COMPARISON OF DRUG BLOCKADE OF A NEURONAL CALCIUM-ACTIVATED POTASSIUM CHANNEL WITH CARDIAC REPOLARIZING POTASSIUM CHANNELS BY POTENTIAL CLASS III AGENTS

by

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ABSTRACT

The aim of the study was to examine the use of a repolarizing calcium dependent potassium current K(Ca) in neurons to describe the actions of a group of novel compounds with potential Class III actions on cardiac cells. This was accomplished by determination of the correlation between potency of the agents to block single channel K(Ca) and potency to prolong effective refractory period (ERP) in heart. If a positive correlation could be established then elucidation of mechanisms of drug actions on single channel K(Ca) could have utility in the description of drug actions on repolarizing K+ currents in heart. At present the low unitary conductance of transient outward and delayed rectifier K+ channels precludes a mechanistic analysis of drug actions on cardiac cells.

Initial experiments included the measurements of the single channel properties of the K(Ca) using inside-out patches obtained from cultured hippocampal neurons. The channel conductances, with physiological (5K+ and 140K+ across patches) and symmetrical (140 K+ across patches) were 110 pS and 170 pS respectively. A requirement of 4 μM internal calcium was necessary to maintain maximal channel activity with a threshold for K(Ca) activation at 0.7 μM. At low internal calcium concentration, depolarization increased the probability of channel openings. The effect was found to be solely dependent on the voltage-sensitive increase of the channel opening frequency; mean open times of the channel were not dependent on patch potential.

The unitary K(Ca) is the microscopic basis for the macroscopic repolarizing current Ic in hippocampal neurons. Ic is responsible for the late repolarization phase and the early afterhyperpolarization (AHP) phase associated with the neuronal action potential. It was of interest to first determine

the effects on Ic of an agent, tedisamil, with known Class III activity in heart. The results showed the drug both prolonged the neuronal action potential and eliminated the subsequent AHP phase.

The primary set of experiments involved the investigations of the effects of 18 RSD novel compounds on unitary K(Ca). All the compounds, except for three, were effective in exhibiting rapid transitions in the K(Ca) from the opening state to a non-conducting state in the inside-out patches. The mean open times of open events were reduced but the closed time durations and the channel amplitudes were not changed. The potency of the effect of the RSD compounds on the K(Ca) was determined as the concentration required to halve the mean open time relative to control value. According to this index the compounds were categorized into five different groups based on potency to decrease mean open time of K(Ca). In addition the actions of five of the RSD compounds on K(Ca) were examined using outside-out patches excised from neurons.

The potency of the RSD compounds on the neuronal K(Ca) was compared with their potency in inhibiting repolarizing K+ currents in the rat whole heart. The index for potency used in the whole heart experiments was the concentrations of the compounds required to increase the effective refractory period by 25%. Of the 18 compounds tested, 3 were found to be inactive (no obvious effect for concentration below 50μM) on properties of K(Ca). These same 3 agents also were ineffective in whole heart (in excess of 20μM). For 13 of the remaining 15 agents, a positive correlation was found (with a correlation coefficient r of 0.71) between potency to block K(Ca) and potency to prolong ERP in whole heart. However, with 2 agents there was no apparent correlation for actions in the neurons and the heart. It was also established that drugs with persistent effects on K(Ca) (likely due to prolonged bonding to membrane sites) were also long-lasting in whole heart experiment.

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1. INTRODUCTON

1.1 The Calcium-activated potassium channels

1.1.1 Calcium-activated potassium conductances

Internal calcium was first demonstrated to be able to regulate potassium flux across membranes of human erythrocytes by Gardos (1958) almost 35 years ago. A more direct approach was given by Meech & Strumwasser (1970), who observed that a microinjection of intracellular calcium activated potassium conductance in *Aplysia* nerve cells and hyperpolarized the cell membrane. Based upon these observations, a Ca^{2+} -activated K^+ conductance, $G_{K(Ca)}$, was postulated (Meech, 1978). Since then, studies using the patch-clamp (Neher and Sakmann eds., 1983) and reconstitution (Miller ed., 1986) techniques have demonstrated several different types of Ca^{2+}-dependent K^+ channels (Blatz and Magleby, 1987). Channel conductance, calcium sensitivity, voltage dependence, and pharmacological properties (i.e. antagonists studies) have been used to distinguish between these channels.

1.1.2 Two types of K(Ca) channels

In general, two major classes of Ca^{2+}-dependent K^+ channels common to the excitable cells have been catergorized. One group are the voltage-dependent K^+ channels of large unit conductance (150-300 pS) (Marty, 1981; Blatz and Magleby, 1984). These are often referred to as the "Big (B)" or "Maxi" K^+ channels, which are also widespread in non-excitable cells. The single channel properties of these have been studied in detail (for example, Pallotta et al., 1981; Moczydlowski and Latorre, 1983), thanks to the large conductance of the channel which yields a high signal-noise ratio in the single channel recordings.

The second group of K^+ channels show little (e.g. *Aplysia*) or no voltage dependence (e.g olfactory neurons) and are of smaller conductance (≤ 80 pS) (Hermann and Erxleben, 1987; Maue and Donne, 1987). They are the "Small (S)" K^+ channels, also known as the AHP channels due to their predominant role in afterhyperpolarization (see later) [Some authors subdivide this class further into the small K^+ channels (conductance < 50pS) and the intermediate K+ channels (50 - 150pS)]. Throughout this paper the high conductance channel will be referred to as the K(Ca) and the small conductance channel as the SK.

1.1.3 Single channel properties of the K(Ca)

1.1.3.1 High permeability

The K(Ca) possess a large conductance that is close to the limit theoretically expected for a pore (Hille, 1984). Depolarization enhances the probability of channel opening, leading to an increased flow of current (Pallota et al., 1981). The conductance also varies with the extracellular $[K^+]$ in a non-linear fashion. In excised patch from cultured rat muscle, the conductance, with symmetrical 140 K^+ across the membrane, is as high as 220 pS. The reduction of $[K^+]_O$ to physiological levels near 5mM results in a decrease in the single channel conductance to about 100 pS (Barrett et al., 1982; McLarnon and Wong, 1991).

1.1.3.2 High selectivity

The K(Ca) also have a high cation selectivity. The situation is somewhat ironical since a high selectivity implies strong interactions of the permeant ion with the selectivity filter (Latorre and Miller, 1983). The channel is strongly selective for K+ over Na^+, with a Na^+/K^+ permeability ratio of less than 0.01

(upper limit 0.03; Yellen, 1984). Thus K(Ca) are as selective as the delayed rectifier of nerve and muscle but with a conductance 10X to 50X bigger. The K(Ca), like most other types of K^+ channels, have a similar ionic selectivity sequence (Blatz and Magleby, 1984; Gitter et al., 1987; Singer and Walsh, 1987): $Ti^+ > K^+ > Rb^+ >> Cs^+, Na^+, Li^+$. The attempts to explain this high selectivity-conductance paradox have given birth to several hypotheses. One of them is the design of an appropriate selectivity filter for the K(Ca), based on the idea that if the entire length of the pore has the same narrowness as the filter part the conductance would be low. Thus, a hypothetical structure has been proposed for the K(Ca) in the SR of mammalian skeletal muscle (Yellen, 1987). He suggested a pore having a wide entry and exit structure, with a very short and narrow connecting tunnel, which acts as the selectivity region. The tunnel would not exceed 0.5nm in length, about one-tenth of the entire membrane thickness, and its cross-sectional area will be reduced to a minimum of around $0.2nm^2$ as compared to the pore average of $0.5nm^2$. A second hypothesis was derived from two pieces of findings: that the K(Ca) are multi-ion channels, and they contain a fixed negative charge in their vestibules (MacKinnon and Miller, 1988). At high permeant ion concentrations, ion repulsion in the multiply-occupied pore increases the rate of K^+ exit, and channel conductance becomes bigger than that in a single-ion pore (Latorre, 1986). At low ion concentrations conductance is still high because the negative charge potential created by the charges concentrates cations at the channel vestibules (Villarroel and Eisenman, 1987).

1.1.3.3 Gating Kinetics

The channel kinetics depend only on the Ca^{2+} activity in the cytoplasm, not on extracellular Ca^{2+}. Several studies have demonstrated that Ca^{2+} acts as a ligand (Latorre et al., 1982; Moczydlowski and Latorre, 1983; McLarnon and

Sawyer, 1993) i.e. the relationship between the calcium concentration and the steady-state open probability is a sigmoidal function. In agreement with these observations it was found that a Ca^{2+} -dependent biochemical pathway, e.g. that involves calmodulin or protein kinase C, is usually not involved in the regulation of the K(Ca) opening. Gating kinetics of K(Ca) requires the binding of several Ca^{2+} ions to fully open a channel, and the number of ions required varies between different tissues. Generally, in the absence of other divalent cations, the Hill coefficients that best describe the probability of opening vs $[Ca2+]_i$ vary between 2 and 4, suggesting that at least 2-4 calcium ions have to be bound before the channel can open (Barrett et al., 1982; Moczydlowski and Latorre, 1983; McLarnon and Sawyer, 1993). Generally, the channel activation occurs at concentrations of Ca^{2+} less than 1 μM.

The studies of the K(Ca) in the cultured rat myotubules have shown that the channel has more than one open state (McManus and Magleby, 1988). Four different modes of kinetic activity were identified: normal, intermediate open, brief open and buzz. For the normal mode which covers about 96% of all transitions, it was found that at least three to four open states and six to eight closed states, including one or more infrequently adopted long-lived shut states, were present. Some divalent ions, including Mg^{2+} and Ni^{2+}, applied intracellularly, potentiates the Ca^{2+} activation of the K(Ca). This result was reflected as a dose-dependent increase of the channel open probability (in muscle: Golowasch et al., 1986; in nerve: McLarnon and Sawyer, 1993). It was suggested that magnesium, by exposing more calcium binding sites which were previously "hidden," increased the interaction between calcium and the binding sites, as well as the coupling between the occupied sites and the opening activity (Golowasch et al., 1986). Several other factors are also thought to affect this Ca^{2+} sensitivity. One of these is the type of lipid surrounding the channel, where

negatively charged lipids are found to increase the apparent Ca2+ sensitivity (Moczydlowski et al., 1985). Another factor is the neuronal development: in mature spinal neurons, raising the $[Ca2+]_i$ increased the probability of channel opening, such a Ca2+ sensitivity was lacking in young neurons (Blair and Dionne, 1985).

1.1.4 Pharmacological Profile

A pharmacological tool to discriminate between the two classes of calcium-activated potassium channels is to use their different sensitivities to the blocking agents charybdotoxin, TEA^+ or apamin. The K(Ca) are blocked by both external and internal TEA (tetraethylammonium) at different sites, with the sensitivity to [TEA]o about 15 times higher than that to [TEA]i (Blatz and Magleby, 1984; Yellen, 1984). The K(Ca) are also blocked by nanomolar concentrations of charybdotoxin (CTX), a venom from the mideastern scorpion *Leirus quinquestriatus* (Miller et al., 1985). The SK, in contrast, are resistant to both TEA and CTX blockade (with the exception in the *Aplysia* neurons; Hermann and Erxleben, 1987), but are blocked by nanomolar concentrations of apamin, a peptide from bee venom (Romey and Lazdunski, 1984; Pennefather et al., 1985). The K(Ca) are resistant to apamin blockade.

1.1.5 Physiological functions of the K(Ca)

Since their original discovery in molluscan neurons (Meech, 1978), Ca^{2+}-activated K^+ channels have been found widely distributed in cell membrane and play a variety of physical roles. Activation of potassium currents can lead to stabilization of the membrane potential; that is, these currents draw the membrane potential closer to the potassium equilibrium potential (E_K) and farther from the firing threshold. Uniquely, the K(Ca) provide a feedback control

of the voltage-dependent influx of calcium, and play important roles in regulating secretion (Peterson and Maruyama, 1984) and smooth muscle contraction (Singer and Walsh, 1984). K(Ca) also play a role in regulating the action potential frequency and duration (the repolarization phase) in neurons and other excitable cells.

1.1.5.1 Repolarization

The K(Ca) are proposed to play important roles in the repolarizing phase of the action potential and in the control of the slow wave activity in some smooth muscle cells (Singer and Walsh, 1984). In many smooth muscles, the complement of ionic currents that generate the electrophysiological responses appears to be very similar. The K(Ca) (> 200pS) are often the predominant cationic outward current, and generally coexist with smaller conductance SK (Tomita, 1988). K(Ca) repolarize smooth muscle cells after an increase in the intracellular Ca^{2+} during, and (or) subsequent to, the upstroke of the action potential (Akbarali et al., 1990a). In tissues where action potentials are rare or nonexistent, such as the trachea (Kirkpatrick, 1975) and the esophagus (Akbarali et al., 1990b), the intracellular Ca2+ that activates the K(Ca) current may come from the SR. A K(Ca) was also found to play a role in the action potential repolarization in the rat vagal motoneurones (Sah and McLachlan, 1992) and in the bullfrog sympathetic ganglion cells (Adams et al., 1982).

1.1.5.2 Afterhyperpolarization

In order to contract spontaneously mammalian myotubes have to generate spontaneous action potentials and an afterhyperpolarization phase (AHP) is an important component of the action potential. It is during the hyperpolarization phase that the sodium channels which have inactivated become reactivated

again, thus allowing the generation of a new action potential. Over the past decade the importance of Ca^{2+} - dependent AHPs in a number of cell types were well documented and the coexistence of two calcium-activated potassium currents have been reported in rat skeletal myotubules (Barrett et al., 1981), Aplysia neurons (Deitmer and Eckert, 1985), bullfrog sympthetic ganglion cells (Pennefather et al, 1985), GH3 cells (Ritchie, 1987), and rat sympathetic neurons (Smart, 1987). Generally, the large conductance, voltage dependent, and TEA sensitive K(Ca) contributes to the early phase of the AHP. The slower Ca^{2+} - dependent K^+ current (SK) is not affected by extracellular TEA but is blocked by apamin in a number of cells (e.g. bullfrog ganglion cells, Pennefather et al. 1985; GH3 cells, Ritchie 1987). The SK current mediates the slow AHP and the prolonged hyperpolarization phase. The accumulating evidence is that each of the two Ca^{2+} - activated K^+ currents underlies a different component of the AHP. Since the K(Ca) current is voltage dependent it tends to turn-off rapidly at voltages close to resting potential and at physiological concentrations of Ca^{2+}. It is thus associated with a fast AHP. On the other hand, since the SK current has little voltage-dependence, its decline may be more closely related to Ca^{2+} diffusion away from the membrane. It is active at lower Ca^{2+} concentrations and underlies the slower long-lasting AHP.

1.1.6 Ca^{2+}-activated channels in hippocampal neurons

In the CA1 hippocampal neurons, the macroscopic current Ic, which corresponds to the activation of K(Ca), is thought to be the major repolarizing current and responsible for the fast AHP (Storm, 1987). Using the single-electrode voltage clamp method on the CA1 pyramidal cells in the rat hippocampal slices, Storm was able to show that both the spike repolarization and the fast AHP were sensitive to external K^+ concentration and were inhibited

by Ca^{2+} -free medium or the divalent cations Co^{2+}, Mn^{2+} and Cd^{2+}. The currents were also sensitive to an inhibition by external TEA (0.5-1mM) and 30nM of charybdotoxin (CTX), but not noradrenaline. Thus CTX and TEA can increase action potential duration (Lancaster et al., 1986; Storm, 1987). A second slower calcium-activated potassium current, thought to be mediated by the activation of SK, was identified as the current responsible for the slow AHP adaptation (I_{AHP}) as well as the spike frequency (Lancaster and Adams, 1986; Lancaster et al., 1991). In contrast to the Ic, the current was blocked by noradrenaline and acetylcholine, but was voltage - and TEA - insensitive (Lancaster and Adams, 1986). Noradrenaline can block the long-lasting I_{AHP} without affecting the spike duration (or the fast AHP) in hippocampal pyramidal cells (Madison and Nicoll, 1982; Madison and Nicoll, 1984). As a result, the spike frequency adaptation, which normally occurs with depolarizing stimuli, is severely reduced. Thus, the number of spikes elicited by a depolarizing stimulus is greatly increased.. At a given temperature, the I_{AHP} is at least an order of magnitude slower than the Ic (Lancaster and Adams, 1986). Similarly in the bullfrog sympathetic ganglion cells (Pennefather et al., 1985), Ic activates rapidly by depolarization beyond about -40mV, and is responsible for the last two thirds of the spike repolarization and the very first AHP (the fast AHP). As the membrane potential returns to rest and beyond, Ic rapidly turns off, although an elevated internal calcium level still remains. However, when most of Ic turns off quickly after the repolarization due to voltage gating, some K(Ca) which are located close to the Ca^{2+} channels may still be activated due to the high internal Ca^{2+} level (Lancaster and Nicoll, 1987). This may explain the possible role of the Ic as a component of the medium AHP and early spike adaptation (Storm, 1990). The I_{AHP} is much better suited to produce prolonged AHP. Activated by Ca^{2+} influx during the action potential, the current activates slowly over a few seconds. The

deactivation is even slower, and is dependent on the size of the current and the membrane potential (Lancaster and Adams, 1986). The I_{AHP} generates the slow but prolonged AHP which follows spike bursts and single spikes, and helps to sustain further discharge by hyperpolarizing the cell. The I_{AHP} is thus crucial in the negative feedback control of the discharge activity of the hippocampal neurons. Forming only a small fraction of the total Ca^{2+} -activated potassium conductance, the I_{AHP} does not contribute to the spike repolarization. However, it is responsible for the spike frequency adaptation in the repetitive firing typical of the pyramidal neurones (Madison and Nicoll, 1984).

Recently, at least six different potassium currents have been identified in the CA1 hippocampal pyramidal cells in slices. Apart from Ic and I_{AHP}, there are also I_A, I_D, I_K and I_M, and the roles of these different potassium channels were more clearly defined (Storm, 1990). The I_A (fast transient current) both activates and inactivates rapidly, and is responsible for the early spike repolarization before Ic becomes predominant. I_D (the delay current) and I_K (the delayed rectifier) are thought to participate in the spike repolarization. The I_M (M current) is slowly activated by depolarizations beyond about -60mV and does not inactivate. It reduces firing rate during spike frequency adaptation and contributes to the medium AHP, together with Ic.

1.2 Class III antiarrhythmic drugs

1.2.1 Outward K^+ current in heart

The duration of a cardiac action potential is mainly determined by its plateau, which is in turn maintained by a fine balance of inward and outward currents. The inward current is mediated through a Na^+ channel, a Ca^{2+} channel and Na^+ -Ca^{2+} exchange. The outward current is carried by a number of

different K^+ channels, a Cl^- channel and the Na^+-K^+ ATPase. Under normal physiological conditions three outward K^+ currents contribute to the net outward cardiac current. The transient outward current (I_{to}) undergoes rapid voltage dependent activation and inactivation, thus is important for the early repolarization phase of the spike potential. In the rat ventricular cells, this current is the major repolarizing current. The delayed rectifier K+ current (I_k) is the predominant outward current throughout the entire plateau phase. It is activated upon depolarization from about -50 mV to 0 mV (Noble, 1984) and two components (slow and fast) have been described in guinea pig atrial and ventricular cells (Sanguinetti and Jurkiewicz, 1990). In other cells, such as the rabbit and cat ventricular myocytes, the I_k has only the faster component. The inward rectifier current (I_{k1}) is important in establishing cell resting potential as well as the final phase of the repolarization. The current is time-dependently activated on hyperpolarization and has an inward rectification - i.e. it passes current only at a membrane potential negative to -20mV, as a result, it has very limited role during the plateau phase of the cardiac action potential. The I_{k1} is also enhanced with increased extracellular K^{2+} concentration.

