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Mode of action of bradycardic agent, S 16257, on ionic currents of rabbit sinoatrial node cells

[1]Patrick Bois, Jocelyn Bescond, Barbara Renaudon & Jacques Lenfant

Laboratoire de Physiologie Générale, U.R.A. CNRS 1869, Université de Poitiers, 40 Av. du Recteur Pineau 86022 Poitiers, France

1 The effect of the bradycardic agent S 16257 on the main ionic mechanisms of diastolic depolarization in sinoatrial node cells isolated from rabbit heart, was investigated by the patch-clamp technique in whole-cell and macro-patch recordings.

2 In whole-cell conditions, S 16257 induced a marked exponential use-dependent blockade of the hyperpolarization-activated I_f current, without shift of the voltage range of its activation curve. The rate of block increased with the drug concentration. The IC_{50} for the block of I_f was 2.8×10^{-6} M.

3 A similar use-dependent decline of I_f was obtained with 3 μM S 16257, in cell-attached and in inside-out macro-patch configurations, suggesting that the bradycardic agent interacts with I_f channels from the inside of the cell.

4 A high concentration of S 16257 (10 μM) had no detectable effect on T-type calcium current and slightly decreased L-type calcium current ($-18.12 \pm 0.66\%$), without significant use-dependent blockade.

5 S 16257 had no effect on the delayed outward potassium current I_K at 3 μM and slightly decreased it only at high concentrations, $-16.3 \pm 1.2\%$ at 10 μM. In contrast, zatebradine, another bradycardic agent, reduced I_K by $20.3 \pm 2.5\%$ at 3 μM.

6 In conclusion, S 16257 may lower heart rate without significant negative inotropic action. In comparison with zatebradine, S 16257 had less effect on I_K suggesting less prolongation of repolarization time.

Keywords: Pacemaker current; S 16257; bradycardic agent; zatebradine; sinoatrial node; calcium current; potassium current; patch-clamp

Introduction

Over the last few years much progress has been made in understanding the ionic mechanisms underlying the generation of the pacemaker activity in sinoatrial (SA) node. Previous work has demonstrated that four time-dependent currents participate in pacemaking of the SA node (Irisawa *et al.*, 1993): (i) decay of the outward potassium current (I_K) activated during the preceding action potential; (ii) activation of the time-dependent inward current, I_f; (iii) activation of two types of calcium currents, the low-threshold transient type current ($I_{Ca,T}$) and the high-threshold long-lasting type current ($I_{Ca,L}$). According to different authors (Irisawa *et al.*, 1993), one of the most important ionic currents for the regulation of pacemaker depolarization in mammalian heart cells is the pacemaker current I_f, a mixed Na^+-K^+ inward current activated by hyperpolarization.

During recent years, drugs with a new pharmacological profile have been described as 'specific bradycardic agents': zatebradine (Goethals *et al.*, 1993) and ZD7288 (BoSmith *et al.*, 1993). These agents decrease the heart rate by direct interaction with the pacemaking cells of the SA node. The major target for this pharmacological control is the hyperpolarization-activated I_f current. Recently, a novel bradycardic agent, S 16257, has been described (Thollon *et al.*, 1994). Its electropharmacological properties have been compared to those of zatebradine, in the rabbit isolated SA node tissue. At low concentrations, S 16257 decreases heart rate by reduction of the speed of diastolic depolarization, which suggests an inhibition of the pacemaker current I_f. At high concentration, it was found that the action potential is slightly prolonged. This compound was described as effective as zatebradine in its bradycardic effect, but more specific as it induced a smaller increase in action potential duration (Thollon *et al.*, 1994). Therefore, it was of interest to examine directly the effect of S 16257 on the current systems of the SA node cells isolated from the rabbit heart, by use of different patch-clamp configurations. The present paper describes the mechanism of action of S 16257 on the I_f current and other ionic currents that also play a role in normal pacemaking of the SA node: $I_{Ca,T}$, $I_{Ca,L}$ and the outward delayed rectifier potassium current (I_K).

[1] Author for correspondence.

Methods

Albino rabbits weighing 500-1200 g were killed by cervical dislocation. The SA node region was isolated and single myocytes were prepared and stored according to the protocol reported previously by Petit-Jacques *et al.* (1993).

Patch-clamp recording

Whole-cell recording with the perforated-patch method To minimize the run-down process, the perforated patch with amphotericin B (Horn & Marty, 1988) was used to record the slow activated currents, I_f and I_K. The amphotericin B was dissolved in dimethylsulphoxide at a concentration of 0.08 M and then added to the internal pipette solution to yield a final concentration of 2.65×10^{-4} M. The internal pipette solution contained (in mM): KCL 30, K_2SO_4 50, $MgCl_2$ 8, $CaCl_2$ 1, HEPES/KOH 10, pH = 7.2. The amphotericin B stock solution was suspended in the internal solution by 60 s ultrasonication. After sonication, the amphotericin B internal solution could be used for about 1-2 h, after which time a fresh solution was prepared. Pipettes were filled by putting the tip directly into amphotericin B-free solution for a few seconds and then back filled with the amphotericin B-containing pipette solution. Series resistance (Rs) commonly decreased to less than 20 MΩ

within 10–30 min after gigaseal formation. On occasion, the perforated patch can spontaneously convert into a conventional whole-cell recording. In the present experiment, such a problem was evident by the appearance of a cell contracture, induced by the presence of the calcium ions in the pipette solution. For recording I_f, the external solution contained (in mM): NaCl 140, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES/NaOH 5, pH = 7.4, $MnCl_2$ (2 mM) and $BaCl_2$ (1 mM) were added to block calcium and potassium currents.

