Effect of Ajmaline on Transient Outward Current in Rat Ventricular Myocytes

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Abstract. The mechanism of ajmaline-induced inhibition of the transient outward current (I_{to}) has been investigated in right ventricular myocytes of rat using the whole cell patch clamp technique. Ajmaline decreased the amplitude and the time integral of I_{to} in a concentration-dependent, but frequency- and use-independent manner. In contrast to the single exponential time course of I_{to}-inactivation in control conditions ($\tau_i = 37.1 \pm 2.7$ ms), the apparent inactivation was fitted by a sum of two exponentials under the effect of ajmaline with concentration-dependent fast and slow components ($\tau_f = 11.7 \pm 0.8$ ms, $\tau_s = 57.6 \pm 2.7$ ms at 10 μmol/l) suggesting block development primarily in the open channel state. An improved expression enabling to calculate the association and dissociation rate constants from the concentration dependence of τ_f and τ_s was derived and resulted in $k_{on} = 4.57\times10^6 \pm 0.32\times10^6$ mol^{-1}·l·s^{-1} and $k_{off} = 20.12 \pm 5.99$ s^{-1}. The value of $K_d = 4.4$ μmol/l calculated as k_{off}/k_{on} was considerably lower than $IC_{50} = 25.9 \pm 2.9$ μmol/l evaluated from the concentration dependence of the integrals of I_{to}. Simulations on a simple model combining Hodgkin–Huxley type gating kinetics and drug-channel interaction entirely in open channel state agreed well with the experimental data including the difference between the K_d and IC_{50}. According to the model, the fraction of blocked channels increases upon depolarization and declines if depolarization is prolonged. The repolarizing step induces recovery from block with time constant of 52 ms. We conclude that in the rat right ventricular myocytes, ajmaline is an open channel blocker with fast recovery from the block at resting voltage.

Key words: Rat ventricular myocytes — Potassium channel — Transient outward current — Ajmaline-induced block — Model simulations

Introduction

One of the characteristic features of class Ia antiarrhythmic drugs according to Vaughan-Williams classification is prolongation of the action potential (AP) dura-

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tion. This effect was also observed in the presence of ajmaline and its derivatives in atrial and ventricular muscle of different species (e.g., Bojorges et al. 1975; Alvarez et al. 1992; Langenfeld et al. 1992; Eckardt et al. 1999) including the rat ventricular myocytes (Bébarová et al. 2004). The predominant outward component of the membrane ionic current in ventricular myocytes of rat is the transient outward current (I_{to}) (Josephson et al. 1984; Apkon and Nerbonne 1991). Its inhibition may account at least partly for the observed ajmaline-induced AP prolongation. However, to our knowledge, the effect of ajmaline on I_{to} has not been reported so far.

The effects of other class Ia antiarrhythmic agents on I_{to} have been studied in various preparations. The amplitude of I_{to} was decreased and the initial apparent inactivation was accelerated by quinidine and disopyramide (Jahnel et al. 1994; Clark et al. 1995; Sanchez-Chapula 1999; Hanada et al. 2003; Iost et al. 2003) but not by procainamide (Iost et al. 2003). The recovery from the block appeared to be very slow in presence of disopyramide (time constant around 7 s, Sanchez-Chapula 1999) but by one order faster in presence of quinidine (time constant of 0.695 s, Jahnelet al. 1994).

An inhibition of I_{to} accompanied by acceleration of apparent inactivation has been observed also under the effect of antiarrhythmic drugs of class III and class IV, e.g. tedisamil (Dukes et al. 1990; Wettwer et al. 1998) or verapamil and nifedipine (Jahnel et al. 1994). The recovery from tedisamil-induced block was slow (12 s) while the recovery from verapamil- and nifedipine-induced block was very fast.

Thus, the acceleration of apparent inactivation is a common feature of I_{to}-block by various antiarrhythmic drugs that suggests prominent block in the open channel state. On the other hand, substantial differences in the velocity of block relief indicate differences of drug-channel interactions in detail. The data concerning the properties of ajmaline are missing. Therefore, we found it useful to describe the effect of ajmaline on I_{to} and to analyse underlying mechanism.

Materials and Methods

Cell isolation

Ventricular myocytes were isolated from right hearts of adult male Wistar rats (250 ± 50 g). We preferred the right ventricle because of considerable heterogeneity related to I_{to} observed in left ventricle (Casis et al. 1998). In cells from right ventricle, we have regularly recorded prominent I_{to} showing mono-exponential time course of inactivation.

The dissociation procedure was similar to that described by Bosteels et al. (1999). After cervical dislocation under mild ether anesthesia, the heart was quickly removed and retrogradely perfused *via* aorta at 37℃ with 0.9 mmol/l $CaCl_2$ Tyrode solution (3–5 min) and then with nominally Ca-free Tyrode solution (4.5 min). During the first digestion step (2–2.5 min), perfusion was continued with nominally

Ca-free Tyrode solution containing collagenase (type S, 0.2 mg/ml; Yakult), protease (type XIV, 0.053 mg/ml; Sigma), bovine serum albumin (fraction V, 2 mg/ml; Sigma), and EGTA (44 μmol/l; Sigma). In the second digestion step (20 min), protease and albumin in the enzyme solution were omitted. The enzyme solution was then washed out in two steps by perfusion with low calcium Tyrode solutions (0.09 and 0.18 mmol/l $CaCl_2$). All solutions were oxygenated with 100 % O_2.

The right ventricular free wall was dissected from the remainder of the heart. The tissue was minced in 30 ml of 0.18 mmol/l $CaCl_2$ Tyrode solution. After filtration, the suspension of cells was exposed to gradually increasing Ca^{2+} concentration in extracellular medium (within 20 min) to the final value of 0.9 mmol/l. The isolation procedure was performed at 37 ℃.

The experiments were carried out in accordance with the institutional guidelines and approved by the local authorities (permit No. 076/2001 – V3).