1.2.2 The class III antiarrhythmics

Even since quinidine was used therapeutically against cardiac arrhythmias, the antiarrhythmic significance of the changes in the refractory period of the cardiac action potential had been recognised (Lewis et al., 1926). However, it was decades later before the effects of the drug were found to be two-fold: quinidine prolongs the repolarization period and also reduces the maximum rate of depolarization (Singh and Nademanee, 1985). This gives rise to two classes of antiarrhythmic compounds: the class Ic agents (e.g. flecainide, encainide) which exhibit antiarrhythmic properties by delaying conduction, and

the class III agents (e.g. sotalol) which exhibit antiarrhythmic properties by prolonging repolarization. The prototype of these latter class III agents, sotalol, prolongs repolarization by inhibiting the delayed rectifier potassium current (with a smaller effect on the I_{k1}), and as a β blocker, it slows heart rate and intranodal conduction, and prolongs the refractory period in the atrioventricular node. The result is the prolongation of the action potential duration and the increase in refractory periods in the atria, ventricles and His-Purkinje system (Singh, 1990). The agent which really defines the class III effect, however, is amiodarone. The major antiarrhythmic effect of amiodarone is lengthening of the action potential duration and an increase in the refractory period. This class III effect is important in pacemaker tissues; lengthening the action potential duration will delay the onset of the next spontaneous diastolic depolarization, slowing down the voltage from reaching the threshold, and the cycle length of tachycardia will be prolonged. On the other hand, in nonpacemaker tissues, the increase in the action potential duration and refractoriness will also slow down the tachycardia. In both cases, the acceleration of tachycardia is slowed down, therefore reducing the chance of the deterioration of the tachycardia into fibrillation. Like sotalol, amiodarone also exerts major effects on the delayed rectifier current (I_k). Although beta blockade is not a major component of amiodarone's actions (unlike sotalol), the inhibitory effects of the drug on heart are not specific. Thus, there is a need to search for "pure" class III antiarrhythmics - drugs which can selectively prolong repolarization (thus lengthening the action potential duration as well as the effective refractory period) without slowing down conduction. Most of these compounds inhibit the delayed rectifier (I_k). UK 68798 (Gwilt et al., 1989), d-sotalol and E-4031 (Sanguinetti & Jurkiewicz, 1990) were shown to inhibit I_k in the guinea pig myocytes, and risotilide (WY 48986) specifically blocks I_k in the cat ventricular

myocytes but have no effects on the inward rectifier (I_{k1}) (Follmer et al., 1989). This class III effect is typically mediated by the inhibition of one or both components of the I_k, although the faster component is the primary target for drugs For example, E-4031 was shown to specificly block the fast component of the I_k in the guinea pig ventricular myocytes (Colatsky et al., 1990). The class III actions of some other agents however are directed against the I_{k1}. For instance, RP 58866 and its enantiomer RP 62719 (terikalant) inhibit I_{k1} in rat myocytes (Escande et al., 1989). These class III antiarrhythmics are attracting major attention since they seem to possess greater efficacy than the class I drugs in those experiments which are most representative of the clinical situation. Also, the class III antiarrhythmic drugs do not possess the negative inotropic effects of all of the class I, II and IV antiarrhythmic agents, as mediated by alterations of intracellular Ca^{2+}. New "pure" class III agents such as sematilide, dofetilide (UK-68798) and E-4031 have been developed and shown to be effective in suppressing programmed stimulation-induced ventricular tachycardia (VT) and preventing the onset of ventricular fibrillation (VF) in the animal models (Katritsis and Camm, 1993). The drugs are being tested clinically and a major concern is the risk of proarrhythmia (i.e. torsade de pointes) associated with these class III drugs. In the development of newer drugs, the balance between the proarrhythmic risk and the antifibrillatory actions will be a major determinant in evaluation of drug utility.

1.2.3 Antiarrhythmic properties of tedisamil (KC8857)

Tedisamil, in a dose-dependent manner, lengthened the effective refractory period in intact rat heart, which prevented both ischaemia and electrically-induced ventricular fribrillation (Walker and Beatch, 1988; Beatch et al., 1991). The ability of the drug to block the two predominant cardiac outward

K^+ currents, the I_k and the transient outward K^+ current (I_{to}), was examined using the voltage clamp technique. (Dukes and Morad, 1989) In a dose-dependent manner, tedisamil was found to block the time-dependent I_k in guinea pig ventricular myocytes and the transient outward K+ current I_{to} in rat ventricular myocytes (Dukes et al., 1990). Dukes and co-workers (1990) also demonstrated that tedisamil was effective in blocking the same two currents (I_{to} and I_k) in mouse astrocytes. Tedisamil also inhibited the I_{to} current in single smooth muscle cells of the guinea-pig portal vein (Pfründer and Kreye, 1992).

1.2.4 Putative class III antiarrhythmics as potent K(Ca) blockers

Using the patch-clamp technique, tedisamil was found to block K(Ca) in smooth muscle cells of the guinea-pig portal vein (Pfrunder and Kreye, 1991). The drug also blocked a K(Ca) in cultured mouse motoneurons (McLarnon et al., 1992). Single channel patch clamp experiments in this laboratory have shown that a number of class III antiarrhythmic agents including tedisamil, UK-68,798 and risotilide inhibit K(Ca) in hippocampal CA1 neurons (McLarnon and Wang, 1991) In addition the tedisamil block of a K(Ca) channel in mouse motoneurons has been documented (McLarnon et al., 1992). A comparison of the tedisamil block of I_k and I_{to} in cardiac cells with that of K(Ca) in hippocampal neurons would suggest a considerable degree of correlation between the actions of the putative class III antiarrhythmics on repolarizing K^+ currents in the cardiac cells and those on the CA1 neurons.

1.2.5 The RSD compounds

Some RSD compounds appear to alter properties of intact rat heart in a manner similar to that of putative class III antiarrhythmics. For example, dose-dependent lengthening of the effective refractory period (ERP) is observed. An

important point of the present work was to examine the possibility that these RSD compounds block K(Ca) in a similar fashion to other putative class III drugs such as tedisamil. The availability of a large number of these compounds also was useful in the examination of the correlation between the effects on prolonging cardiac action potentials with the inhibition of the CA1 neuronal K(Ca).

2. METHOD

2.1 Tissue culture preparation

The procedures carried out to obtain Wistar rat hippocampal cultures were generally based on the method employed by Banker and Cowan (1977). Briefly, the hippocampi of day 18 fetal rats were dissected out and dissociated into single cells by enzymatic (trypsinization) and mechanical (repeated pipetting through Pasteur pipette) treatments. Once counted, the cell suspension was diluted down to approximately 105 cells/cm^2 in Dulbecco's Modified Eagle's Medium (DMEM), and plated onto 18 mm laminin-coated coverslips, which had been treated with poly-D-lysine to inhibit the proliferation of non-neuronal cells. These coverslips were then inserted into 6-well plates and incubated, with the growth side downwards, in DMEM and 5% CO^2 at 37°C until use. By experience, it was best to perform patch clamp on hippocampal cultures within two weeks after their isolation. Thus, experiments were carried out on the cultured neurons 3-14 days after cells had been placed on coverslips, since the K(Ca) are only found to be expressed in hippocampal cell cultures that were at least three days old, and patching becomes difficult when cells begin to deteriorate after about two weeks of isolation.

2.2 Eletrophysiology - patch-clamp recordings

2.2.1 Pipette preparation

The patch clamp electrodes were made from Corning #7052 borosilicate glass (A-M Systems, Washington), which has the advantage of reducing energy loss when voltage is being applied. The glass had a diameter of either 1.2 mm (0.68 mm inner diameter) or 1.65 mm (inner diameter of 1.2 mm). The

electrodes were prepared with a standard two-pull technique using a Narishige PP-83 vertical glass microelectrode puller. For each size of glass, fixed pulling length and fixed settings for the two heaters were generally maintained to minimize the differences between different electrodes. The resultant electrodes had tips with diameters of about 1~2 μm, corresponding to a pipette resistance of 4-8 MΩ. These pipettes were then fire-polished on a homemade microphorge consisting of a glass-coated U-shaped platinum filament connected to a D.C. voltage supply. Under a light microscope (× 200 magnification), the pipette was brought close to the heated platinum filament and polished to create a smooth tip. An airstream was directed at the filament during polishing, controlling the temperature of the filament as well as confining the polishing to the very tip of the glass pipette. The pipettes were then filled with the appropriate pipette solutions. For inside-out patches, the pipette solution contained (in mM) NaCl 140, KCl 5, CaCl2 0.2 and 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) 10, at pH 7.3; for outside-out patches, it contained (in mM) NaCl 5, KCl 140, CaCl2 0.2 and HEPES 10, also at pH 7.3. The pipette was usually filled only half way up or just enough for the reference wire to be immersed.

2.2.2 Pipette Mounting

The pipette was inserted into the pipette holder, which was connected to the headstage amplifier - a current to voltage converter with a 50 GΩ feedback resistance (Axon model CV-3 with 1/100 gain) for the patch clamp amplifer (Axopatch). These were mounted onto an Optikon lab jack, which contained a Newport motor drive micromanipulator for fine upward and downward movement of the pipette and also another manipulator for coarse three-dimensional movements. After mounting onto the pipette holder, a small positive pressure was applied to the pipette through a rubber tubing to prevent the

building up of debris around the pipette tip when it was immersed into the bath solution. The pipette was then lowered into the bath solution and positioned above the cell chosen for patching.

2.2.3 Patching

A coverslip of hippocampal neurons was removed from the 6-well plate and placed in the Perspex circular recording chamber. For inside-out patches the bath solution used both before and during the patch excision contained (in mM): NaCl 140, KCl 5, CaCl2 0.2 and HEPES 10, at pH 7.3 (same as the pipette solution). Immediately after an excised patch had been obtained, the bath solution was changed to one that contained (in mM): NaCl 5, KCl 140, CaCl2 0.2 and HEPES 10, also at pH 7.3. For outside-out patches, the high K^+ solution (140 mM) was used for the pipette solution and the low K^+ solution (5 mM) was used as the bath solution both before and after the excision. In one experiment, the $[Ca^{2+}]$ in bath was varied and controlled by using EGTA for measuring the $[Ca^{2+}]$ dependency of K(Ca). A 0.15M KCl agar-filled plastic bridge, connected to the ground, was submerged into the bath solution and completed the circuit (when the pipette tip was immersed into the solution). The chamber, affixed to the stage of a phase contrast microscope (Nikon), allowed the neurons to be viewed at a ×300 magnification. At such a magnification both the viable cells and the pipette were visible allowing the entire experiment to be monitored visually. After being lowered down to just above the chosen cell, a test pulse of 0.2mV was applied to indicate the change in seal resistance together with an audio signal which served the same purpose. The pipette was then slowly lowered onto the soma of the chosen cell using the Newport MPH-1 micromanipulator. Once the pipette was touching the cell as indicated visually, electrically and audibly, a negative pressure was applied to the pipette through a

mouth suction tube which was connected to the pipette holder. This procedure usually resulted in the formation of a giga-ohm (> 10 GΩ) seal. The presence of such a seal was confirmed with the disappearance of the 0.2 mV test pulse. The output electrical signal was amplified with the Axopatch 1B patch clamp amplifier (Axon Instruments, Inc.), visible on a Kikusui 5020A oscilloscope. Now the electrical activity of the patch could be observed as rectangular pulses on the oscilloscope screen.

2.2.4 Patch excision: Inside-out and Outside out

After the formation of the cell-attached seals (the gigaohm seals), which allowed a 10-fold reduction in background noise, the stage was set for manipulations to isolate membrane patches which could lead to one of the two differerent cell-free recording configurations. If the pipette was quickly withdrawn with the microdriver, a patch of the membrane could be isolated in the inside-out mode (cystolic side of the cell membrane exposed to the bath solution). The mode was especially useful for measuring the effects of agonists acting on receptors found on the cellular face of the cell membrane. Alternatively, instead of excising the patch at the cell-attached mode, a whole-cell patch could be produced by the disruption of the patch membrane with extra suction. If the pipette was then pulled away from the whole-cell patch an outside-out patch (with extracellular side of the cell membrane facing the bath solution), was obtained.

2.2.5 Single channel recordings

After the excision to the inside-out mode, channel activities in the patch generally required triggering by drawing the patch briefly out of the solution with exposure to air. It is presumed that the action would remove any vesicle

that had been formed at the tip of the pipette during the excision of the patch from the cell membrane. The loss of channel activities during recording may sometimes be reversed using the same method along with the application of positive and/or negative potential. Since it is very easy to lose the patch during the take-out time, the procedure must be carried out very carefully and precisely. The procedure is usually not useful in the case of outside-out patches as the action of taking the patch out into the air will in most cases end up in the loss of the patch.

The unitary currents of K(Ca) were generally recorded with the patch clamp amplifier (Axopatch, Axon Instruments), digitized at sampling rates of 5 kHz, with a low-pass filter set at 2kHz. The low-pass filter removed the high frequency component of the background electrical noise of the signal allowing the single channel current to be resolved. Since the reference ground was the bath, the extracellular side of the channel had zero potential. Thus, when positive K^+ ions entered the pipette through the K(Ca) in an inside-out patch (140 K^+ in bath, 5 K^+ in pipette), a downward deflection from the baseline was produced. Conversely, the same K(Ca) currents were recorded as an upward deflection from the baseline in an outside-out patch (5 K^+ in bath, 140 K^+ in bath). For the pharmacological experiments, all the events were obtained with the patch held at 0 mV, where the driving force for the K^+ currents was generated by the concentration gradient of K^+ across the patch. Steps of depolarizations and hyperpolarizations were also applied to determine the voltage dependence of the channel.

The patch clamp data were recorded onto an IBM-compatible PC (486 DX33) using the programme pCLAMP V.5.0 (Axon Instruments) for off-line analysis. For backup recording an Instrutech VR-10 digital data recorder was connected to a Panasonic Omnivision Hi-Fi VHS, model PV-4760-K. When the

channel open times were very short (e.g. rapid channel flickerings produced during a channel inhibition by an K(Ca) blocker), the currents were digitized at a higher sampling rate of 20 kHz, with the low-pass filter set at 5 kHz. When the frequency of openings was low, an AI 2020 event detector could also be used to start recording only when an event of the proper amplitude had occurred.

2.2.6 Data Analysis

Off-line analysis of the data were performed with pCLAMP V.5 (Axon Instruments). Three major bits of information about the single channel data were obtained: the amplitudes of the single channel events, the durations of the events, and the length of time between two consecutive events. After determination of the baseline (closed channel level) and the threshold (estimated amplitude of the events), the computer programme would detect the openings using a half-amplitude threshold criterion. Namely, the initiation and termination of an event (opening and closing of a channel) were defined by the crossings of the half threshold level by the current trace. The half-amplitude threshold method is a common procedure used in the collection of single channel events. The sampling rate of 5 kHz allowed resolution of channel openings with open durations at 400 μs or greater. The minimum resolvable closing duration was set at 200 μs.

The amplitude, open time and interval duration between two consecutive opens of the selected single channel events were saved on a separate record and were used to generate mean values and histograms, using the subprogramme pSTAT V.5.0 (Axon Instruments). Diagrams of the distributions of the amplitude, open intervals and closed intervals could be produced by this subprogramme. Plots of this analysis were output on a HP plotter.

2.3 Current clamp experiment on the hippocampal slice

Rat hippocampal slices (400 μM) were prepared according to standard techniques (Church and McLennan, 1989) and placed in a recording chamber at 34.5°C at the interface between a humidified atmosphere (95% O_2: 5% CO_2) and control artificial cerebrospinal fluid (ACSF) which contained (mM): NaCl 125.5, KCl 3, $NaHCO_3$ 21, NaH_2PO_4 1.5, $MgSO_4$ 1.5, $CaCl_2$ 2, D-glucose 10 (pH 7.4 after equilibration with 95% O_2: 5% CO_2). Intracellular recordings were measured using current clamp. The current clamp pulses were 5 ms in duration and increased in 0.1 μA steps from rest. The hippocampal action potentials were elicited first in control. Tedisamil was added to the perfusing solution and 30 minutes were allowed for the drug to act on the slice. With this procedure the concentration of tedisamil at the active site was not known and had to be estimated.

2.4 Measurements of the ERP (effective refractory period)

The ERP measurements belong to part 2 of the four standard screens used to examine the RSD compounds, where screen I includes the measurement of the effects of the administration of cumulative iv (intravascular) doses of the drugs on the heart rate, blood pressure and ECG of the intact animal. Male Sprague-Dawley rats weighing from 150 to 350g were used, and were anaesthetised with sodium pentobarbital (60 mg/kg i.p.). The right carotid artery was cannulated for the measurement of the blood pressure. Stimulating electrodes, placed 1-2 mm apart, were implanted in the left ventricle to accomplish electrical stimulation. The ECG was recorded with a special chest lead configuration (Penz et al., 1992) where two electrodes were used. The superior and the lower electrodes were placed 0.5 cm from the midline of the trachea, at the level of the right clavicle and that of the 9th and 10th ribs

respectively. ERP, as part of the ECG, was obtained while the electrical stimulation was applied. After obtaining the stable control values (2 identical consecutive readings or the average of 3 similar ones), the RSD drugs were administered as iv infusions through the cannulated right jugular vein over a period of 5 minutes. Starting at a volume of about 0.05ml/kg/5 min. the infusion rate is doubled as the experiment proceeds but not to exceed 10ml/kg/5 min. The ECG was recorded on a Grass polygraph (model 7D) at a chart speed of 100 mm/sec. With screen I of the examination giving a general picture of the cardiovascular actions of the RSD compounds, the screen II experiments allows an assessments of the actions of the drugs (if effective) on the myocardial ionic (Na^+ and K^+). Drugs known to affect the sodium and/or the potassium channels have been shown to produce clear profile of actions in these screen II tests. With our interests in the potassium channel blocking ability of the RSD drugs, the ERP value, which is a good representative of the repolarization activity of the animal heart, was measured for a number of RSD compounds.

3. RESULTS

3.1 Single channel properties of K(Ca)

3.1.1 Channel conductance

Unitary currents were recorded primarily from inside-out patches of the cultured rat CA1 hippocampal neurons. With a physiological K^+ gradient of 140 mM K^+ internal and 5 mM K^+ external across these patches, a channel with conductance of 80 - 110 pS could be obtained. Typical unitary activity is shown in Fig. 1, where (by convention) upward deflection indicates the outward flow of positive K^+ ions. Starting from -40 mV the holding potential of the patch was increased with each depolarizing step of 20mV, up to 60 mV. The variation in channel activity is also show in Fig. 1, using representative portions of traces at each indicated voltage. As the patch was depolarized from - 40 mV to 60 mV, the amplitude of the opens increased accordingly. The current-voltage relation of this K(Ca) is expressed by the I/V plot shown in Fig.2. The conductance of the channel, given by the equation $I_m = C.V_m + V_o$ (where I_m is the patch current, C the conductance, V_m the patch potential, and V_o the zero-current potential), is determined by the straight portion of the slope of the curve (-20 mV to +60 mV). The conductance is found to be 110pS, which is close to the value measured for a high conductance K(Ca) in cultured rat muscle (Barrett, et al., 1982). The use of non-symmetrical K^+ across the patch results in a significant non-linearity in the curve (as predicted from the Goldman equation), and prevents an estimate of the zero current potential from the curve.

Fig. 1 *Typical unitary currents of K(Ca) measured in the cultured CA1 hippocampal neurons.*

By convention, the channel openings are the upward rectangular deflections from the baseline. Currents were recorded from an inside-out patch with 140 mM K^+ in the bath solution and 5 mM K^+ in the pipette solution. Starting from the top trace recorded at a Vm of -40 mV, the traces shown represent depolarizing steps of 20 mV, to + 60 mV. The amplitude of the channel activity increased with depolarization.