To record I_K, the external solution contained (in mM): NaCl 140, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 0.9, HEPES/KOH 10, pH = 7.4, $CdCl_2$ (0.1 mM) was added to suppress calcium currents.

Conventional whole-cell recording Considering the large access resistance with the perforated patch method, the relatively fast currents, $I_{Ca,T}$ and $I_{Ca,L}$, were recorded by the classical ruptured-patch method. The pipette contained (in mM): CsOH 120, TEACl 20, MgATP 5, Na_2 GTP 0.1, EGTA 5, $CaCl_2$ 1.2, HEPES 10 and pH was adjusted to 7.2 with aspartic acid. The external solution contained (in mM): TEACl 140, $CaCl_2$ 1.8, $MgCl_2$ 0.5, HEPES/CsOH 10, pH = 7.4. To investigate T-type calcium current, 2 μM nifedipine was added to suppress L-type current. Moreover, the resolution of $I_{Ca,T}$ was amplified by increasing Ca^{2+} from 1.8 to 5 mM (Hagiwara *et al.*, 1988).

Macro-patches To analyse the molecular mode of S 16257 action, the I_f current was also recorded in cell-free and cell-attached macro-patch configurations. Macro-patches containing a large number of I_f channels were obtained by use of large-tipped pipettes (diameter of 1–1.5 μm and resistance of 1–2 MΩ), as reported previously (Yatani & Brown, 1990). In cell-attached configuration, the bathing solution contained (in mM): KCl 130, NaCl 10, $CaCl_2$ 2, EGTA 5, HEPES/KOH 10, pH = 7.4. This high potassium solution was used to reduce the resting membrane potential to zero. The standard patch pipette solution was (in mM) : KCl 70, NaCl 70, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES/KOH 5, pH = 7.4. $MnCl_2$ (2 mM) and $BaCl_2$ (1 mM) were added to block other currents. In inside-out patch configuration the external solution was (in mM): NaCl 10, K-aspartate 90, KCl 40, $CaCl_2$ 2, EGTA 5, MgATP 2, GTP 0.1, HEPES/KOH 10, pH = 7.4. The pipette solution is the same as the one used in cell-attached configuration.

Control and test solutions were delivered through a wide-tipped pipette placed in close proximity to the cell under study. Experiments were carried out at the temperature of 25 ± 1°C in order to minimize current noises, particularly under macro-patch conditions. In some experiments, membrane capacitance was recorded to allow measurements of current density. The average membrane capacitance was 45 ± 1.37 pF (n = 51).

