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Block of gating currents related to K^+ channels as a mechanism of action of clofilium and d-sotalol in isolated guinea-pig ventricular heart cells

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1 The possibility that the class III antiarrhythmic drugs clofilium and d-sotalol might affect delayed rectifier potassium channels at the level of their gating currents was assessed with the whole-cell patch-clamp technique in guinea-pig isolated ventricular heart cells.

2 Clofilium (up to 20 μM) and d-sotalol (1 μM) did not decrease the Na current, the L-type Ca current or the background K current i_{K1}, but significantly depressed the time-dependent delayed outward K current i_K.

3 Clofilium partially decreased in a dose-dependent manner (1–20 μM) Q_{ON} of intramembrane charge movements (ICM) elicited by a depolarizing pulse applied from a holding potential of −110 mV or following a 100 ms inactivating prepulse to −50 mV. D-sotalol (1 μM) also decreased Q_{ON}. Channel density estimated from the clofilium-sensitive ICM closely matched that of the delayed rectifier channels.

4 Clofilium and d-sotalol decreased Q_{OFF} seen on repolarization in a dose- and voltage-dependent manner. The kinetics of the decay of the OFF gating currents were not affected, and only the fast phase was depressed.

5 In control conditions, Q_{ON} availability with voltage was most of the time well described by two inactivating components. In the presence of clofilium and d-sotalol, a complex behaviour of Q_{ON} availability was observed, unmasking additional components. The reactivation kinetics of Q_{ON} after a 500 ms inactivating pulse to 0 mV was not affected.

6 We conclude that delayed rectifier K channels significantly contribute to Q_{ON} and Q_{OFF} of ICM in guinea-pig ventricular heart cells, besides Na and Ca channels, and that clofilium and d-sotalol directly interact with these K channels proteins by affecting their gating properties.

Keywords: Intramembrane charge movements; K^+ channels; native cardiac cells; class III antiarrhythmic drugs; clofilium, d-sotalol

Abbreviations: ICM, intramembrane charge movements, IPP50, inactivating prepulse to −50 mV

Introduction

Intramembrane charge movements (ICM) in excitable cells result from molecular rearrangements of ion channel proteins under the impetus changes in the membrane potential. In mammalian cardiac cells, only sodium and calcium channels have been thought to give rise to ICM recorded with short depolarizing pulses, because of the much slower activation kinetics of the delayed rectifier potassium current (see Field *et al*., 1988; Bean & Rios, 1989; Hadley & Lederer, 1989, 1991a; Hanck *et al*., 1990; Shirokov *et al*., 1992; 1993). However, we have found more than two components of ICM in guinea-pig ventricular myocytes (Malécot & Argibay, 1996) which could not be entirely suppressed by the Na and Ca channels blockers lidocaine, tetracaine, nifedipine and D600 (Malécot *et al*., 1997a,b), although the underlying currents are blocked. However, compounds such as D600 or nitrendipine have been shown to be able to suppress all of the gating currents in heart cells, but at high concentrations that also affect potassium currents (e.g., Hadley & Lederer, 1991a). These results suggest that other channels might also contribute to ICM in heart cells. Moreover, recent studies of different expressed cloned potassium channels have revealed their fast gating currents (Larsson *et al*., 1996; Fedida, 1997), which are very similar in shape to those recorded in isolated heart cells. Therefore, we have re-investigated the pharmacological properties of ICM in freshly isolated guinea-pig heart cells and, in particular, we have tested the hypothesis of the presence of a component of ICM originating from the voltage-activated delayed rectifier K channels. Because several antiarrhythmic drugs have been shown to affect gating currents in many preparations, we have tested in this paper the possibility that the class III antiarrythmic drugs, clofilium and d-sotalol, blockers of i_{Ks} and of i_{Kr} (for review see Baró & Escande, 1993; Snyders, 1995), might affect delayed rectifier potassium channels at the level of their gating currents. We show here that ICM activating at positive potentials are sensitive to clofilium and d-sotalol at concentrations not inhibiting the Na or the L-type Ca channels but depressing the delayed outward K current in our recording conditions. To our knowledge, this is the first time that K channels are shown to significantly contribute to ICM in freshly isolated heart cells, besides Na and Ca channels. This new finding opens the door to a better understanding of the relationships between ICM and membrane excitability and to the future development of more powerful pharmacological compounds. A preliminary note on these experiments has been published (Malécot & Argibay, 1998).

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Methods

Cell isolation

Guinea-pig heart cells were enzymatically isolated from the left ventricle as described elsewhere (Le Guennec *et al*., 1993). The experimental procedure was performed in accordance with the French ethical guidelines (authorization no 7741). Dissociated cells were placed in a small chamber, on the stage of an inverted microscope (Olympus IX70, Tokyo, Japan), continuously superfused by gravity with normal Tyrode solution containing (in mM): NaCl 140, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 1.8, NaH_2PO_4 0.33, HEPES buffer 10, glucose 11, pH adjusted to 7.3 with NaOH.

Solutions

The cells were locally superfused by gravity (small capillaries positioned within 50 μm of the cell and allowing fast solution changes). For the calcium current (I_{Ca}) experiments, the local superfusing saline contained (in mM): TEACl 140, $MgCl_2$ 1, HEPES buffer 10, $CaCl_2$ 1.8, and Glucose 11 (pH 7.3 with TEAOH). To record the fast sodium current I_{Na}, the saline was composed of (in mM): NaCl 30, TEACl 110, $CaCl_2$ 1, $MgCl_2$ 1, $CoCl_2$ 2, $GdCl_3$ 0.1, HEPES buffer 10, and Glucose 11 (pH 7.3 with TEAOH). The potassium currents were recorded in normal Tyrode solution to which 0.1 mM $CdCl_2$ was added to block the Ca current and to reduce the Na current. During the gating currents experiments, all ionic currents were blocked with a solution containing (in mM): TEACl 140, $MgCl_2$ 1, HEPES buffer 10, $CaCl_2$ 1, $CdCl_2$ 2, $GdCl_3$ 0.1, Tetrodotoxin (TTX) 0.001 and Glucose 11 (pH 7.3 with TEAOH). The pipette solution used to record I_{Na}, I_{Ca} or the gating currents contained (in mM): CsCl 110, TEACl 30, HEPES buffer 10, EGTA 10, Mg^{2+}-ATP 5 (pH 7.3 with CsOH). For the K currents, the pipette solution consisted of (in mM): NaCl 5, KCl 140, Mg^{2+}-ATP 5, EGTA 5, HEPES buffer 10 (pH 7.3 with KOH). Clofilium tosylate (Lilly, Indianapolis, IN, U.S.A.) was prepared as a 10^{-2} M stock solution in DMSO and then added to the external superfusing saline to reach the final concentration. Equivalent amounts of DMSO were added to the external control superfusing solutions. The highest final DMSO concentration was 0.5%. D-sotalol hydrochloride (kindly supplied by Bristol-Myers Squibb, Princeton, NJ, U.S.A.), prepared as a 10^{-2} M stock solution in distilled water, was directly added to the external superfusing solution.

Electrophysiological recordings and analysis

The whole-cell patch-clamp technique was used to study the ionic or the gating currents at room temperature (22–25°C). Patch pipettes (2.3 ± 0.08 MΩ, mean ± s.e., $n = 41$; range 1.5–2.9 MΩ) were pulled from thick wall borosilicate glass capillaries (Plowden & Thompson Ltd., Stourbridge, U.K.) with a Narishighe PB7 puller (Narishige, Tokyo, Japan) and were coated with dental wax to decrease their capacitances. An Axopatch 200A amplifier (Axon Instruments Inc., Foster City, CA, U.S.A.), connected to a Pentium 75 computer equipped with pClamp 6.0.3. software (Axon Instruments), through a Digidata 1200A interface (Axon Instruments), was used to control voltage and record currents. Data were analysed with Clampfit. The pipette and cell capacitances were compensated.

In order to have an adequate control of the membrane voltage and to improve the clamp speed, especially for the Na current and gating currents recordings, care was taken to select relatively small cells (membrane capacitance $C_m = 82.3 \pm 3.2$ pF, $n = 36$; range 46.7–120 pF) to carry out the experiments. Cells for which the membrane capacitance varied by more than 2% during the time course of the experiments were discarded and not included in the analysis. The series resistance was also compensated by 80%: this value was chosen to avoid saturation of the electronical circuit of the Axopatch 200A with the large voltage commands used. The mean time constant of the clamp speed, determined from the residual series resistance after compensation (0.86 ± 0.04 MΩ, $n = 41$) and the mean cell capacitance, was estimated to 70.8 μs. Thus, in most of the cells, more than 98% of the command voltage was seen by the membrane in less than 300 μs. For the Na current and gating currents experiments, the data were acquired at 25 kHz and filtered with a 4-pole lowpass Bessel filter at 5 kHz (Na current) or 1 kHz (gating currents): these recording conditions of the gating currents have been successfully used in heart cells by others (e.g., Shirokov *et al*., 1992). It should be noted that due to the residual R_s and cell capacitance, the preparation intrinsic mean filter frequency was about 2.25 kHz. Control experiments made with the filter frequency set at 10 kHz and the use of an over-sampling data acquisition frequency (200 kHz) showed that no information regarding the charge measurements (maximum charge moved Q_{max}, and voltage dependence) was lost with the 25 kHz sampling rate and 1 kHz filter frequency (not shown). Thus, the 1 kHz setting was routinely used, as the recordings were much less noisy than with the 10 kHz setting.