Solutions and chemicals

The composition of the Ca-free Tyrode solution was (in mmol/l): NaCl 135, KCl 5.4, $MgCl_2$ 0.9, HEPES 10, NaH_2PO_4 0.33, glucose 10 (pH was adjusted to 7.4 with NaOH). $CaCl_2$ (0.9 mmol/l) and the calcium channel blocker $CoCl_2$ (2 mmol/l) were administered in the course of the experiments. The patch electrode filling solution contained (in mmol/l): L-aspartic acid 130, KCl 25, $MgCl_2$ 1, Na_2ATP 5, EGTA 1, HEPES 5, GTP 0.1, Na_2-phosphocreatine 3 (pH 7.25 adjusted with KOH).

$CoCl_2$ (Sigma) was prepared as 1 mol/l stock solution in deionized water. Ajmaline (Gilurytmal® 10, Solvay Pharmaceuticals) was applied in concentrations ranging between 0.3 μmol/l and 5 mmol/l.

Electrophysiological measurements

Single rod-shaped cells with well apparent striation were used for the membrane current recordings at room temperature applying the whole cell patch clamp technique. The patch pipettes were pulled from borosilicate glass capillary tubes and heat polished on a programmable horizontal puller (Zeitz-Instrumente). The resistance of the filled glass electrodes was below 1.5 MΩ to keep the access resistance as low as possible. For generation of experimental protocols and data acquisition, the Axopatch 200A equipment (Axon Instruments, Inc.) and pCLAMP program (version 6.0.4) were used. The currents were filtrated with a four-pole Bessel filter at 5 kHz, digitally sampled at 4 kHz and stored on the hard disk of a computer.

Statistical analysis

Results are presented as means ± S.E. Curve fitting and the paired t-test (used to assess the significance ($p < 0.05$) of the effects of ajmaline with the drug-free conditions as control) were performed using GraphPad Prism, version 4.0 (GraphPad Software, Inc.).

The time course of inactivation of I_{to} was approximated tentatively by single exponential function and by a sum of two exponential functions using the least

square fitting procedure. Inactivation was regarded as double exponential if introduction of the second exponential function resulted in the decrease in sum of squares (χ^2) by more than 5 %.

Quantitative modelling

A kinetic diagram of I_{to}-channel gating supplemented by drug-channel interaction in the open state was proposed and described by a set of linear differential equations. Simulation of experiments was performed using the numeric computation software MATLAB, version 6 (The Math Works, Inc.).

Results

Depolarization-induced block

Fig. 1 illustrates the concentration dependence of the effect of ajmaline on I_{to}, quantified as the time integral of the current response to 250 ms pulse from −75 mV to +60 mV after subtraction of the constant current component measured at the end of the pulse. Using the least square best fit procedure, the pooled data were approximated by the Hill equation. The resulting data of concentration inducing 50 % inhibition *(IC_{50})* and the Hill coefficient were 25.9±2.9 μmol/l and 1.07±0.15, respectively.

Fig. 2A shows the superimposed original records of I_{to} in response to 250 ms lasting impulses from −75 mV to +60 mV in control conditions and in the presence of ajmaline (10 μmol/l). The block developed with a delay after an early phase of activation suggesting no block in the resting state. In control, the time course of inactivation of I_{to} could be well fitted by a single exponential function with the time constant $\tau_i = 37.1 \pm 2.7$ ms ($n = 8$). Under the effect of 10 μmol/l ajmaline, the course of apparent inactivation became biphasic and could be approximated by a sum of two exponentials (fast and slow) with time constants $\tau_f = 11.7 \pm 0.8$ and $\tau_s = 57.6 \pm 2.7$ ms ($n = 8$, decrease of χ^2 by 50 % at average in comparison with single exponential approximation). This behaviour suggests that I_{to}-block could

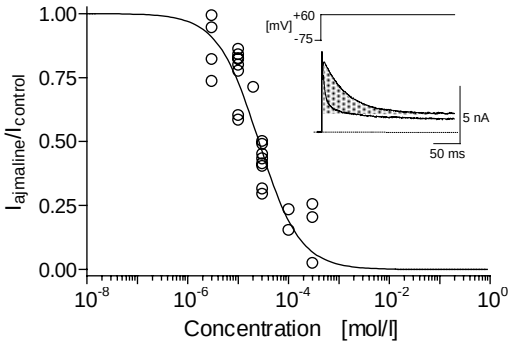


Figure 1. Concentration dependence of normalized ajmaline-induced I_{to}-block. I_{to} was measured in response to depolarizing pulses from −75 mV to +60 mV applied at 0.1 Hz and evaluated as an integral after subtraction of the current at the end of 250 ms impulse (inset). Calcium current was blocked by Co^{2+} (2 mmol/l).

