Use-Dependent Block and Use-Dependent Unblock of the Delayed Rectifier $K^+$ Current by Almokalant in Rabbit Ventricular Myocytes

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A voltage-clamp analysis of the effect of almokalant on the delayed rectifier $K^+$ current ($I_{K}$) was made in rabbit ventricular myocytes. The two-suction pipette method was used, and appropriate voltage-clamp protocols were used to study more specifically use dependence, block development, and recovery from block. Almokalant interacted with the $I_{K}$ in two ways: it shifted the activation curve in the hyperpolarizing direction (stimulatory effect) and blocked the open $I_{K}$ channel in a use-dependent way (inhibitory effect). For 2-second voltage clamps to $+20$ mV, half-maximum block was obtained at $5 \times 10^{-8}$ mol/L, with a Hill coefficient of 1.76. Use-dependent block was related to an open-channel block that occurred at 0 mV with a time constant of 1.07 second and a rather slow recovery from block: at $-50$ mV, recovery time constant was $\approx 10$ seconds; at $-75$ mV, recovery was practically absent. The absence of an important recovery at negative membrane potentials is consistent with the hypothesis of the drug being trapped in the channel. A limited frequency-dependent block could be demonstrated. Use-dependent unblock was demonstrated by a rapid recovery from block during stimulation following complete washout of the drug. It is concluded that almokalant shifts the activation curve of $I_{K}$ in the hyperpolarizing direction, blocks the open channel, and is trapped by the closure of the activation gate. (Circ Res. 1993;73:857-868.)

KEY WORDS • delayed $K^+$ current • block • use dependence • cardiac cells • antiarrhythmic drugs • prolongation of action potential • class III activity

Antiarrhythmic drugs play an important role in preventing sudden cardiac death. Drugs that block the Na$^+$ channel act by reducing conduction velocity and increasing the wavelength of the impulse in the reentry pathway. When the excitable gap in the reentry pathway is short, the same result can be obtained by increasing the effective refractory period, a result provided by substances that prolong the action potential duration.1 Prolongation of the action potential can be caused by reducing the rate of inactivation of Na$^+$ or Ca$^{2+}$ current or by blocking outward $K^+$ currents and, more specifically, the delayed rectifier $K^+$ current ($I_{K}$). All these mechanisms can be arrhythmogenic. However, the danger of causing Ca$^{2+}$ overload is more pronounced with substances acting on the Na$^+$ and Ca$^{2+}$ currents. The most promising mechanism thus seems to be a blockade of $K^+$ outward movement.

To be effective against tachyarrhythmias, a drug should preferentially block conduction or prolong refractoriness at high rates of stimulation. A minimum requirement to exert a frequency-dependent block is to preferentially block activated and/or inactivated channels, an effect that has been called use dependent because block develops during the use of the channel.2,3

To selectively block at high frequencies with no effect at low frequencies, the development of block as well as the recovery from block should occur with the appropriate time constants. In the case of Na$^+$ channel blockers, the slow rate constants for block and recovery explain the less favorable results obtained with so-called class Ic agents.4 Similar restraints with respect to the kinetics of block have to be imposed on drugs acting on the $I_{K}$ channel.5 To prolong the action potential at high frequencies with no effect a low frequencies, the drug should block the open channel with a time constant in the order of seconds and show a recovery at the resting potential with a time constant of $\approx 100$ milliseconds. In the evaluation of the antiarrhythmic activity of a drug, it is thus necessary to include a study of use-dependence, development of block, and recovery from block.

4-[3-[[Ethyl[3-(propylsulfinyl)propyl]amino]-2-hydroxypropoxy]-benzonitrile (almokalant, Fig 1) is a novel antiarrhythmic agent that selectively lengthens the action potential duration and increases refractoriness in dog atria and ventricular muscle,6 in cat papillary muscle,7 and in human ventricular muscle.8 In addition to its electrophysiological effects, the drug has been shown to increase the maximum rate of pressure development in the left ventricle and to increase the peak developed force of contraction in isolated cat and human ventricular muscle.7,8

In the present study, a voltage-clamp analysis of the changes in $I_{K}$ by almokalant is presented. Evidence will be given that interaction of almokalant with the channel results in two effects: a change in the activation kinetics of $I_{K}$ with a shift in the hyperpolarizing direction of activation, and block of the open $I_{K}$ channel in a use-dependent

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way. The time constant for block development is in the order of 1 second. At hyperpolarized levels, the drug is trapped by the channel, recovery from block is rather slow, and although unblock is also use-dependent, frequency-dependent block remains small.

Materials and Methods

Cell Preparation

Single ventricular myocytes of the rabbit were dissociated by enzymatic dispersion, following a procedure described in detail in a previous publication.9 In brief, the heart was quickly removed after decerebration of the animal and was mounted on a Langendorff perfusion system. The aorta was cannulated, and the heart was perfused at 37°C with (1) Ca2+-free standard solution (see “Solutions”) for 10 minutes. (2) Ca2+-free standard solution containing 35 mg/50 mL collagenase A (Boehringer-Mannheim, Mannheim, Germany) for 15 minutes, (3) a Ca2+-free solution containing collagenase and 6.5 mg/50 mL protease XIV (Sigma Chemical Co, St Louis, Mo) for 35 minutes, and (4) 0.2 mmol/L Ca2+ containing solution for an extra 10 minutes. After the isolation of cells, they were stored at room temperature in HEPES-Tyrode solution until used.

