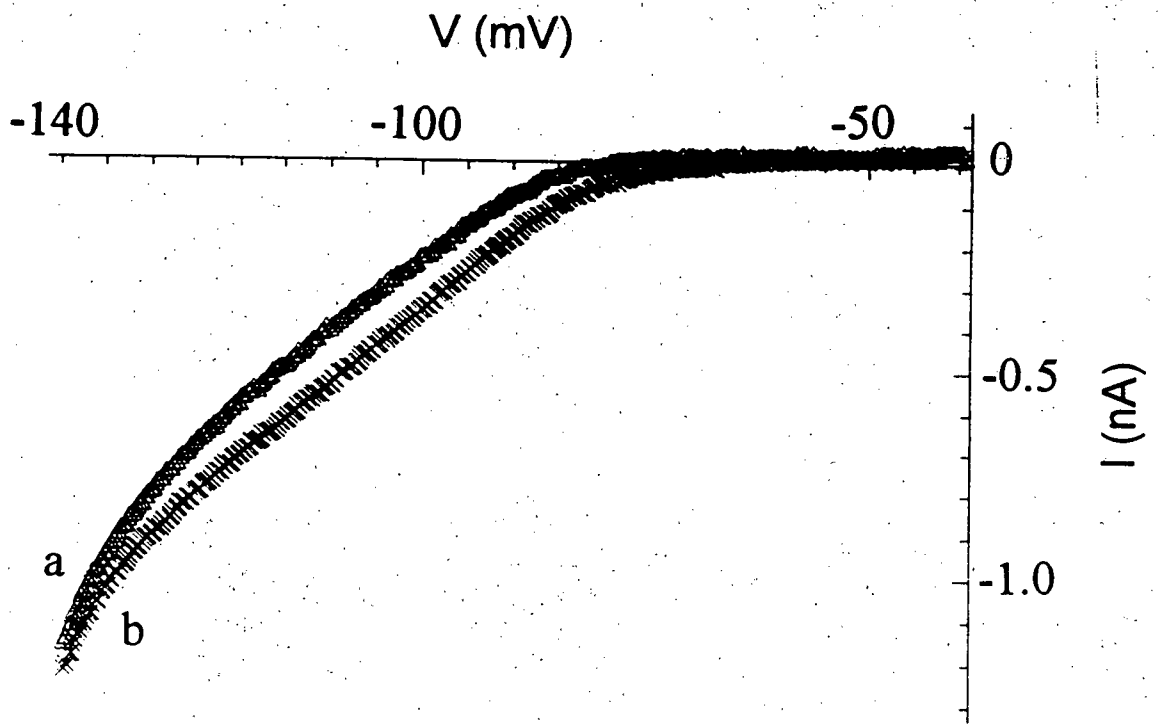


Figure 21

Figure. 22 Effects of RSD1015 on I_{Ca}. I_{Ca} was recorded from a single cell with depolarizing steps to -10, 0 and +10 mV. The results in control (A) and in the presence of 20 μM RSD1015 (B) are shown. The I/V relationship of I_{Ca} is shown in (C) with control (open circles) and 20 μM RSD1015 (closed circles).