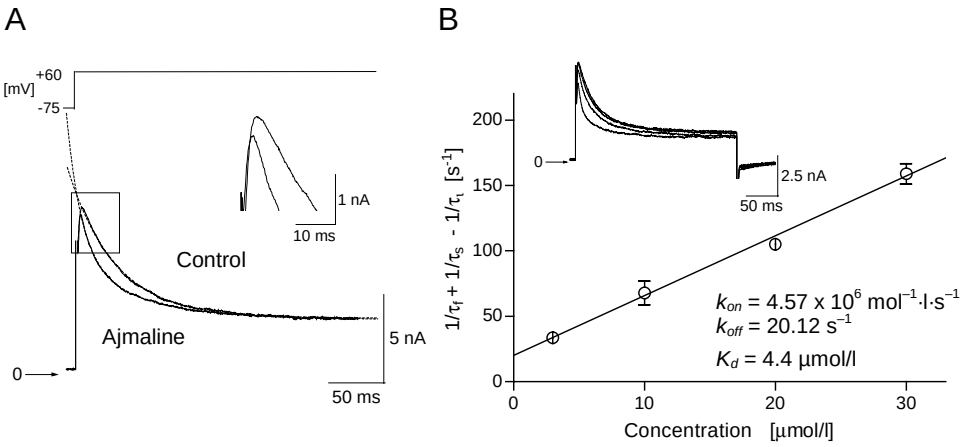


Figure 2. Kinetics of ajmaline-induced I_{to}-block. **A.** Superimposed current tracings in response to steps from −75 mV to +60 mV (0.1 Hz). In control conditions, the time course of inactivation was fitted by exponential function with $\tau_i = 37.1 \pm 2.7$ ms. Under the effect of ajmaline (10 μmol/l), the apparent inactivation was fitted by a sum of two exponentials with $\tau_f = 11.7 \pm 0.8$ ms and $\tau_s = 57.6 \pm 2.7$ ms (mean ± S.E., $n = 8$). Inset: the augmented detail illustrates that initial activation of I_{to} is not affected by ajmaline. **B.** The term $1/\tau_f + 1/\tau_s - 1/\tau_i$ as a function of ajmaline concentration (see Eq. (2)). The straight line resulted from the linear fit and yielded values of the apparent association and dissociation rate constants (k_{on} and k_{off}). K_d was calculated as k_{off}/k_{on}. Inset: superimposed currents recorded at drug concentrations of 0, 3, 10 and 30 μmol/l.

occur primarily in the open channel state (Castle 1990) and becomes manifest as initial acceleration of the apparent inactivation. τ_f has been interpreted as the time constant of block in the open state

$$\tau_f = 1/(k_{off} + [D]k_{on})$$

where k_{off}, k_{on} are the rate constants of the open channel block and $[D]$ denotes drug concentration (Snyders et al. 1992; Sanchez-Chapula 1999). Thus, reciprocal of τ_f plotted as a function of $[D]$ represents equation of a straight line

$$\frac{1}{\tau_f} = k_{off} + [D]k_{on} \tag{1}$$

from which k_{off} and k_{on} can be evaluated. Based on simplifying assumptions, this model introduces an error particularly at low drug concentrations as discussed by Snyders et al. (1992). Therefore, we based the estimation of open channel block kinetics on the analysis of kinetic diagram (see Appendix) that resulted in an improved equation

$$\frac{1}{\tau_f} + \frac{1}{\tau_s} - \frac{1}{\tau_i} = k_{off} + [D]k_{on} \tag{2}$$

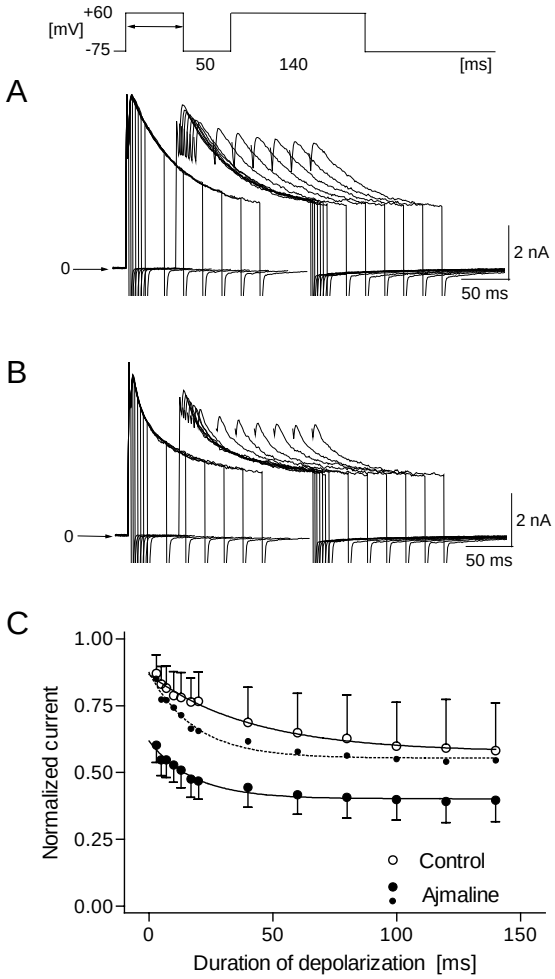


Figure 3. Development of ajmaline-induced I_{to}-block during depolarization. Experimental protocol: a 30 s period of rest was followed by a conditioning pulse of variable duration (between 3 and 140 ms), a 50 ms rest interval, and a uniform test pulse (140 ms). Superimposed current tracings recorded in control conditions (A) and under the effect of 10 μmol/l ajmaline (B). C. Normalized I_{to}-magnitude in response to the test pulse plotted against the duration of a conditioning depolarization. Both the control data (open circles) and ajmaline data (filled circles) were normalized to I_{to} on the conditioning pulse in control. The data were fitted by exponential functions with time constants 41.1 ± 5.6 ms in control and 17.9 ± 2.8 ms in the presence of ajmaline ($n = 5$, $p < 0.001$). Dashed line: the ajmaline data normalized to I_{to} on the conditioning pulse in the presence of ajmaline, for better comparison.