Data Acquisition and Analysis

Aliquots of cells were allowed to settle on the glass bottom of a tissue bath (volume, <0.5 mL) and then superfused (3 mL/min, 37°C) with buffer solution. The two-electrode voltage clamp (Axoclamp 2-A amplifier, Axon Instruments, Foster City, Calif) was applied using suction pipettes with resistance ranging from 2 to 5 MΩ. After a 10-minute dialysis, stable recordings were obtained; I K was a single current and was not subject to any rundown. Pclamp software (Axon Instruments) was used to generate voltage-pulse protocols and to acquire and analyze data. In some experiments in which recordings longer than 4 seconds were needed, currents were directly recorded on a Gould Brush recorder 220 (−3 dB at 100 Hz, Simac, Puteuil, Belgium) and analyzed manually. Computer-recorded data were sampled at a frequency of 100 Hz and filtered at a frequency of 50 Hz. To obtain the half-maximum activation voltage (V1/2) and the slope factor (s) for the activation curve, the data were fitted by the following Boltzmann equation:

\[ I/I_{max} = \frac{1}{1 + \exp\left[\frac{V - V_{1/2}}{s}\right]} \]

where \( I_{max} \) corresponds to the maximum plateau value of the tail currents and \( V \) is the test voltage. Concentration effects were quantified by fitting the results by the following equation:

\[ I + (D/K_{eq})^n \]

where [D] is the drug concentration, \( K_{eq} \) is the drug concentration for 50% efficiency, and n is the Hill coefficient.

Solutions

The standard external solution contained (mmol/L) NaCl, 137.6; KCl, 5.4; MgCl2, 0.5; CaCl2, 1.8; HEPES, 11.6; and glucose, 5; NaOH was added to pH 7.4. For cell isolation, CaCl2 was omitted. Intracellular solution contained (mmol/L) KCl, 120; MgCl2, 6; CaCl2, 0.154; Na2-ATP, 5; EGTA, 5; and HEPES, 10 with KOH added until pH 7.2.

Drugs

Almokalant was obtained from Astra Hässle, Göteborg, Sweden, as a 10–2 mol/L stock solution; pKa of the drug is 7.8, and the log P (partition coefficient of octanol-water) for the base is 1.2. Nisoldipine (Bayer, Wuppertal, Germany) was used to block the L-type Ca2+ current in a final concentration of 0.2×10–4 mol/L prepared from a stock solution of 10–4 mol/L in dimethyl sulfoxide.

Statistics

Results are expressed as mean±SEM.

Results

Almokalant Blocks a Single K+ Current

In the experiment illustrated in Fig 2, voltage-clamp steps of variable duration were applied from a holding potential of −50 to −10 mV, under control conditions and in the presence of a very high concentration of almokalant (10−5 mol/L). During the clamp at −10 mV, the current is inward with respect to the holding current at −50 mV but gradually shifts in the outward direction (Fig 2A); in the presence of the drug, current is more inward and is practically stable during the whole clamp of 3.4 seconds (Fig 2B). The difference current obtained by subtracting the current in Fig 2B from that in Fig 2A is shown in Fig 2C. Tail currents at −50 mV under control conditions gradually increased in amplitude with increasing duration of the clamp. In the presence of 10−5 mol/L almokalant, they were completely suppressed. A plot of the tail current amplitude is superimposed on the difference tracing. The fact that the time course of the difference current and the evolution of the tail currents are similar indicates that a single current is affected by the drug. The interrupted line in Fig 2C is a single exponential with a time constant of 510 milliseconds. Small deviations from this theoretical line may suggest a biexponential time course. The main point in the argumentation, however, is that both the difference current and the change in tail currents with time are superimposable. Compared with the two components of \( I_K \) in the guinea pig ventricle, activation of the current in the rabbit is faster than the slowly activating component of \( I_K \) (\( I_{K_s} \)) but slower than the rapidly activating component of \( I_K \) (\( I_{K_r} \)).10 The results with almokalant are consistent with previous findings for the same preparation in the presence of dofetilide, another agent with class III activity. In these experiments, the current blocked by dofetilide activated with a time constant of 750 milliseconds at −10 mV, 375 milliseconds at 0 mV, and 150 milliseconds at +10 mV.11 Since there is only one delayed \( K^+ \) current in the rabbit ventricular muscle, the current has been designated as \( I_K \).

Activation: Voltage Shift and Block

A quantitative evaluation of the activation and its modification by the drug was studied by applying 2-sec-
Fig 2. Almokalant blocks a single outward current in rabbit ventricular myocytes. Voltage clamps of variable duration were applied from a holding potential of -50 to -10 mV. Superimposed current recordings are shown under control conditions (A) and in the presence of 10^{-5} mol/L almokalant (B). C is the plot of the almokalant-sensitive current during a depolarizing pulse of 3.4 seconds obtained by subtraction of the current in the presence of the drug from the current under control conditions. i relative indicates relative current. Dots indicate tail current amplitudes following depolarizations of different durations. Tail current amplitude in this figure and in all following figures was measured as change from holding current before application of the depolarization. Currents are expressed in relative units as a function of time. Maximal value was 86 pA for the difference current and 122 pA for the tail current. The interrupted line is an exponential with a time constant of 510 milliseconds.