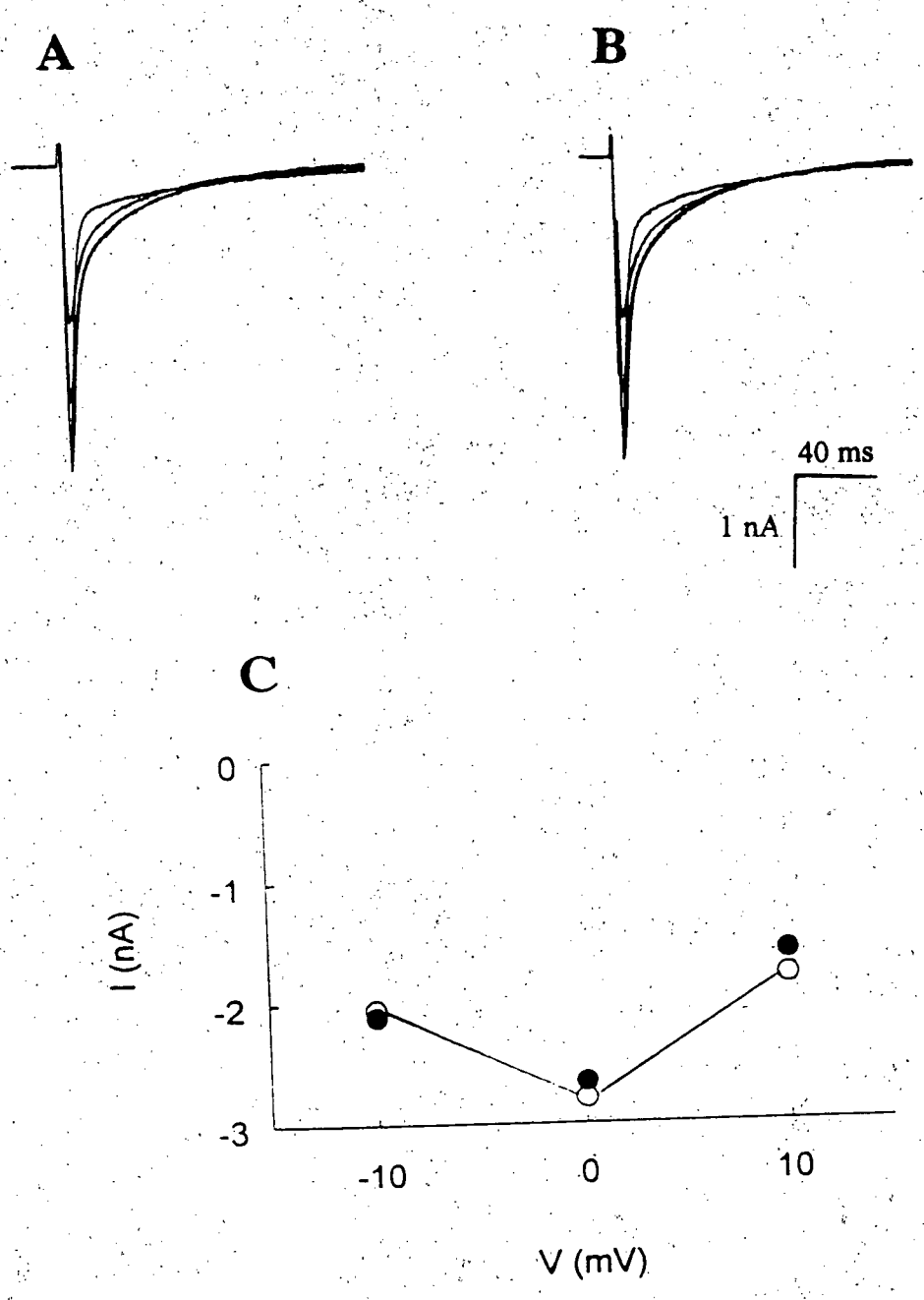


Figure 22

3.5. The *in vivo* study of RSD1015 and RSD1000

The effects of RSD1015 and RSD1000 in isolated rat heart, and in electrically- and ischemical-induced arrhythmia rat were studied. RSD1015 and RSD1000 dose-dependently decreased mean blood pressure (BP), heart rate and prolonged both PR and QT intervals. RSD1015 and RSD1000 also increased the threshold currents for capture (iT), effective refractory period (ERP) and ventricular fibrillation threshold (VFt) in a dose-related manner. The antiarrhythmic actions of RSD1015 and RSD1000 against ischemia-induced arrhythmias in the intact rat are presented as AA_{50} in Table 12.