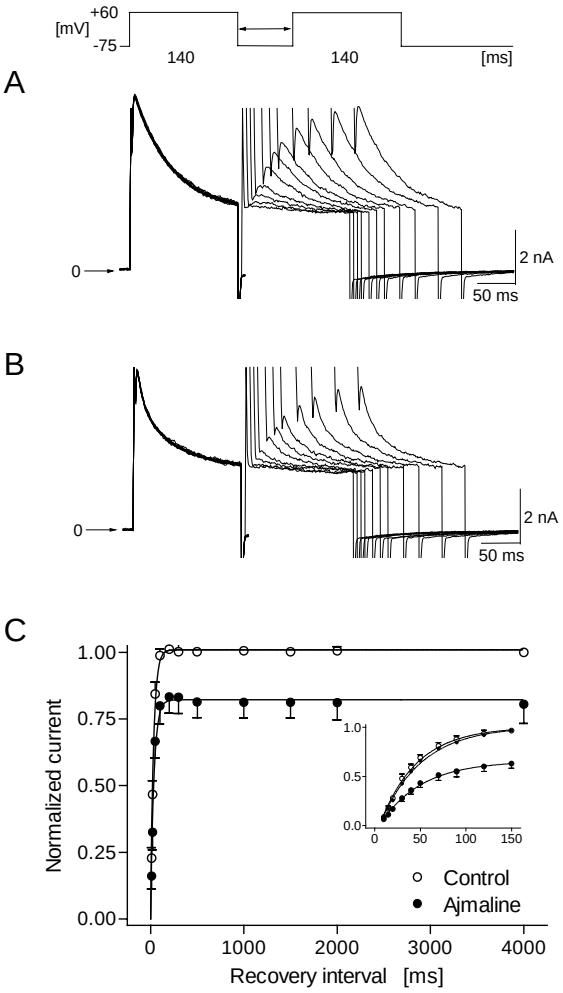


Figure 4. Recovery of I_{to} from inactivation in control (A) and from inactivation and block induced by 10 μmol/l ajmaline (B). Experimental protocol: a 30 s period of rest was followed by two uniform depolarizing pulses separated by a variable recovery interval (between 11 and 4000 ms). C. Normalized magnitude of I_{to} in response to the test pulse *versus* the duration of variable interval. Both the control data (open circles) and ajmaline data (filled circles) were normalized to I_{to} recorded in response to conditioning pulse in control. The data were fitted by exponential functions with time constants 31.9 ± 1.9 ms in control and 35.8 ± 2.8 ms in presence of ajmaline ($n = 5$, $p < 0.05$). Inset: first 150 ms of the plot; the ajmaline-affected data renormalized to current recorded after 4000 ms recovery interval were hardly distinguishable from the control curve.

The Eq. (2) differed from the accustomed relation (1) by the term $1/\tau_s - 1/\tau_i$. The plot of the expression on the left side of Eq. (2) against drug concentration for data obtained from four experiments is shown in Fig. 2B. The straight line is the best fit to the Eq. (2) resulting from least squares procedure. Its intercept point with ordinate and its slope give values of dissociation and association rate constants $k_{off} = 20.12 \pm 5.99$ s^{-1} and $k_{on} = 4.57 \times 10^6 \pm 0.32 \times 10^6$ mol^{-1}·l·s^{-1}, respectively, of the ajmaline-channel interaction in the open state. The value of $K_d = k_{off}/k_{on} = 4.4$ μmol/l differs considerably from IC_{50} (see Discussion).

To obtain more data related to the development of ajmaline-induced I_{to}-block, standard double-pulse protocols were applied at holding potential of −75 mV. The onset of block during depolarization was studied at variable duration of conditioning pulse between 3 and 140 ms (Fig. 3). The interval between pulses was set to 50 ms as a compromise to allow time for considerable fraction of unblocked I_{to}-channels to recover from inactivation (see Discussion). With regard to variable rate of apparent inactivation, magnitude of I_{to} measured 10 ms after the onset of the test pulse appeared to be a suitable simple indicator of I_{to}. The value measured in this way exhibited approximately the same degree of ajmaline-induced I_{to}-block as the time integral. Shorter times of reading led to underestimation while longer times to overestimation of the ajmaline effect. In control conditions (Fig. 3A,C), the magnitude of I_{to} in response to the test pulses gradually decreased with prolongation of the conditioning pulses by about 33 %. The decrease was exponential with the time constant 41.1 ± 5.6 ms that was close to the time constant of I_{to}-inactivation ($\tau_i =$ 37.1 ms). In the presence of 10 μmol/l ajmaline (Fig. 3B,C), the gradual decrease of the current magnitude was more than twice faster (time constant 17.9 ± 2.8 ms) in accordance with acceleration of the apparent inactivation.

Recovery from block

To investigate the recovery from inactivation and block, a double-pulse protocol was used. Two 140 ms impulses from holding potential −75 mV to +60 mV were separated by a variable rest interval in the range between 11 and 4000 ms (Fig. 4). The recovery from inactivation under control conditions (Fig. 4A,C) could be fitted with a single exponential function (the time constant of recovery from inactivation, $\tau_{rec} = 31.9 \pm 1.9$ ms). Under the effect of ajmaline (10 μmol/l), the magnitude of I_{to} was reduced (Fig. 4B,C). However, the time course of recovery was not significantly altered. Single exponential approximation resulted in a slightly prolonged time constant (35.8 ± 2.8 ms) suggesting that the rate of recovery from block was not markedly slower than the recovery from inactivation. As a consequence, the ajmaline-induced I_{to}-block could be expected frequency- and use-independent provided that the intervals between impulses at the maximal frequency of the measured range were sufficiently long in comparison with the time constant of I_{to}-recovery from block.

The frequency dependence of I_{to}-block induced by ajmaline (10 μmol/l) was examined by 140 ms lasting pulses from −75 mV to +60 mV at regular steady-state stimulation ranging from 20 to 200 min^{-1} (Fig. 5A). The ratio of I_{to} magnitude