Fig 3. Activation curve for delayed rectifier K⁺ current (i_K) in five rabbit ventricular myocytes and its change by almokalant (5×10^{-8} mol/L). Relative amplitude of tail currents obtained for clamps of 2-second duration is shown as a function of test potential. Holding potential was -50 mV. Amplitude of tail current for depolarizations to +20 mV was taken as 100%. Values are mean±SEM. In the range of voltages at which activation increases, block also increases. Lines through the experimental values were drawn by eye.

of depolarization (-10 and -20 mV) were applied. The figure shows the currents during the depolarizing step and the tails on return to the holding potential of -50 mV in a superimposed way. Measurements were made under control conditions, in the presence of 3×10^{-8} mol/L almokalant, and on washout. In the presence of the drug, tail currents are increased after short depolarizing pulses, whereas long pulses result in a decrease of the tail current. For pulses of 1.0 second (not shown), tails had practically the same amplitude in the control condition and in the presence of the drug. The increase in tail current for short pulses was accompanied by an apparent faster increase of the current during the depolarizing step; the smaller tail amplitude following long pulses was correlated to a gradual decrease in outward current during the pulse compared with the control value. Reversibility was obtained on washout. Quantitative data on tail currents for a broader range of voltages in the same experiment are given in Fig 4B. They illustrate the gradual change from initial enhancement to final reduction of the tail current at levels positive to -20 mV. At levels negative to -20 mV, the increase due to the shift was balanced by the later decrease due to block. Similar results were obtained in five experiments. Although less complete (eg, only one or two test potentials), all experiments confirmed the occurrence of an initial increase in current. Results in other types of experiments to be described later (see Fig 7) are consistent with these observations.

The main conclusion from these control experiments is that interaction of the drug with the channel results in (1) a hyperpolarizing shift of the activation curve (stimulatory effect) and (2) a block of the conduction pathway (inhibitory effect). The shift can be seen independent of block, especially for short and small depolarizations; block is
better seen for longer durations of depolarization. The shift in activation was estimated by measuring the change in amplitude of the current. However, such a shift also implies a shift in the kinetics. No attempt was made to measure this shift because changes in block (increases as well as decreases) during the open state affect the time course of the current. The existence of open channel block is already evident, as seen in Fig 4, and will be analyzed in more detail (Figs 8 through 10); later experiments (Fig 10) will show that the decay rate of tail currents is increased after short pulses (increase of block) and decreased after long pulses (decrease of block).

Is the block intrinsically voltage dependent? Although a shift of the activation curve represents a voltage-dependent modulation of \( I_K \), the question of whether the block is intrinsically voltage dependent is not answered. To answer this question, clamps were applied to different voltages above the level of maximal activation. An example of such an experiment is given in Fig 5 and shows superimposed recordings of currents during depolarizing clamps to levels between 0 and +60 mV for control conditions (Fig 5A) and in the presence of \( 3 \times 10^{-8} \) mol/L (Fig 5C) and of \( 10^{-5} \) mol/L (Fig 5B) almokalant. Steady-state current values for the three conditions are illustrated in Fig 5D. It is clear that under control conditions all tail currents for depolarizations positive to 0 mV are identical, demonstrating that activation was maximal; they were absent in \( 10^{-5} \) mol/L almokalant (complete block) and reduced to the same extent in \( 3 \times 10^{-8} \) mol/L almokalant. This latter result (Fig 5C) shows that there is no intrinsic voltage dependence of the block. In other words, access of the charged drug to the blocking site occurs outside the electrical field of the channel, or the channel is blocked by the uncharged form. The experiment further shows that (1) \( I_K \) is inwardly rectifying: the difference current (control minus current in the presence of \( 10^{-5} \) mol/L of the drug) becomes smaller at more positive potentials (Fig 5D), and (2) a substantial outward current different from \( I_K \) is present at these positive potentials. The nature of this current was not further examined.

Concentration dependence. The blocking effect was dependent on concentration. In six cells, increasing drug concentrations were applied in a cumulative way, each concentration for 10 minutes. Depolarizing pulses were applied from -50 to +10 mV for 2 seconds every 10 seconds. The decrease in tail current as a function of drug concentration is shown in Fig 6. The theoretical curve was drawn according to the general equation of receptor occupancy (see "Materials and Methods") with a Hill coefficient \( n \) of 1.76±0.10 and a \( K_{50} \) of 4.7×10^{-4}±0.74 mol/L. Later experiments will show that a 2-second duration of pulse is not long enough to obtain steady-state block, especially at lower concentrations. For this reason, the potency of the drug is slightly underestimated, and the Hill coefficient is overestimated.
Use Dependence

Fig 7 illustrates the result of an experiment in which the existence of use dependence for $5 \times 10^{-8}$ mol/L almokalant was tested. After a long (>1-minute) rest period at the holding potential of $-50$ mV, a series of short (0.2-second) depolarizing clamps to $+10$ mV were applied at a repetition frequency of 1.33 Hz. Under control conditions, the amplitude of the tail currents rapidly increased with successive depolarizations to a saturating value. A steady state is obtained when the amount of activation during the pulse equals the amount of deactivation during the time at the holding potential. In the presence of the drug, the picture was different. Repetition of the clamps resulted in a negative staircase of the tail current amplitude. Instead of gradually increasing, tail currents steadily decreased to reach a steady-state value after $\approx 10$ pulses. In this example, the first tail current in the presence of the drug was greater than in control conditions, a result that is consistent with the stimulatory effect described in the previous section.