Since the residual leak current was found to be linear between −140 and +20 mV in the presence of the blockers of ionic currents used to record the gating currents (not shown), a P/5 subtraction protocol (Bezanilla & Armstrong, 1977), used to remove leak and linear capacitive currents, was applied before the test pulse or before the conditioning pulse from a sub-holding potential of −120 mV (regular holding potential of −110 mV). Control pulses were of the same polarity as the test pulses. Considering the mean time constant of recovery from inactivation of the charges at −120 mV, following a 500 ms inactivating pulse, a 100 ms settling time (more than 99.9% of recovery) was used. The stimulation frequency was 0.1 Hz. These recording conditions have been shown by Shirokov and co-workers (Shirokov *et al*., 1992) to avoid contamination of the control recordings by non linear charge movements of the charge 2 type. The Na current was recorded in the same conditions. For the calcium and the potassium currents experiments, the P/5 subtraction protocol was not used. Calcium and potassium currents were recorded from a holding potential of −80 mV by applying depolarizing pulses at 0.1 Hz for the Ca current and at 0.05 Hz (I-V curves) and 0.033 Hz (drug application) for the potassium current. The background K current was recorded by applying a slow (10.18 mV/s) depolarizing ramp from −80 to +60 mV. The acquisition frequencies were usually of 1 and 6.6 kHz for the potassium and for the calcium currents experiments, respectively.

Mobile charges were measured by integrating the first 20 ms of gating currents during depolarization (Q_{ON}) or repolarization (Q_{OFF}), after defining the steady-state current level for at least 5 ms as baseline. The voltage dependence of charge movements was quantified according to a two-state system, as discussed in details by Rios & Pizzarro (1991) and used by several authors to describe charge movements in heart cells (e.g., Bean & Rios, 1989; Hadley & Lederer, 1989; 1991a,b; Hanck *et al*, 1990; Shirokov *et al*., 1992; 1993). Activation curves were obtained by fitting the data with the following double Boltzman equation:

$$Q_{ON} = Q_{max,1}/[1+\exp\{(V-V_{1/2,1})/K_1\}] + Q_{max,2}/[1+\exp\{(V-V_{1/2,2})/K2\}] \quad (1)$$

where $Q_{max,1}$ and $Q_{max,2}$ are the maximal charge of the two components of charges (with no assumption regarding their origin) displaced by strong depolarizations and are proportional to the number (n) of mobile electron charges (e^-) and to the apparent valence of the charge moved (z′): $Q_{max} = nz'e^-$. Subscripts 1 and 2 refer to voltage-dependent components activating firstly (1 or 'negative') and secondly (2 or 'positive') with increasing the voltage. z′ is related to the real charge z by the relation $z' = z\delta$, with δ ($0 \leqslant \delta \leqslant 1$) representing the fractional distance over which the charge moves within the membrane. The slope factors K_1 and K_2 are inversely proportional to z′ ($K = kT/z'e^-$ where k is the Boltzman constant and T the absolute temperature). $V_{1/2,1}$ and $V_{1/2,2}$ are the voltages for the half maximal activation of the negative and positive (i.e., less negative) components, respectively. A similar equation was used to describe the inactivation of the charge with voltage. Q_{ON} and Q_{OFF} were normalized to the cell capacitance C_m and are expressed in nC/μF when quantified. More details are given in the text when appropriate.

Statistics

Data are presented as mean values ± s.e., with the number of experiments indicated as *n*. Statistical significance was assessed with the paired Student's *t*-test or one-way analysis of variance (ANOVA). The choice of the model used to fit the experimental data with Boltzman equations (1 *vs* 2 or 3 components) was determined by least square fitting and statistical testing using the theory of nested models, as proposed by Horn (1987). A *P* value less than 0.05 was considered as statistically significant.

Results

Clofilium and d-sotalol effects on ionic currents

To determine the relative specificity of the class III antiarrhythmic drug clofilium and d-sotalol in our recording conditions, we first tested their potential effects on the main ionic currents present in guinea-pig ventricular myocytes, i.e., the sodium, L-type calcium, background and delayed outward potassium currents. Typical examples of the effects of clofilium are shown in Figure 1. Clofilium at concentrations up to 20 μM (Figure 1A) had no significant effect on the peak Na current (10 μM: +2.7 ± 2.2%, $n = 4$; 20 μM: −1.4 ± 0.6%, $n = 7$), nor on its voltage dependence (control characteristics: threshold of activation: −53.9 ± 1.4 mV; voltage of maximum peak current: −15.4 ± 1.5 mV; reversal potential: +54.6 ± 2.6 mV: $n = 24$) or steady-state voltage-dependent inactivation (not shown; half inactivating potential: −66.3 ± 0.14 mV, $n = 12$). Under our experimental conditions, (i.e., in the presence of DMSO both in control and drug-containing solutions), clofilium at 20 μM (DMSO concentration of 0.2%) consistently induced an unexpected increase of I_{Ca} elicited from −80 to 0 mV (Figure 1B; the mean increase observed in four cells was +44.9 ± 8.1%), which was fully reversible upon wash-out. We have also observed in six other cells an increase of I_{Ca} in the presence of 10 μM clofilium (not shown). Figure 1C illustrates that clofilium at 10 μM had no effect on the background K current (inward rectifier) elicited with a slow voltage ramp (see legend; potentials between −80 and −15 mV), but markedly decreased the outward current for potentials more positive than −15 mV, consistent with its inhibitory effect on the time-dependent delayed outward K current elicited with depolarizing voltage pulses, as shown in Figure 1D. Total tail K current measured on repolarization to −40 mV following a 3 s

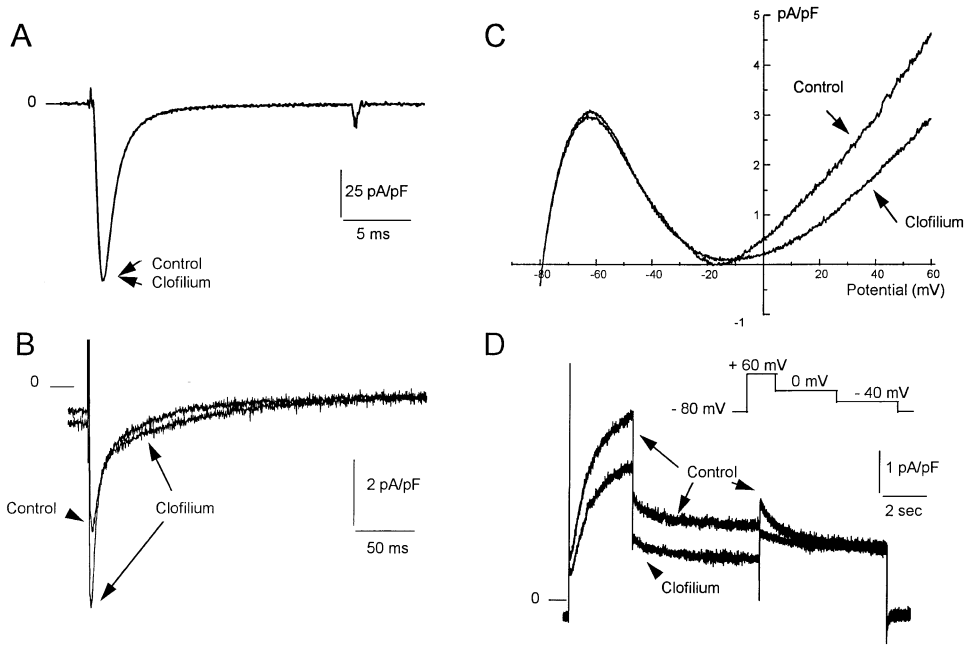

Figure 1 Effects of clofilium on the Na current (A), the Ca current (B), the background K current (i_{K1}, C), and the delayed rectifier K current (i_K, D) recorded in guinea-pig ventricular heart cells. (A) Na current recorded with 20 ms depolarizing pulse applied from −110 to −15 mV under control conditions and in the presence of 20 μM clofilium (cell C2110). (B) Ca current recorded with a 250 ms depolarizing pulse applied from −80 to 0 mV under control conditions and in the presence of 20 μM clofilium (cell E509). (C) background i_{K1} current recorded with a voltage ramp (10.18 mV/s) applied from −80 to +60 mV, under control conditions and in the presence of 10 μM clofilium (cell A1910). (D) delayed rectifier i_K current recorded, under control conditions and in the presence of 10 μM clofilium, with the protocol shown in inset. The pulses durations were 3 s at +60 mV to activate i_K, and 6 s at 0 and −40 mV to record mainly the slow i_{Ks} and the fast i_{Kr} tail currents, respectively (although part of i_{Ks} also contributes to the tail current at −40 mV: cell A1910).