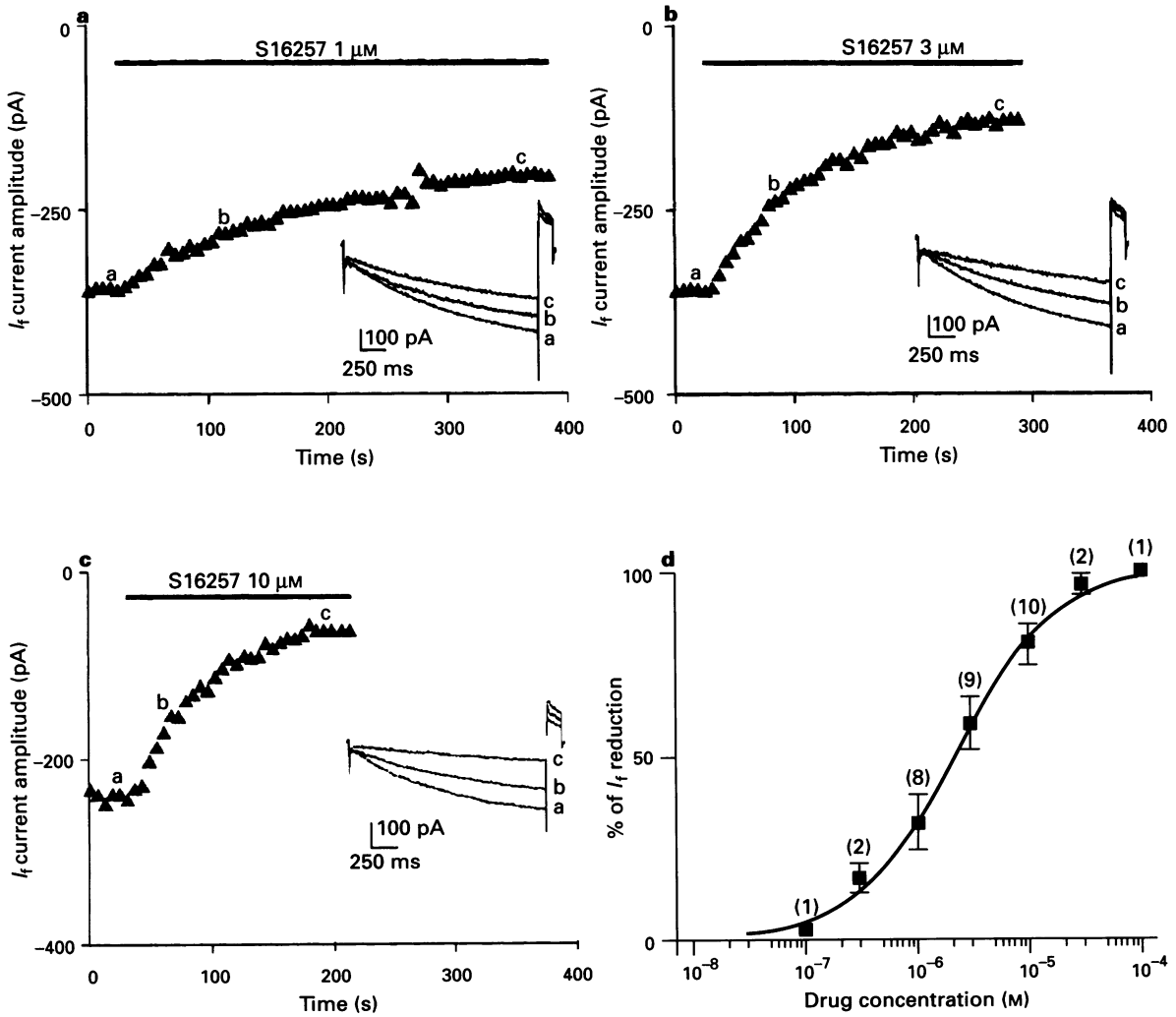

Figure 1 Use-dependent block of I_f by S 16257 at 1 μM (a), 3 μM (b) and 10 μM (c). I_f current was elicited by hyperpolarizing steps to −100 mV from a holding potential of −30 mV at 1/6 Hz. The graphs plot the amplitude of the time-dependent inward current (which was taken as the difference between final and initial current) before and during cell perfusion with S 16257. The inset figures show a set of three I_f traces recorded in sequence just before (a) and during cell perfusion with S 16257 (b,c). (d) Illustration of the effect of various doses of S 16257 on I_f. The data were fitted with the expression $f(x) = 100/[1 + (IC_{50}/x)^{n_H}]$ where IC_{50} (2.18×10^{-6} M) is the drug concentration at which inhibition is half-maximum and n_H (0.96) is the Hill coefficient. The correlation coefficient was 0.9987. The number of cells (n) is indicated above each point of the curve.

Electrical recording and analysis

Experiments were performed using a patch clamp amplifier (RK300, Biologic, Grenoble, France) driven by an IBM-PC-AT compatible microcomputer (IPC, Essex Electric PTE, Singapore), equipped with an A–D, D–A conversion board (Labmaster TM 40, Scientific solutions, Solon, USA). Voltage clamp procedures and stored experiment results were respectively programmed and analysed with specific software (pClamp V5–5, Axon). All data were presented as mean values ± s.e. mean.

Drugs

S 16257 (7,8-dimethoxy 3-{3-{[1S)-(4, 5-dimethoxybenzocyclobutan-1-yl) methyl] methyl-amino} propyl}-1,3,4,5-tetrahydro-2*H*-3-benzazepin-2-one hydrochloride) and zatebradine were kindly provided by Institut de Recherches Servier, Suresnes, France. The drugs were added to the solution from stock solution (10^{-2} or 10^{-3} M in H_2O).

Results

Use-dependent block of I_f by S 16257

Figure 1 shows the effects of three concentrations of S 16257 (1 μM, 3 μM and 10 μM) on I_f current. To examine the time course of the block induced by the S 16257, a train of pulses from −30 to −100 mV, with 1.8 s duration, was applied at the frequency of 1/6 Hz. The drug was exposed during the steps after stable I_f current responses had been established. At every concentration, S 16257 induced a marked use-dependent blockade of I_f. The pacemaker current decreased in an exponential manner (Figure 1 a, b and c). The rate of block increased with the drug concentration. The time constants of block, as deduced from single exponential fitting, were 94.3 ± 3.9 s (n = 8), 73.7 ± 1.3 s (n = 9) and 45.17 ± 0.66 s (n = 10) with 1, 3 and 10 μM S 16257, respectively. A log concentration-response curve for the use-dependent block of I_f is shown in Figure 1d. The percentage of I_f reduction was measured at the steady-state of blockade. The estimated IC_{50}-value was 2.18×10^{-6} M. At every drug concentration, the effects were partially reversible. At 3 μM S 16257, full recovery of drug-induced effects was reached only after 20 min of washout, in 3 out of 9 cells. In other experiments, the membrane potential was held at −30 mV, at which most I_f channels are expected to be closed, and perfusion with 10 μM S 16257 was started. In this case, no tonic block was observed (data not shown). Such a result indicates that the blockade of I_f was use-dependent and requires the I_f channel to be open to produce the inhibitory effect of the compound.