The observation that the tail current amplitude was not changed or even increased at the start of a series of depolarizing pulses indicates that the drug does not exert a tonic block. The decrease in tail amplitude with repetition of the depolarizing clamp on the other hand shows that the block develops during activation and does not recover completely during the rest period at the holding potential and thus accumulates with time. In the next series of experiments, the development of block, as well as the recovery from block, will be analyzed in more detail.

Fig 6. Concentration-response curve. Relative block was estimated by measuring the decrease in tail current amplitude for clamp depolarizations of 2 seconds from $-50$ to $+10$ mV. $i_K$ indicates the delayed rectifier $K^+$ current. The theoretical curve was drawn according to the following equation: $[1 + ([D]/K_m)]^{-1}$, where $[D]$ is the drug concentration, $n$ is the Hill coefficient (1.76), and $K_m$ is the drug concentration for 50% efficiency ($4.7 \times 10^{-8}$ mol/L). Values are mean±SEM for six rabbit ventricular myocytes.

Fig 5. Absence of intrinsic voltage-dependent block. Clamps were applied from a holding potential of $-50$ mV to levels between 0 and $+60$ mV. A, B, and C, Superimposed current recordings are shown in control conditions and in the presence of $10^{-8}$ and $3 \times 10^{-8}$ mol/L almokalant, respectively. Note identical tail currents in $3 \times 10^{-8}$ mol/L of the drug, demonstrating the absence of voltage-dependent block between 0 and $+60$ mV. The tail currents vanished completely in the presence of $10^{-8}$ mol/L almokalant. Note, in the beginning of the recording, the presence of a transient outward current that varies in amplitude within the level of depolarization. D, Steady-state currents are plotted as a function of membrane potential under control conditions (○) and in the presence of $3 \times 10^{-8}$ mol/L (●) and $10^{-5}$ mol/L (■) of the drug. The current blocked by almokalant shows inward rectification, whereas the remaining outward current, which at 0 mV is of amplitude equal to the delayed rectifier $K^+$ current, increases with depolarization.

Fig 7. Illustration of use-dependent block induced by $5 \times 10^{-8}$ mol/L almokalant. Currents obtained for 0.2-second depolarizations to $+10$ mV and applied at a frequency of 1.33 Hz are shown. Holding potential was $-50$ mV. Under control conditions (A), tail currents show summation with repetition of the clamp depolarization. In the presence of the drug (B), summation is not only absent, but the reverse sequence is seen, with tails declining with repetition. ○ indicates current at $+10$ mV; ●, tail current at $-50$ mV.
Development of Block

Development of block has been measured in three different types of experiments. In the first type, the change in current during the depolarization was compared in the presence and absence of the drug. In the second type, the change in the amplitude of tail currents after clamps of different duration was measured. In the third type, the change in decay time of tail currents after short depolarizing clamps was recorded.

An example of the first method is given in Fig 8. A depolarizing clamp of 2 seconds is applied from -50 to 0 mV in the absence and in the presence of 6x10^-5 and 10^-5 mol/L almokalant. As shown in Fig 2, the high concentration of 10^-5 mol/L almokalant blocks a single current. The difference currents between control (tracing 1) or the presence of 6x10^-5 mol/L almokalant (tracing 2) and current in 10^-5 mol/L almokalant (tracing 3) are given in Fig 8B and represent the total drug-sensitive current or total I_K (tracing a) and the I_K component remaining in a solution containing 6x10^-8 mol/L (tracing b). By constructing the ratio of b to a (Fig 8C), one then directly obtains an image of the block development in the presence of 6x10^-8 mol/L almokalant. In this particular experiment, the current decay or block development could be described by an exponential with a time constant of 1.22 seconds. Block did not attain steady state during the 2-second depolarization. The figure further shows that the ratio of tails, and thus block, did not change to any extent during the 2 seconds at -50 mV. Since binding of the drug also results in a voltage shift of the activation, and thus of the kinetics, estimation of the time constant may be in error. The error concerns the initial transient current but not the steady state; at 0 mV, full activation is obtained, and the shift has no effect on the amplitude of the current at this voltage level. These considerations should be taken into account when interpreting the actual time constant. The fact that the ratio of currents can be described by a single exponential suggests, however, that the change in block is the main determinant of the change in current.