activating pulse to +60 mV was decreased by 72.4±12.0% ($n=5$) after exposure to 10 μM clofilium. This inhibitory effect was barely reversible after 10 min of wash-out. Thus, under our experimental conditions, only the delayed outward K current appeared to be depressed by clofilium. Figure 2 illustrates typical results obtained in the presence of 1 μM d-sotalol. We observed no significant effect on the peak Na (Figure 2A; $n=5$ cells) and L-type Ca currents (Figure 2B; $n=6$ cells), as well as on the background K current (inward rectifier, Figure 2C; potentials between −80 and −15 mV). The only inhibitory effect observed in our recording conditions was on the time-dependent outward K current (Figure 1D). Total tail K current measured on repolarization to −40 mV following a 3 s activating pulse to +60 mV was decreased by 15.9±3.0% ($n=7$) after exposure to 1 μM d-sotalol.

Clofilium decreases a component of ICM

Figure 3 shows a typical example of the effects of 10 μM of clofilium on the gating currents recorded when all ionic currents are blocked. Figure 3A shows families of gating currents recorded by applying 20 ms depolarizing pulses to various voltages from an HP of −110 mV. The current in response to depolarizing pulses consisted of a transient outward current (Q_{ON}) corresponding to the displacement of intramembrane charges across the transmembrane electric field. On repolarization, all the charges that have moved during the depolarization returned to their initial position, giving rise to a transient inward current (Q_{OFF}). Both Q_{ON} and Q_{OFF} components increased with increasing the test potential, as shown in Figure 3A left, but reached saturation at positive potentials (see Figure 4A), as expected from charges displacements, as opposed to charging of the membrane capacitance (which does not saturate with potential). In the presence of 10 μM clofilium (Figure 3A right), both Q_{ON} and Q_{OFF} components are decreased, especially for the more positive test potentials. Thus, clofilium apparently decreased the gating currents with voltage.

Figure 3B illustrates the effect of 10 μM clofilium when the pulse length is increased. In control conditions, when the cell is depolarized by applying a voltage pulse from a holding potential (HP) of −110 to +20 mV and the pulse duration increased from 2 to 38 ms, there is little variation in the amplitude of the Q_{OFF} seen on repolarization to −110 mV (Figure 3Ba left). In the presence of clofilium, not only the Q_{ON} component is depressed, but also Q_{OFF} markedly decreased with increasing the pulse length (Figure 3Ba right), an effect consistent with the reported clofilium-induced time-dependent block of K channels (e.g., Snyders, 1995). No evidence for a use-dependent effect of clofilium was observed (not shown). This depressing effect of clofilium on ICM is better seen in Figure 3Bb which shows on an expanded time scale the Q_{ON} and Q_{OFF} of the 10th pulse (i.e., 20 ms). Normalization of Q_{OFF} in the presence of clofilium to that in control conditions revealed no changes in its kinetics of decay (not shown; such an approach was made possible because of the clamp speed time constant in the cells used: 87.3 μs in the example presented in Figure 3B.). This was confirmed by direct kinetics analysis of the decay of the OFF component at −110 mV by adjusting a double exponential. The mean fast and slow time constant of decay of the OFF gating current were 0.4±0.04 and 5.4±0.16 ms in control conditions and 0.4±0.04 and 5.8±0.61 ms in the presence of clofilium, respectively ($P>0.5$, $n=4$). As shown below, only the amplitude of the fast phase of decay was significantly affected: from −1170.7±83.7 to −1007.4±68.7 pA at the peak gating current for a test pulse to +20 mV, $n=4$. At this potential the slow phase amplitude determined also at the peak gating current was −77.7±10.7 pA in control conditions and −67.5±5.7 pA in the presence of clofilium (see Figure 4Ba).

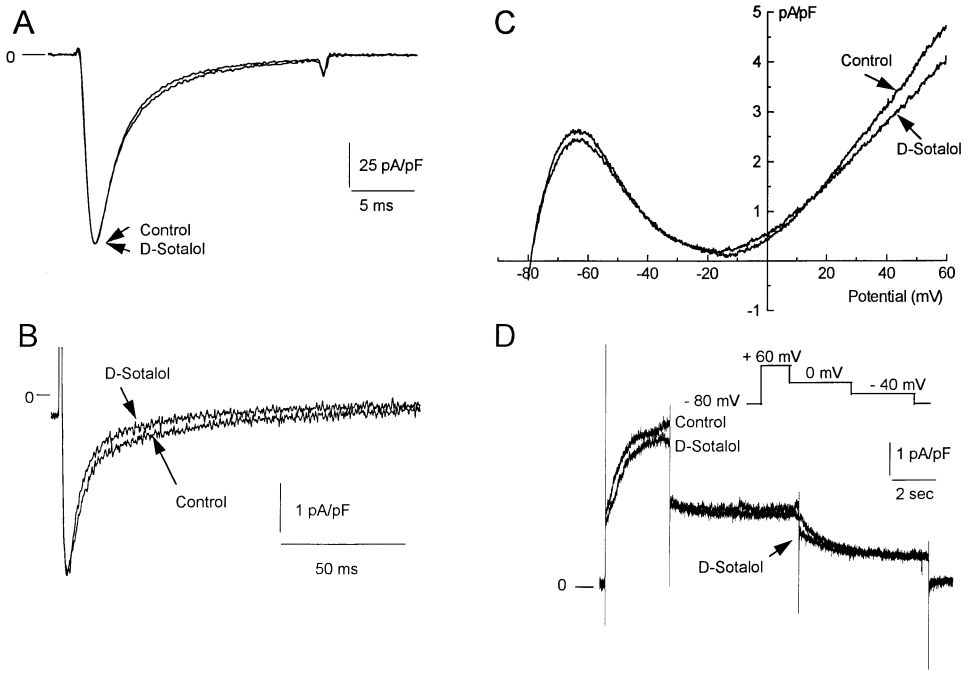

Figure 2 Typical examples of the effects of d-sotalol on the Na current (A), the Ca current (B), the background K current (i_{K1}, C), and the delayed rectifier K current (i_K, D) recorded in guinea-pig ventricular heart cells with the same protocols as those described in Figure 1. Lack of effect of 1 μM d-sotalol on the amplitude of the Na current (A; test pulse to −15 mV, cell E2710) and of the Ca current (B; test pulse to 0 mV, cell C1610). (C) background i_{K1} current recorded under control conditions and in the presence of 1 μM d-sotalol (cell C1711). (D) delayed rectifier i_K current recorded under control conditions and in the presence of 1 μM d-sotalol (cell B2511).

The lack of effect of clofilium on the amplitude of the slow phase of decay of the OFF charges (which has been shown by Shirokov *et al*. (1992) to correspond to the charge 2, i.e., charges in the inactivated mode) suggests that clofilium did not induce a mere interconversion between charge 1 and charge 2 (if this were the case, such an interconversion should have resulted in an increase of the amplitude of the slow phase of decay of the OFF component), but more likely a true partial block of the charges. However, the exact mechanism by which clofilium decreases the gating currents (immobilization or charge 1-charge 2 interconversion) awaits further studies.

The mean voltage dependence of Q_{ON} activation determined in four cells is shown in Figure 4A. Clofilium (10 μM) decreased Q_{ON} for potentials more positive than -30 mV, both from an HP of -110 mV (Figure 4Aa), or following a 100 ms inactivating prepulse to -50 mV (100 ms IPP50) applied from the same HP (Figure 4Ab) to inactivate most ICM originating from sodium channels, as reported by others (i.e., Bean & Rios, 1989; however, see below). This can also be seen with the curve (open diamonds) illustrating the voltage dependence of the clofilium-sensitive component. From the HP of -110 mV, the clofilium-sensitive charge movements saturated with potentials and amounted to 2.13 nC/μF. As discussed later, this value is close to the amount of charge that could be expected for the guinea-pig delayed rectifier channels (see Discussion).

The voltage dependences of the mean amplitudes ($n=4$ cells) of the decaying phases of Q_{OFF} gating currents elicited on repolarization to either -110 mV (HP $=-110$ mV) or to -50 mV (following a 100 ms IPP50) from various potentials, are illustrated in Figure 4B. The amplitudes of the fast and slow decaying phases of Q_{OFF} have been determined from a two-exponential fit extrapolated to time of peak OFF gating current on repolarization. Clofilium had no significant effect on the slow phase, whereas it markedly reduced the fast phase for potentials more positive than -20 mV. Clofilium effect was more pronounced at positive voltages, especially on repolarization to -50 mV (Figure 4Bb) suggesting a voltage-dependent decrease of Q_{OFF}.