Effect of S 16257 on the I_f activation curve

To study the effects of S 16257 on I_f conductance, the compound was tested on the activation curve. In Figure 2, I_f was recorded during hyperpolarizing steps from −45 to −110 mV. The duration of the steps was decreased as the steady state activation of the inward current was reached faster. The hyperpolarizing pulses were followed by a short depolarizing pulse to +20 mV.

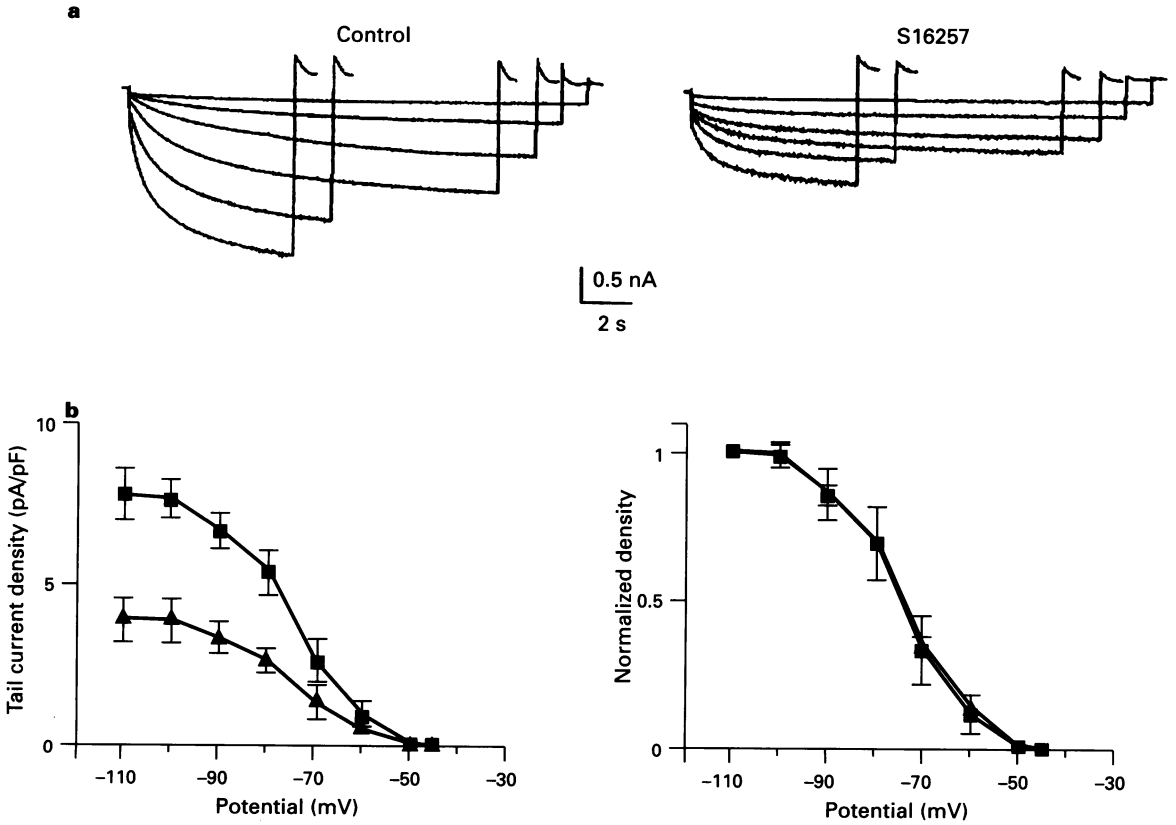

Figure 2 Effect of S 16257 on I_f activation curve. The membrane was held at −30 mV and pulses up to +20 mV were given after prepulses of different duration and amplitude lying between −45 and −110 mV. (a) Examples of currents in control (left) and after the steady state use dependent block of I_f in the presence of 3 μM S 16257 (right). (b) The I_f activation curve was constructed by measuring tail current density values at +20 mV in 3 cells. Absolute amplitude (left graph) and relative amplitude (right graph) values were plotted against prepulses in control conditions (■) and in the presence of 3 μM S 16257 (▲). Note that the S 16257 decreases the steady state current density without shift of the activation curve, as is illustrated by the superimposition of the normalized curves in the right panel.

Figure 2a shows an example of the current traces in control solution (left panel) and after the steady state use-dependent block of I_f by 3 μM S 16257 (right panel). The I_f activation curve is shown for three cells by measuring tail current density values at +20 mV in control and in the presence of drug (Figure 2b). S 16257 decreased the steady state current density without shifting the activation curve as is illustrated by the superimposition of the normalized curves in the right panel. The average conductance was reduced by 50.4±2.3% (n=3) in the presence of 3 μM S 16257.