In most experiments, block development was measured using the second method of tail currents and their ratios. Examples of such recordings for depolarizing steps to 0 mV are given in Fig 9A. Under control conditions, interruption of the depolarizing pulse at different times yields tail currents of increasing amplitude that reach a plateau after 1.5 seconds. During the depolarizing pulse, the current first increases in the inward direction, because of the inactivation of the transient outward current, and later slightly increases in the outward direction. In the presence of 5x10^-8 mol/L almokalant, the current during the pulse shifts in the inward direction (see dotted line); the tails are hardly changed during the first hundred milliseconds but decrease gradually afterwards. In some experiments, the tails at short duration even increased before decreasing. Fig 9B gives a plot of the amplitude of the tail currents as a function of the duration of the depolarizing pulse. These results show that block of I_K by the drug increases with time. The relative amount of block can be estimated by calculating the ratio of tail amplitude in the presence and in the absence of the drug. The time course of block development was quantified by plotting these ratios as a function of clamp duration for four experiments in which 5x10^-8 mol/L almokalant was tested (Fig 9C). An exponential was fitted to the results and shows that, after a 4-second depolarization, block approached a steady-state value of 73% with a time constant of 1.07 second. Quantification of the change in current during the depolarization was not made. In absolute terms, the change in amplitude of the tail current was greater than of the current during the pulse, which is evidence for the inward rectifying properties of this current (see also Fig 5).

Further evidence for open-channel block was obtained by measuring the decay time of the tail current at
Fig 9. Development of open-state block estimated by tail current amplitude. The voltage-clamp protocol consisted of clamp depolarizations from −50 to 0 mV for various durations. A, Superimposed currents during and after the depolarizing pulses (tails) are shown for control conditions (top tracing) and in the presence of $5 \times 10^{-8}$ mol/L almokalant (bottom tracing). Under control conditions, tail currents increased with time and reached a plateau after ≈1.5 second. In the presence of the drug, current during the depolarization became more inward (control current, indicated by the dotted line), and tail currents decreased (control tail currents, indicated by the dotted line). Note the presence of a transient outward current in the beginning of the recording; its apparent change is caused by the block of the delayed rectifier K⁺ current ($I_{K}$). B, Graph illustrates tail current amplitudes in the control condition (○) and in the presence of $5 \times 10^{-8}$ mol/L almokalant (●). Amplitude of tail currents was measured as the difference with the holding current before application of the depolarizing pulse. C, Time course of block development at 0 mV is shown. Ratios of tail current amplitude in the presence of drug ($5 \times 10^{-8}$ mol/L) and in control conditions were calculated for four experiments and plotted as a function of duration of the clamp depolarization. An exponential with a time constant of 1.07 second was fitted to the results.

Fig 10. A, The development of block increased the rate of decay of the tail current following a short (150-millisecond) depolarization to +10 mV from a holding potential of −50 mV. Superimposed recordings of the tail currents are shown for control conditions (1) and in the presence of $6 \times 10^{-8}$ (2) and $10^{-7}$ mol/L (3) almokalant. From the ratios of tail currents in the presence and absence of the drug (ie, 2/1 and 3/1), time constants for development of block were estimated to be 1.33 second in $6 \times 10^{-8}$ mol/L and 0.62 second in $10^{-7}$ mol/L almokalant. B, Recovery from block slows the decay of the tail current following a long (6-second) depolarization to 0 mV from a holding potential of −50 mV. Superimposed recordings are shown during control conditions (dots) and in the presence of $3 \times 10^{-8}$ mol/L almokalant (jagged line). The two tail currents cross at ≈2 seconds after the return to −50 mV. Since 6 seconds is sufficient to obtain steady-state block at 0 mV, the greater probability of the blocked channel to open at −50 mV results in a slow escape of the drug from the channel and generates an outward current.

−50 mV following short (150-millisecond) depolarizing pulses to +10 mV. This type of pulse is adequate to activate almost all the channels but is too short to obtain a steady-state block. If almokalant blocks open channels, and the block is itself not voltage dependent, block should continue to increase during the tail current at −50 mV. This prediction was fulfilled as shown by the faster decline of the tail current in Fig 10A. By constructing the ratios of the tail current in the presence of the drug over the tail current under control conditions, the time course of block development was estimated by an exponential through the experimental data. In the example shown, the time constant was 1.33 second for $6 \times 10^{-8}$ mol/L and 0.615 second for $10^{-7}$ mol/L almokal-
Recovery From Block

Recovery from block has been measured by applying a test pulse at variable times following induction of block by a conditioning pulse protocol. The change in tail current of the test pulse compared with the control value is a measure of the recovery process. We used two different conditioning protocols to induce block.

In a first series, induction of steady-state block was made by applying short clamps of 0.2 second to +10 mV at a frequency of 1 Hz during 1 minute. The potential was then clamped at −50 or −75 mV for a variable period. After this variable period, a test pulse of 0.2-second duration to +10 mV was given, and the tail current at the end of this pulse was measured at −50 mV. These measurements were done in the absence (control) and presence of the drug. The amplitude of the tail current compared with the control or tail current ratio is a measure of the recovery from block occurring at the holding potential. The results were different at −50 and −75 mV (Fig. 11). At −50 mV, the tail current amplitude gradually increased with time, and complete recovery was obtained after 30 to 60 seconds of rest at −50 mV. The time course could be described by an exponential with a time constant of 13.9 seconds (data from three cells). The tail current returned to its initial value, although the drug remained in the bath medium. At −75 mV, recovery only attained 20% even after 30 seconds. In five other experiments, the difference in recovery at −50 and −75 mV has been confirmed by measuring the tail current after 1 minute of rest at the two membrane potentials: at −50 mV, recovery was complete; at −75 mV, recovery was small or absent (see mean values at 60 seconds in Fig. 11).