Fig. 1

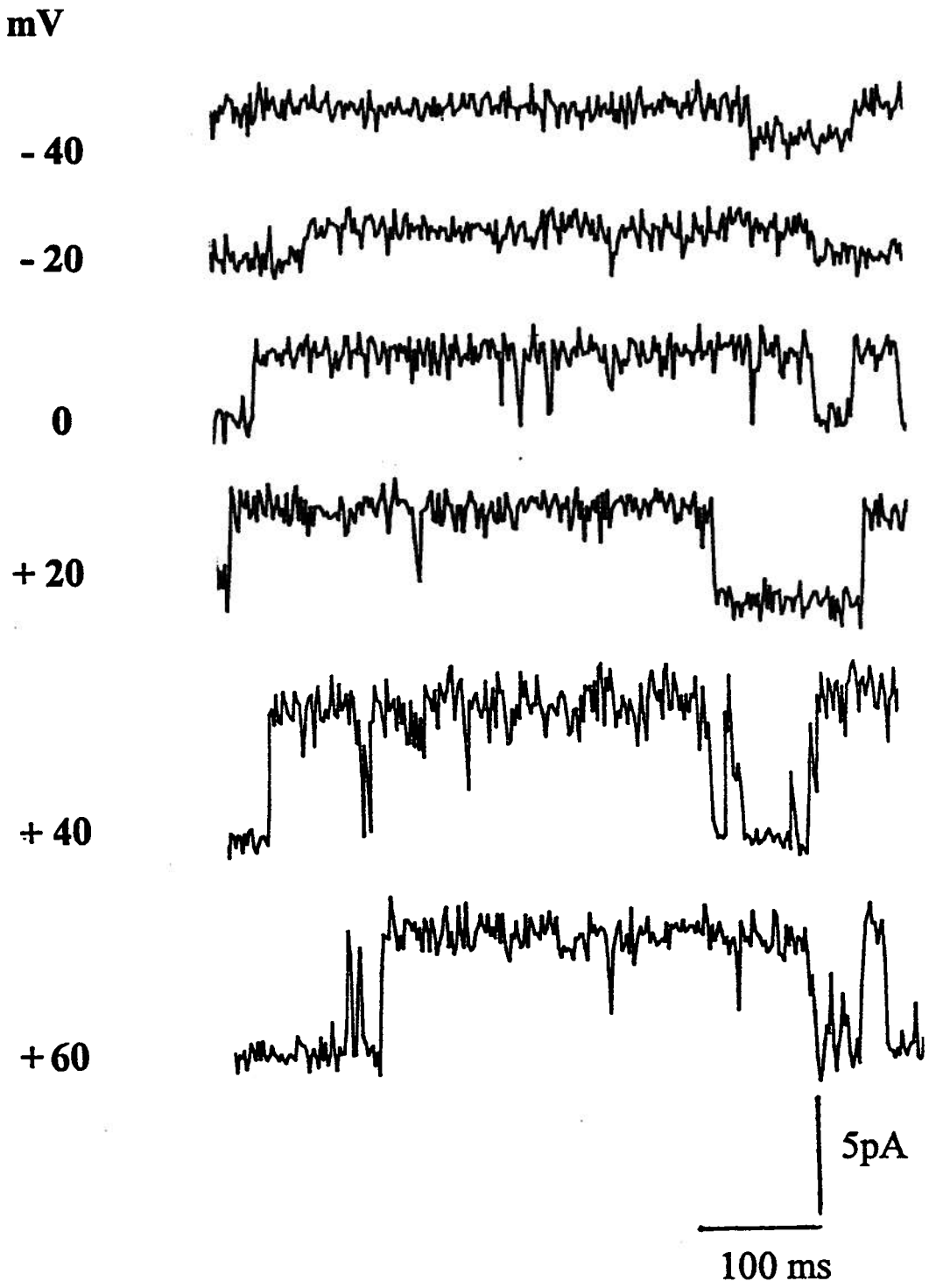
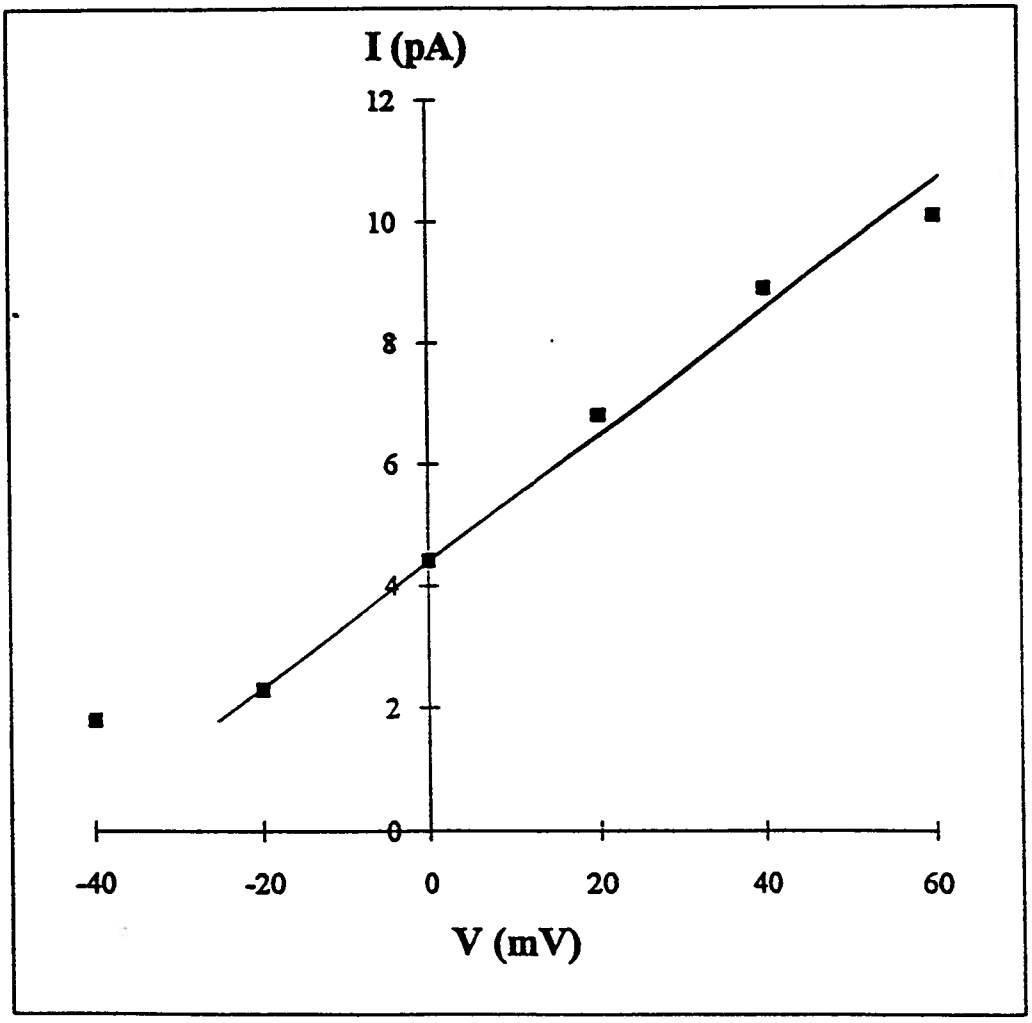


Fig. 2 *Current (I) - voltage (V) plot*

I-V plot for K(Ca) openings recorded in an excised inside-out patch with 140 mM K^+ in the bath solution and 5mM K^+ in the pipette solution. The curve is a visual fit to the data, and the linear portion gave a slope of 110 pS.

Fig. 2



Most measurements of K(Ca) channels have used symmetrical 140 mM K^+ across patches since the increase in extracellular [K^+] can increase unitary conductance. The results of changing the external solution to the high 140 mM K^+ is shown in Fig. 3. With symmetrical K^+ concentration across the patch the zero-current (reversal) potential was 0 mV. Thus the only driving force for the current flow was the holding potential, which was changed (increments of 20 mV) from -40 mV to +60 mV. The direction of the current flow changes as the holding potential varies from above the zero-current potential to below it: a negative holding potential causes an influx of K^+ and a positive one drives an efflux of K^+. The amplitude of the current also varies with the holding potential which increases as the absolute value of the holding potential increases. Traces showing the variation of the channel activity with the holding potential (varying from -40 mV to +60 mV with a depolarizing step of 20 mV) are shown in Fig.3. The current-voltage plot is given in Fig. 4. The slope conductance for this I-V curve was found to be approximately
170 pS.

3.1.2 Calcium dependence

In our experiments we have demonstrated the dependence of the K(Ca) on internal calcium by varying the calcium concentration of the bath solution. Throughout our experiments with the inside-out patches, a concentration of internal calcium (0.2mM) was used to measure the K(Ca) channel activities. It was found that decreasing the calcium concentration from 0.2 mM to about 10 μM had little or no effect on the patch activity and decreasing intracellular calcium to 0.07μM totally abolished the openings. In order to investigate the

Fig. 3 *Unitary currents measured in a patch with no [K+] gradient*

Currents were recorded from an inside-out patch with 140 mM K^+ in both the bath and the pipette solutions. Starting from a Vm = -40 mV and moving down in 20 mV depolarizing steps to + 60 mV, traces of channel activity are shown. For a Vm < 0 mV, the channel openings are downward deflections from the baseline; for a Vm > 0 mV, the openings are upward deflections. The amplitude of the channel activity increases as $|Vm|$ increases (i.e. away from zero-current potential).

Fig. 3

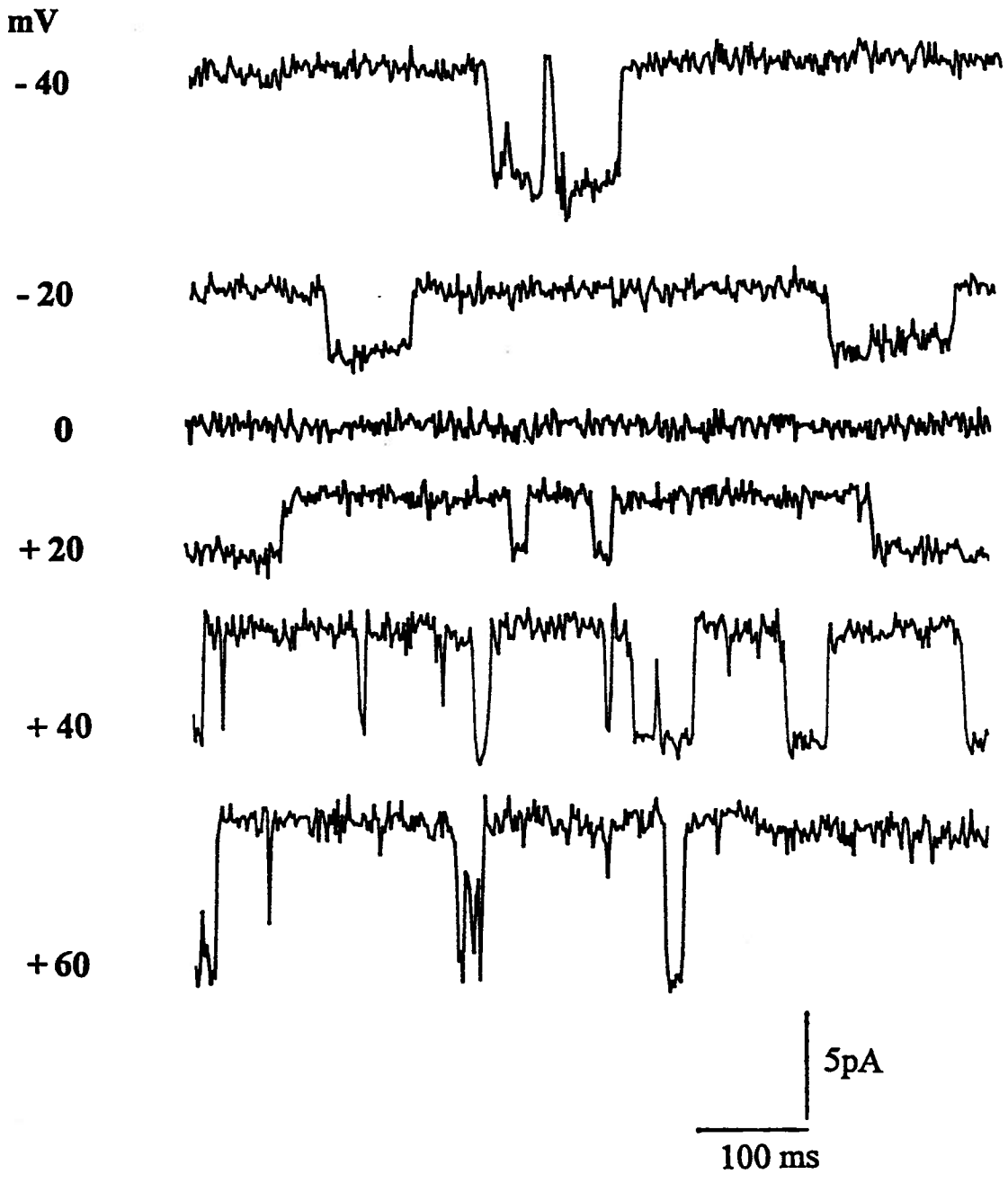
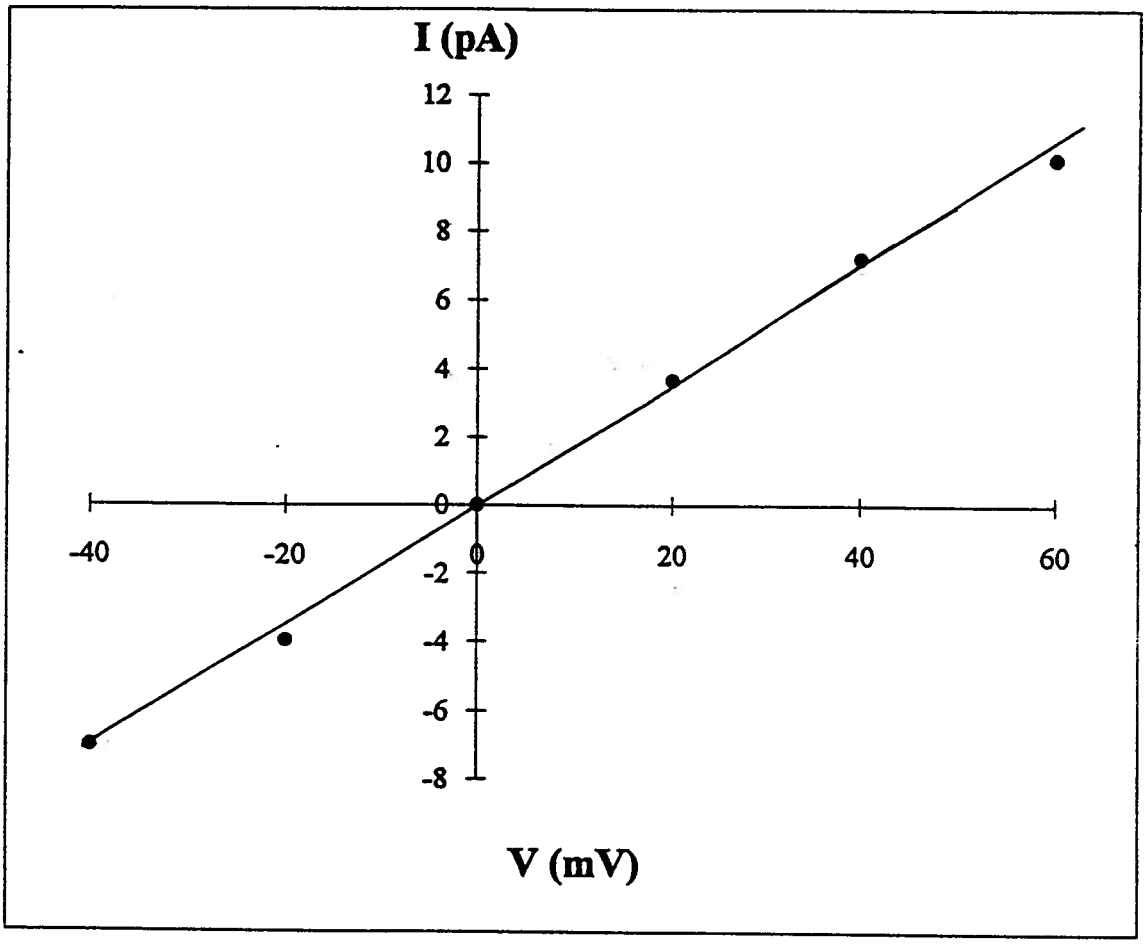


Fig. 4 *I - V plot*

I - V plot for K(Ca) openings recorded in an inside-out patch with both bath and pipette solutions containing 140 mM K^+. The curve is a linear fit to the data, with a slope of 170 pS.

Fig. 4



calcium-dependence of the channel activity, a number of bath solutions containing different concentrations of calcium were prepared. The procedures used EGTA added to a stock solution of Ca^{2+} and calculations using a computer program outlined in Fabiato and Fabiato (1979). By increasing the concentrations of free calcium gradually from the minimum of 0.07μM to higher values, an estimate for the threshold of internal calcium concentration required for channel activity was made. This value was near 2.2 μM, which was the minimum concentration at which channel activity was evident. Upon the return of internal Ca^{2+} above 4 μM the channel activity was maximal as judged by no further increase in the P_{open}. The variation of the channel activity with respect to different $[Ca^{2+}]_i$ is represented by the traces of unitary activity in Fig. 5. Similar dependence of the CA1 hippocampal K(Ca) on internal Ca^{2+} has been demonstrated in this laboratory previously and a sigmoidal relationship between the channel open probability and $[Ca^{2+}]_i$ has been obtained (McLarnon and Sawyer, 1993). The internal calcium-induced variation in the hippocampal K(Ca) open probability was primarily due to changes in mean open time of the channel and a slower closed time component.

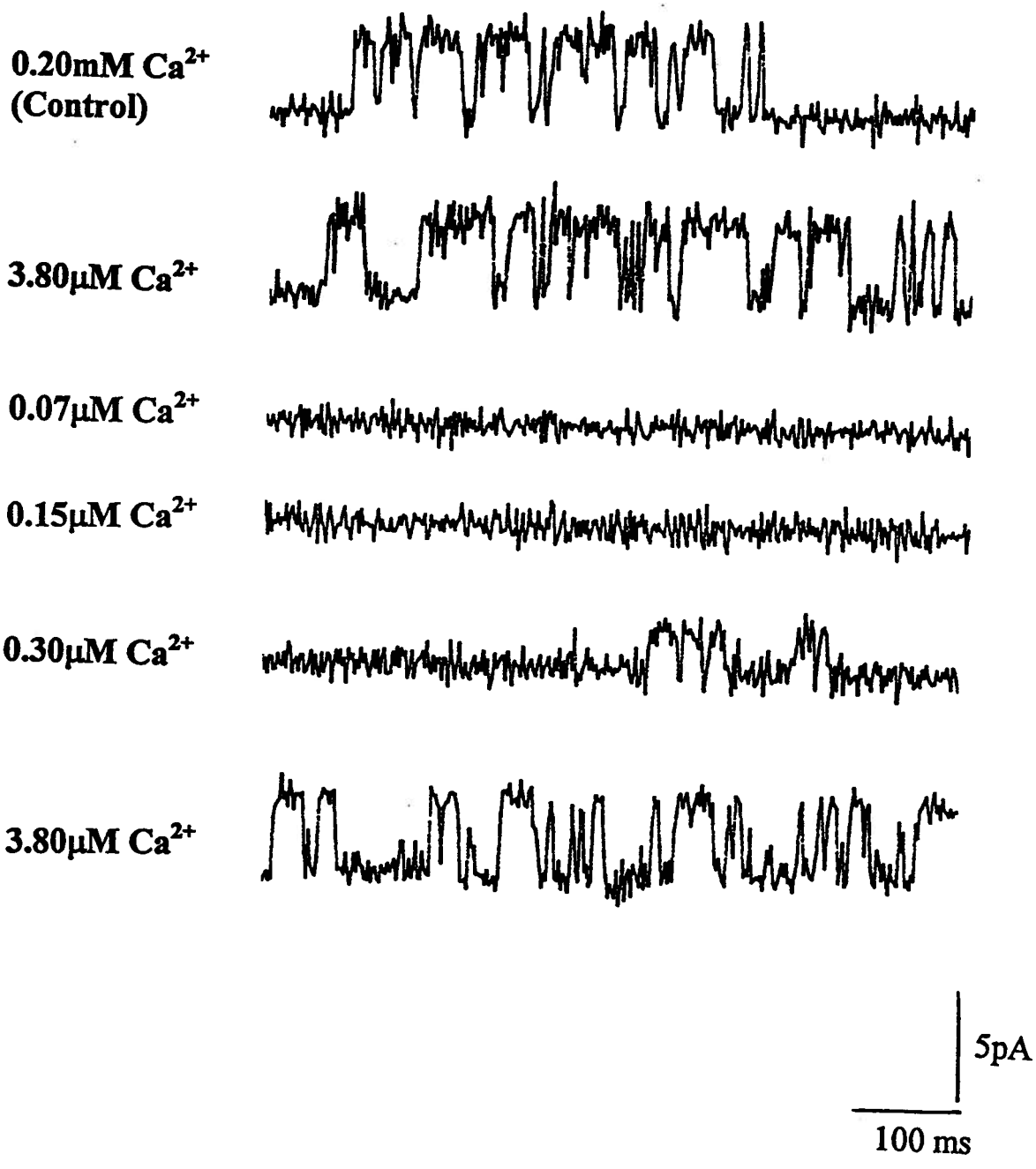
3.1.3 Voltage dependence

In experiments to examine the voltage dependency of P_{open}, the patch potential was changed between 0 mV and +20 mV. These studies used a calcium concentration of 0.7 μM Ca^{2+} to keep P_{open} low. The P_{open} (open probability) was measured as the probability of the channel in the open state (in percentage) and was obtained as a product of the mean open time and the

Fig. 5 *Unitary currents showing Ca^{2+} dependence of K(Ca)*

Currents recorded from an excised inside-out patch showing the effects of changing the internal Ca^{2+} concentration on K(Ca). The Ca^{2+} concentration decreases down the traces from a control of 0.20 mM to 3.80 μM [0.2 mM EGTA] (with normal channel activities) and to 0.07 μM [0.4 mM EGTA] (total abolishment of openings). The fourth trace shows the increase of Ca^{2+} to 0.15 μM [0.3 mM EGTA], with still no openings. An increase to 0.30 μM [0.25 mM EGTA] induces some channel activity but of a smaller amplitude, and an increase back to 3.8 μM results in the return of the normal channel activities.