The dose dependence of clofilium was assessed with concentrations ranging from 1 to 50 μM. In each cell, Q_{ON} activation with voltage was best described ($P<0.001$) by a double Boltzman relationship (Equation 1), as described in the methods section, either from the HP of -110 mV or following a 100 ms IPP50, in agreement with previous findings (Bean & Rios, 1989; Hadley & Lederer, 1989; 1991a; Malécot & Argibay, 1996). The parameters used to fit the experimental data in every cell were averaged for each concentration and are given in Figures 5 and 6 for the first (i.e., activating at negative potentials, hatched bars; this component will be referred to as 'negative') and for the second (i.e., activating at potentials more positive – or less negative than – the first component, grey bars; this component will be refered to as 'positive') components of Q_{ON} activation from the HP of -110 mV (Figure 5) and following a 100 ms IPP50 (Figure 6).

From an HP of -110 mV, the negative ($V_{1/2}=-26$ mV, $n=51$ cells) and positive ($V_{1/2}=-1.3$ mV, $n=51$) components were previously characterised as ICM originating mostly from Na channels and from L-type Ca channels, respectively. Following a 100 ms IPP50, the first negative component ($V_{1/2}=-27$ mV, $n=51$) was found to correspond to non inactivated Na channels and to L-type Ca channels, and the positive one ($V_{1/2}=2.7$ mV, $n=51$) mostly to L-type Ca channels (Malécot & Argibay, 1996; Malécot *et al*., 1997a,b). In the presence of clofilium, from an HP of -110 mV, the maximum Q_{ON} components ($Q_{max,1}$ and $Q_{max,2}$) were depressed (Figure 5A) for concentrations higher than 1 μM, the effect being mostly on the negative component (component 1, hatched bars). This effect was accompanied by either a positive (1 and 10 μM) or a negative (20 and 50 μM) shift of the $V_{1/2}$ of activation (Figure 5B), and by a decrease in the slope factor K for concentrations above 1 μM (Figure 5C). This last effect,

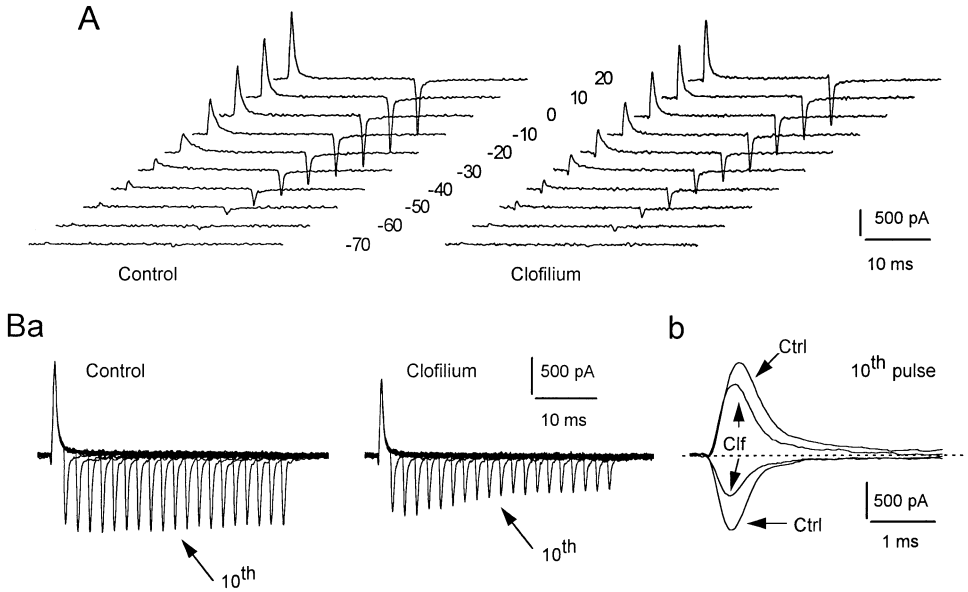

Figure 3 Effect of 10 μM clofilium on gating currents recorded in a guinea-pig ventricular heart cell. (A) Typical recordings of gating currents under control conditions (left) and after application of 10 μM clofilium (right). The currents were elicited by depolarizing the membrane for 20 ms to the potentials (mV) indicated with each trace ($C_m=73$ pF; residual $R_s=1.2$ MΩ; clamp time-constant $=87.6$ μs). (B) Superimposed currents elicited by depolarizing the cell from -110 V to $+20$ mV and increasing the pulse duration from 2 to 38 ms in 2 ms steps (a). The 10th pulse (20 ms depolarization) is shown on an expanded time scale in (b). Ctrl: control conditions; Clf: in the presence of 10 μM clofilium. The dotted line corresponds to the zero current level. In (a) and (b), upwards and downwards deflections correspond to the Q_{ON} and to the Q_{OFF} components of gating currents seen on depolarization and on repolarization, respectively ($C_m=74$ pF; residual $R_s=1.18$ MΩ; clamp time constant $=87.3$ μs).

explained by an increase of the apparent valency z′ of the charge displaced during depolarisation (since $K = kT/z'e$, see Methods), and the decreases of $Q_{max,1}$ and of $Q_{max,2}$ indicate that clofilium decreased the number n of elementary charges moving during depolarization, and this, for the two components of charges activation.

Following a 100 ms IPP50, clofilium had no effect on the negative component of Q_{ON} (i.e., on that corresponding to ICM originating from non inactivated Na channels and from Ca channels), but induced a dose-dependent decrease of the positive one at all concentrations tested (Figure 6A). This inhibitory effect was accompanied by a decrease in the slope factor of the Boltzman relationship (Figure 6C), indicating again an increase of the apparent valency z′ of the charge moving with voltage. Because $Q_{max,2}$ also decreased, this implies that the number n of charges displaced during depolarization decreased in the presence of clofilium. At 1 μM, clofilium induced a 12 mV positive shift of the $V_{1/2}$ of the positive component. No shift could be detected at higher concentrations. Thus, clofilium decreases the maximum of charges moved during membrane depolarization under our experimental conditions. Because of the K channel blocking properties of clofilium, the effects we observed might indeed correspond to a partial block of ICM originating from the delayed rectifier K channels (see also Discussion). On the contrary to its effect on the Ca current (see below and Figure 1B), all these effects of clofilium on the gating currents were barely reversible, in agreement with the reported lack of reversibility of the K current block (Arena & Kass, 1988; this study).

Effects of d-sotalol on ICM

The difference of effects of clofilium on the negative or positive Q_{ON} components activated either from HP = −110 mV, or after a 100 ms IPP50, could be taken as an indication that the potassium channels involved might be different. This prompted us to look at the effects of d-sotalol, a selective blocker of i_{Kr}, the fast component of the delayed rectifier potassium current. Figure 7A shows the typical effect of 1 μM d-sotalol on the gating currents. A representation similar to that of the effects of clofilium (Figure 3B) was used. From the HP of −110 mV, d-sotalol had a small depressing effect on Q_{ON} elicited at +20 mV (Figure 7Ab), but like clofilium, induced a time-