Analysis of S 16257 effect in macro-patch configurations

For more direct evaluation of the molecular mode of action of S 16257, the compound was tested in cell-attached and inside-out macro-patch conditions (see Methods). In both patch conditions (Figure 3), I_f does not arise from single channels but appears to be induced by a large number of channels per patch (Yatani & Brown, 1991). The activation protocol consisted of hyperpolarizing steps to −100 mV (a, cell-attached) or −110 mV (b, inside-out) applied every 6 s. It can be seen that I_f amplitude recorded in both configurations decreased during a perfusion with 3 μM S 16257. In both cases, an exponential use-dependent decline in I_f was observed, like that recorded in the whole-cell condition. Such a result suggests that the bradycardic agent (S 16257) interacts with the I_f channel from the inside of the cell.

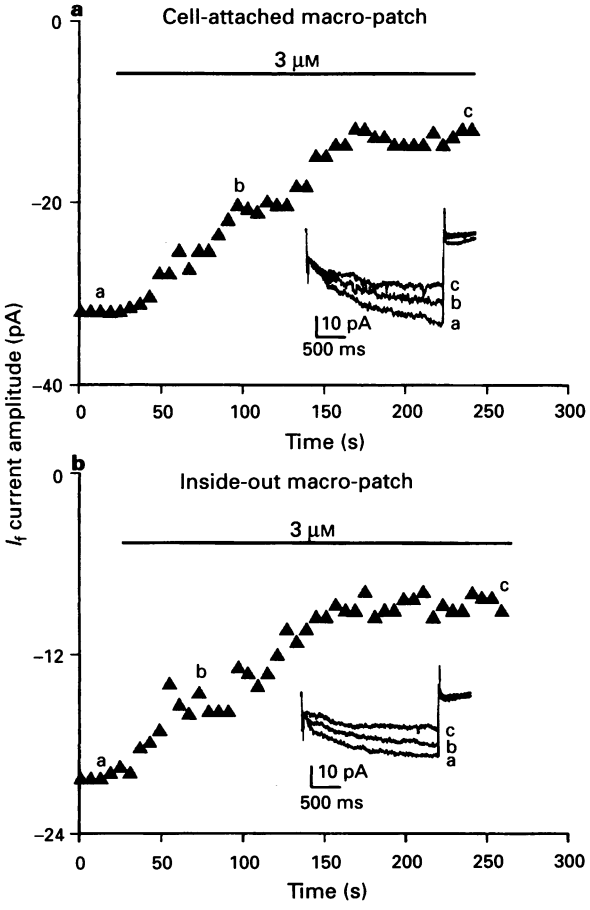

Figure 3 Use-dependent block of I_f in cell-attached (a) and inside-out (b) macro-patch conditions. From a holding potential of −30 mV, −100 mV (cell-attached) or −110 mV (inside-out) pulses were applied as a train at a rate of 1/6 Hz. The amplitude of the time-dependent inward current is plotted in cell-attached (a) and in inside-out (b) macro-patch conditions. Inset panels show a set of three I_f traces recorded in sequence just before (a) and during cell perfusion with 3 μM S 16257 (b,c).

Effect of S 16257 on the two types of calcium currents

To verify the possibility that S 16257 may affect ionic currents other than I_f, the action of S 16257 was investigated on the two types of calcium currents (I_{CaT}, $I_{Ca,L}$) present in pacemaker cells (Hagiwara *et al.*, 1988; Bois & Lenfant, 1991). The currents can be separated by their different voltage and pharmacological properties. In Figure 4, $I_{Ca,L}$ was activated by depolarizing pulses to 0 mV from a holding potential of −45 mV (Figure 4a) whereas the T-type calcium current was elicited from −80 to −20 mV with the presence of selective L-type current blocker, nifedipine (Figure 4b). In both experiments the voltage-clamp pulses train was applied at a frequency of 1/6 Hz. In the presence of 10 μM S 16257, no detectable effect was observed on T-type current. In contrast, at the same concentration, the L-type calcium current was slightly decreased (−17%). However, no significant use-dependent block was observed. The percentage of reduction of $I_{Ca,L}$ was −18.12±0.66% in 8 experiments. These changes were partially reversible after 15 min of washout in 5 out of 8 cells. The right panels of Figure 4a and b show the current-density of $I_{Ca,L}$ and $I_{Ca,T}$ respectively as a function of membrane potential average from 5 cells. S 16257 (10 μM) depressed $I_{Ca,L}$ current density at every potential without shifting the peak along the voltage axis, but failed to change the $I_{Ca,T}$ current density-voltage relation.