It is also noted that the antiarrhythmic dose-response curve of RSD1000 is a "sigmodial" shape, which indicated dose-related antiarrhythmic effects, the highest dose of 8.0 μmol/kg/min RSD1000 provided complete antiarrhythmic protection against ventricular tachycardia and fibrillation; while that of RSD1015 is an "N" shaped, such "N-shaped" dose-response curve is an antiarrhythmic dose-response curve with doses following a contiguous dose-related response at low doses of 0.4-1 μmol/kg/min, but at high doses of 2 and 4 μmol/kg/min failing to provide antiarrhythmic protection.

TABLE 12 The *in vivo* effects of RSD1015 and RSD1000

	RSD1015	RSD1000
D_{25} of BP (μmol/kg/min)	2.8 ± 0.7	3.0 ± 0.8
D_{25} of PR (μmol/kg/min)	6 ± 1.7	13 ± 2.1
D_{25} of ERP (μmol/kg/min)	2.9 ± 0.9	4.2 ±1.3
AA_{50} (μmol/kg/min)	3.5 ± 1.5	2.5 ± 0.6

The values were obtained from dose-response curves. Results are expressed as means ± SEM. D_{25} is the dose producing a 25% change from control in a variable. AA_{50} is the doses producing a 50% change from pre-drug arrhythmia score (AS) values.

4. DISCUSSION

4.1. Effects of RSD1015 and RSD1000 on macroscopic cardiac currents

The present study has shown that two new antiarrhythmic agents RSD1015 and RSD1000 exhibit potent mixed blockade of I_{to} and I_{Na} with little or no effects on other currents including I_{K1} and I_{Ca}. The mixed inhibition of RSD1015 and RSD1000 on I_{to} and I_{Na} in isolated rat ventricular myocytes is consistent with findings from *in vivo* studies. In rats, RSD1015 and RSD1000 dose-dependently increased the potassium-dependent variables, such as QT interval in ECG measurements and ERP in electrical-stimulated experiments, and the sodium-dependent measurements PR, QRS and iT with the perfusion of agents (Yong *et al* 1997; unpublished observed data). It is noteworthy that the EC_{50} values of RSD1015 and RSD1000 for mixed block of I_{to} and I_{Na} are lower than those of any other compounds which are known inhibitors of both currents, such as KC8851 (Xu *et al* 1996; McLarnon and Xu, 1997) and quinidine (Clark *et a.,* 1995). The mechanisms of drugs actions on different states of channels were investigated and the effects of RSD compounds on both I_{to} and I_{Na} in solutions of different pH were also studied. The relationship of physicochemical properties of RSD1000 and RSD1015 to the potency and selectivity of agents is discussed.

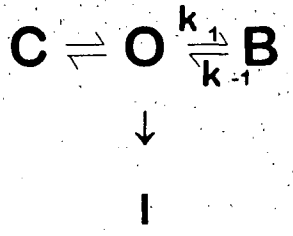
4.2. The interactions of RSD1015 and RSD1000 with transient outward potassium current (I_{to})

4.2.1. Mechanisms of drug actions on different states of I_{to}

RSD1015 and RSD1000 share some characteristics for blockade of I_{to}, such as the agents increase the rate of decay of the current in a concentration-dependent

manner (Figure 3 and 4), without significant change in the activation of current (Figure 5), steady-state inactivation (Figure 6) or recovery from inactivation (Figure 7). The inhibition of RSD1015 and 1000 was time-dependent (see Figure 9). The effects are very different from closed channel blockers such as 4-AP (Giles and Imaizumi, 1988; Jahnel *et al* 1994), and indicated that RSD1015 and RSD1000 mainly interact with the open state of the I_{to} channel. At the onset of a depolarizing pulse, there was little inhibition of I_{to} with low concentration of drugs. This result would suggest that these two agents do not interact with the resting state of the channel. In addition, the lack of effect of RSD compounds on the voltage-dependence of steady-state inactivation and the recovery from inactivation suggests that the agents do not bind to the inactivated state of the channel. With continued depolarization (during channel opening) inhibition develops in an exponential manner, with the rate and magnitude of inhibition dependent on drug concentration (Figure 9B). This effect can be explained that these two agents interact preferentially with the open state of the channel leading to what is called "open channel block" (Armstrong, 1969). Based on this mode of action, it would be predicted that these agents do not alter the single-channel conductance but reduce the duration of a single opening or a burst of openings.