Fig. 5


0.20mM Ca^{2+} (Control)
3.80μM Ca^{2+}
0.07μM Ca^{2+}
0.15μM Ca^{2+}
0.30μM Ca^{2+}
3.80μM Ca^{2+}
5pA
100 ms

frequency of opens, divided by the overall time of measurement (10s). The results for one patch gave P_{open} of 0.57 ± 0.10 % (at 0 mV) and P_{open} of 0.91 ± 0.18 % (at +20 mV). These measurements were based on analysis of 10 separate applications of the two potentials. The increase in P_{open} was then studied to determine if potential altered the mean open time, the frequency of openings, or both properties. It was found that mean open time was not significantly changed (2.0 ± 0.12 ms at 0 mV to 2.07 ± 0.09 ms at +20 mV). The increase in open frequency was significant (from 27.6 ± 4.5 per 10 seconds at 0 mV to 44.4 ± 7.0 per 10 seconds at +20 mV). The results showed that the mean open time was not affected by the change in voltage. The increase in open probability from 0 to +20 mV, obtained at a low calcium concentration of 0.7 μM, was solely due to an increase in the frequency of channel openings. An illustration of the difference in channel activities at 0 mV and +20 mV is shown in Fig. 6, with the increase in the number of openings in the traces recorded at +20 mV. A comparision of the changes in the number of opens, mean open time and open probability, as affected by the voltage change, is given in Fig. 7.

3.2 Macroscopic currents with tedisamil on hippocampal slices

The large conductance K(Ca) is the unitary basis of the macroscopic Ic described in hippocampal neurons which is important in the later two thirds of the spike repolarization and the fast and the middle afterhyperpolarization phases. It was therefore of interest to determine the possible effects of antiarrhythmic drugs on this hippocampal Ic using current clamp technique on the hippocampal slice preparation. Tedisamil, a possible class III antiarrhythmic

Fig. 6 *Unitary currents showing the voltage dependence of K(Ca)*

Traces of unitary currents obtained from excised inside-out patches are chosen to represent the actual situation of the channel activities. Changing between 0 mV to + 20 mV consecutively, an increase in the number of channel openings is observed at + 20 mV. Notice also that the channel openings are larger at + 20 mV due to the enhanced driving force.

Fig. 6

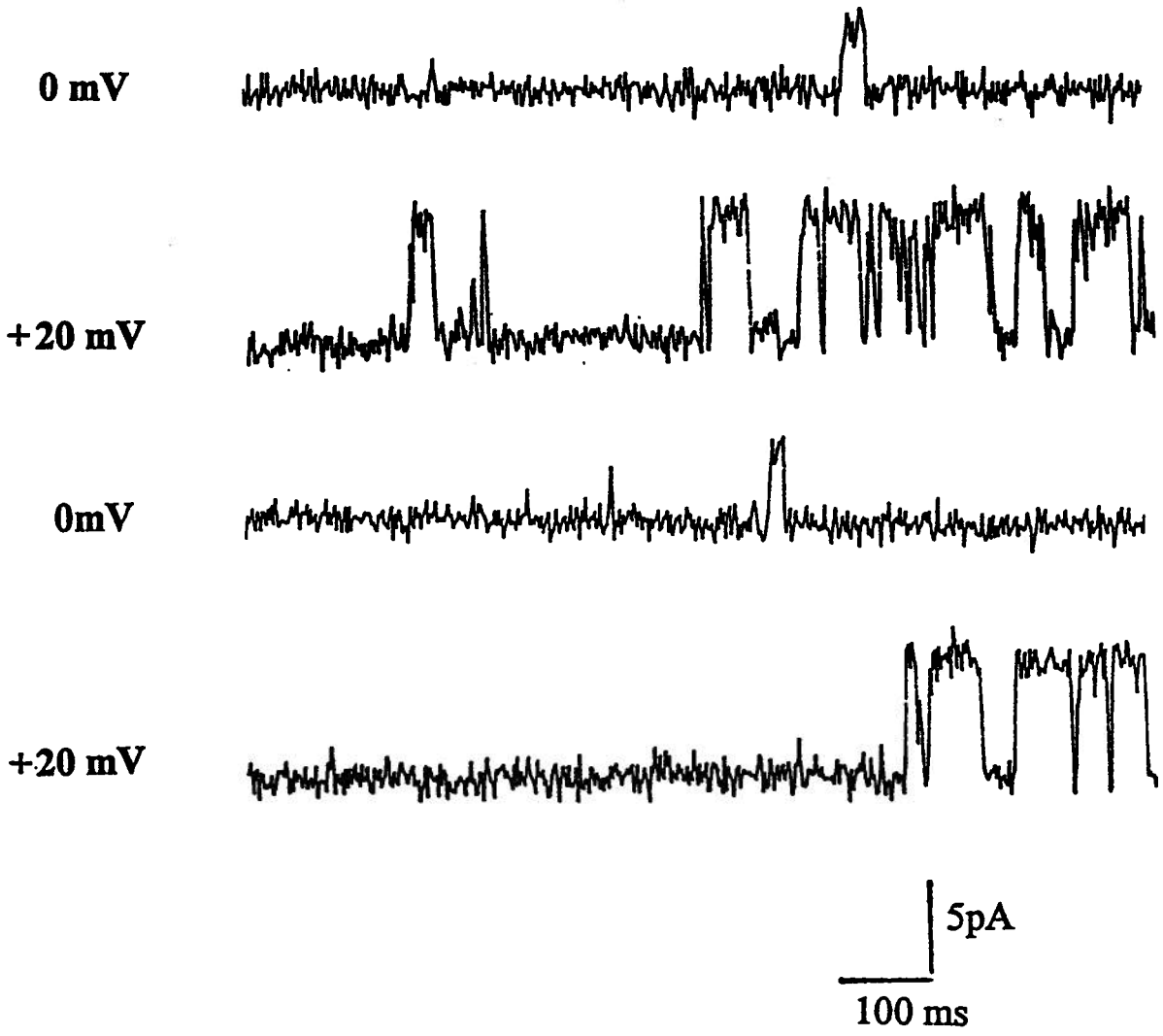


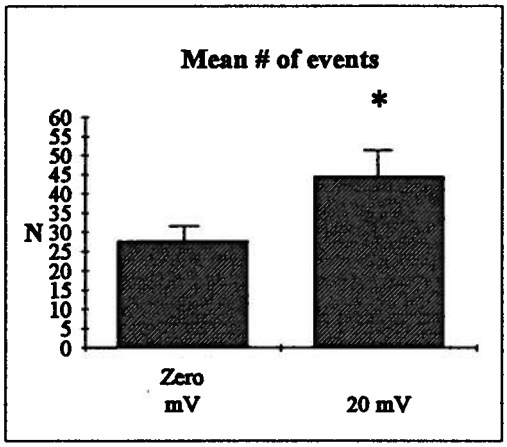
Fig. 7 Voltage dependence of K(Ca)

A comparision of the A) frequency of channel openings, B) mean open times, and C) Open probability between channel activities recorded at 0 mV and + 20 mV from an inside-out patch. Significant differences exist between activities recorded at the two Vm in terms of the frequency of openings and open probability ($P < 0.05$; one-tailed t-test), while no such differences between the mean open time values. The increase in Vm increases the open probability by increasing the frequency of openings but not the mean open times.

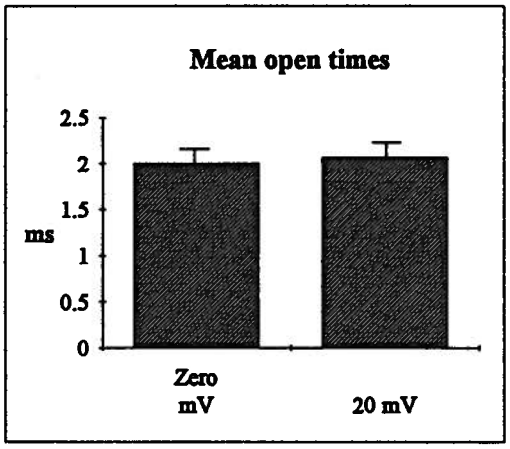
Fig. 7

* $P < 0.05$

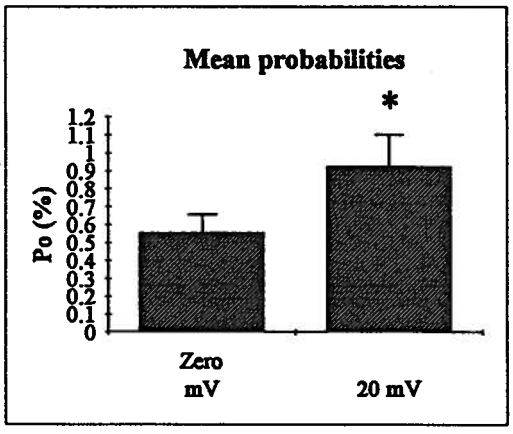
A



B



C



agent and known to block K(Ca) in the hippocampal neurons (McLarnon and Wang, 1991), was used in this study. Tedisamil (at 5 μM) lengthened action potential duration as recorded from the hippocampal slice by prolonging the later phase of the action potential repolarization (Fig. 8). In addition the afterhyperpolarization (AHP) phase was reduced. This result suggests that tedisamil blocked the K(Ca)-mediated Ic in the hippocampal neurons, causing a prolongation in the repolarization phase of the action potential in the hippocampal slice. These results would indicate that putative class III antiarrhythmic agents could block repolarizing K^+ channels in excitable membrane. Thus the measurements of drug actions, at the single channel level on neuronal K^+ currents, may be useful in the characterization of mechanisms of actions of antiarrhythmic agents. At present, similar studies on cardiac K^+ channels are severly limited by the low unitary conductance of repolarizing K^+ channels in cardiac cells such as I_{to} (transient outward K^+ current) and I_k (delayed rectifier K^+ current). A critical point however was to determine if potency for drug block of K(Ca) in hippocampal neurons was correlated with drug potency to alter properties in whole heart. This point served as a focus for the subsequent experiments

3.3 Pharmacology of K(Ca)

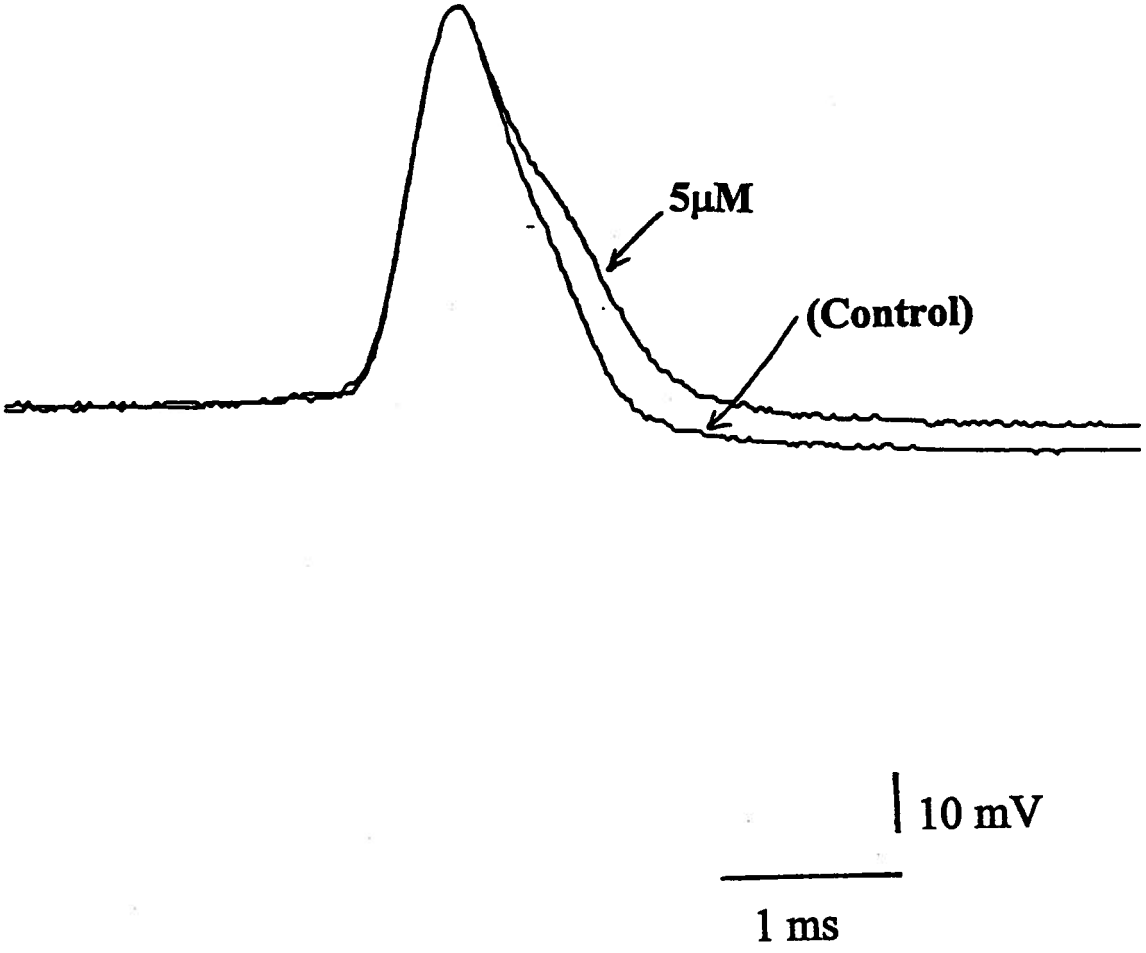
3.3.1 The RSD compounds - potential class III agents

The class III antiarrhythmic compounds (the K^+ blockers such as UK-68798, tedisamil and risotilide) have been demonstrated to be effective in blocking the K(Ca) in a dose-dependent manner (McLarnon and Wang, 1991).

Fig. 8 Effects of 5 μM tedisamil on the action potentials elicited in the hippocampal slice

The traces show the intracellular recordings of a hippocampal slice using current clamp. Current clamp pulses were of 5 ms duration and increased in the steps of 0.1 μA from rest. An action potential first elicited in the slice is shown as the control (bottom trace). The top trace shows the addition of tedisamil (5 μM) into perfusing solution after 30 minutes of waiting time. The prolongation of the late 2/3 of the repolarizing phase and an abolishment of the early afterhyperpolarization are evident.

Fig. 8



The action of some of the newly developed RSD compounds in diminishing the K(Ca) is the subject of investigation in this section. These drugs act on K^+ and Na^+ channels in cardiac cells with the potency for K^+ and Na^+ inhibition different and variable among the RSD compounds. A total of 18 RSD compounds were used and they are listed, in an ascending numerical order, as follows: 921, 935, 939, 942, 949, 952, 956, 959, 968, 969, 971, 973, 974, 979, 983, 984, 986 and 987.

3.3.2 Inside-out patch clamp experiments

The RSD compounds were tested for their actions in inhibiting K(Ca). After measurements of channel properties in control solutions the compounds were applied to the bath solution at an initial concentration of 1 μM. If channel unitary properties such as open times or opening frequency were altered by compounds applied at 1 μM then a concentration range between 1 - 10 μM was generally studied. If a compound showed little or no effect on properties of K(Ca) at a concentraton of 1 μM, the concentration was then increased to higher values (i.e. 10, 20, 50 μM), to a maximum value of 100 μM. The blockage of K(Ca) was evident from the increased flickering (channel transitions) of the unitary activity from open to closed (blocked) states. On the basis of single channel records it was not possible to distinguish between closed or blocked states. However, bursts of channel closures, not present in control records, were taken as evidence for drug block. In most cases wash-off of drug was possible yielding values for channel kinetics close to that found prior to drug application.

The RSD compound RSD 971 was the most potent blocker of K(Ca); a typical action of this agent is shown in Fig. 9. The top trace indicates the channel

activity in control. With addition of the compound, the flickeriness of the channel increased with a decrease in the time of the openings (i.e. shorter openings) as the concentration of the drug increased from 1μM to 4μM. The bottom trace showed the recovery of the long openings as the compound was washed away by re-perfusing the bath with the control solution. The measurement of these channel activities can yield some useful analysis of the data: the unitary amplitude of the channel, the closed and open duration and the frequency of channel events.

3.3.2.1 The open time analysis

Typical open time distributions are shown for another potent K(Ca) blocker (RSD 939) in Fig. 10 A. The distributions were fit with single exponential curves as indicated on the figures. After comparing the goodness of fit, it was found that the single component (compared with fits using multi-components) gave the best fit; this point was determined by comparing errors in the fits using different numbers of components. The arithmetic mean open time was also useful in estimating this corrected τ_0. The two measurements (fit vs mean value) were identical except for a factor of the system resolution time. (Colquhoun and Sigworth, 1983). For most of the τ_0 measurements in our experiments, the corrected τ_0 was smaller than the arithmetic τ_0 by a consistent time (in the range of 0.2 - 0.6 ms). The single exponential fit indicated that there is only one channel opening state of the hippocampal neuronal K(Ca) which was also confirmed by the amplitude distributions (see 3.3.2.3).

Fig. 9 *Effects of a RSD compound (971) on K(Ca)*

Unitary currents obtained from an inside-out patch at 0 mV are shown to be affected by the administration of RSD 971(concentrations as shown). The compound was the most potent of the 18 compounds tested in the investigation. A dose-dependent decrease of the mean open times (as well as the increase of flickering) of the channel activities is evident. A return to the control solution resulted in kinetic behavior close to that found prior to compound application.