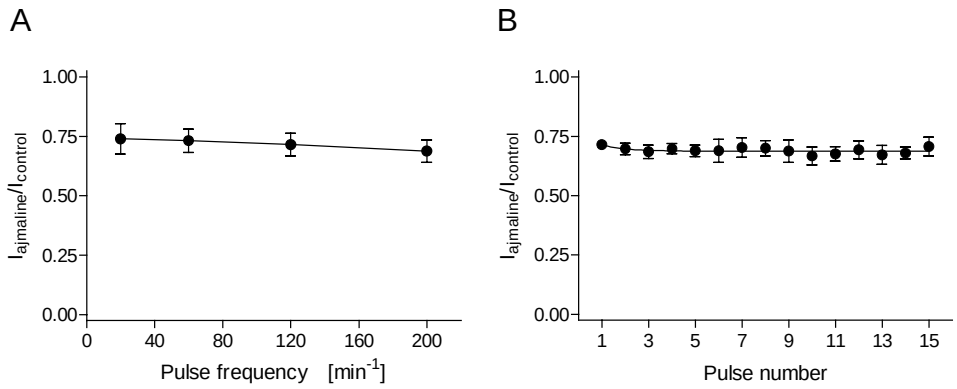


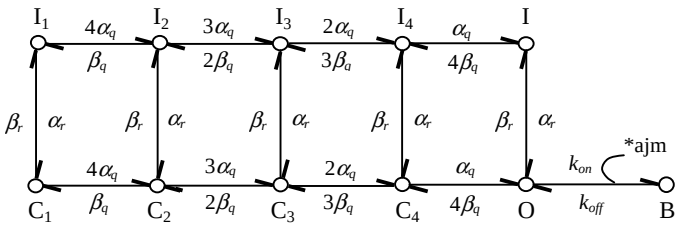
Figure 5. The effect of ajmaline on I_{to} exhibits neither frequency nor use dependence. Ordinate: relative I_{to} defined as a ratio of I_{to} in presence of ajmaline/I_{to} in control. Test pulses: 140 ms from −75 mV to +60 mV. **A.** Steady-state frequency dependence of I_{to}-block in the range between 20 and 200 min^{-1} (mean ± S.E., $n = 4$, $p > 0.05$). **B.** I_{to}-block during a sequence of depolarizing pulses preceded by 30 s rest period (mean ± S.E., $n = 5$). The test pulses were applied at 200 min^{-1}.

measured under the effect of ajmaline and in control was regarded a measure of the fraction of unblocked channels. The observed slight increase in block with increasing frequency was not statistically significant at $p < 0.05$. Similarly, the fraction of unblocked channels remained virtually constant during the sequence of 140 ms pulses at 200 min^{-1} preceded by a 30 s rest (Fig. 5B).

Quantitative model

A simple model composed of activation and inactivation gating of Hodgkin–Huxley type and drug-channel interaction entirely in the open channel state was used to verify whether the experimental results were compatible with the idea that ajmaline is a pure open channel state blocker of I_{to} (Fig. 6).

The association and dissociation rate constants (k_{on}, k_{off}) and rate constants related to inactivation (α_r, β_r) were set to the values estimated in Fig. 2. α_r and β_r were determined from the time constant of inactivation ($\tau_i = 1/(\alpha_r + \beta_r) = 37.1$ ms) considering nearly complete inactivation ($\alpha_r \ll \beta_r$) at +60 mV in control. At −75 mV $\alpha_r \gg \beta_r$ whereas $\alpha_r + \beta_r$ remained unaltered in agreement with the experimental results (τ_i and τ_{rec} nearly equalled). From the viewpoint of our simulations, the description of fast kinetics of activation and deactivation (α_q, β_q) was not critical. The data were adopted from Šimurda et al. (2001). The ajmaline-induced I_{to}-block was regarded voltage-independent. None or only a slight increase in block at positive membrane voltages was detected in a few experiments (not shown). Under the effect of other open channel blockers, the inhibition of I_{to} was reported either to be slightly voltage-dependent (the fractional electrical field about 0.18) (Snyders et al. 1992; Clark et al. 1995; Sanchez-Chapula 1999), or voltage-independent



V_m [mV]	α_q [s^{-1}]	β_q [s^{-1}]	α_r [s^{-1}]	β_r [s^{-1}]	K_{on} [$mol^{-1}\cdot l\cdot s^{-1}$]	k_{off} [s^{-1}]
–75	0.78	269.9	27	0.27	4.57×10^6	20.12
+60	313.7	9.63	0.27	27		

Figure 6. Kinetic diagram of I_{to}-channel gating and ajmaline-induced block in open state. C_n, closed states; O, open state; I_n, inactivated states; B, blocked state; *ajm, molecule of ajmaline. Table: values of the rate constants used for simulations of experiments; V_m, membrane voltage.

(Dukes et al. 1990). A tentative introduction of voltage-dependent block (fractional electrical field, $\delta = 0.18$) into the model did not considerably affect the present results.

Fig. 7A,B shows the time courses of I_{to} calculated as solution of differential equations describing the model presented in Fig. 6. I_{to} is represented by the fraction of open channels. The results exhibit characteristic features of the experimentally obtained currents including the biphasic apparent inactivation in the presence of ajmaline (see also Fig. 10A). The double-pulse experimental protocols (Figs. 3 and 4) were simulated and the calculated currents were evaluated in the same way as the experimental data. The normalized magnitude of I_{to} plotted both as a function of the duration of the conditioning pulse (Fig. 7C, left) and as a function of the resting interval prior to the test pulse (Fig. 7C, right) agreed fairly well with the experimental data (Figs. 3C, 4C).

A clue for understanding a likely mechanism of the ajmaline-induced I_{to}-block yielded simulation of block development in response to 140 and 1000 ms lasting depolarizing pulses (Fig. 8). Fig. 8B and C show the time courses of the fractions of open and blocked channels, respectively. On early depolarization, the channel opening was followed by an increase in a fraction of blocked channels. In the later course, the fraction of channels was gradually unblocked and appeared transiently in the open state before being inactivated (transitions B → O → I in the kinetic diagram in Fig. 6). This process was responsible for the decline of the fraction of blocked channels during sustained depolarization (Fig. 8C) and for the slow phase of apparent inactivation (Fig. 8B). It was absent in the presence of drug in high con-