In order to model I_{to}, it is assumed that with membrane depolarization, potassium channels change from a closed state into an open state and then to either an inactivated state or a drug-occluded state in the presence of drug. The kinetic model of transient outward current can be written as:



The C, O and I are closed, open and inactivated states of the I_{to} channels, respectively. B is a non-conducting, drug-blocked channel. This model is simplified from the complex channel states in that it only has one closed state and one inactivated state (Aldrich, 1981; Clark *et al.*, 1988, 1995). It is understandable that the normal decay of the transient outward potassium current will be accelerated by RSD1015 or RSD1000 using this kinetic model. Because the drug-occluded state competes with the endogenous inactivation process, then the open channel can be followed by two pathways; namely, inactivation of the potassium channel (O $\rightarrow$ I) or by drug block (O $\rightarrow$ B).

The interactions of RSD1015 and RSD1000 with the potassium channel can be summarized by k_1, k_{-1} and k_d , which are the onset, offset rate and dissociation constant (k_{-1}/k_1) respectively (Table 6). The decrease in peak amplitude of I_{to} as shown in Figure 3 and 4 is still consistent with rapid open channel block. The net onward rate constant, product of k_1 and [D], is about 80 s^{-1} with 5 μM RSD1015 or 10 μM RSD1000. Inverting this product gives a time of 12.5 ms. This time can be considered as an estimate for equilibration of the drug during channel blockade. If the time taken for I_{to} to peak is near 5 ms (Jahnel *et al.*, 1994), then a significant portion of channels will be blocked during the activation phase. Drug block will then be recorded as an effect to diminish peak amplitude during the activation phase, especially in the presence of high

concentrations of RSD compounds. However, at lower concentrations of agents, the product of k_1[D] is quite small so that few channels would be blocked during channel activation. In this case, some contributions from tonic block by the agents may also occur.

The relatively slow onset of drug actions and the observations of only partial recovery of I_{to} after wash-off suggest an intramembrane or intracellular site for actions of RSD1015 and RSD1000. This point was studied by including the compounds (at 30 μM) in the patch pipette solution. The results, from analysis of several cells with RSD1015 (n=5) and RSD1000 (n=4), showed that the drug had little or no effects on the amplitudes or the kinetic behavior of I_{to} for long durations (up to 20 min) after the establishment of the whole-cell mode. The lack of effect with internal application of compounds would indicate that either very little agent was released from the patch pipette, or if any agent was released, was buffered by internal cytoplasmic factors such as anionic protein. This type of behavior has been previously described in study using cationic potentiometric dyes (Waggoner, 1979). This result may apply to the positively charged RSD1015 and RSD1000 molecules which may accumulate in a localized area of the intracellular compartment and not access the channel. Other studies have shown the effects of intracellular drug application. For example, some agents that blocked I_{to} with external perfusion, such as tedisamil (Dukes *et al* 1990) and clofilium (Castle, 1991), were also effective blockers when applied intracellularly. An internal action site is consistent with the idea that agents may move into the inner opening of the channel and mimic inactivation. But, the absence of either voltage- (Figure 8) or use-dependence of I_{to}, unlike the effects of quinidine (Clark *et al* 1995), suggested that

the agents do not sense changes in the membrane voltage field. In any event, the specific pathway for block of I_{to} can not be ascertained from out data.

4.2.2. The relationship of RSD compounds structure and I_{to} block actions

There are no significant differences of the EC_{50} values of RSD1015 and RSD1000 in normal solution compared to acid bath solution (Table 10). This effect indicated that the actions of RSD compounds on I_{to} are insensitive to changes on external pH. The pH-independent I_{to} blocking actions of RSD1015 and RSD1000 suggested that I_{to} blockade may provide a background antiarrhythmic action, which may not related to the pKa or other structural properties of compounds.