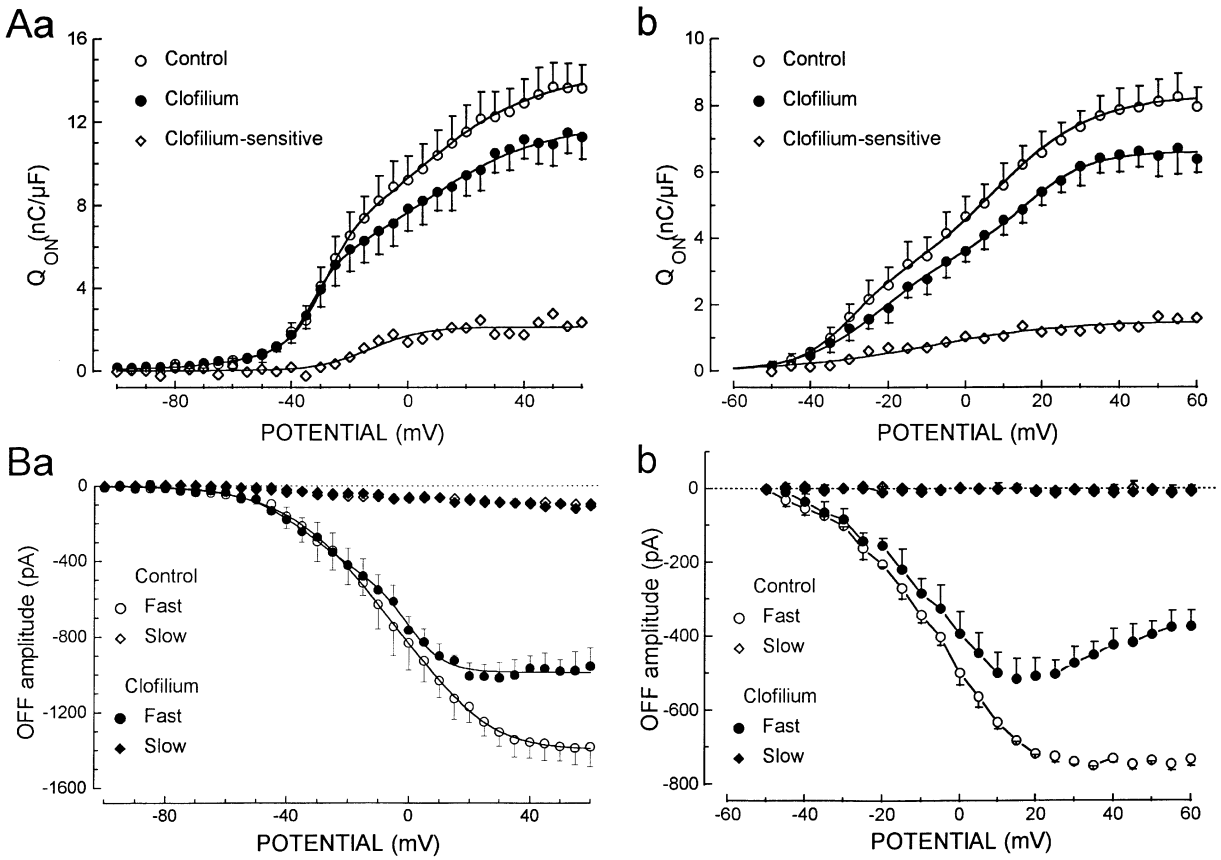

Figure 4 (A) Mean charge-voltage relationships of Q_{ON} ($n = 4$ cells) elicited from an HP of −110 mV (a) and after a 100 ms IPP50 (b) in control conditions (open circles) and in the presence of 10 μM clofilium (filled circles). Open diamonds: voltage dependence of the mean clofilium-sensitive Q_{ON} component. The smooth curves are best fits to the mean data points with equation (1) for the control conditions and in the presence of clofilium, with a single Boltzman relationship for the clofilium-sensitive Q_{ON} component. (a) control: $Q_{max,1} = 4.8$ nC/μF, $V_{1/2,1} = -29$ mV, $K_1 = 5.3$ mV, $Q_{max,2} = 9.6$ nC/μF, $V_{1/2,2} = 2.3$ mV, $K_2 = 21$ mV; clofilium: $Q_{max,1} = 3.7$ nC/μF, $V_{1/2,1} = -33$ mV, $K_1 = 4.5$ mV, $Q_{max,2} = 8.3$ nC/μF, $V_{1/2,2} = 1.8$ mV, $K_2 = 22$ mV; clofilium-sensitive: $Q_{max} = 2.13$ nC/μF, $V_{1/2} = -12.7$ mV, $K = 9.1$ mV. (b) control: $Q_{max,1} = 2.5$ nC/μF, $V_{1/2,1} = -30$ mV, $K_1 = 6.5$ mV, $Q_{max,2} = 5.8$ nC/μF, $V_{1/2,2} = 7.5$ mV, $K_2 = 13$ mV; clofilium: $Q_{max,1} = 3.6$ nC/μF, $V_{1/2,1} = -22$ mV, $K_1 = 10$ mV, $Q_{max,2} = 3.0$ nC/μF, $V_{1/2,2} = 16$ mV, $K_2 = 8$ mV; clofilium-sensitive: $Q_{max} = 1.49$ nC/μF, $V_{1/2} = -9.6$ mV, $K = 18$ mV. (B) Mean ($n = 4$) current-voltage relationships of the amplitudes of the fast (circles) and slow (diamonds) phases of decay of the OFF gating current determined by double exponential fits (current amplitudes at the time of the peak of the OFF gating current) in control conditions (open symbols) and in the presence of 10 μM clofilium (filled symbols). (Ba) on repolarization to −110 mV following a 20 ms depolarizing pulse applied from −110 mV to voltages shown in abscissa. (Bb) on repolarization to −50 mV from voltages indicated in abscissa, following a 100 ms IPP50 applied from −110 mV. Data points represent mean values (n = four different cells) and vertical bars ± s.e.

dependent inhibition of the Q_{OFF} (Figure 7Aa and Ab). The mean effect of 1 μM d-sotalol on the voltage dependence of Q_{ON} activation observed in four cells is given in Figure 7B. Like clofilium, d-sotalol decreased the amount of Q_{ON} at potentials more positive than −30 mV, both from the HP of −110 mV (Figure 7Ba) or following a 100 ms IPP50 (Figure 7Bb). It also decreased the Q_{OFF} component, seen on repolarization to −50 mV, for potentials more positive than 0 mV (Figure 7Bc), suggesting again a voltage-dependent effect on the OFF component. Figures 5 and 6 summarize these effects of d-sotalol on the two Q_{ON} components elicited from an HP of −110 mV without (Figure 5) or with (Figure 6) a 100 ms IPP50. At 1 μM, d-sotalol significantly decreased the negative (hatched bars) and positive (grey bars) components

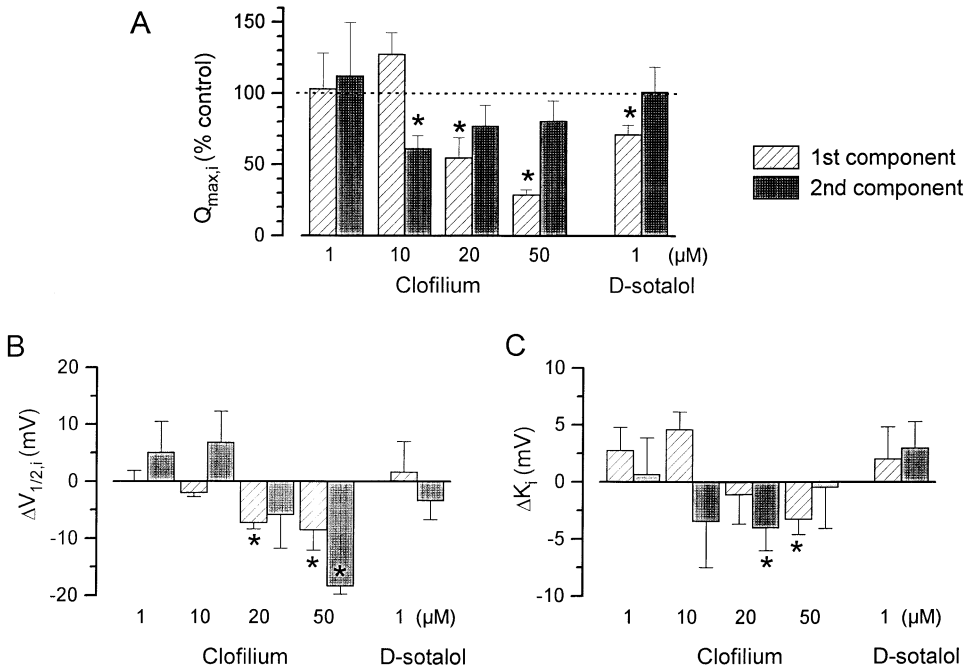

Figure 5 Histograms of the mean effects of clofilium and of d-sotalol on the two components of Q_{ON} activation obtained from an HP of −110 mV. The figure shows the mean effects on the maximum amplitudes (A) of the two Q_{ON} components ($Q_{max,1}$ and $Q_{max,\ 2}$ are expressed as percent of their control values), the mean changes on the half-activating potentials $V_{1/2,i}$. (B) and the mean changes in the slopes K_i (C) of the Boltzman equations used to fit the experimental data points for each cell. Hatched bars and grey bars correspond to the variations of the parameters of the negative (1) and of the positive (2) components, respectively. In (B) and (C), positive values correspond to a positive shift of $V_{1/2}$ and to an increase of K. The vertical bars represent s.e. and the stars indicate statistical difference from control. $n=4$ for each concentration, except for 50 μM ($n=2$).