The presence of recovery at −50 mV was confirmed in a second series of 13 preparations in which block was induced by holding the potential at +10 V for 30 seconds. The time constant for recovery was 9.10±1.22 seconds. At −75 mV, recovery was practically absent. The finding of complete recovery at −50 mV suggests the absence of block for rested channels. The absence of a substantial recovery at hyperpolarized levels, on the other hand, favors the hypothesis of trapping of the drug. Both observations can be explained by assuming that the drug only blocks open or activated channels and cannot leave the channel when it is closed.

If steady-state block is obtained during a depolarizing pulse and unblocking occurs at −50 mV, tail current at −50 mV in the presence of the drug should be slower than in control conditions. Since the rate of this unblocking reaction is very slow, the expected change is small. An attempt to measure this phenomenon was made in six experiments. In two experiments, a shift in holding current precluded any conclusion. In four experiments, the decline of the tail current in the presence of the drug was slower, and an example is shown in Fig. 10B. In this example, it is clear that the two tail currents cross at about 2 seconds after the return to −50 mV. Quantification of the process was not made. The description of the tail current requires more than one exponential, and the small currents during the late part of the tail current may be contaminated by other processes than decline of $I_{K}$, such as the electrogenic pump current, the Na+-Ca$^{2+}$ exchange current, and the Cl$^{-}$ current.

Frequency Dependence of Block

Any frequency-dependent block of $I_{K}$ in steady state depends on the amount of block developed during the depolarizing pulse and the extent of recovery during the time at the holding potential. The effect of frequency was tested by applying clamp depolarizations of 0.2 second to +10 mV, with a variable total interval of 0.5, 1, 2, and 5 seconds. Each frequency was applied for 1 minute. The holding potential was −50 mV. The concentration of almokalant was $6\times10^{-8}$ mol/L.

Since the time constant of recovery is 10 seconds at −50 mV, a substantial block should already be seen at a stimulus interval of 5 seconds, with a limited increase at the higher frequencies. Results for five cells are shown in Fig. 12. $I_{K}$ was reduced to 65±5% for the 5-second interval and decreased to 34±4% for the 0.5-second interval. A small frequency-dependent increase in block was thus superimposed on a relatively large tonic block.

Use-Dependent Unblock: Further Evidence for Trapping

A direct demonstration of use-dependent unblock was given by comparing the rate of block disappearance during washout of the drug with or without repetitive
stimulation. The rationale of the experiments is as follows. If the drug cannot leave the channel when it is closed, washout of the drug from the bathing medium with the channels closed should also have no consequence for the block. In other words, for a closed channel, block should remain at the same level whether the drug is present or not in the extracellular medium. Furthermore, after washout of the drug from the extracellular medium, stimulation should result in a fast recovery from block.

Fig 12 first shows the evolution of change in $I_K$ during washin and washout of $5 \times 10^{-8}$ mol/L almokalant when the cell is repetitively depolarized to $+20$ mV for 0.2 second at a rate of 1 Hz. A steady-state block was obtained within 2 minutes of superfusion, and the block completely disappeared after 3 minutes of washout. Washout of the drug from the extracellular space and from the membrane or intracellular space is thus possible within 3 minutes. To investigate whether this fast recovery was related to the repetitive stimulation used, the following washout experiments were done. An example is illustrated in Fig 14. The preparation was subjected to successive periods of rest and repetitive voltage clamps in the absence and in the presence of $6 \times 10^{-8}$ mol/L almokalant. Clamps were applied from $-50$ or $-75$ mV to $+20$ mV for 0.2 second in order to measure the tail current. In control conditions, the tail amplitude was 100 pA (horizontal line). The drug was added to the superfusion medium under continuous repetitive depolarizations. Gradually, $I_K$ became blocked (not shown), and in steady state, $I_K$ was reduced to $\approx 50$ pA (filled symbols). The preparation was then rested for 1 minute at $-50$ mV, and $I_K$ recovered practically to the control value. The cell was again subjected to a series of depolarizing pulses from $-50$ mV; block developed again. A second 1-minute rest period now at $-75$ mV was not accompanied by any recovery in this example. During application of a new series of voltage clamps, no change occurred in the block. At the end, the membrane was clamped at $-75$ mV, and washout started. Washout was continued for 5 minutes in the absence of stimulation, which is a time more than sufficient to clear the extracellular space from all drug (see Fig 13). Application of one stimulus revealed the presence of an important block, although the extracellular space did not contain any drug. In four preparations, mean recovery was 17±5.5%. The drug thus seemed to still be present in the channel. Repetitive stimulation now, however, resulted in a fast recovery, and block disappeared completely within 20 to 30 seconds. Similar results were obtained in the three other experiments. These results show that (1) the drug cannot leave the channel in the rested closed state even when the drug is washed out of the medium (the drug is trapped), and (2) activation of the channel allows the drug to exit from the channel (recovery is use dependent).

**Discussion**

The present analysis shows that almokalant affects $I_K$ and causes two effects: (1) a shift of the activation curve
in the hyperpolarized direction and (2) block of the channel.