The magnitudes of the k_d of RSD1015 and RSD1000 (Table 6) are quite low compared with the following values for other compound: KC8851 (2.1 μM) (McLarnon and Xu, 1997); tedisamil (2-5 μM) (Dukes *et al*, 1990); terikalant (17 μM) (McLarnon and Xu, 1995), bupivacaine (23 μM) and octylacaine (3.5 μM) (Castle, 1990). However, the k_d of RSD1015 on I_{to} is lower than that of RSD1000 (Table 6), which may relate to the high hydrophobicity of RSD1015, it has been suggested that increasing the hydrophobicity enhances the potency (Castle, 1990),

4.3. The interactions of RSD1015 and RSD1000 with inward sodium current (I_{Na})

The results show that RSD1015 and RSD1000 have similar mechanisms of I_{Na} blockade. They produce a concentration-dependent, reversible block of the I_{Na} in rat ventricular myocytes and shift the inactivation curve of I_{Na} to more hyperpolarized potentials (Figure 13) with no shift in the activation curve (Figure 12). The I_{Na} inhibition also showed tonic and use-dependent block, which is very similar to most class I

antiarrhythmic agents described previously (Campbell, 1992; Nattel, 1993; Wu *et al* 1995; Pugsley and Saint, 1995; Nawada *et al.*, 1995).

According to the modulated receptor theory mentioned previously (section 1.4.1.1), tonic block is composed of a resting and an inactivated state block of the sodium channel. From the results in this study, it is difficult to make a clear distinction between the rest- and the inactivated-state block of RSD1015 and RSD1000. However, it has been suggested that these two agents have high affinities for the inactivated state because there are significant negative shifts of the voltage-dependent inactivation curves (Figure 13), especially RSD1000, which has a more negative hyperpolarizing shift than that of RSD1015. If the compound unbinding rate is quite slow, the inhibition in inactivated state would decrease in the number of sodium channels available in the steady state condition, since considerable agents are still associated with the channel when the next action potential occurs.

In the presence of RSD1015 or RSD1000, a train of depolarization pulses produced use-dependent I_{Na} block which was enhanced at high stimulation frequencies (Figure 16). This use-dependent blockade occurs because either the compounds molecule accesses its binding site only when the Na^+ channel is in an open state or the recovery from block is incomplete during channel inactivation. There was no significant difference of use-dependent block with long or short depolarization pulses (Figure 17), which suggested that the onset of the use-dependent block is rapid and the use-dependent blockade of I_{Na} was mainly due to binding in the open state. This open state blockade can play an important role in inhibition of I_{Na}. In practice, use-dependence blockade is a useful property for an antiarrhythmic drug since rapid firing of action

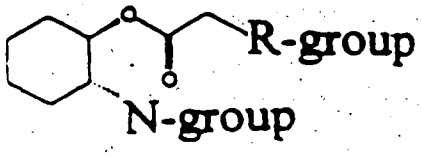
potentials will be suppressed in tachycardia, but the normal firing of action potentials will not be greatly affected by the agent.

4.4. The physicochemical properties of RSD compounds and their actions on I_{Na}

There are some significant differences between the actions of RSD1015 and RSD1000 on potassium and sodium currents including the EC_{50} values for I_{to} and I_{Na} block (Figure 3 and 4; Figure 10 and 11), the magnitudes of use-dependent blockade of I_{Na}, and the high ischemic-selectivity of RSD1000 on sodium current in acid conditions. These two agents also showed different effects against ischemia-induced arrhythmias *in vivo* (section 3.5). The physicochemical characteristics of RSD1015 and RSD1000 are discussed to possibly explain their roles in determining the efficacy of the agents.