Modulation of the delayed potassium current by S 16257 and zatebradine

Figure 5 shows the depressant effects of S 16257 and zatebradine on I_K when 2 s depolarizing pulses were applied from a holding potential of −45 mV to a test potential of +40 mV at a frequency of 1/6 Hz. In Figure 5a, the application of S 16257 at 10 μM decreased I_K by 16%. No effect was observed at 3 μM. At 10 μM the average reduction obtained in 12 cells was −16.29±1.18%. Figure 5b, c illustrates the effect of S 16257 on I_K activation curve in 6 cells. The amplitude of tail current density, recorded before and during the perfusion of S 16257 was plotted against test potentials. The analysis of activation curves before (Figure 5b) and after (Figure 5c) normalizing them, indicates that S 16257 decreases the steady state current density without shift of the voltage range of I_K activation. In these experiments, the average conductance was reduced by 15.48±4.21%, with 10 μM S 16257. By comparison, the effect of zatebradine was tested on I_K in the same experimental conditions (Figure 5d). Unlike S 16257, 3 μM zatebradine was sufficient to reduce the amplitude of I_K significantly. At this concentration, the percentage of reduction on I_K was −20.31±2.47% (in 5 experiments). The effects of the drugs were partially reversible after 5 min of washout in 5 and 3 cells with S 16257 and zatebradine respectively.

Discussion

The purpose of the present study was to investigate the effects of S 16257, a bradycardic agent, on the ionic currents involved in spontaneous activity of the isolated sinoatrial node cells. The results indicate that S 16257 selectively blocks the pacemaker current I_f in use-dependent manner.

The effects on the hyperpolarization-activated current were observed at concentrations which reduced the rate of diastolic depolarization recorded with an intracellular microelectrode, in isolated SA node tissue (Thollon *et al.*, 1994). Unlike cholinergic effects (DiFrancesco & Tromba, 1987) or development of the run down process (DiFrancesco *et al.*, 1986) (which is eliminated here by using the perforated patch configuration), the reduction of I_f by S 16257 is not caused by a shift of its activation voltage range but rather by a reduction of fully-activated I_f conductance. The decrease in the activation curve amplitude may reflect either a reduction in the channel conductance and/or a reduced probability of opening. S 16257