Although the block in itself is voltage independent (there is no further block at positive potentials once all channels are activated) (Fig 5), the shift of the activation parameter provides a voltage-dependent modulation of the channel activity. Shifts of the activation or inactivation curves can occur when a drug preferentially blocks the channel in a given state, even when the block itself is voltage independent. If the whole process can be written as a series of reactions proceeding in a cycle, the principle of microscopic reversibility predicts that the product of the rate constants going clockwise must equal the product of rate constants in the counterclockwise direction. Application of this rule to a diagram in which closed, open, blocked open, and blocked closed channels are connected in a cycle predicts a shift in the hyperpolarized direction. In the present study, evidence has been presented that this principle cannot be applied because the cycle is open: rested blocked channels do not recover from block via the closed form (Fig 14); the drug is trapped, and the channel has to be activated again to allow escape of the drug. Our experiments furthermore have shown that the shift can occur without block and should be considered a characteristic of the conductive channel (Fig 4). For small depolarizations, the shift in activation curve counteracts the effect of an eventual block and may even show up as a net increase in \( I_K \) instead of a decrease. It should be stressed that these results cannot be explained by an artifactual increase in tail current due to interference with the transient outward current. If this were the case, the increase in tail amplitude should have been more prominent the greater the depolarization; however, the opposite observation was made. Also, in many cells the transient outward current was absent or small. To a certain degree the effect of almokalant on \( I_K \) can be compared with the effect of dihydropyridines on the \( Ca^{2+} \) current. Dihydropyridines cause a shift in the gating process and may also block the channel. The stimulatory effect of dihydropyridines results from a shift of the current-voltage relation in the hyperpolarized direction together with an increase in the maximum current. At the single channel level, no change has been observed in the single-channel conductance, only in the open probability. The effect has been modeled assuming a shift in the voltage dependence of the rate constant for channel closure.

To explain the stimulatory and inhibitory effects, the drug is assumed to bind to two different sites. Binding to the site responsible for the stimulatory effect occurs in the closed or activated state, but in this last case, it should be a fast reaction. The stimulatory effect was indeed seen for short depolarizations. It was also seen preferentially at lower concentrations, suggesting that the two binding sites for shift and block show different affinities. Extrapolating to in vivo conditions in which the cardiac cells are continuously stimulated at a given frequency, the stimulatory effect will have no practical consequences; it will not exert a shortening effect on the action potential duration because of the existence of a use-dependent increase in block.

The second effect of almokalant is block of the channel. Such a block does not seem to occur via the rested state. The observation that complete recovery from a pronounced block by almokalant was possible at the holding potential of \(-50 \text{ mV}\), although the drug was present in the bathing medium (Figs 11 and 14), makes a blocking reaction with the rested-state configuration very unlikely. The blocking effect requires activation as shown by the demonstration of use dependence; the measurement of a time constant for block during the depolarization pulse further indicates that block is possible via the open state. This observation does not exclude, however, that block already starts during transitional states between the rested and the open state. In favor of this proposition, it should be mentioned that at higher concentrations no stimulatory effect was seen, and block increased from the beginning of the depolarizing pulse.

The time constant for block development during the open state was estimated by different methods. The
most reliable results were obtained using pulses of different durations and measuring the amplitude of the tail currents (Fig 9). Since the stimulatory effect already exists at the beginning of the pulse or develops very quickly, it will not disturb, to any large degree, the estimation of the exponential time course. The time constant for block development estimated in this way was in the order of 1 second for pulses to 0 mV and a drug concentration of $5 \times 10^{-8}$ mol/L. This means that block during a single action potential is substantial but far from complete. Estimation of development or decrease of block by measuring the rate of tail decay may be in error. The error is due to the existence of a voltage shift of the activation curve, which on a theoretical basis should also change the kinetics. At $-50$ mV, however, the rate of deactivation is not very sensitive to a shift in the voltage dependence of the kinetics, because the time constants show near maximal values between $-60$ and $-40$ mV (author’s unpublished observations). The results in Fig 10, in which an increase as well as a decrease in rate of decay was seen, should be interpreted taking these considerations into account.

The block itself is voltage independent. Since almokalant at pH 7.4 is 72% in the charged form, the voltage independence of the block means that the charged form interacts with the channel outside the electric field (in this case the trapping should be considered to occur outside the pore) or that the uncharged form is responsible for the block.

Open-state block has been described for other substances acting on K+ channels. It has been shown, for instance, for internal tetraethylammonium (TEA) block of IK in the squid axon and for the A-type shaker current in Drosophila.15 Open-state block of IK in heart has been demonstrated for quinidine,16 flecaïnide and encainide,17,18 and dofetilide.11 For quinidine, for instance, the time course of block development has been measured in rabbit nodal cells16 and in a cloned human K+ channel (HK2)19; at the K+ concentration, the time constant for block development was in the order of tens of milliseconds. Such a rapid time course means that steady state will be reached during a single action potential and that the block will not show any frequency-dependent change. The cloned human HK2 current shows a very fast activation time course (order of milliseconds) and a slow inactivation; it resembles a recently described current in rat atrial myocytes.20 Whether present knowledge regarding kinetics in nodal cells and the cloned HK2 channel is applicable to the much slower activated IK in ventricular cells is not known. Differences in blocking mechanisms indeed are known for K+ channels in the same cell and species. In the guinea pig ventricle, the IKr, or rapidly activated component, is more sensitive to blockade than the IK, or slowly activated component. A differential sensitivity has been shown for the block by E403110 and dofetilide.11

Recovery from block does not seem to occur via the closed channel but requires the open state: (1) In the presence of the drug, recovery at $-75$ mV was inconstant or very small (Figs 11 and 14). (2) Even after washout of the drug, recovery of block was nil when the membrane was kept hyperpolarized at $-75$ mV and no depolarizations were applied (Fig 14). The drug thus seems to be trapped by the closure of the activation gate. Transition between the blocked open state and the blocked rested state may be possible, but exit from the rested state does not occur. The phenomenon of trapping is not exceptional and has been first described for block of the K+ channel in the squid giant axon by quaternary ammonium derivatives14 and later for neuronal and cardiac Na+ channels (see References 21 and 22).