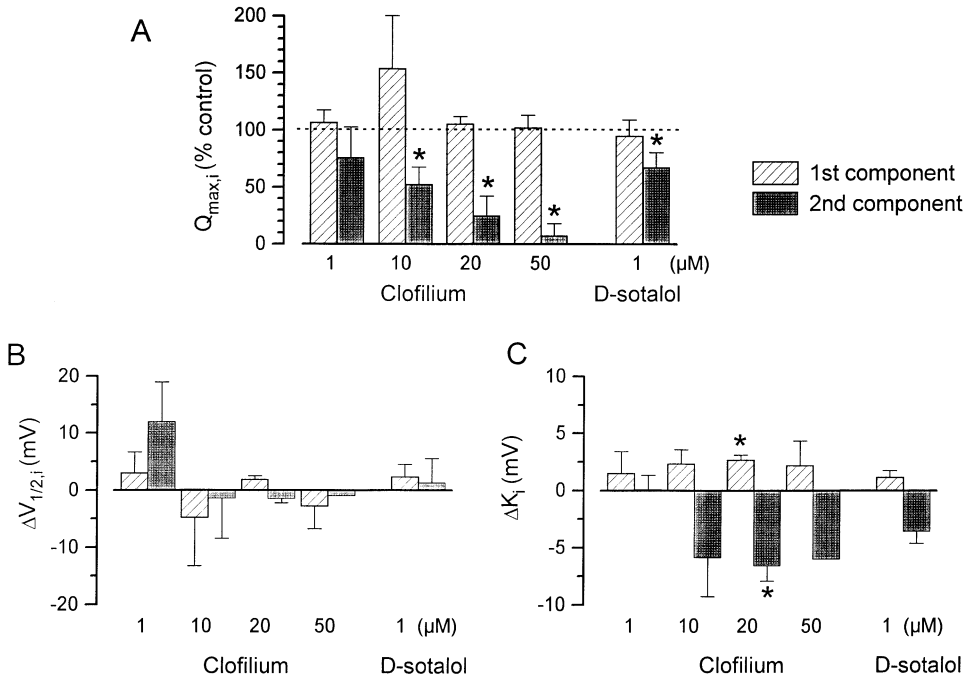

Figure 6 Histograms of the mean effects of clofilium and of d-sotalol on the two components of Q_{ON} activation obtained after a 100 ms IPP50. Same representation as in Figure 5 (see legend for details).

elicited respectively from the HP of −110 mV and after a 100 ms IPP50. No significant effects were observed on their $V_{1/2}$ or K. At this concentration, d-sotalol had an effect similar to that of 10 μM clofilium on Q_{OFF} seen on repolarization to −50 mV (Figure 4Bb). Thus, since no significant changes in the slope factor K were observed, the depressing effects of d-sotalol can be explained by a decrease in the number *n* of elementary charges moving during depolarization.

Effects of clofilium and d-sotalol on Q_{ON} and Q_{OFF} availability with voltage

The voltage dependence of Q_{ON} availability, or inactivation to parallel the terminology used for the ionic currents, was assessed as described in the legend of Figure 8, i.e., using a 1 ms return to the holding potential between the 500 ms conditioning pulse and the test pulse to +20 mV (see also Figure 8 inset). Complete inactivation of Q_{ON} occurred in these conditions (Figure 8), was biphasic and well described by a double Boltzman function. (Note: The observation of a full (this study) or of a partial inactivation of Q_{ON}, as commonly reported (e.g., Bean & Rios, 1989; Hadley & Lederer, 1989; 1991a) strongly and only depends on the interpulse duration used, because of the very fast reactivation kinetics of the gating currents at −100 mV ($\tau_{fast} = 3.1 \pm 0.9$ ms; $\tau_{slow} = 28.6 \pm 7.3$ ms; $n = 9$; the fast component representing ≈50% of the recovery process. This is about two times faster than the recovery process of the sodium current measured in the same conditions – not shown). The time at which the P/5 subtraction protocol is applied (before the conditioning prepulse or after the test pulse; not shown) had no influence on the measured Q_{ON}, thus excluding the possibility that full inactivation of Q_{ON} with voltage might be due to an interconversion charge 1-charge 2 occurring during the 500 ms conditioning prepulse. A cell damage can also be excluded, as there is indeed a very good agreement between the size of the measured non inactivating component for a given cell with a given interpulse duration and that of the expected non inactivating one determined by the calculus made from the time constants of recovery of its gating current (not shown)).

In control conditions, using a 1 ms interpulse duration, the mean $V_{1/2}$ and slope factors K calculated for 21 cells were -68.2 ± 0.97 and -9.2 ± 0.53 mV for the first

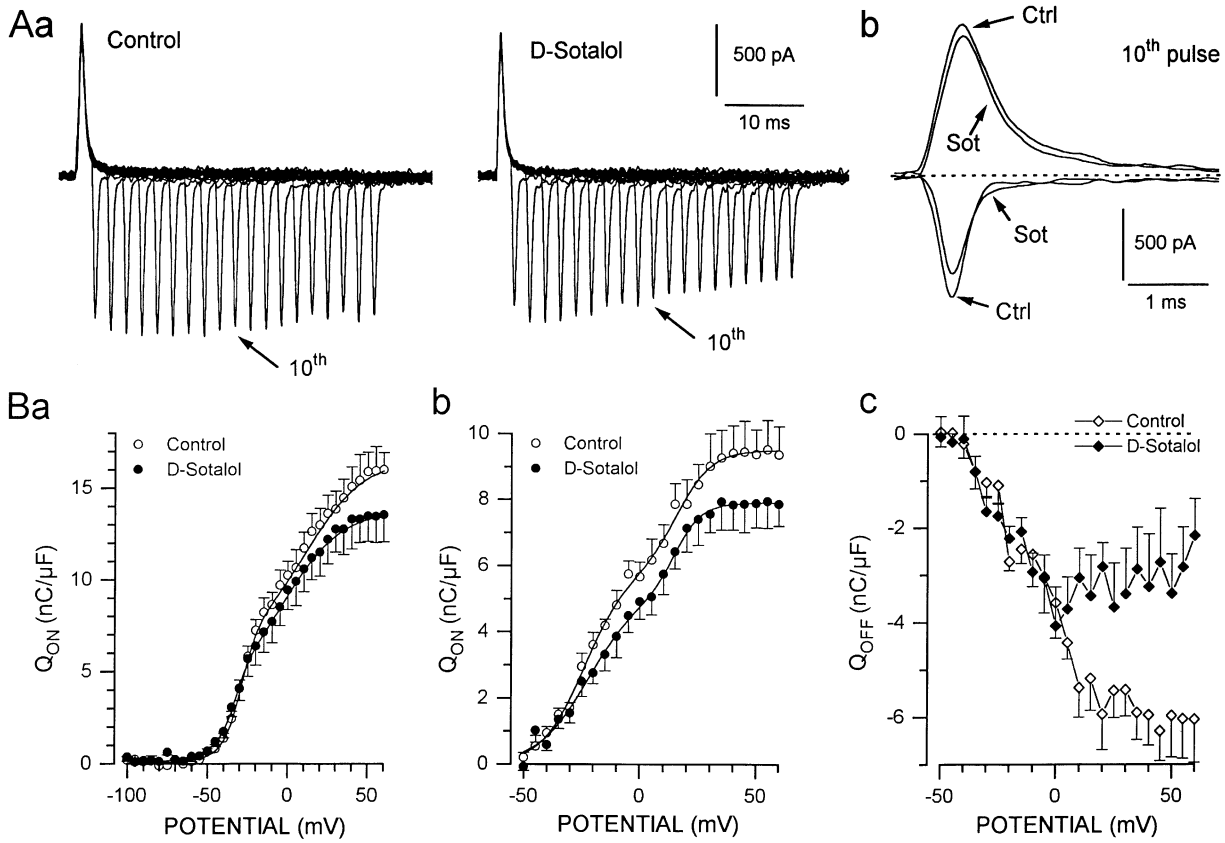

Figure 7 Effects of 1 μM d-sotalol on the gating currents of guinea-pig ventricular heart cells and their voltage dependence. (A) Currents elicited by depolarizing the cell from −110 to +20 mV and increasing the pulse duration from 2 to 38 ms in 2 ms steps (a). The 10th pulse (20 ms depolarization) is shown on an expanded time scale in (b). Ctrl : control conditions; Sot : in the presence of d-sotalol. The dotted line corresponds to the zero current level. In (a) and (b), upwards and downwards deflections correspond to the Q_{ON} and to the Q_{OFF} components of gating currents seen on depolarization and on repolarization, respectively (cell A1902; $C_m = 80$ pF; residual $R_s = 1.02$ MΩ; clamp time constant = 81.6 μs). (B) Mean charge-voltage relationships of Q_{ON} elicited from an HP of −110 mV (a) and after a 100 ms IPP50 (b) in control conditions (open circles) and in the presence of 1 μM d-sotalol (filled circles). Data points are mean values of four cells and vertical bars represent s.e. The smooth curves are best fits to the mean data points with equation (1). (a) Control: $Q_{max,1} = 6.6$ nC/μF, $V_{1/2,1} = -29$ mV, $K_1 = 5.4$ mV, $Q_{max,2} = 10$ nC/μF, $V_{1/2,2} = 12$ mV, $K_2 = 18$ mV: d-sotalol: $Q_{max,1} = 3.8$ nC/μF, $V_{1/2,1} = -33$ mV, $K_1 = 4.7$ mV, $Q_{max,2} = 10$ nC/μF, $V_{1/2,2} = -2.6$ mV, $K_2 = 18$ mV. (b) Control: $Q_{max,1} = 5.8$ nC/μF, $V_{1/2,1} = -24$ mV, $K_1 = 9.2$ mV, $Q_{max,2} = 3.7$ nC/μF, $V_{1/2,2} = 16$ mV, $K_2 = 7.5$ mV; d-sotalol: $Q_{max,1} = 5.2$ nC/μF, $V_{1/2,1} = -22$ mV, $K_1 = 11$ mV, $Q_{max,2} = 2.7$ nC/μF $V_{1/2,2} = 15$ mV, $K_2 = 5.6$ mV. (c) Mean charge-voltage relationships of Q_{OFF} recorded on repolarization to −50 mV from voltages indicated in abscissa, following a 100 ms IPP50 in control conditions (open diamonds) and in the presence of 1 μM d-sotalol (filled diamonds). Data points represent mean values ($n = 4$ different cells) and vertical bars ± s.e.