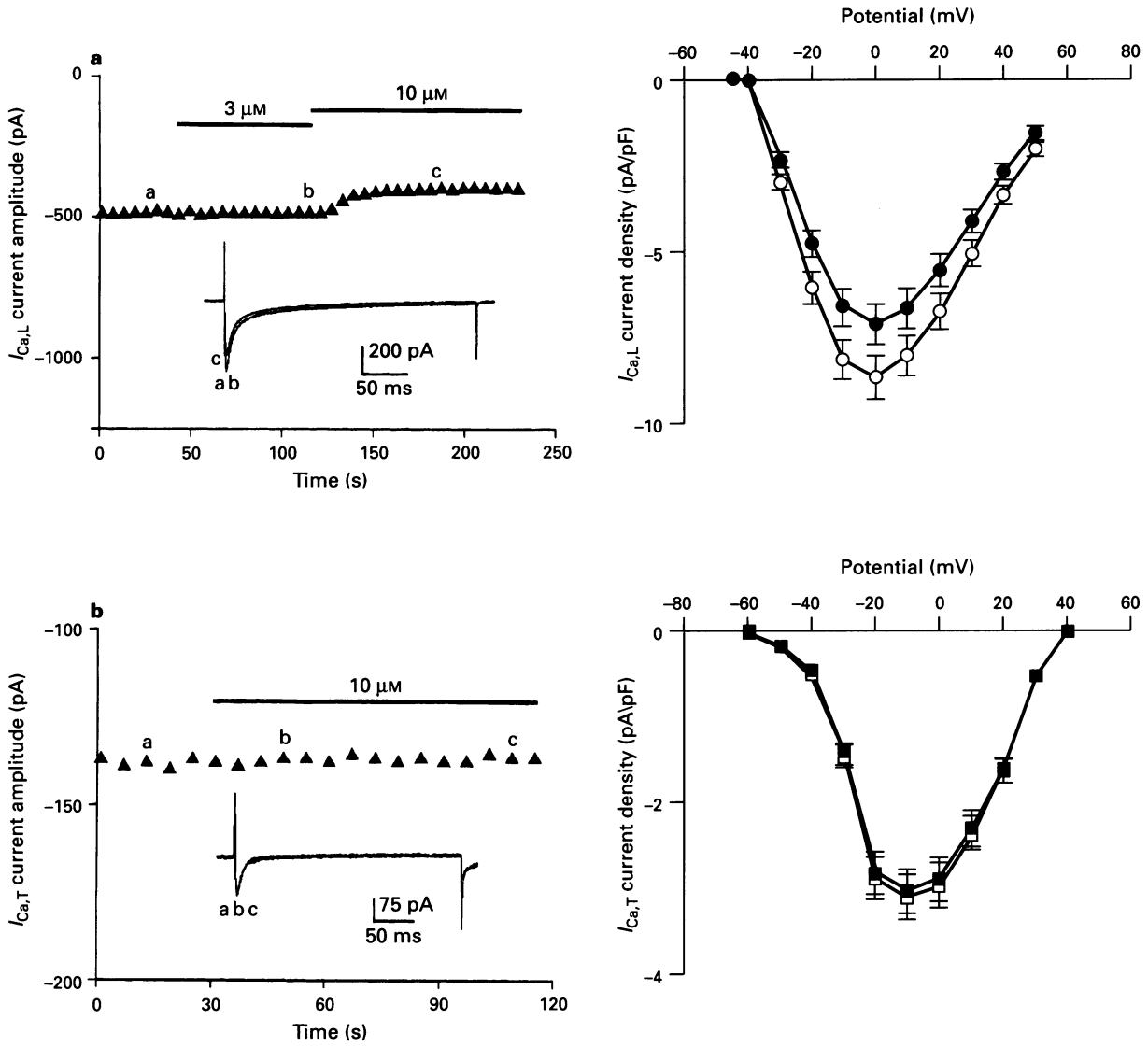

Figure 4 Effect of S 16257 on two types of calcium currents ($I_{Ca,L}$ and $I_{Ca,T}$). Voltage steps applied from −45 to 0 mV and from −80 to −20 mV elicited L-type current (a, left panel) and T-type current (b, left panel), respectively. The pulses were applied at a rate of 1/6 Hz. Peak current amplitudes (▲) were reported before and during different S 16257 concentrations (horizontal bars). The insets show the traces recorded at various time before (a) and during perfusion with 10 μM S 16257 (b,c). Right panels (a,b) show the effect of S 16257 on current density-voltage relations of L (in 5 cells) and T-type currents (in 5 cells). The calcium currents were activated by various depolarizing steps from −45 mV (for $I_{Ca,L}$) and −80 mV (for $I_{Ca,T}$) in the absence (○,□) and in the presence (●,■) of 10 μM S 16257.

action on I_f resembles that of zatebradine (Goethals *et al.*, 1993) but differs from the effect of ZD7288 (BoSmith *et al.*, 1993) in sinoatrial cells. Indeed, zatebradine has been reported to show a use-dependent blockade of I_f, without causing a shift of its activation curve. On the other hand, ZD7288 causes a tonic block of I_f by shifting its activation range to more negative potentials. The presence of a use-dependent blockade on I_f by S 16257 indicates that, in contrast to ZD7288, the compound has an affinity for the open I_f channel. Moreover, the exponential decline of the I_f current observed in cell-attached and inside-out macro-patch configurations suggests that S 16257 passes the cell membrane and interacts with the I_f channel in its open state. A similar molecular mode of action has also been proposed for zatebradine in the SA node cells (Goethals *et al.*, 1993) and in sheep cardiac Purkinje fibres (van Bogaert & Goethals, 1992).

Two types of calcium currents have been described in patch-clamp recording from cardiac pacemaker cells (Hagiwara *et al.*, 1988; Bois & Lenfant, 1991). It has been suggested that the T-type current contributes to pacemaker depolarization. Contrary to the effect recorded on pacemaker current, no detectable blockade was recorded on T-type current in presence of 10 μM S 16257. It is therefore most unlikely that the bradycardic effect of S 16257 is mediated via inhibition of $I_{Ca,T}$. At a similar concentration, L-type calcium current is slightly reduced (−18%). Nevertheless, this reduction appears with a concentration which markedly inhibits I_f. Thus, at low concentrations neither $I_{Ca,L}$ nor $I_{Ca,T}$ block seems to play an important role in the bradycardic effect induced by S 16257. On the other hand, the relative lack of effect on L-type current suggests an absence of negative inotropy induced by the compound. A similar discriminatory effect has also been reported for zatebradine in SA node cells (Goethals *et al.*, 1993).

In rabbit SA node cells, the delayed rectifier potassium current is considered to be related to the repolarization phase of the action potential. The present experiments show that zatebradine was more effective than S 16257 in causing the reduction of outward current amplitude at similar concentrations. Zatebradine (3 μM), was sufficient to reduce the amplitude of I_K by 21%. At this concentration, no effect