4.4.1. The physicochemical properties of RSD1015 and RSD1000

RSD1015 and RSD1000 are developed from a basic structure, (±)-trans-[2-(4-Morpholinyl)cyclohexyl] acetate (see Figure 2 for detailed structure of RSD1015 and RSD1000). The compounds are in the family of esters with a naphthalene R-group.



The important physicochemical properties of RSD1015 and RSD1000 are presented in Table 13.

TABLE 13 The physicochemical properties of RSD1015 and RSD1000

	RSD1015	RSD1000
N-group	N-methylpiperizine	N-morpholino
R-group	2-naphthalene	1-naphthalene
Molecular weight	439.42	390
pKa	8.9	6.1

Although it has been suggested that some physicochemical characteristics, such as molecular weight, lipid solubility and especially the type/position of the aryl R-group can act as determinants for efficacy of RSD agents, this study focused on pKa as one of the important factors for the difference of antiarrhythmic actions of RSD1015 and RSD1000.

4.4.2. The pKa of RSD1015 and RSD1000 and their I_{Na} blockade

The interactions of local anaesthetics and antiarrhythmic agents with nerve and cardiac sodium channels have been well studied (Hille, 1977). From these studies, the modulated receptor hypothesis was formulated (section 1.4.1.1). The following hypothesis are proposed for interactions of antiarrhythmic agents with cardiac sodium channels.

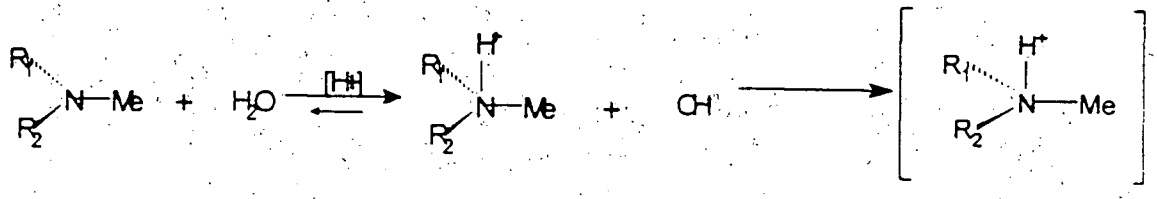
1. Antiarrhythmic agents bind to a specific intracellular site on or near the sodium channel.
2. The neutral agent can gain access to the binding site through the hydrophobic pathway extracellular (i.e., via the lipid layer), whereas the

charged form must use the hydrophilic pathway intracellular (i.e., through the open pore) (Hille, 1977).

3. The cationic molecule interacts preferentially with the sodium channel, although both protonated and unprotonated forms have some activities on channels (Butterworth and Strichartz, 1990).

Some properties of RSD1015 and RSD1000 and their actions on sodium channel are considered:

1. The pKa values of RSD1015 and RSD1000 are mainly determined by the nitrogen (N) group. In solution, the following equilibrium between the neutral and charged form can be established for these agents with different N-groups.



2. The possible pathways of RSD compounds to interact on an intracellular channel binding site from extracellular to intracellular milieu are illustrated below (Figure 23):
3. In acid conditions with external solution pH of 6.4, the internal pH of cells also decreased.

4. At a solution pH of 7.4 and 6.4, 97% and 99% of the RSD1015 molecule are protonated because of its pKa of 8.9.
5. At a solution pH of 7.4 and 6.4, 5% and 25% of RSD1000 (pKa=6.1) are positively charged.
6. The concentration of neutral species is in equilibrium between the extracellular and intracellular compartments.

Using these values, in acid solution of pH 6.4, more RSD1015 will be in the cationic form than it in a solution of pH 7.4, which could not access into intercellular pool via the open channel pore ("trapped") or the hydrophobic pathway (the lipid membrane layer). This decreases the concentration of neutral species (N_i) in the intracellular compartment and then the intracellular cation (C_i) concentration which interacts preferentially with sodium channel binding site. In other words, less charged forms of RSD1015 are available for intracellular binding to sodium channels. This model can explain the significantly lower use-dependent inhibition of I_{Na} by RSD1015 in acid solution.