(negative) component of inactivation, and -14.2 ± 1.1 and -7.8 ± 0.46 mV for the second (positive) one, respectively. These two components have been previously characterized, in a pharmacological study using lidocaine, tetracaine, nifedipine and D600 (Malécot *et al.*, 1997a,b), as ICM originating mostly from Na channels for the negative one, and mostly from Ca channels for the positive one, in agreement with results from other laboratories (e.g., Bean & Rios, 1989). The respective participation of both components varied considerably from cell to cell, as can be seen in Figure 8Aa and Ba. In the presence of 1 μM clofilium (Figure 8Aa), the amplitude of component 2 was decreased by about 16.5% with no changes in the slope factor K, as can be better seen in Figure 8Ab where the two Boltzman components are individually shown in control conditions (dotted lines) and in the presence of clofilium (solid lines). No changes in component 1 occurred. However, a more complex figure was revealed in the presence of 10 μM clofilium (Figure 8B): three Boltzman functions (1′, 1″ and 2′; Figure 8Bb) are needed to best describe the voltage dependence of Q_{ON} inactivation. In the presence of clofilium, component 1 ($V_{1/2}$ of -69.1 mV) is split into two components, 1′ and 1″, with respective $V_{1/2}$ of -80.7 and -46.3 mV, and component 2 is decreased by 44.6%, with no significant changes in its $V_{1/2}$ (from -11.1 to -12.2 mV). This decrease is similar to that of the second component of Q_{ON} activation from the HP of -110 mV observed in the presence of the same concentration of clofilium (38–47%, Figure 5A). Component 1″ might correspond to a third component of Q_{ON} inactivation with voltage, which was masked in control conditions by the large component 1 (this component 1″ has been found to be present in some cells in control conditions; not shown). For higher clofilium concentrations (20 μM), only two components of inactivation can be discerned, likely because of the large negative shift of both components associated with a more pronounced decrease of the second control component (not shown). These effects of clofilium on Q_{ON} availability with voltage do not appear to be an artifact arising from our experimental protocol (1 ms repolarizing pulse to -100 mV) as the time constant of reactivation of Q_{ON} were not significantly affected in the presence of 10 μM clofilium [τ_{fast}: 3.0 ± 0.5 ms (control) and 2.4 ± 0.2 ms (clofilium; $P=0.071$); τ_{slow}: 30.4 ± 5.3 ms (control) and 19.5 ± 3.6 (clofilium; $P=0.16$); $n=3$]. It should be noted that the 500 ms conditioning prepulse used had no

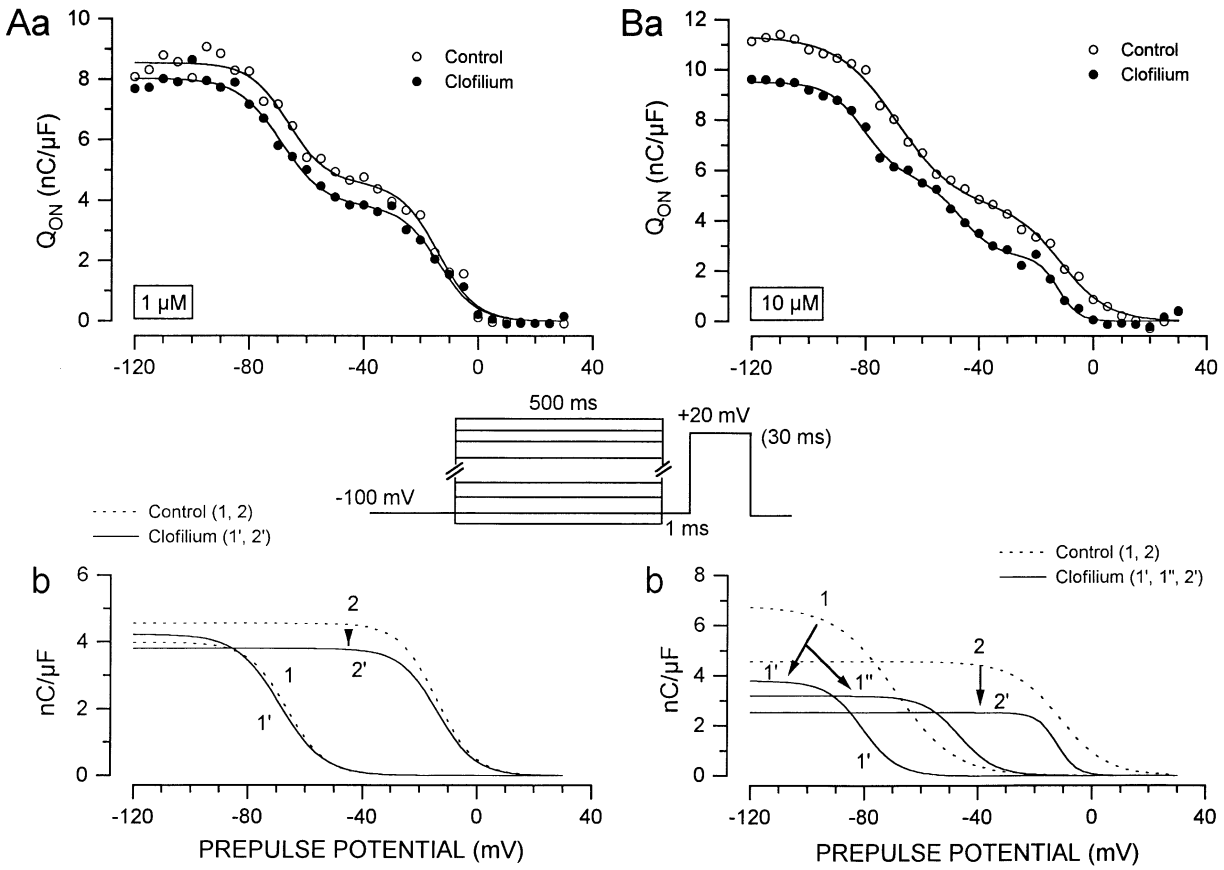

Figure 8 Effects of 1 μM (A) and of 10 μM (B) clofilium on the voltage dependencies of inactivation of the Q_{ON} component of ICM. The cells were polarized from -100 mV to between -120 and $+30$ mV for 500 ms (5 mV steps) to inactivate the charges and then briefly repolarised to -100 mV for 1 ms before applying a test pulse to $+20$ mV (see inset). In A and B, panels (a) represent the mean inactivation curves in control conditions (open circles) and in the presence of clofilium (filled circles), and panels (b) the decomposition into single Boltzman functions (dotted lines: control ; solid lines: clofilium) of the smooth curves shown in (a). Data points are mean values obtained in four cells for 1 μM and in four other cells for 10 μM clofilium. The vertical bars representing s.e. are omitted for clarity. Fit parameters best describing Q_{ON} inactivation: (A) control : $Q_{max,1}=3.9$ nC/μF, $V_{1/2,1}=-66.3$ mV, $K_1=-6.7$ mV, $Q_{max,2}=4.6$ nC/μF, $V_{1/2,2}=-13.9$ mV, $K_2=-6.6$ mV; 1 μM clofilium: $Q_{max,1}=4.2$ nC/μF, $V_{1/2,1}=-68.8$ mV, $K_1=-7.5$ mV, $Q_{max,2}=3.8$nC/μF, $V_{1/2,2}=-13.6$ mV, $K_2=-6.5$ mV; (B) Control : $Q_{max,1}=6.8$ nC/μF, $V_{1/2,1}=-69.1$ mV, $K_1=-10.5$ mV, $Q_{max,2}=4.6$ nC/μF, $V_{1/2,2}=-11.1$ mV, $K_2=-8.3$ mV; 10 μM clofilium: $Q_{max,1}=3.8$ nC/μF, $V_{1/2,1}=-80.7$ mV, $K_1=-6.2$ mV, $Q_{max,2}=3.2$ nC/μF, $V_{1/2,2}=-46.3$ mV, $K_2=-6.37$ mV, $Q_{max,3}=2.5$ nC/μF, $V_{1/2,3}=-12.2$ mV, $K_3=-3.5$ mV.

significant effect on the Q_{OFF} seen on repolarization to -110 mV from the test pulse of $+20$ mV, both in control conditions and in the presence of 1 or 10 μM clofilium (not shown).