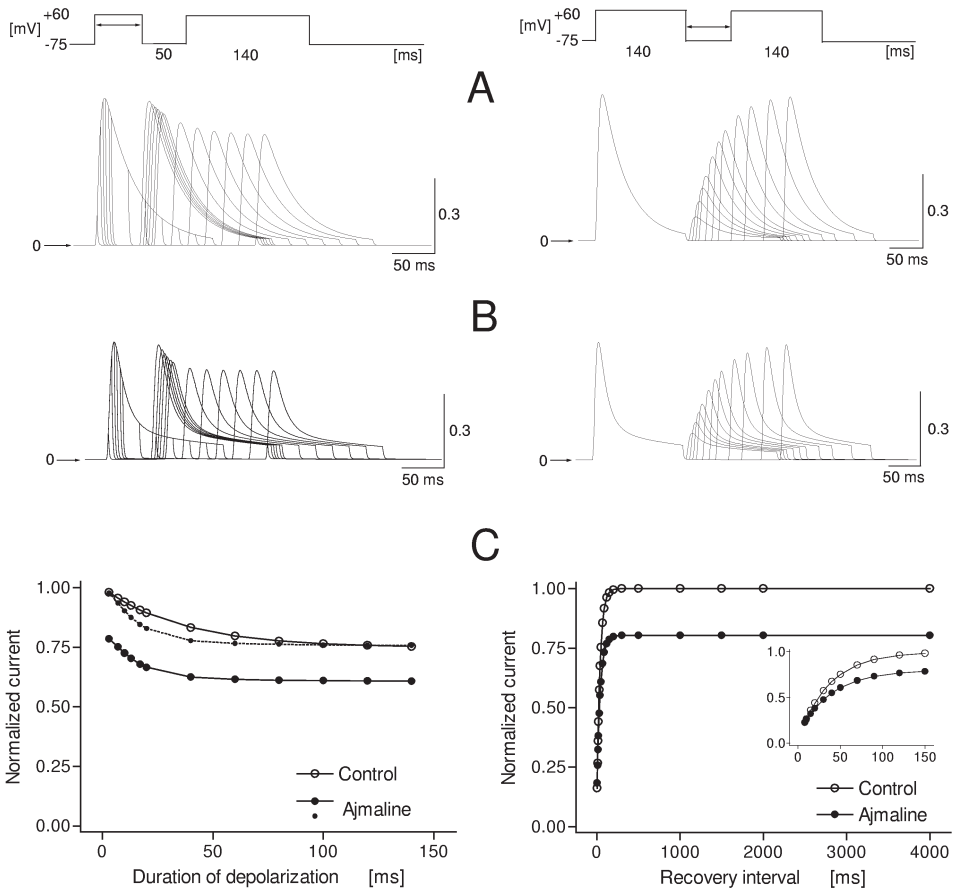


Figure 7. Simulations of experiments presented in Figs. 3 and 4. The calculated currents (represented by fractions of open channels) were evaluated in the same way as the experimental data.

centrations (not shown). Upon repolarization (140 ms pulse), the open unblocked channels were closed very fast (Fig. 8B). The fraction of blocked channels declined exponentially (Fig. 8C). The time constant of recovery from block amounted to 52 ms.

As a consequence of fast unblock, the ajmaline-induced I_{to}-block could not show frequency or use dependence. The interval between 140 ms pulses at 200 min^{-1} was 160 ms representing approximately three time constants of recovery from the block (52 ms). Thus, the recovery interval was sufficiently long to explain the results presented in Fig. 5 in agreement with simulations in Fig. 9.

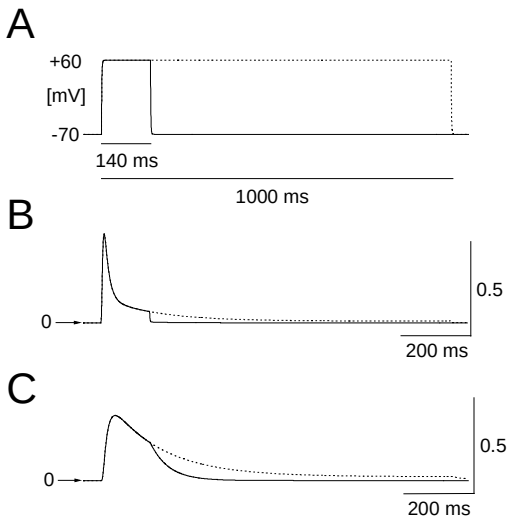


Figure 8. Simulated development of I_{to}-block induced by ajmaline (10 μmol/l) in response to short (full lines) and long (dotted lines) depolarizing pulse. **A.** Depolarizing pulses. **B.** Development of the fraction of open channels. **C.** Development of the fraction of blocked channels.

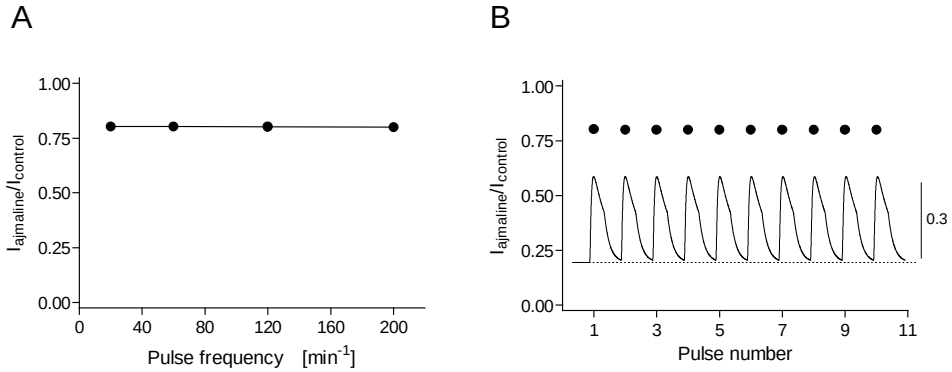


Figure 9. Simulation of experiments presented in Fig. 5. The model was able to reproduce both frequency independence of I_{to}-block between 20 and 200 min^{-1} (A) and use independence at 200 min^{-1} (B). Inset in B shows the time course of the fraction of blocked channels that vanished during each interval between depolarizing pulses.

Discussion

Kv4.2 potassium channels expressed in *Xenopus* oocytes were reported to be moderately blocked by 100 μmol/l ajmaline (Rolf et al. 2000) which is well above the

therapeutic concentration. As yet, the transient outward current I_{to} has not been included in the electrophysiological studies of the effect of ajmaline on ionic currents of cardiac cells. The goal of the present study was to fill up this blank in understanding the ajmaline effects.