Fig. 9

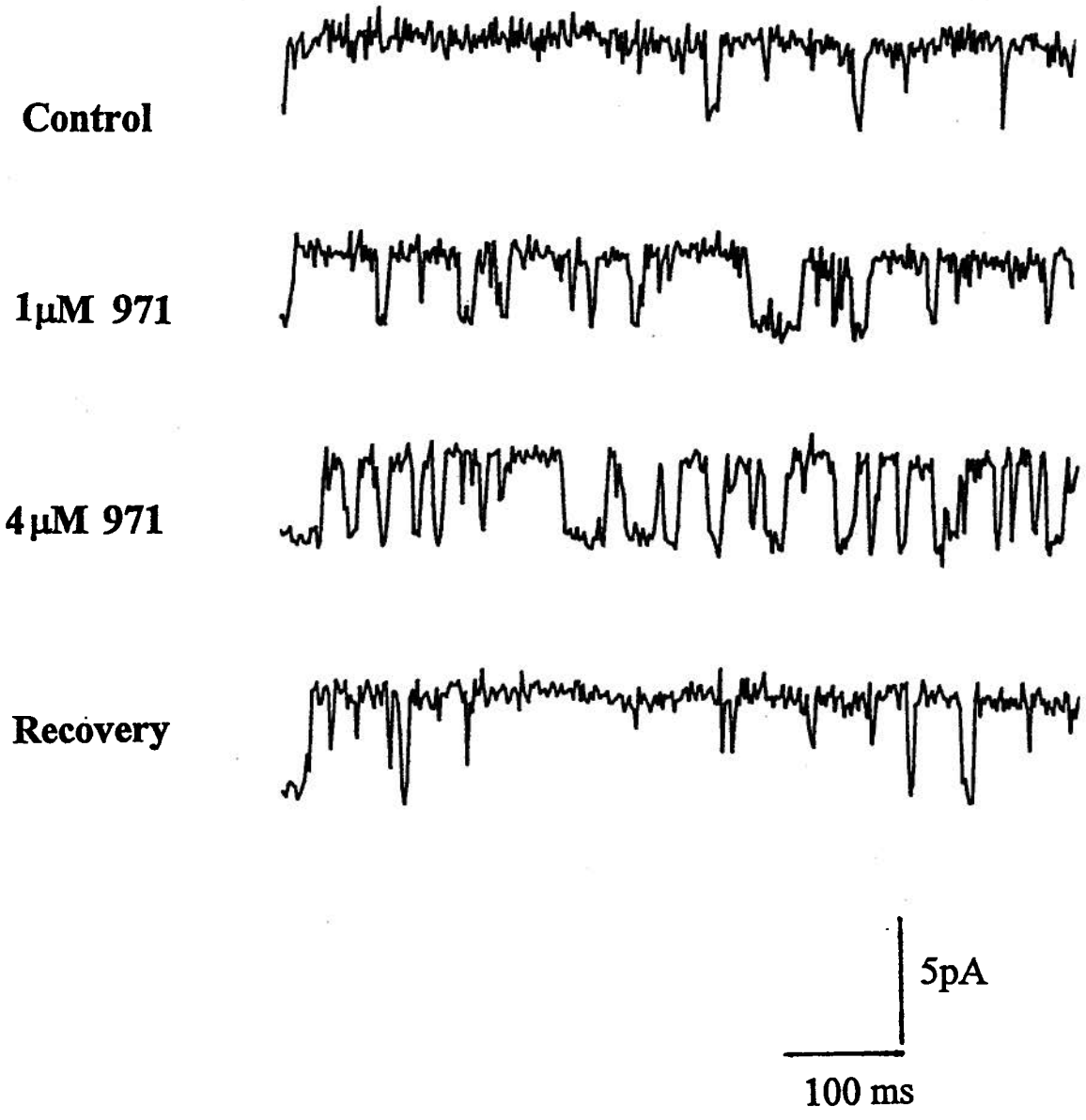


Fig. 10 *Effects of 4μM 939 on the open times, closed times and amplitude distributions*

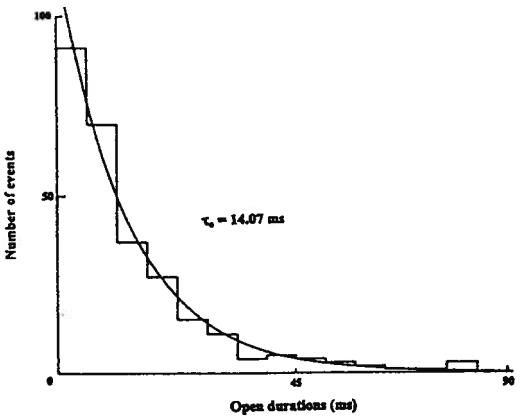
Histograms of the open times (A), closed times (B) and amplitudes (C) distributions were constructed to show the effects of 4μM RSD 939. The open time distributions were fitted by a single exponential component and the closed time distributions by two components. For the control solution: the mean open time was 14.07±0.98 ms (A), the closed time had components 0.86±0.06 ms and 4.13±1.25 ms (B). For 4μM 939: the mean open time was 6.84±0.77 ms, with the closed times components 0.77±0.03 ms and 4.79±0.83 ms. The results, which indicate reduction in the mean open time with the compound and no significant changes in both the amplitude and closed time distributions, suggests that 939 blocks K(Ca) in an uncompetitive manner.

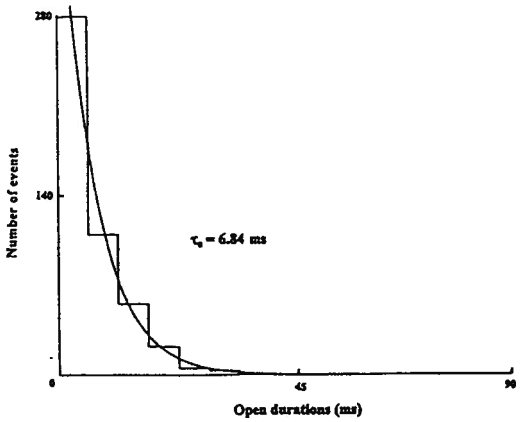
Fig. 10

Control

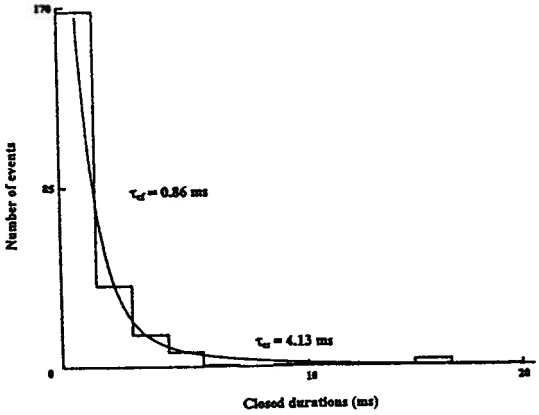
4 μM 939

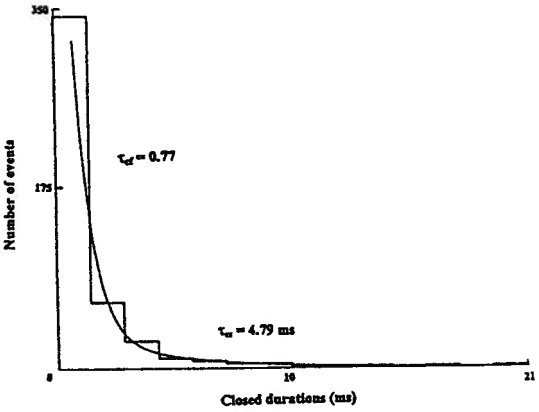
A





B





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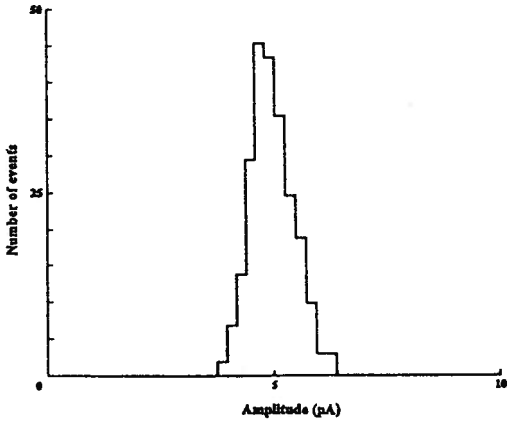
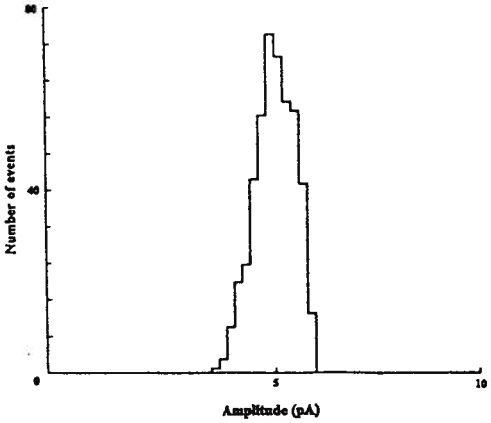




In Fig. 10 A, compound RSD 939 (4 μM) was shown to decrease the τ_O (roughly halving it), reflecting a significant reduction in the mean open times of the channel. Most other RSD compounds showed a similar effect in decreasing the mean open times but with varying potency. An investigation and comparison of this effect of the compounds is given in the later section (3.3).

3.3.2.2 The closed time analysis

Typical closed time distributions are shown in Fig. 10 B. The closed time distributions could be best fit with a two-component exponential function. The two components were related to the single channel records discussed previously: on this basis the fast component (τ_{cf}) represents the closures within a single burst, largely due to the block of the channel by the compound. The slow component (τ_{cs}), on the other hand, represents closures between individual bursts. The presence of 939 had little or no effects on both τ_{cf} and τ_{cs} (Fig. 10 B). Other RSD compounds have been found to act in a similar manner and had no significant effects at any concentrations on the closed time distributions (either component) of the K(Ca).

3.3.2.3 The amplitude analysis

Typical amplitude histograms are shown in Fig. 10 C (both recorded at zero mV). The single peak distribution indicates that i) there is only one type of channel activity in the patch, and ii) no channel subconductance state was present. The amplitude showed little variation between patches (within 0.5 pA) and amplitudes of unitary currents were not altered with any of the RSD compounds at any concentration studied. This result indicated that the

compounds did not exhibit very fast channel block since in such cases unitary current amplitudes can be reduced by the compound. In this case the full current amplitudes cannot be resolved due to limitations in amplifier band width.

3.3.2.4 The open channel block

The decrease in the mean open time, with very little change in the amplitude and closed time duration distributions, are typical characteristics of an open channel block. An open channel block model has been useful in describing the actions of putative class III antiarrhythmics in blocking K(Ca) in CA1 hippocampal neurons including KC 8851 (McLarnon, 1990) and UK 68798, tedisamil and risotilide (McLarnon and Wang, 1991). In addition tedisamil blocked K(Ca) in motoneurons (McLarnon et al., 1992). Fig. 10 represents the general situation of the actions of the RSD compounds tested on the three major aspects of the channel kinetics (open and closed times and amplitude). The open channel block model can be represented by the scheme shown below:

$$\underset{}{\overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}}} \text{Closed} \quad \text{Open} \quad \underset{}{\overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}}} \text{Blocked}$$

where k_1 is the onward rate constant from the closed state to the open state and k_{-1} represent the transition from open to closed state. k_2 is the onward rate constant for the association of the drug, and k_{-2} is the off (unblocking) rate

constant. The dissociation rate constant k_D is defined as k_{-2}/k_2. The drug-induced dose-dependent decrease in the mean open times of K(Ca) can be expressed as a linear function of the concentration of the blocker, in the form of the rate of decay (i.e, the reciprocal of the mean open time, τ^{-1}). The equation is given by:

$$\tau^{-1} = k_2 [D] + k_{-1}$$

A plot of τ^{-1} vs [D] can thus be used to determine the blocking rate constant k_2. If such a plot is not fit by a linear relation, then the data would suggest inapplicability of an open channel block model to describe the actions of the RSD compounds. Otherwise, the effects on the mean open times can be used as an index of the potency of the RSD compounds.

3.3.3 A comparison of the class III antiarrhythmics as potent K(Ca) blockers

As noted above the RSD compounds examined, like other putative class III antiarrhythmics, block K(Ca) with evident increase in channel transitions from the open to a non-conducting state. Thus, measurements of mean open time provides an index for drug block of K(Ca). In particular, I have used the concentration which caused mean open time in control to be halved as a measurement of the potency of the RSD compounds in blocking K(Ca). This value is denoted as MOT_{50}. The quantitative assessment of channel block has utilized the specific open channel block scheme already described. According to this model, a plot of the inverse of mean open time in presence of the compound

against the compound concentration should be linear. The slope of the graph is the onward blocking rate constant.

3.3.3.1. A plot of τ^{-1} against [D]

The *k2* value (blocking rate constant) gives an estimate of the blocking potency of the drugs. The *k2* can be determined by plotting τ^{-1} (calculated as the inverse of the mean open time) against the concentration of the agonist ([D]). The slope of the fitted straight line gives *k2*, while the fitted line can be extrapolated to intercept the y-axis at the point yielding k_{-1} . Five drugs were chosen for the analysis of the linearity of the relationship between mean open time and compound concentration and for calculating *k2*. As noted above the check of linearity is essentially a check for applicability of the blocking model. The results are summarized in Table 1 and the plots are shown in Fig. 11. The linear relationship between the compound concentration and the inverse of mean open times justifies the use of the open block scheme to describe the blocking action of the RSD compounds. It is also the basis to determine the potency of the compounds in terms of concentration required to exert a given change in the mean open time (in this case, the mean open time to be one-half of the control value). The k_{-1} values, which are independent of the presence of drugs, were found to be similar for the five RSD compounds used. This result suggests that the kinetics of channel closing were similar in control from patch to patch.

An analysis for the value of k_{-2} (the concentration-independent off-rate constant from the blocked state to the open state) was done as follows. Records of unitary events were scanned and regions of rapid closures were analysed for time durations of closed state transitions. This analysis assumed such closures

Table 1. *Onward blocking rate constants (k_2) for five drugs*

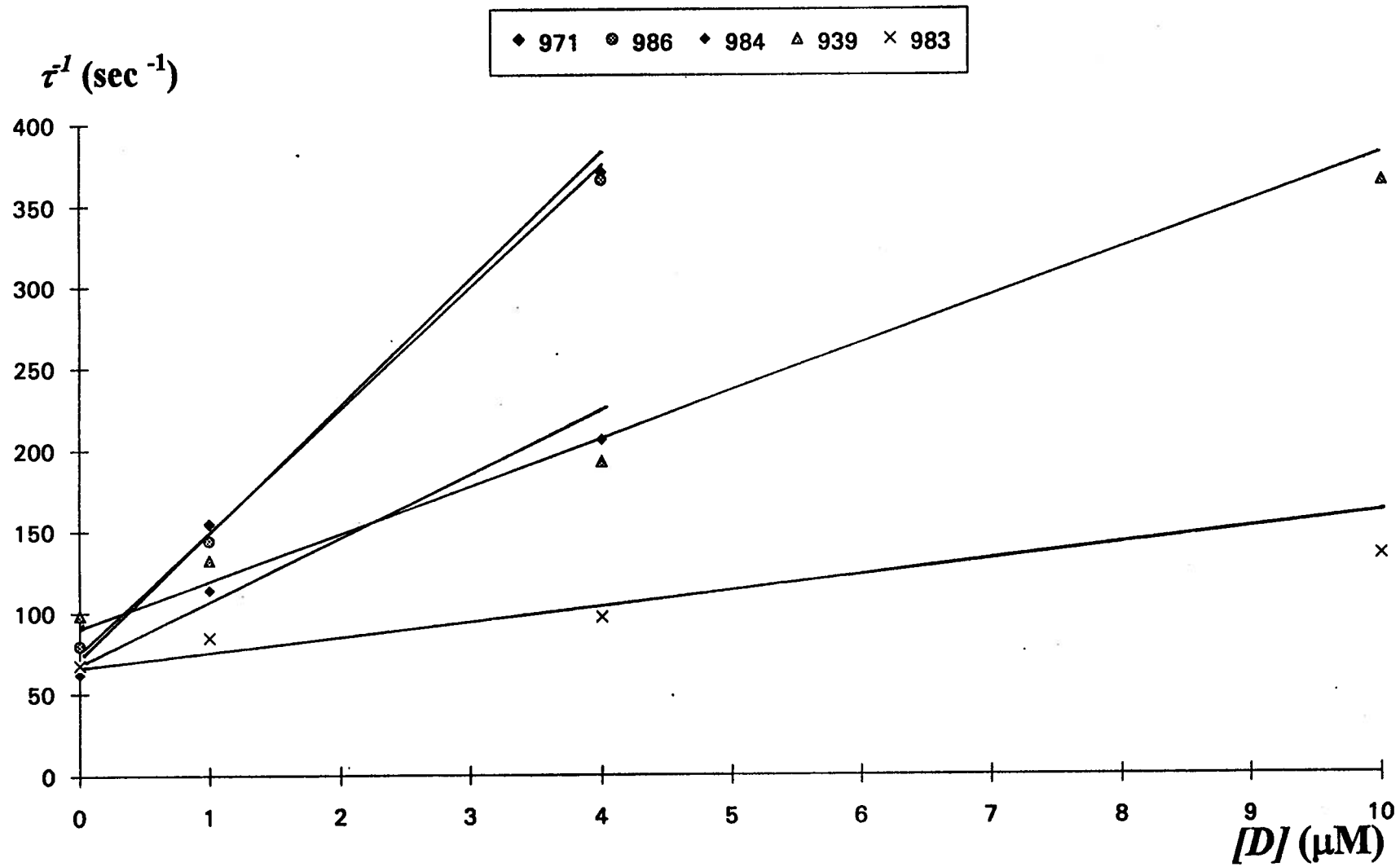
The τ^{-1}, calculated as the inverse of the mean open time in the presence of drugs, is related to k_2 by the equation $\tau^{-1} = k_2$ [D] + k_{-1} . The k_2 is defined as the slope of the graph of τ^{-1} against [D] (concentration of RSD compound), and the five slopes are shown in Fig. 11.

RSD	n	[D] (μM)	τ^{-1} (sec^{-1})	k_2 (10^7 M^{-1} sec^{-1})
971	4	0	65.89 ± 8.48	7.60
		1	154.6 ± 12.17	
		4	369.9 ± 19.10	
986	4	0	79.50 ± 8.38	7.14
		1	143.8 ± 26.68	
		4	365.2 ± 72.20	
984	3	0	61.83 ± 4.19	3.60
		1	113.8 ± 7.70	
		4	205.9 ± 7.96	
939	4	0	98.40 ± 12.54	2.35
		1	132.0 ± 15.88	
		4	192.6 ± 18.10	
		10	363.5 ± 12.55	
983	3	0	67.60 ± 2.70	0.80
		1	84.40 ± 4.95	
		4	96.80 ± 9.04	
		10	133.9 ± 3.18	

Fig. 11 *The plots of τ^{-1} against [D]*

The mean open time and concentration relationship of five RSD compounds are shown. The RSD compounds acted by causing a dose-dependent decrease of the mean open time, which can be expressed by the open channel block scheme $\tau^{-1} = k_2 [D] + k_{-1}$. The plots of τ^{-1} vs [D] are thus linear. For data please refer to Table 1.

Fig. 11


971
986
984
939
983
τ^{-1} (sec $^{-1}$)
400
350
300
250
200
150
100
50
0
0
1
2
3
4
5
6
7
8
9
10
[D] (μM)

were channel blocking sojourns. This procedure yielded only an estimate for k_{-2} (inverse of the times) since it was difficult to differentiate between blocked and closed state transitions. For RSD 971 the off-rate constant was estimated to be approximately 1/ 1000 sec^{-1}, which would give a k_D value near 0.1 μM.

3.3.3.2. Index of the potency of the compounds - MOT50

The MOT_{50} values of 15 RSD compounds are listed in Table 2. The n values indicate number of experiments with given drugs and was at least n=3 for all agents with the exception of RSD 952 (with n=2). A MOT_{50} value was obtained for 15 out of 18 compounds with the remaining 3 agents having MOT_{50} values higher than the maximum concentration used in these experiments (set at 0.1mM). A system of subdividing the RSD compounds according to their potency for blocking K(Ca) allowed grouping them into 4 concentration ranges. These ranges were based on concentrations of the compounds to cause mean open time to be half of control values. These were as follows:

1) Highly potent (0.5 μM - 1.5 μM)	-	971, 959, 986, 979, 921
2) Potent (1.6μM - 2.5 μM)	-	949, 984, 987, 969
3) Intermediate (2.6 μM - 5 μM)	-	968, 974, 939
4) Low potency (6 μM - 20 μM)	-	952, 983, 973
5) Not effective	-	935, 942, 956

Table 2. *MOT50 values of the RSD compounds.*

The MOT50 values are defined as the concentration (in μM) of the compound required to reduce the mean open time to 50% of its original value. The values are catorgorized into 4 group according to the potency of the compounds (1 - highly potent; 2 - potent; 3 - intermediate; 4 - low potency). 15 compounds are listed below, with the exception of RSD compounds 935, 942 and 956, which were ineffective at a concentration as high as 100 μM.