Although there are 25% of RSD1000 protonated in the solution of pH 6.4, there are still 75% of agent in the hydrophobic form, which can cross the lipid cell membrane layer for channel binding site without a partitioning-limiting effects. In the acid intracellular condition caused by external acid solution, more RSD1000 molecules will be ionized ($N_i \rightarrow C_i$), which lowers the intracellular concentration of neutral forms(N_i), drives more neutral species from extracellular compartment into intracellular pool ($N_o \rightarrow N_i$) and become charged ($N_i \rightarrow C_i$) species for preferentially channel binding. The high concentration of more active ionized compound in the intracellular compartment could

cause the I_{Na} use-dependence blockade and high ischemia-selectivity of RSD1000. This hypothesis of pH-dependent interactions of tertiary amine sodium channel blockers has been suggested to be the mechanism of pH modulated effects of RSD1000 (Yong *et al.*, 1997). Other studies have indicated that only the quaternary analogues and tertiary amine analogues existing predominantly as the charged molecular form produce use-dependent block in nerve (Narahashi, *et al.*, 1970, Frazier, *et al.*, 1970) and cardiac tissue(Gintant, *et al.*, 1983).

4.5. The *in vivo* actions of RSD1015 and RSD1000

4.5.1. The actions of RSD1015 and RSD1000 on cardiac currents

Both RSD1015 and RSD1000 exhibit potent inhibition effects on I_{to} *in vitro* and *in vivo* (Figure 3 and Figure 4, Table 12). The plateau (phase 2) of action potential is determined by a equilibrium which exists between the inward and outward currents in this phase. In rat and human atrial and ventricular myocytes, I_{to} channel is the main repolarizing current and plays an important role during the early repolarization (Apkon and Nerbonne, 1991; Nabauer *et al.*, 1993). Therefore, I_{to} blockers, such as RSD1015 and RSD1000, would cause a prolongation of the action potential duration, especially the early repolarization period (APD_{50}) and increase the refractoriness *in vivo*. Such modification of both atrial and ventricular electrophysiological properties will have a wide variety of antiarrhythmic effects in human. It is well known that K^+ outward currents suppressers prolong the action potential duration, increase the refractoriness of the conduction system and thereby exert their antiarrhythmic effects (Hondeghem and Katzung, 1987; Gwilt *et al.*,1991; Hondeghem, 1992; Noble, 1992; Wu, *et al.*, 1995).

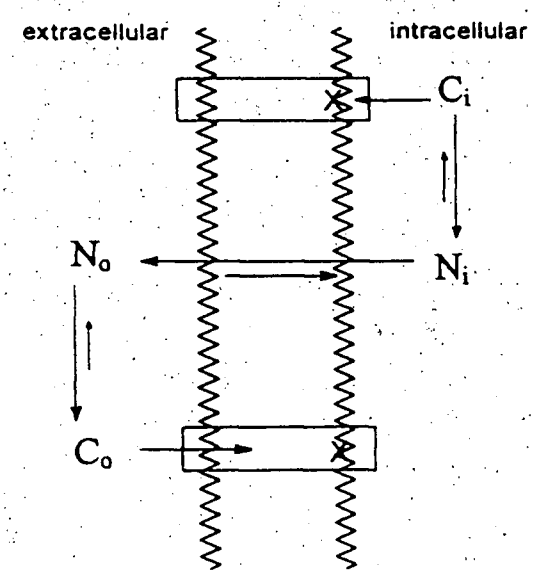


Figure 23 The model illustrates the partitioning of neutral (N) and cation (C) agents between extracellular and intracellular milieu and acting at an intracellular binding site (X) on the sodium channel, the subscript of "o" and "I" indicate extracellular and intracellular compartments, respectively.