In the presence of 1 μM d-sotalol, a slightly more complex behaviour of Q_{ON} inactivation with voltage was observed. The decrease of the negative component (about 23%) and its negative shift (4.9 mV) allowed a better individualization of the component with intermediate voltage dependence (corresponding to component 1″ seen in the presence of 10 μM clofilium (see Figure 8B). Some of the charges participating to the positive component 2 had their voltage dependence negatively shifted, resulting in the appearance of two positive components (not shown).

Discussion

The main results of our study are that, under our experimental conditions: (i) clofilium and d-sotalol decrease only the delayed rectifier K currents; (ii) intramembrane charge movements (ICM) sensitive to the class III antiarrhythmic (K channels blockers) drugs clofilium and d-sotalol are present in freshly isolated guinea-pig ventricular heart cells; (iii) these ICM can be observed during short depolarizing pulses and consequently (iv) the kinetics of potassium channel ICM bears little relation with that of the channel opening in guinea-pig ventricular heart cells, as delayed rectifier Kr and Ks channels activate relatively slowly at room temperature (Sanguinetti & Jurkiewicz, 1990; they did not observe at 22°C significant time-dependent outward K current during 225 ms depolarizing pulses), in contrast with those of Na and Ca channels. Thus, clofilium and d-sotalol appear to directly interact with the K channels proteins and to affect their gating properties.

The first question raised is whether the gating currents we recorded could be contaminated by an outward ionic current sensitive to class III antiarrythmic agents. Although this seems unlikely considering the 'cocktail' of blockers used, the potential candidates would be residual outward currents through Na, Ca, K, Cl and/or non selective channels. Na channels and Ca channels are blocked by the presence of high concentrations of $CdCl_2$ and of $GdCl_3$. Increasing the TTX concentration in the external saline to 25 μM did not decrease the gating current (not shown). Currents through K channels should be blocked by the presence of TEACl and CsCl in the external and internal solutions. Because the gating currents we recorded saturate at depolarized potentials, ion fluxes through other channels are also unlikely. Moreover, the maximal total amount of charges displaced from an HP of -110 mV ($Q_{max} = 14.0 \pm 0.6$ nC/μF, $n = 22$ cells) found in this study is comparable to values of charge 1 reported by several authors for the same preparation (for example: Bean & Rios, 1989: 11 nC/μF; Hadley & Lederer, 1989: 11.7 nC/μF; Shirokov *et al*., 1992: 14–14.5 nC/μF). Thus, we can assume that the currents recorded indeed correspond to 'pure' ICM.

The next question deals with the specificity towards potassium channels of the blockers used. Indeed, the partial block of ICM observed might be a blockade of charges originating from Na or Ca channels, since only these channels were reported by several authors to give rise to ICM in cardiac cells (e.g.: Bean & Rios, 1989; Hadley & Lederer, 1989; 1991a,b). To assess this point, we have studied the effect of clofilium (10–20 μM) and of d-sotalol (1 μM) on the Na current (I_{Na}) and on the L-type Ca current (I_{Ca}), because most of the clofilium and d-sotalol effects occurred in the potential range of Na and Ca channels activation, and on the positive Q_{ON} components of ICM activation (mostly Ca channels). Our results presented in Figure 1 and 2 clearly show that at the concentrations used in the present study both clofilium and d-sotalol do not decrease these two currents, in contrast to results reported by Li *et al*. (1996) who found a reversible block of Na and of L-type Ca channels by clofilium (although at a higher concentration: 30 μM, and with different recording conditions). Thus, a blocking effect of Na and of Ca channels in the presence of clofilium (1–20 μM) in our experimental conditions can be ruled out. A similar conclusion applies also in the presence of 1 μM d-sotalol, since no significant effects were observed on the Na current (Figure 2A) and on the calcium current elicited from -80 to 0 mV (Figure 2B). Therefore, because significant depressing effects were observed on Q_{ON} with low concentrations of clofilium (1–20 μM) and of d-sotalol (1 μM) which do not affect the Na and Ca currents (this study: Carmeliet, 1984; Baró & Escande, 1993; Singh *et al*., 1993), but decreased the delayed outward current (this study) the most likely explanation is that these effects should result from a partial blockade of gating charges from the delayed rectifier potassium channels.

Although K channels seem to significantly contribute to the gating currents that can be recorded in guinea-pig heart cells under our experimental conditions, the exact quantification of ICM originating from K channels is difficult, because of the decrease of the specificity of these antiarrhythmic drugs at doses reported to maximally affect the corresponding K channels (concentrations not used in our study). However the potassium channel density calculated from the clofilium-sensitive charge movements from an HP of -110 mV (2.13 nC/μF: Figure 4A), i.e., 3.92 channels μm^2 (assuming 12 e^- per channel) is compatible with that of the delayed rectifier channels calculated from the I_{Ks} current density of 11 pA/pF (Sanguinetti & Jurkiewicz, 1990; 1991), and from the single channel conductance of 20 pS and open probability P_o of 0.0037 (Duchatelle-Gourdon & Hartzell, 1990). Nevertheless, the dose dependence of the inhibitory effects of these blocking agents might also differ for ICM or current block (ICM might be less sensitive than the ionic current in itself): this will be true especially for those drugs which are open channel blockers, like quinidine which does not affect Q_{ON}, at concentrations between 10 and 100 μM, of expressed cloned human Kv1.5 channels (Fedida, 1997).

The fact that three Boltzman functions are necessary to describe the voltage dependence of Q_{ON} inactivation in the presence of 10 μM clofilium is interesting as it reveals the presence of a component of ICM which has not previously been shown. However, this component can also be observed in some cells in control conditions (not shown). Under control conditions, the voltage dependences of ICM originating from Na and from K channels are probably similar and cannot be distinguished just by adjusting Boltzman functions to the data. The same applies also for Ca and K channels. However, in the presence of clofilium, the positive component of Q_{ON} activated from -110 mV is not only decreased (Figure 5A), but also slightly shifted towards positive potentials (Figure 5B). These two effects will contribute to decrease the total amount of charges activated at $+20$ mV. Thus, we propose that inactivating component 1′ (Figure 8Bb) might correspond to ICM originating from Na channels (as we have shown that component 1 was sensitive to lidocaine and to tetracaine: Malécot *et al*., 1997a,b), component 1″, in part, to unmasked ICM originating from channels that need to be characterized, and that components 2 and 2′ might originate from both K and Ca channels, as we have shown that component 2 was

depressed, although not entirely blocked, by nifedipine and D600 (Malécot *et al*., 1997a,b).

Because all the effects of clofilium and of d-sotalol occurred at concentrations not affecting the Na or the Ca channels, our results suggest that the rapid delayed rectifier Kr channels might contribute to the negative Q_{ON} component activated during depolarization from -110 mV and also to the positive one activated after a 100 ms IPP50 (Kr channels are not fully inactivated at -50 mV), and that slow Ks delayed rectifier channels might mostly contribute to the positive Q_{ON} component activated during the two voltage clamp protocols used.

It should be noted that following the 100 ms IPP50, the respective participation of the two components of Q_{ON} to the total ICM activation decreased from 5.4 ± 0.4 nC/μF (HP $= -110$ mV) to 3.7 ± 0.4 nC/μF (negative component) and from 8.6 ± 0.5 nC/μF (HP $= -110$ mV) to 4.9 ± 0.4 nC/μF (positive component). Thus, during the 100 ms IPP50, almost 41% of the total amount of ICM are activated. It is noteworthy that these prepulse-sensitive ICM are not significantly affected by the compounds used in this study (not shown), indicating that they do not apparently originate from K channels, but more likely from Na channels, as previously suggested (Malécot & Argibay, 1996).

The finding in heart cell of ICM related to delayed rectifier potassium channels is not really surprising, as ICM have been described as molecular rearrangements of ion channel proteins which have to occur before the channel can actually open. Thus, the kinetics of ICM should only reflect transitions between the closed states of the channel, and there is apparently no reason to believe that these transitions are slower for K channels than for Na or Ca channels. In fact, the limiting factor for the channel to open slowly can be either the transition between the last two closed states of the channel, or even the final step, i.e., the transition between the last closed state and the open state (for review, see Bezanilla, 1985). Nevertheless, our results should give a new insight into the 'mechanistic' of the channel and into the mode of action of antiarrhythmic drugs: knowledge of the exact relationships existing between the ionic current and the ICM of ions channels will bring understanding into their function, and thus indicate new directions for the discovery of new more efficient and/or more specific pharmacological compounds for the treatment of heart diseases.

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