1	RSD	n	MOT50 (μM)
	971	4	0.78 ± 0.12
	959	4	1.03 ± 0.16
	986	4	1.25 ± 0.17
	979	6	1.30 ± 0.16
	921	5	1.40 ± 0.17

2	RSD	n	MOT50 (μM)
	949	5	1.80 ± 0.12
	984	4	1.88 ± 0.24
	987	4	2.00 ± 0.35
	969	5	2.34 ± 0.14

3	RSD	n	MOT50 (μM)
	968	3	3.60 ± 0.31
	974	5	4.20 ± 0.46
	939	4	4.38 ± 0.24

4	RSD	n	MOT50 (μM)
	952	2	6.50 ± 0.50
	983	4	9.00 ± 0.41
	973	3	15.30 ±1.45

The effects of the RSD drugs on open time durations (n=1) are shown in Fig.12 - Fig.19 as mean open time distributions fitted with a single exponential curve along with the τ_0 value given on each graph. Compounds chosen from each of the first four groups of potency are shown: from Fig. 12 to Fig.14, drugs 971, 986 and 979; from Fig. 15 to Fig. 16, drugs 984 and 987; from Fig. 17 to Fig. 18, drugs 939 and 983; in Fig. 19, drug 973. Open time distributions of the control and two concentrations of the compound are shown in each figure with concentrations being 1 μM and 4 μM for most of the compounds. Higher concentrations of the less potent drugs are shown (4 μM and 10 μM of 983, 10 μM and 20 μM of 973).

3.3.3.3. Comparison of MOT50 with putative class III antiarrhythmics

The MOT50 values determined for RSD compounds can be compared with other class III antiarrhythmics (McLarnon and Wang, 1991). It was found that both the sulfonamide compound UK 68798 (MOT50 = 0.4μM) and the dihydrochloride derivative tedisamil (MOT50 = 1μM) belong to group 1, with UK 68798 even more potent than RSD 971(MOT50 = 0.78μM). Risotilide (7.5 μM) was low in potency compared to these RSD compounds. RP 62719, a benzopyran compound (personal communication from this laboratory) with an MOT50 value of 3.5 μM, was intermediate in its potency.

3.3.3.4. Comparison of the MOT50 and the ERP25 values

A comparison was made between the inhibiting actions of the RSD compounds on the neuronal K(Ca) and their actions on repolarizing K^+ currents in whole heart. The potency of the RSD compounds in inhibiting the repolarizing K^+ currents in the whole heart was determined by measuring the concentration of the compounds required to increase the effective refractory period (ERP) by

Fig. 12 - Fig. 14 *Effects of 971, 986, 979 on the mean open time*

Three RSD drugs of group 1 (very potent) are effective in decreasing the mean open time, shown here (n=1) as histograms of open time distributions, fitted with a single-exponential curve.

Fig. 12 -

A) Control, with mean open time = 12.97±0.45 ms
B) 1μM 971, with mean open time = 6.58±0.46 ms
C) 4μM 971, with mean open time = 2.76±0.19 ms

Fig. 13 -

A) Control, with mean open time = 13.36±0.71 ms
B) 1μM 986, with mean open time = 8.78±0.62 ms
C) 4μM 986, with mean open time = 5.56±0.34 ms

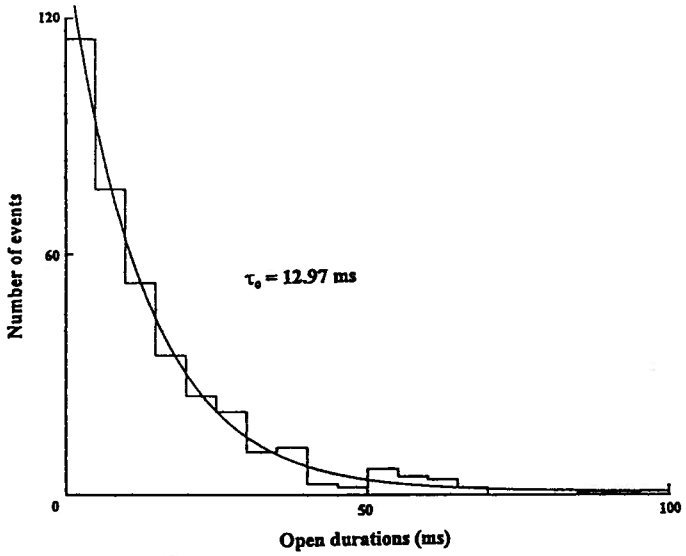
Fig. 14 -

A) Control, with mean open time = 12.06±0.99 ms
B) 1μM 979, with mean open time = 6.09±0.31 ms
C) 4μM 979, with mean open time = 3.77±0.20 ms

Fig. 12

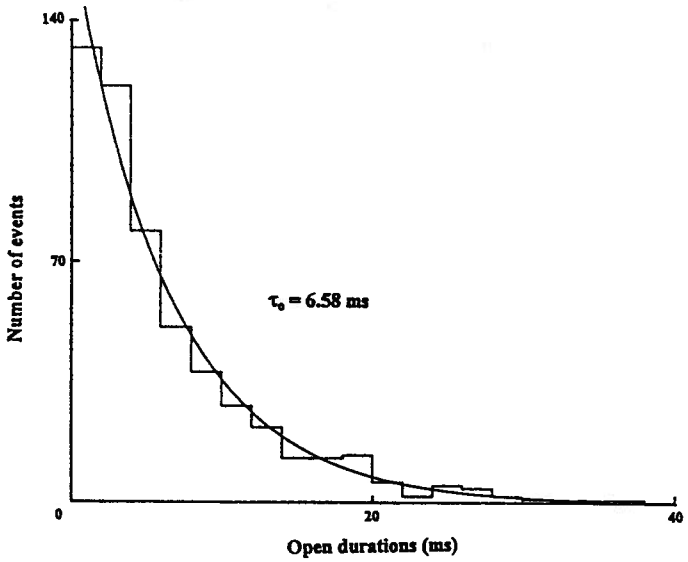
A

Control



B

1 μM 971



C

4 μM 971

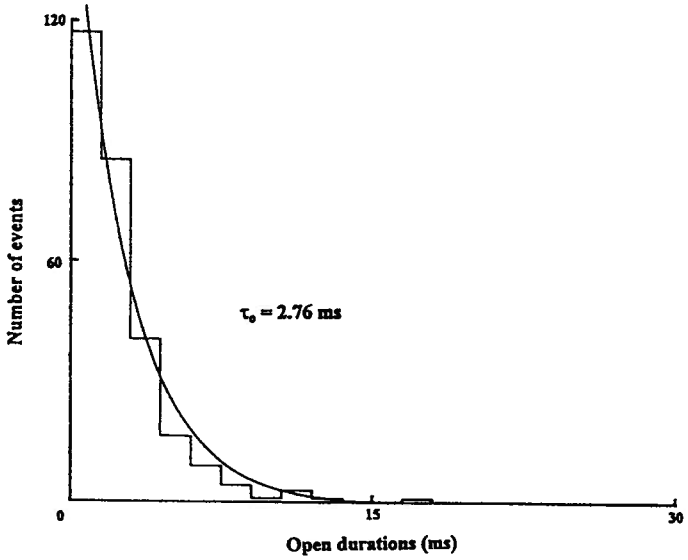
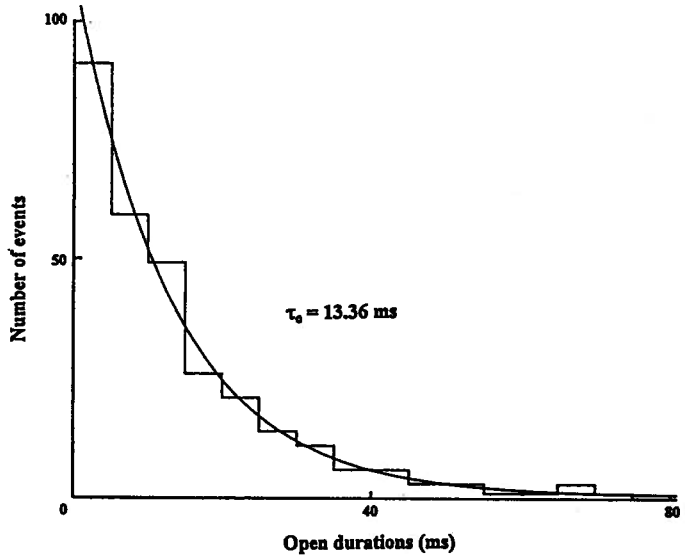


Fig. 13

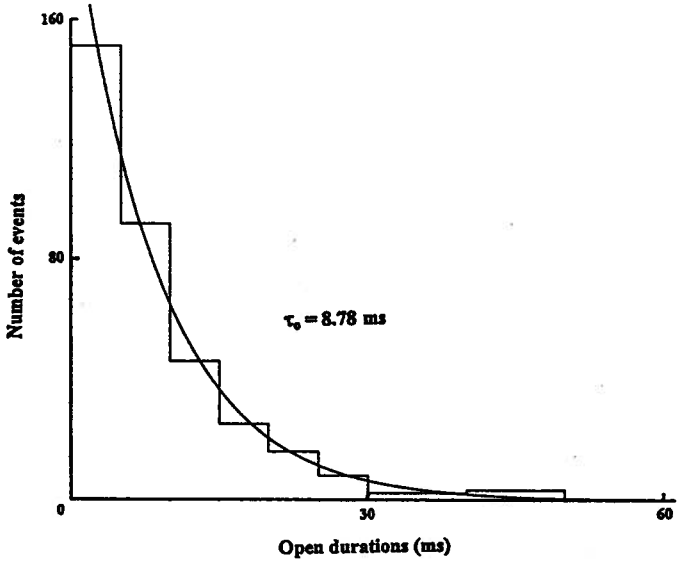
A

Control



B

1 μM 986



C

4 μM 986

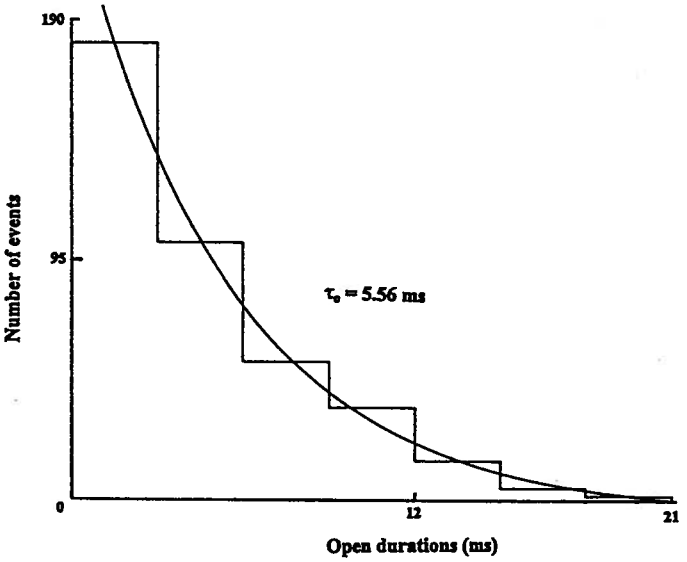
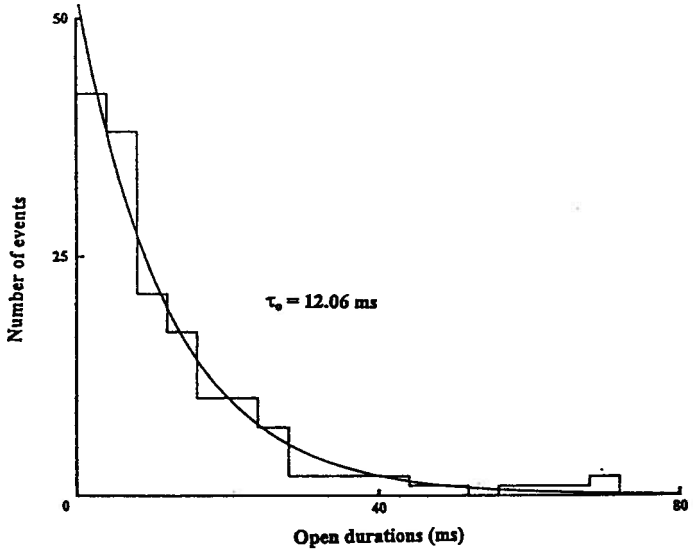


Fig. 14

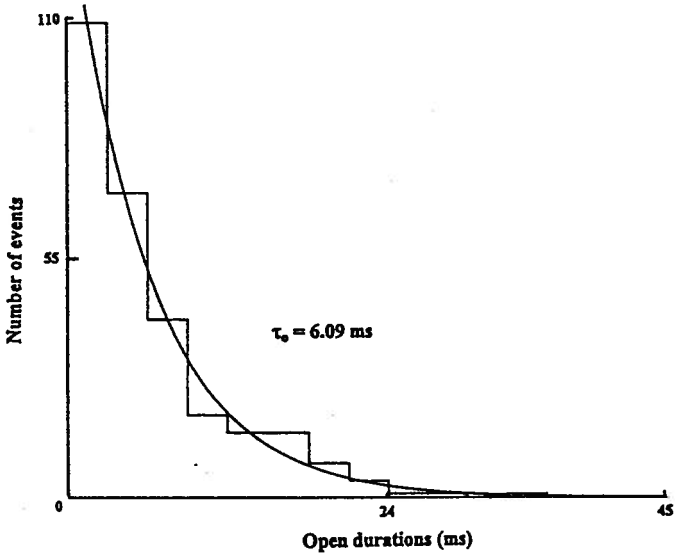
A

Control



B

1 μM 979



C

4 μM 979

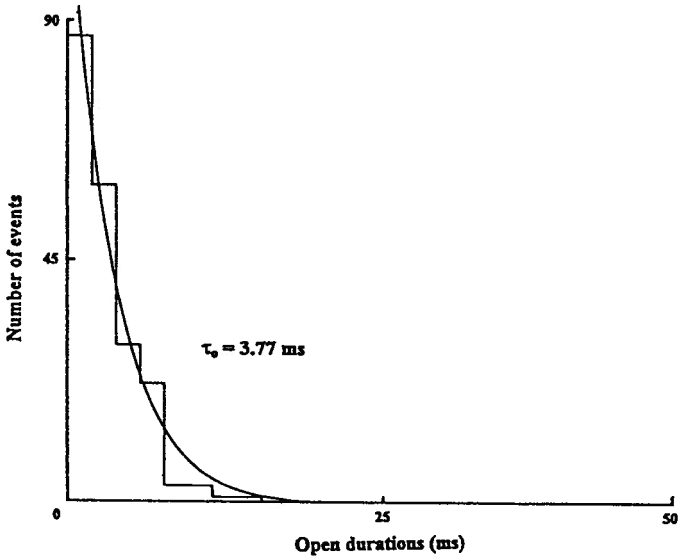


Fig. 15- Fig. 16 *Effects of 984 and 987 (group 2: potent) on the mean open time (n=1)*

Fig. 15 -

A) Control, with mean open time = 12.87±0.76 ms
B) 1μM 984, with mean open time = 7.63±0.27 ms
C) 4μM 984, with mean open time = 4.45±0.21 ms

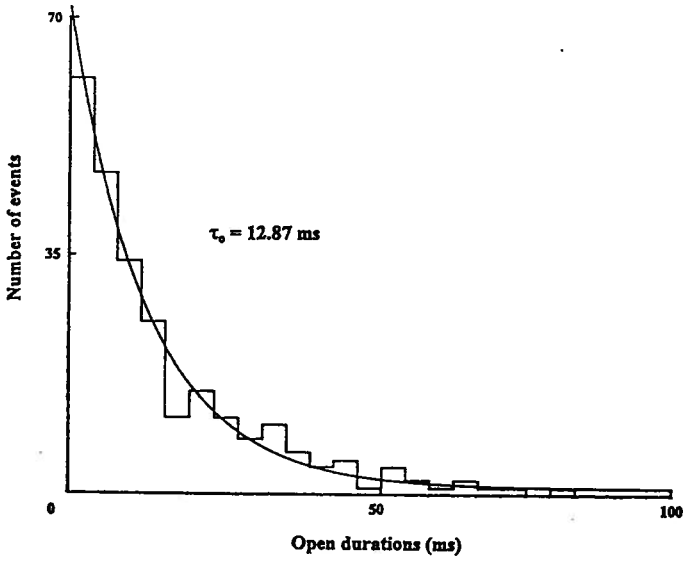
Fig. 16 -

A) Control, with mean open time = 11.69±0.46 ms
B) 1μM 987, with mean open time = 7.84±0.34 ms
C) 4μM 987, with mean open time = 4.49±0.26 ms

Fig. 15

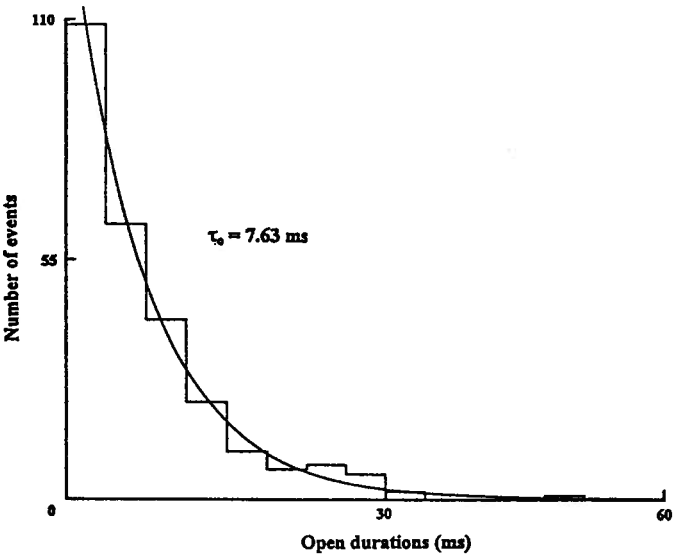
A

Control



B

1 μM 984



C

4 μM 984

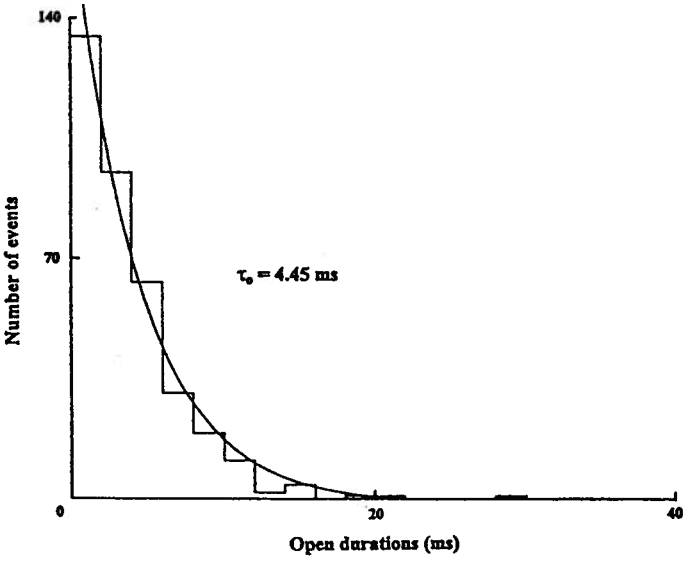
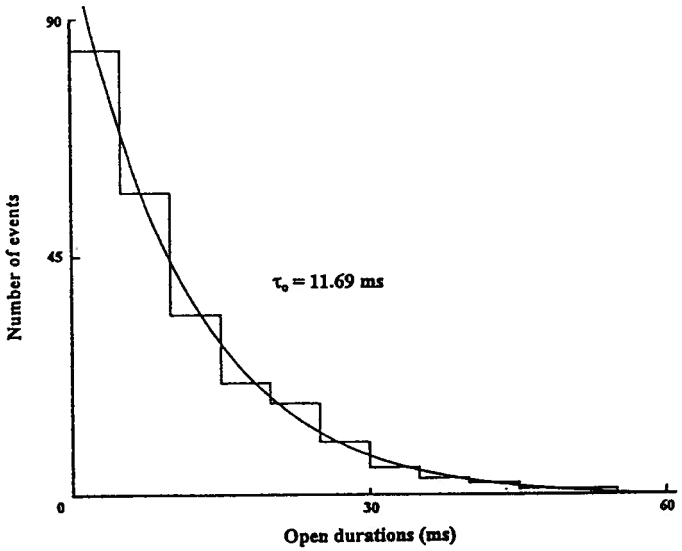