RSD1015 and RSD1000 also blocked variables dependent on sodium currents (Figure 10 and 11, Table 12). When Na^+ channels are blocked, threshold for excitability is increased, *i.e.*, greater membrane depolarization is required to bring Na^+ channels from the rest to open states. By increasing threshold, Na^+ channel block decreases automaticity and can inhibit triggered activity arising from delayed afterdepolarizations or early afterdepolarizations. In anatomically defined re-entry, Na^+ channel blockers may decrease conduction sufficiently to extinguish the propagating re-entrant. However, conduction slowing due to Na^+ channel block may exacerbate re-entry and promote arrhythmias (proarrhythmia).

4.5.2. The ischemic selectivity of RSD1015 and RSD1000

The selectivity effects of RSD1015 and RSD1000 over ischemic tissue were measured using bath solution of pH 6.4, which is similar to the conditions in ischemic cardiac tissue. The potency of I_{Na} block (EC_{50}) and the use-dependent of RSD1000 was significantly enhanced in acid solution (Figure 19, Table 10). This increased potency of RSD1000 in acid condition is termed "ischemia-selectivity".

One of the interesting results from *in* vivo study is that although both agents can abolish occlusion-induced arrhythmias, RSD1000 produced a "sigmodial" antiarrhythmic dose-response curve, while RSD1015 produces a "N-shaped" curve. Such "N-shaped" dose-response curve of RSD1015 may indicate proarrhythmic effects at higher concentrations of 2 and 4 μmol/kg/min. From these results, the inhibition of RSD compounds on I_{to} is one of the important portion of their antiarrhythmic activities, but their selective sodium channel blockade in acid solution affect the selectivity for ischemic myocardium with no or little contribution from I_{to} blockade.

There is no change in the potency and the use-dependent blockade of RSD1015 on I_{Na}, which indicated a low ischemic-selectivity for this agent. In the case of re-entry induced arrhythmias, antiarrhythmic agents with low ischemic selectivity may cause proarrhythmias by decreasing conduction in both ischemic and normal tissue, and thereby facilitate or produce re-entrant circuits for arrhythmogenesis (Hondeghem, 1987; Janse, 1992; Roden, 1994).

The selectivity of ischemic conditions for RSD1000 appears to be the function of ischemic selective effects of sodium channel block according to its pKa value (section 4. 4. 2). The high ischemia-selectivity of lidocaine was contributed to its protection against ischemia-induced arrhythmias (Davis, *et al* 1985; Campbell and Hemsworth, 1990; Barrett *et al* 1995). It had been hypothesised that the lack of ischemia-selectivity of class I antiarrhythmic agents may be one of the important factors contributing to their limited efficacy against ischemia-induced arrhythmias (Barrett *et al* 1995). The lack of selectivity against ischemic tissue could be one of the important factors for the increased mortality in the CAST study (Tamargo *et al.*, 1992). In this way, the high selectivity of RSD1000 in acid condition may provide great clinical usefulness.

The modulation by pH of I_{Na} block with RSD1000 can also be caused by a rate-limiting step of drug dissociation from blocked channels (section 1.5.1). The slowed recovery from blockade at low pH is believed to come from an increase in the fraction of charged drug at the receptor site (Hille, 1977; Schwarz *et al.*, 1977). This is because the charged agent must first undergo deprotonation before the neutral species can escape via the hydrophobic membrane route. However, without evidence on the recovery kinetics of RSD compounds on I_{Na} block, there is little to be said about the pH-

dependence of drug recovery from I_{Na} blockade. Other structural properties, such as the type/position of the aryl R-group, have been suggested as an important determinant for ischemic selectivity (unpublished data).

It also necessary to mention that the conclusion of pKa as an important determinant of ischemia-selective antiarrhythmic activity, comes from the study with only two agents. Future studies with other structurally-related RSD compounds but different pKa's and compounds with similar pKa and different structures will be required to evaluate and optimize factors important in determining antiarrhythmic efficacy.

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