The effect of ajmaline on apparent inactivation

The analyses of the time course of I_{to}-inactivation in rat ventricular myocytes presented so far yielded controversial results. The current decline has been described by single exponential function (e.g., Josephson et al. 1984; Apkon and Nerbonne 1991; Jourdon and Feuvray 1993; Wettwer et al. 1993; Slawsky and Castle 1994; Hung et al. 1998; Sanchez-Chapula 1999; Aimond et al. 2000; Gallego and Casis 2001) or by more complex (usually biexponential) function (e.g., Xu and Rozanski 1997; Tsuchida and Watajima 1997; Donohoe et al. 2000; Liu et al. 2000; Martinez and Delgado 2000; Štengl and Barták 2000; Bru-Mercier et al. 2002; James et al. 2001). Apart from the possibility of contamination by other current components, the latter observation can be interpreted as a result of co-existence of two types of I_{to}-channels, $I_{to,f}$ and $I_{to,s}$ (Weis et al. 1993; Berger et al. 1998) although transitions between inactivated states of the same channel populations also come into consideration (Bähring et al. 2001; Beck and Covarrubias 2001; Shahidullah and Covarrubias 2003). In our experiments on right ventricular myocytes, the time course of I_{to}-inactivation in control conditions could be well fitted by a single exponential function. Its time constant ($\tau_i = 37.1 \pm 2.7$ ms at +60 mV) falls into the range of reported values. In two of fifteen cells, we observed biexponential time course of I_{to}-inactivation. These cells were not included in this study.

Under the effect of ajmaline, the time course of apparent inactivation became biphasic and could be approximated by a sum of fast and slow exponential functions (at 10 μmol/l $\tau_f = 11.7$ ms, $\tau_s = 57.6$ ms). In comparison with monoexponential time course in the control, the early phase of the apparent inactivation was accelerated while the later phase was slowed down. This property has been described in various heart muscle preparations (Imaizumi and Giles 1987; Dukes et al. 1990; Snyders et al. 1992) and has been considered a specific feature of the block in open channel state (Castle 1990; Snyders et al. 1992; Clark et al. 1995; Sanchez-Chapula 1999). The faster exponential function has been interpreted as a manifestation of the onset of block; its time constant (τ_f) measured as a function of drug concentration has been used to evaluate the rate constants of open channel block (Eq. (1)). One of the significant results of this study is a proposal of a more precise relation (Eq. (2)) including also concentration dependence of the slower time constant τ_s beside the fast one τ_f (see Appendix).

Reconstruction of block development

With the aim to assess the kinetics of block development in response to depolarization and following repolarization, standard protocols were first applied (Figs. 3 and 4). However, the reconstruction of the block development requires much slower recovery from the block than recovery from the inactivation. This condition was

not met in the case of I_{to}-block induced by ajmaline. The rest interval between the conditioning and the test pulse (Fig. 3) could not be adjusted so as to be sufficiently long for most channels to recover from inactivation and at the same time short enough to keep the level of block virtually unaltered. As follows from the inset in Fig. 4C, only about 70 % of channels were recovered from inactivation during 50 ms at rest while the recovery from block was so fast that the normalized time courses of recovery from apparent inactivation in control and under the effect of 10 μmol/l ajmaline were nearly identical. It follows that the development of block cannot be reconstructed from these experiments directly. However, the consistency of the present results with the idea of open channel block could be verified by means of quantitative modelling.

To describe the gating in Kv4 potassium channels, several model variants have been proposed which differ mainly in specification of transitions to inactivated states (e.g., Liu and Rasmunsson 1998; Jerng et al. 1999; Greenstein et al. 2000; Bähring et al. 2001; Beck and Covarrubias 2001). Our results obtained in rat right ventricular myocytes (particularly the monoexponential time course of inactivation and fast recovery from inactivation) could be well described by a simple model of Hodgkin–Huxley type. We used this description for the sake of simplicity taking into account that the exact description of fast kinetics of activation and deactivation phases was not critical for the results of simulations of drug-channel interaction in the open state.

The results summarised in Figs. 7–10 demonstrate that the observed features of ajmaline-induced I_{to}-block may be satisfactorily simulated by the model exclusively based on the block in open channel state. This fact justifies inference of the values of kinetic parameters of I_{to}-block from the model simulations. The time course of the increase and of the following slow decrease of block in response to a depolarizing pulse, that could not be estimated directly from the results of experiments presented in Fig. 3, is shown in Figs. 8C and 10B. Similarly, the recovery from block at −75 mV that could not be estimated from the results of Fig. 4 appears upon termination of the 140 ms depolarizing pulse in Fig. 8C. The time constant 52 ms, which results from an approximation by exponential function, is close to the value of $1/k_{off}$.

IC_{50} and K_d

The value of K_d (4.4 μmol/l) calculated from the Eq. (2) (after insertion of the parameters of the straight line in Fig. 2) appeared to be substantially lower than the value of IC_{50} (25.9 μmol/l) estimated from the concentration response of the integrated currents (Fig. 1). Assuming an open channel block, this difference is a consequence of insufficient time for equilibrium between the open and the blocked states to be reached in the course of fast inactivation. Both values would become close to each other if the channels were only partially inactivated (Snyders et al. 1992) or when the open channel block was very fast in comparison with inactivation.

The quantitative model made it possible to verify this interpretation. The binding constants of the drug-channel interaction were set after the experimental results so that $K_d = 4.82$ μmol/l in the open state. To estimate IC_{50}, the