Fig. 16

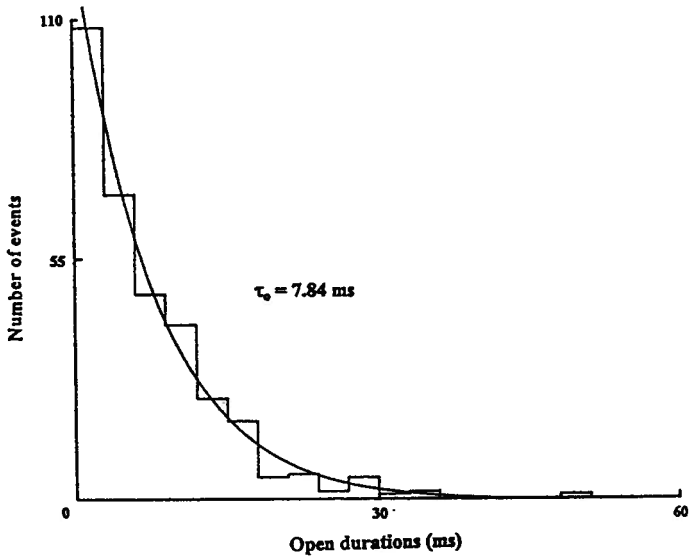
A

Control



B

1 μM 987



C

4 μM 987

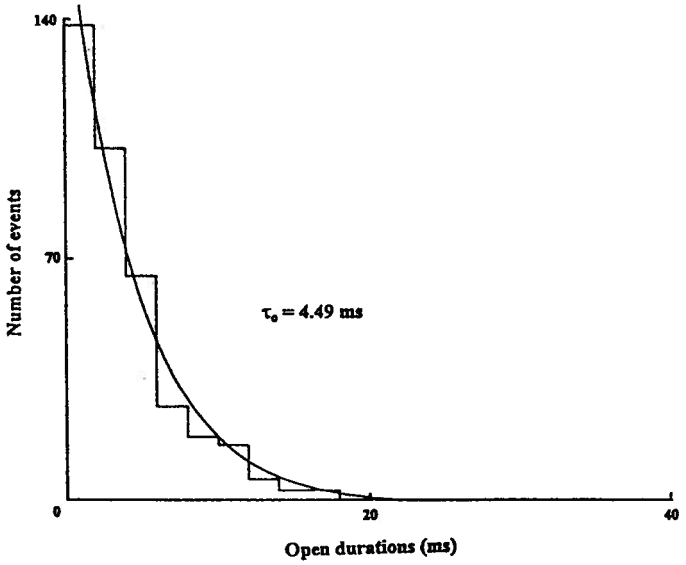


Fig. 17- Fig. 18 *Effects of 939 and 983 (group 3: intermediate) on the mean open time (n=1)*

Fig. 17 -

A) Control, with mean open time = 14.69±0.91 ms
B) 1μM 939, with mean open time = 12.43±0.60 ms
C) 4μM 939, with mean open time = 7.13±0.14 ms

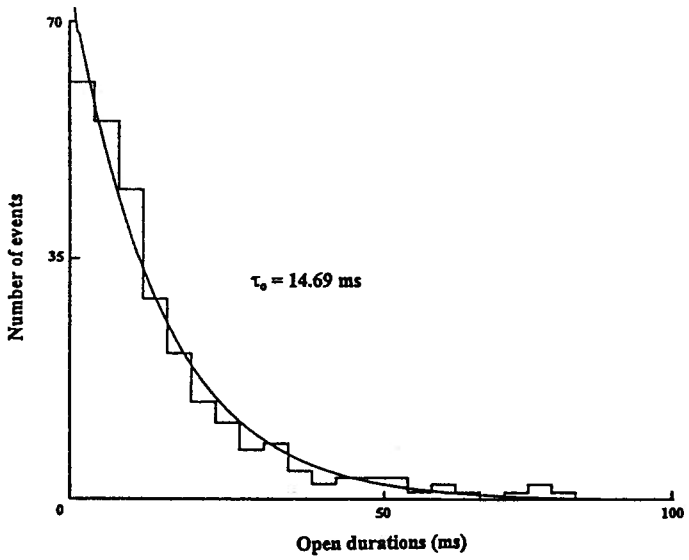
Fig. 18 -

A) Control, with mean open time = 15.09±1.05 ms
B) 4μM 983, with mean open time = 10.58±0.43 ms
C) 10μM 983, with mean open time = 7.99±0.42 ms

Fig. 17

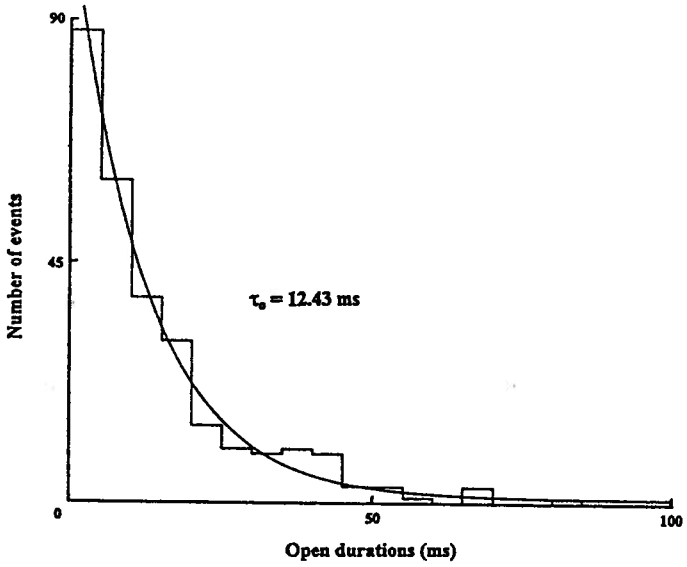
A

Control



B

1 μM 939



C

4 μM 939

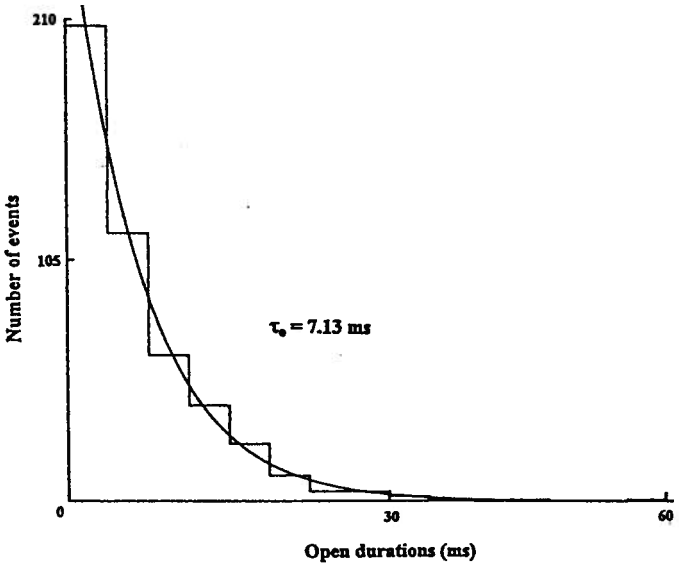
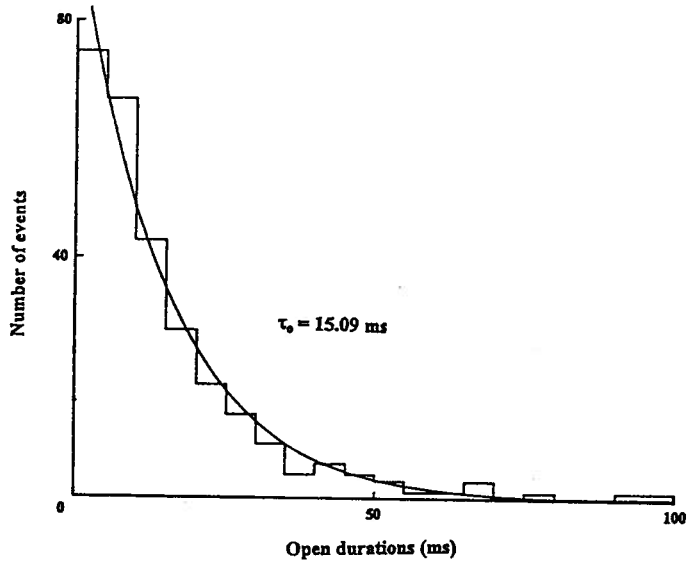


Fig. 18

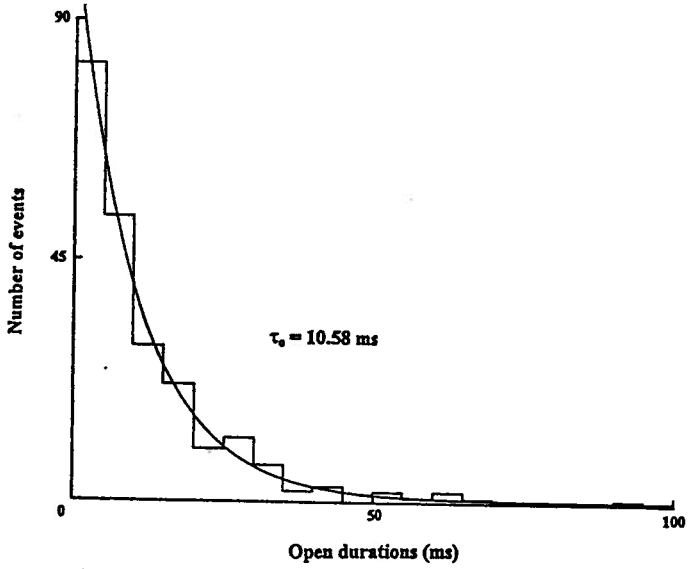
A

Control



B

4 μM 983



C

10 μM 983

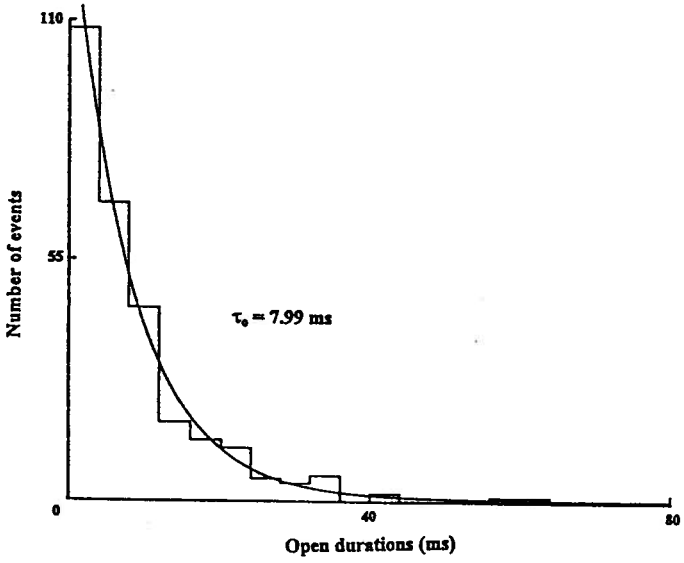


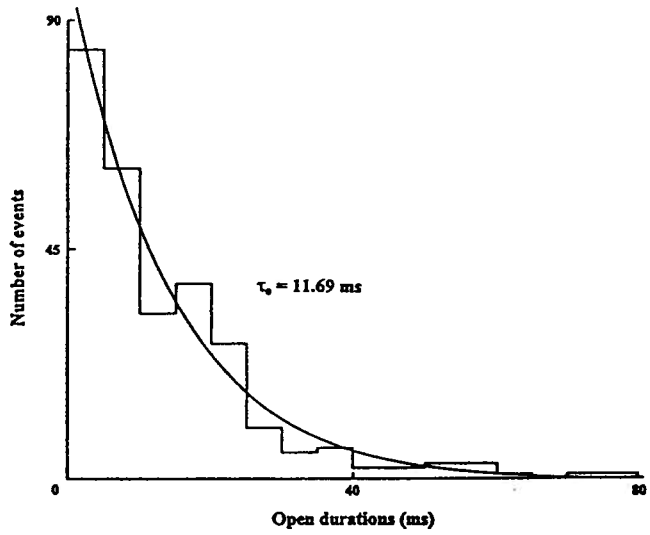
Fig. 19 *Effects of 973 (group 4: low potency) on the mean open time (n=1)*

A) Control, with mean open time = 11.69±1.55 ms
B) 10μM 973, with mean open time = 6.05±0.38 ms
C) 20μM 973, with mean open time = 4.47±0.14 ms

Fig. 19

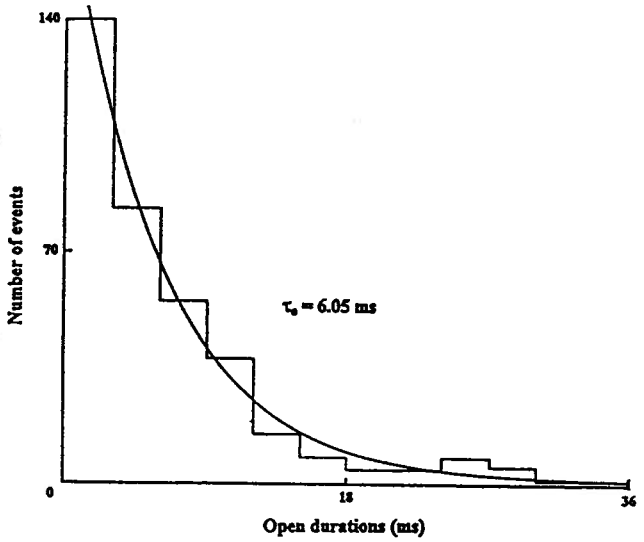
A

Control



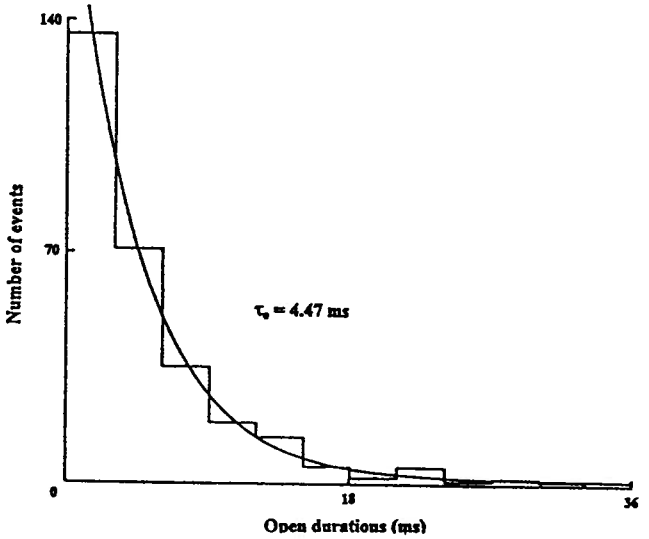
B

10μM 973



C

20μM 973



25% per kg weight of the animal per minute. These concentrations, represented by ERP_{25} were obtained from experiments performed in Dr. M.J.A. Walker's laboratory. Since the actions of the compounds reach a steady state in the whole rat experiment, we can compare the ERP_{25} values with the MOT_{50} values. A table summarizing the two sets of values are given in Table 3, and the graph showing the correlation between these data are given in Fig. 20. Fig. 20 includes all of the 18 compounds tested in both laboratories. The data suggests the existence of a general correlation between the actions of the compounds, except for the two outliers RSD 979 and 971. Using simple linear correlation theory it was found that with the outliers, the MOT_{50} compares poorly with the ERP_{25} and gave a correlation coefficient (r) of 0.04. By excluding the two outliers, the correlation was greatly improved with $r = 0.71$ (thus yielding correlation index, r^2, of 0.504). See Fig. 21. The correlation was statistically significant with $P<0.05$ in committing a type I error. All three RSD compounds (935, 942, 956) which were found to be ineffective in inhibiting the K(Ca) (with a $MOT_{50} > 0.1$ mM) were also not potent in inhibiting the repolarizing K+ currents in the isolated heart (with $ERP_{25} \geq 20$ μM), and are shown on the right hand corner of Fig. 20. The general conclusion was that the effects of the drugs to block the repolarizing K(Ca) in neurons showed a positive correlation with drug effects to prolong ERP in whole heart.

3.3.4. The wash-off/recovery times

One interesting area of the study was to determine the wash-off/recovery times for different compounds. Although the inside-out patch clamp set-up may be a poor representation of the actual physiological situation, the ability of the

Table 3. *A comparison of the MOT_{50} and ERP_{25} values*

The MOT_{50} values (in μM) and the ERP_{25} values (in μM/kg/min.) of 15 RSD compounds are listed below. The MOT_{50} values are obtained as described in Table 1. The ERP_{25} values were calculated from the ECG traces obtained in the screen II test of the RSD drugs on the whole rat heart.

RSD	MOT50 (μM)	ERP25 (μM/kg/min.)
971	0.78	8.40
959	1.03	1.30
986	1.25	0.30
979	1.30	18.0
921	1.40	0.50
949	1.80	1.30
984	1.88	1.20
987	2.00	0.90
969	2.34	4.40
968	3.60	3.50
974	4.20	4.40
939	4.38	2.30
952	6.50	3.60
983	9.00	1.50
973	15.3	6.80

Fig. 20 *A comparison of the MOT_{50} and ERP_{25}*

The graph of MOT_{50} vs ERP_{25} is shown for the 18 RSD compounds tested . Two outliers, RSD 971 and 979, could be seen at the bottom of the graph. The estimated positions of the three RSD compounds (935, 942, 956) indicated on the righthand corner of the plot were found to be ineffective in both inhibiting the K(Ca) (with $MOT_{50} > 0.1$ mM) and the repolarizing K^+ currents in the isolated heart (with $ERP_{25} \geq 20$ μM/kg/min.).