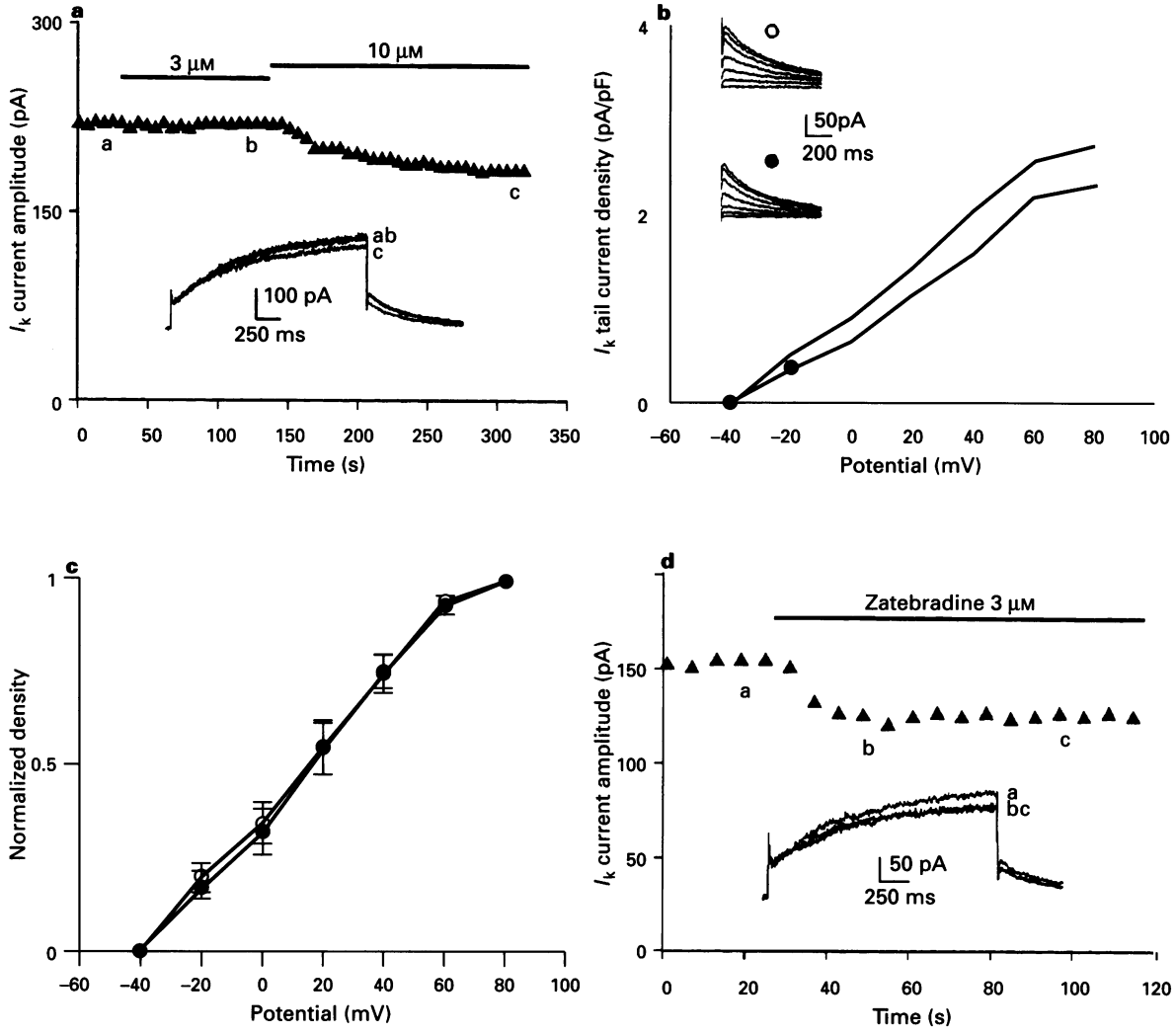

Figure 5 Effect of S 16257 and zatebradine on the delayed outward current (I_K). (a) Depolarizing voltage-clamp pulses were applied from −45 mV to +40 mV to activate I_K (at 1/6 Hz). The amplitudes of time-dependent outward current (measured between initial and final current) were plotted against time in control and in the presence of S 16257 (3 μM and 10 μM). (b and c) Effect of 10 μM S 16257 on the I_K activation curve. Absolute (b) and relative (c) amplitudes of tail current-density (measured at −45 mV, see inset) of I_K were plotted against test potentials before (○) and after (●) the addition of 10 μM S 16257. The data were obtained from six experiments. (d) Effect of 3 μM zatebradine on I_K activated with the same protocol as this illustrated in (a). Note the significant effect of 3 μM zatebradine on I_K.

was observed in presence of S 16257. Higher concentrations of S 16257 (10 μM) were needed to obtain a reduction of I_K amplitude similar to that observed with zatebradine. Moreover, Goethals *et al.* (1993) found a partial reduction of outward current by 1 μM zatebradine. In fact, the comparison between the effect of S 16257 and that of zatebradine in SA node tissue, papillary muscles and Purkinje fibres, shows that S 16257 and zatebradine similarly reduce the rate of diastolic depolarization of the cells. However, S 16257 causes less prolongation of the action potential duration than zatebradine (Thollon *et al.*, 1994). A prolongation of the action potential, associated with a slowing of the heart rate, can induce early after-depolarizations which might be a cause of polymorphic ventricular tachyarrhythmias. The effect of S 16257 on I_K seems to be temperature-independent, since similar results were obtained at 30°C in three cells (data not shown). Also Thollon *et al.* (1994), analysing the effect of different S 16257 concentrations (1 to 10 μM) on the action potentials of guinea-pig papillary muscles, have reported that at 36°C, a significant prolongation of AP duration was obtained only with 10 μM S 16257. Such observations are in favour of the absence of significant class III antiarrhythmic activity of the drug.

Pérez *et al.* (1995) reported that the fast sodium inward current was blocked by S 16257 at concentrations higher than 5×10^{-6} M. This block probably plays a role in the effects of S 16257 on the activity of Purkinje cells and sinus node subsidiary cells, where I_{Na} is present. In the primary pacemaker cells, it has been reported that the fast sodium inward current is absent or not normally activated (Irisawa *et al.*, 1993). Thus, a block of I_{Na} cannot be involved in the bradycardic action of S 16257 on these cells.

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