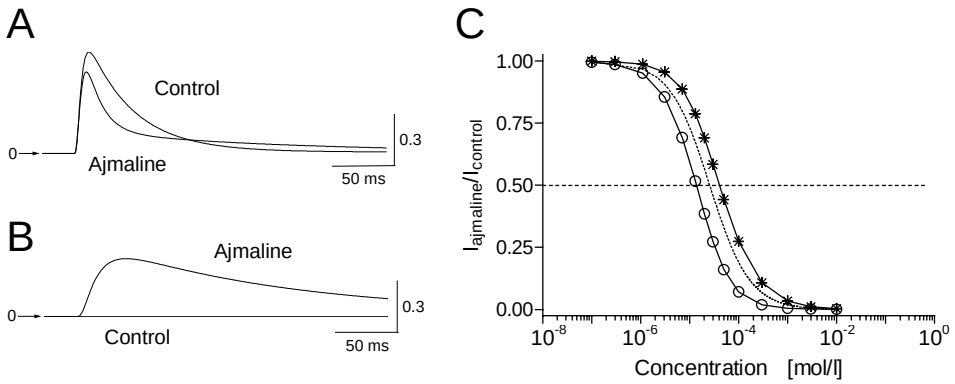


Figure 10. Simulations of the concentration dependence of I_{to}-block. Time courses of the fraction of open channels (A) and blocked channels (B) in control and under the effect of 10 μmol/l ajmaline calculated in response to 250 ms depolarizing pulse. C. Relative time integrals of the fraction of open channels plotted against concentration of ajmaline. Integrated were both the total fractions of open channels ($*$, IC_{50} = 40.5 μmol/l) and the fractions of open channels after subtraction of the residual value at the end of the depolarizing pulse ($\circ$, IC_{50} = 13.7 μmol/l) to match I_{to} reading used in the experiments (Fig. 1). The experimentally obtained curve is plotted for comparison (dotted line).

experimental protocol described in legend to Fig. 1 was simulated. The plot of integrals of I_{to} against drug concentration (Fig. 10C) approximated with Hill equation yielded IC_{50} = 40.5 μmol/l which is about 8 times higher than the value of K_d. In voltage clamp experiments, however, I_{to} was read from the level of current at the end of 250 ms pulse that was long enough for I_{to} to inactivate in control but might be insufficient for the slow component of the drug-affected current to decay completely. Thus, the integrals of ajmaline-affected traces could have been underestimated. This suspicion was supported by superimposed responses of the fraction of open I_{to}-channels to depolarizing pulse (250 ms, +60 mV) in control and under 10 μmol/l ajmaline (Fig. 10A). The curve of concentration dependence of I_{to}-block was therefore alternatively constructed from the integrals calculated after subtraction of the values attained at the end of 250 ms pulses. The resulting concentration dependence (Fig. 10C) was shifted to the left giving IC_{50} = 13.7 μmol/l which is still triple of K_d. The experimental value 25.9 μmol/l falls between both results of model simulations. Thus, the model confirmed the difference between values of K_d and IC_{50}. The surprisingly high sensitivity of IC_{50} to the way of reading the current may be one of the main reasons of great diversity of published experimental data.

I_{to}-channels are blocked by ajmaline in open state

Besides acceleration of the early apparent inactivation, there are other features of ajmaline-induced I_{to}-block that support the idea of the drug-channel interaction

in the open state. The time course of the current during activation of I_{to} is not affected by ajmaline indicating no block in the resting states (inset in Fig. 2A). The slow phase of apparent inactivation (time constant τ_s) reflects very likely a partial unblock of the channels during sustained depolarization and their transition *via* open channel state into inactivated state in agreement with the simulated results (Fig. 10A,B).

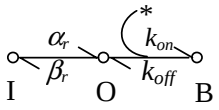
It can be concluded that in the rat ventricular myocytes, ajmaline appears to be an effective blocker of I_{to} that exhibits the properties attributed to the pure open state blockers with fast recovery kinetics. The partial unblock during sustained depolarization together with fast unblock at rest offers explanation for the frequency and use independence of ajmaline-induced I_{to}-block even at relatively high frequencies.

As an antiarrhythmic drug of class I according to Vaughan–Williams classification, ajmaline is primarily regarded a sodium current blocking agent. Results of our measurements suggested approximately the same values of IC_{50} for I_{to} and I_{Na} as will be documented in our next comparative study of the effect of ajmaline on ionic current components.

Acknowledgements. We thank to Prof. P. Bravený and Dr. G. Christé for reading the manuscript and comments and to Mrs. B. Vyoralová for technical assistance. This work was supported by the grant project MSM141100004 and MSM0021622402 from the Ministry of Education, Youth and Sports of the Czech Republic and by the grant No. 204/02/D129 from the Grant Agency of the Czech Republic.

Appendix

Taking into account fast an full activation of I_{to}-channels in response to depolarizing pulse to +60 mV, the time course of the apparent inactivation can be described by a simplified three-state model including open (O), inactivated (I) and drug occupied (blocked – B) channel state.



The kinetic diagram is described by a set of three first order homogenous linear differential equations that can be written in a vector form

$$X' = AX \tag{3}$$

where X and A are, respectively, vector of states and matrix of coefficients that are constant at constant membrane voltage.

$$X = \begin{bmatrix} I \\ O \\ B \end{bmatrix} \qquad A = \begin{bmatrix} -\alpha_r & \beta_r & 0 \\ \alpha_r & -(\beta_r + [D]k_{on}) & k_{off} \\ 0 & [D]k_{on} & -k_{off} \end{bmatrix} \tag{4}$$

The characteristic equation related to Eq. (3) has one root zero ($\lambda_3 = 0$). The two remaining roots are solutions of the quadratic equation

$$\lambda^2 + (\alpha_r + \beta_r + k_{off} + [D]k_{on})\lambda + (k_{off}\beta_r + k_{off}\alpha_r + [D]k_{on}\alpha_r) = 0 \tag{5}$$

Taking into account that the sum of roots of a quadratic equation (written in this form) equals the coefficient at λ with opposite sign, we obtain

$$-(\lambda_1 + \lambda_2) = (\alpha_r + \beta_r + k_{off} + [D]k_{on})$$

Considering the relations between the roots of the characteristic equation and time constants of exponential terms of the solution of Eq. (3) $-\lambda_1 = 1/\tau_f$, $-\lambda_2 = 1/\tau_s$ and the relation $\alpha_r + \beta_r = 1/\tau_i$ (where τ_i is time constant of I_{to}-inactivation measured in the absence of the drug), we finaly obtain the expression (2) suitable for practical use because all time constants on the left side are measurable quantities

$$\frac{1}{\tau_f} + \frac{1}{\tau_s} - \frac{1}{\tau_i} = k_{off} + [D]k_{on} \tag{2}$$

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Final version accepted: November 24, 2004