Fig. 20

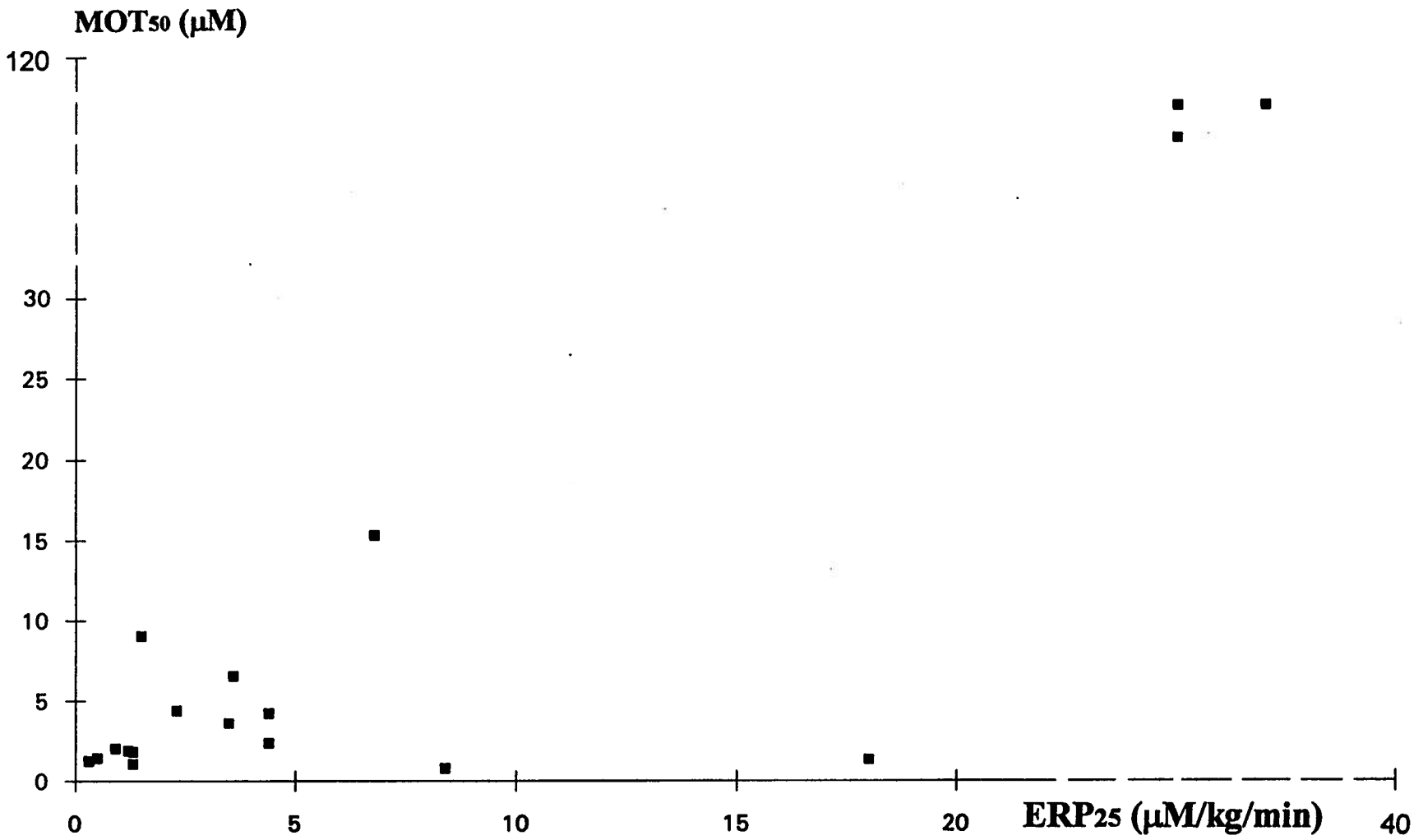
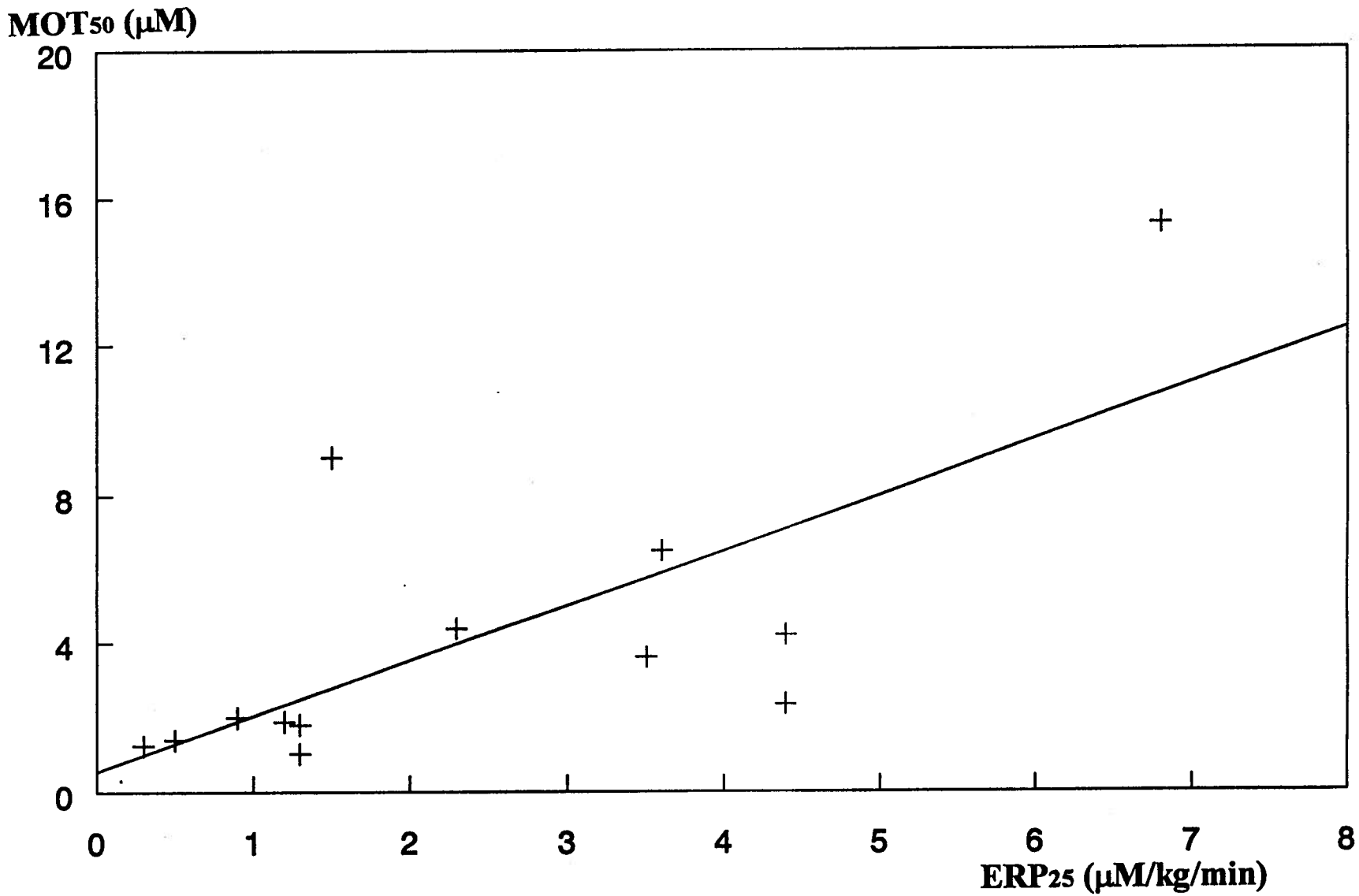


Fig. 21 *The correlation of MOT_{50} and ERP_{25}*

The simple linear correlation plot of MOT_{50} vs ERP_{25} is shown. The three non-responsive RSD compounds (935, 942, 956) as well as the two outliers (971, 979) were excluded. With all of the 15 compounds correlated, a correlation coefficient (r) of 0.04 was obtained. By removing the two outliers, r became statistically significant ($P<0.05$) with a value of 0.71.

Fig. 21



compounds to remain at sites associated with the channel may still gives clues to the actual behavior of the drugs *in vivo*. Apart from the ability of the compound to stay on the membrane (the magnitude of its off-rate constant), the position of the patch relative to the fluid inlet can also significantly affect the time taken by the reperfusing control solution to remove the compounds. If the patch lies close to the path of the flow of the solution wash-off may be more rapid; otherwise the solution will have to first fill up the bath before the compounds can be washed off. Eight drugs were observed for their washing-off phenomena including 959, 968, 969, 971, 983, 984, 986 and 987. The index used for the determination of the wash-off time was as follows. With the average flow rate of 1 ml in every 17 seconds, a 50% recovery of the control mean open time within 20 seconds was considered to be fast. A 50% recovery within 30 - 90 seconds was considered as medium, and a 50 % recovery that came after 2 minutes was considered to be slow. The results showed that 983, 984 and 987 can be washed off easily (fast recovery). 968, 969, 971 and 986 were considered to give intermediate recovery, and 959 was found to be persistent against being washed off (slow recovery). The wash-off/recovery time of these compounds were compared to their wash-off times found in the isolated heart experiments in Dr. M.J.A. Walker's laboratory. A recovery of the ERP was considered to be fast when it occurred within 30 seconds. Between 1 and 5 minutes it was rendered slow and extremely slow for a period of more than 5 minutes. The results showed that 983, 984, 987, 968 and 969 all gave fast recovery. and 971 and 986 were slow in recovery and 959 being extremely slow. A comparison of the recovery phenomena of these compounds in the two set-ups indicate a certain degree of correlation in the compound affinity. Further experiments however, will be

required for a more detailed comparison of the recovery times. The comparison of these times are shown in Table 4.

3.3.5. Outside-out patches

The actions of five RSD compounds were studied by adding the compounds to the outside of the membrane in the outside-out patches. Long openings were observed in the control solution. Upon the application of the compounds rapid flickering transitions to the non-conducting state and characteristic of open channel block were observed. The potency of the compounds, expressed in MOT_{50} , are given in Table 5. It was found that two of the compounds, RSD 974 and 979, has a MOT_{50} value similar to that found with inside-out patches. The other three compounds, 949, 959 and 969, all showed a reduction in potency in the outside-out patches. At present it is impossible to differentiate between an internal and external site of action for these compounds. However, the similarity of the actions of the compounds in both the inside-out and outside-out patches suggest that the same site may be involved in both types of patches.

Table 4. *A comparison of the recovery times*

In the inside-out patch experiments, the recovery times were classified as fast (within 20 s), intermediate (between 30 - 90 s) and slow (more than 2 minutes). In the isolated heart experiments, the recovery times were categorized as fast (within 30 s), slow (between 1 and 5 minutes), and extremely slow (more than 5 minutes).

Recovery mode	Inside-out patch (K(Ca))	Isolated heart (I_{K+})
A) Fast	983 984 987	983 984 987 968 969
B) Intermediate for patch clamp experiments and slow for the isolated heart experiments	968 969 971 986	971 986
C) Slow for patch clamp experiments and extremely slow for the isolated heart experiments	959	959

Table 5. *MOT50 values of five RSD compounds in outside-out patches*

The MOT50 values are defined as the concentration (in μM) of the compound required to reduce the mean open time to 50% of its original value. The MOT50 values found in the inside-out patches for the same compounds are given also for comparison.

RSD compounds	*n*	*MOT50 values for outside-out patch (μM)*	*MOT50 values for inside-out patch (μM)*
949	2	5.85	1.80
959	2	5.10	1.03
969	3	6.23 ± 1.50	2.34 ± 0.14
974	2	4.50	4.20
979	3	1.10 ± 0.20	1.30 ± 0.16

4. DISCUSSION

The essential question forming the basis for the present studies concerned the applicability of a repolarizing K^+ channel in neurons (K(Ca)) to serve as a target for putative class III antiarrhythmic compounds. If this point could be shown then K(Ca), which is readily isolated and active in both inside-out and outside-out patches, could serve a role as a plausible model for drug interactions with K^+ currents in cardiac cells. Previous studies have indeed shown that a number of putative class III antiarrhythmic compounds, with a diversity on structures, blocked K(Ca) at concentrations similar to values which altered properties in whole heart preparations. In order to test the hypothesis that K(Ca) could serve as a surrogate for repolarizing K^+ in ventricular cells, an experimental program was carried out in a systematic fashion. First, a known agent with class III actions, tedisamil, was applied to hippocampal slices. The experiment was designed to measure drug effects on Ic which is the macroscopic repolarizing current in hippocampal neurons and is built on K(Ca). Secondly, unitary properties of K(Ca) were established by recording from inside-out and outside-out patches. Finally, the critical experiments were carried out to measure actions of 18 RSD compounds on K(Ca) and results correlated with drug actions in whole heart.

4.1 Single channel properties of CA1 hippocampal K(Ca)

The single channel properties of the K(Ca) were examined. Under physiological conditions of K+ (140 mM intracellular and 5 mM extracellular), the channel conductance was found to be 110 pS. By putting symmetrical K^+ (140 mM) across the patches, a higher value of conductance of 170 pS was

obtained. The dependence of the CA1 K(Ca) on internal Ca^{2+} can be expressed by a sigmoidal dose-response curve with channel open probability against internal Ca^{2+} concentration (McLarnon and Sawyer, 1993). An internal Ca^{2+} concentration of 4 μM was required for maximal channel activity as judged by no further increase in the P_{open} with increased Ca^{2+}. At a low internal Ca^{2+} concentration of 0.7μM, the P_{open} was increased from 0.57 % to 0.91 % by a depolarization step of 20 mV. The increase in P_{open} was found to be the sole result of a voltage-sensitive increase of the opening frequency and the mean open times of the unitary current remained unaffected by the depolarization.

Current clamp experiments were carried out to examine the effect of tedisamil on the elicited action potentials in the hippocampal slices. Tedisamil (5 μM) prolonged the action potential by prolonging the repolarization phase and abolishing the afterhyperpolarization phase (Fig. 8). The result has demonstrated that class III agents like tedisamil could be useful in blocking repolarizing K^+ currents in excitable membranes and the measurements of drug actions at the single channel level may be useful in the characterization of the actions of antiarrhythmic agents on repolarizing K^+ currents.

4.2 Correlation of the drug effects on K(Ca) and repolarizing K^+ currents

Previous findings have appeared to suggest the similarity between the inhibitory effects on the repolarizing K^+ currents in cardiac cells with drug actions on the rat K(Ca). In order to test this possibility, a number of potential class III agents were tested separately for their actions on the neuronal K(Ca) and compared with actions on whole heart. A total of 18 RSD compounds were used in these

experiments. All of the compounds were examined for their inhibitory actions on K(Ca) in the cultured rat CA1 neurons using inside-out patches; in a few cases the effects on outside-out patches were also examined. In the presence of most of the compounds the unitary activity showed increased transitions from the opening state to a non-conducting state. In the presence of the compounds the mean open time of the channel decreased with the closed time duration and the amplitude of the current remaining unchanged. This result was consistent in all of the compounds tested which showed significant inhibitory effects on the K(Ca); 3 of the compounds, RSD 935, 942 and 956 showed no significant inhibitory effects on the K(Ca) at concentrations as high as 0.1 mM. The data suggested open channel blockade as the mechanism of action for these RSD compounds on the CA1 K(Ca). The open channel blockade scheme relates the mean open time of the channel to the concentration of the drug by the following equation: $\tau^{-1} = k_2$ [D] + k_{-1}. The plot of τ^{-1} against [D] thus should give a straight line with a slope equal to the onward (blocking) rate constant k_2. The τ^{-1} vs [D] plots for 5 RSD compounds are shown in Fig. 11. The linearity of the data suggests that these RSD compounds inhibit K(Ca) consistent with an open channel blockade model. The results were consistent with the identification of the mechanism of action on the CA1 K(Ca) for a number of other class III agents as open channel block (KC 8851 - McLarnon, 1990; UK 68798, tedisamil and risotilide - McLarnon and Wang, 1991).

The index of the potency of the RSD compounds was chosen as the concentration of the compound which caused mean open time in control to be halved. This value is denoted as MOT_{50}. By using this index, a group of 18 compounds can be subdivided into 5 categories according to their potency in inhibiting the K(Ca) (see RESULTS). These data were then used to compare with the potencies of these compounds in inhibiting the repolarizing K^+ currents

in rat whole heart. The index of potency for these compounds in blocking the outward K^+ currents was the concentration of the compound per kg weight of animal per minute required to increase the effective refractory period (ERP) by 25% and represented by ERP_{25}. The correlation of the MOT_{50} and the ERP_{25} is given in Fig. 20. Initial inspection of the available data seems to suggest no correlation between the responses of neuronal K(Ca) and of cardiac repolarizing K^+ channels to these Class III agents. Compounds highly effective in prolonging the ERP in whole rat (e.g. 971 and 979) are not found to be comparatively effective in the neuronal situation, which seems to confirm that no correlation exists. The present data only allow a correlation within the range of roughly 0 - 16 μM in MOT_{50} and around 0 - 8 μM/kg/min. in ERP_{25}, far too narrow for a good correlation system. On the other hand, however, the plot does show a general correlation between the two sets of values without the two outliers (RSD 979 and 971). By excluding these outliers the simple correlation yields a correlation coefficient of 0.71 which is statistically significant ($P<0.05$) although only a coefficient of 0.04 is obtained by including them. By using the Chauvenet's criterion, we can legitimately reject any data points of ERP_{25} which exceed 3.89 + 7.07 = 10.98 μM/kg/min. in value (with a Chauvenet's Criterion value of 8%, any values beyond ±1.55 standard deviations of the mean in a normal distribution are rejected). In this way we can exclude 979, which gives us a correlation coefficient of 0.375 with the remaining 14 agents, which is statistically significant.

Several points should be considered in the results. First the ERP values for drug effects in whole animals may be subject to errors. For example, the pharmacokinetic behavior of the drugs may lead to drug potencies in whole animal which do not reflect actions only on repolarizing K^+ currents. Secondly, the two sets of data do correlate with the two outliers removed. Furthermore,

analysis of both ERP_{25} and MOT_{50} values show agreement in the identification of RSD drugs which are not potent (see Fig. 20). Thus, unitary K(Ca) can serve as a reasonable model for drug actions in cardiac cells. Since single channel studies on the cardiac K+ channels are limited by the low unitary conductance of repolarizing K+ channels (such as I_{to} and I_k), the K(Ca) in neurons have utility as a screening device for putative class III agents.

4.2.1. A comparison of the recovery times

In addition to the drug potency, the K(Ca) experiments can also be useful in determining the offset time for drugs. Eight RSD compounds were tested for the recovery time in the inside-out patches. A 50 % recovery of the control mean open time within first 20 seconds of reperfusion was considered as fast, intermediate recovery was defined as 50% recovery between 30-90 seconds, and slow recovery, if it occurred, after 2 minutes. The same RSD compounds were tested for the recovery period in the whole heart set-up (see description in RESULTS) and the comparison of the two sets of recovery time are given in table 4. The comparison shows a general agreement between the two sets of recovery times. All of 983, 984 and 987 were found to be fast in recovery (i.e. washed off quickly) in both set-ups, and 959 was persistent in both single channel and whole heart experiments. Two of the intermediate compounds in the inside-out patch clamp experiments, 968 and 969, were found to wash-off rapidly in the isolated heart experiment. Although a detailed comparison between the recovery times was not possible, valuable information can be obtained from the K(Ca) experiments. Most evidently was 959 which clearly, relative to other RSD compounds, was persistent in action. Little recovery from drug actions was observed even after reperfusion of control solution for long

periods. This compound, in heart, has been found to be essentially irreversible. Thus K(Ca) would also seem to be useful in the assessment of drug wash-off.

4.2.2. Inside-out vs. outside-out patches

Outside-out experiments (n=2) were carried out on five RSD compounds (949, 959, 969, 974, 979). A comparison of the MOT_{50} values obtained from inside-out patches and outside-out patches showed that 974 and 979 had similar potencies in inhibiting the K(Ca). This result was consistent with that obtained with some other class III agents (McLarnon and Wang, 1991). However 949, 959 and 969 were found to be somewhat less potent when applied to the outside of the patch membrane. The low number of patches studied in the outside-out configuration (n=2 or 3) may have contributed to the measured differences in potency. The use of inside-out vs outside-out patches is theoretically very useful in determining whether the active site is internal or external. Unfortunately in our system a rapid perfusion system which can apply drugs in the order of ms was not used. Thus, it was not possible to differentiate between an internal or external active site. Previous work with tedisamil on the cardiac I_{to} also found that tedisamil blocked this cardiac current with both internal and external application (Dukes and Morad, 1989).

5. CONCLUSIONS

A detailed investigation of the structure-activity relationship for RSD compound was not possible at this stage since drug structures were not published. The establishment of a correlation between MOT_{50} and ERP_{25} is important since the patch clamp technique allows the measurement of drug actions on single channel K(Ca) activity. This allows a rapid method for examination of potential Class III compounds and also gives important information on sites of action and wash-off phenomena. At present similar experiments on unitary currents in cardiac cells are not feasible, in part due to the low unitary conductance of repolarizing K+ channels in heart. The correlation between drug actions on K(Ca) and ERP values in heart would suggest former data have relevance to a description of drug mechanisms of action. The investigation suggests the utility of more detailed single channel examination of these potential class III agents in the future. The procedure may also provide a rapid screening device in the development of putative class III antiarrhythmic compounds.

Some caution should be exercised in the application of drug effects on K(Ca) in neurons to describe drug actions in heart. The primary repolarizing K^+ channel in rat myocytes is I_{to} and although this current is modulated by calcium it is not dependent on calcium for activation as is K(Ca) in neurons. Indeed, the analog of Ito in neurons is most likely to be I_A (the transient rapid repolarizing K^+ current) and it is well known that the pharmacological profile of I_A and K(Ca) are different. It would seem evident from the present work, however, that considerable homology occurs for repolarizing K^+ channels in different tissues.

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