Trapping of the drug with no intrinsic voltage dependence of the block, however, poses the problem of recovery from block. How is recovery possible when no transition exists between the blocked rested state and the rested state? In the case of block with trapping of intracellularly applied TEA in the squid axon, recovery of the delayed K+ channel occurs during deactivation at hyperpolarized levels because block by TEA is intrinsically voltage dependent. Block as well as unblock requires the open-channel state, but entry of the positively charged TEA molecule in the channel is favored by depolarization and exit by hyperpolarization. Furthermore, Armstrong14 showed that the greater influx of potassium ions at hyperpolarized levels was a second factor that favored unblocking.

For almokalant, this explanation cannot be given because there is no intrinsic voltage-dependent block. To explain the voltage dependence of the recovery from block, the shift of the activation curve is of primary importance. The shift in the hyperpolarized direction means that channels to which the drug has been bound will show a higher probability to be activated at a potential level negative to the maximal activation level. At $-50$ mV, for instance, occasional openings of the blocked rested-state channel will be more frequent than those of the nonbound rested-state channels, and this will allow the drug to escape from the channel. At $-75$ mV, activation of the blocked channels will be very improbable, and recovery is thus extremely slow.

Although the final equilibrium at $-50$ mV is disappearance of block, the decay of the tail currents at $-50$ mV after a short depolarization was faster in the presence of the drug compared with control conditions. This result was considered to be due to an increase in block. The existence at $-50$ mV of an increase in block during a tail and the complete disappearance of block at rest are not contradictory. After a short depolarization, a large number of channels are activated, but block is still far from equilibrium for open channels, a phenomenon that is voltage independent and occurs with a time constant of 1.0 second. When deactivation has occurred, the effect of the voltage shift becomes important, and the higher probability of the blocked channels to become activated allows the drug to escape. Since openings at $-50$ mV are rare, the reaction proceeds slowly, with a time constant of $\approx 10$ seconds.

A drug that blocks IK will prolong the action potential duration. Selective prolongation at high frequencies of stimulation offers the advantage of being efficient against tachyarrhythmias with a minimum risk of prolonging the action potential at low frequencies and provoking “torsade de pointes” arrhythmia.23 Almokalant prolongs the action potential duration, but the effect is not selective at high frequencies; rather, the reverse effect is seen,8,10 although, as demonstrated in the present study, almokalant shows that a normal use-dependent increase in block of current and in steady-state block was more pronounced at higher frequencies. To
emphasize the difference in effects of the rate on the current on one hand and the action potential duration on the other hand, the latter effect could be called “reverse” rate-dependent lengthening of the action potential, as proposed by Jurkiewicz and Sanguinetti.24

To understand the apparent paradox between effects on current and action potential duration, one should realize that the action potential duration is a complex phenomenon and is the result of the interplay between a number of tonic currents. During the plateau, a fine balance exists between inward (Na+, Ca2+, and Na+-Ca2+ exchange) currents and outward (transient outward, delayed K+, Cl-, Na+-K+ pump, and eventually the acetylcholine-induced K+) currents. The relative contribution to the plateau of all these currents changes with frequency. As a typical example, we may mention the increase with frequency of the two components of Ik in the guinea pig ventricle. Because of the difference in their time constants of activation, the slower component will be more important at high frequencies, whereas the fast component will already saturate at moderate frequencies. Since the faster component is selectively blocked by a number of drugs, eg, dofetilide,11,24 the effect of the use-dependent block by this drug will be offset by the summation of the slower component.24

A direct quantitative translation of a given change in Ik into a change of action potential duration is not possible without careful modeling. Two considerations can be made to understand why a drug with a positive use dependence can cause a greater prolongation of the action potential at low frequencies. First, at low frequencies the rate of repolarization during the plateau is slower than at high frequencies, indicating a small net current. Even a minimal change in Ik will then be very effective in prolonging the action potential. Second, at low frequencies, not only is the rate of repolarization slower but the total duration of the action potential is longer. Since the drug is an open-channel blocker, the amount of block depends on the duration during which the cell is depolarized, and block during a single action potential can thus be considerable.

In the search of the “ideal” K+ channel blocker,4 selection should be based on the combination of slow development during depolarization and fast recovery of block during diastole. At the present time, such a combination is not available in the drugs presently used. Either block development is too fast (eg, quinidine6,19 and probably encainide17) so that block at slow rates is already maximal, or recovery from block is too slow (dofetilide11 and almokalant in the present study) so that most of the block appears as a tonic block. In both cases, frequency-dependent block in the steady state is small or nonexistent.

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References

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