British Journal of Pharmacology (2000) 131, 569–577
www.nature.com/bjp

The bee venom peptide tertiapin underlines the role of I_{KACh} in acetylcholine-induced atrioventricular blocks

[1]Milou-Daniel Drici, [1]Sylvie Diochot, [1]Cécile Terrenoire, [1]Georges Romey & *,[1]Michel Lazdunski

[1]Institut de Pharmacologie Moléculaire et Cellulaire, CNRS, 660 route des Lucioles, Sophia Antipolis, 06560 Valbonne, France

1 Acetylcholine (ACh) is an important neuromodulator of cardiac function that is released upon stimulation of the vagus nerve. Despite numerous reports on activation of I_{KACh} by acetylcholine in cardiomyocytes, it has yet to be demonstrated what role this channel plays in cardiac conduction. We studied the effect of tertiapin, a bee venom peptide blocking I_{KACh}, to evaluate the role of I_{KACh} in Langendorff preparations challenged with ACh.

2 ACh (0.5 μM) reproducibly and reversibly induced complete atrioventricular (AV) blocks in retroperfused guinea-pig isolated hearts ($n=12$).

3 Tertiapin (10 to 300 nM) dose-dependently and reversibly prevented the AV conduction decrements and the complete blocks in unpaced hearts ($n=8$, $P<0.01$).

4 Tertiapin dose-dependently blunted the ACh-induced negative chronotropic response from an ACh-induced decrease in heart rate of $39\pm16\%$ in control conditions to $3\pm3\%$ after 300 nM tertiapin ($P=0.01$). These effects were not accompanied by any significant change in QT intervals.

5 Tertiapin blocked I_{KACh} with an IC_{50} of 30 ± 4 nM with no significant effect on the major currents classically associated with cardiac repolarisation process (I_{Kr}, I_{Ks}, I_{to1}, I_{sus}, I_{K1} or I_{KATP}) or AV conduction (I_{Na} and $I_{Ca(L)}$).

6 In summary, tertiapin prevents dose-dependently ACh-induced AV blocks in mammalian hearts by inhibiting I_{KACh}.

British Journal of Pharmacology (2000) **131,** 569–577

Keywords: Tertiapin; atrioventricular conduction; Kir; acetylcholine; cardiac I_{KACh}; complete heart block

Abbreviations: HERG, human ether-a-go-go-related gene; $I_{Ca(L)}$, L type voltage dependent Ca^{2+} current; I_{KATP}; ATP sensitive K^+ current; I_{K1}, inward rectifier K^+ current; I_{Kr}, rapid component of the delayed rectifier K^+ current; I_{Ks}, slow component of the delayed rectifier K^+ current; I_{Na}, voltage dependent Na^+ current; I_{to1}, transient outward K^+ current

Introduction

Despite numerous reports on activation of I_{KACh} by acetylcholine in cardiac myocytes (Fischmeister & Hartzell, 1986; Hartzell & Simmons, 1987), it has yet to be demonstrated what role this channel plays in cardiac conduction. Negative chronotropic and dromotropic effects (Di Francesco *et al*., 1989; Goyal, 1989; Loewi, 1921; Loewi & Navratil, 1926) are cardiovascular features associated with acetylcholine (ACh) release upon parasympathetic stimulation. In the mammalian heart, cholinergic parasympathetic fibres are extensively distributed to the sinus node, to the atria and to the atrioventricular (AV) node (Heller Brown & Taylor, 1996), where muscarinic M2 receptors mediate the effects of ACh (Goyal, 1989).

Parasympathetic stimulation is relayed by several transduction pathways (for review see Yamada *et al*., 1998), and particularly through activation of G proteins and modulation of ion channels. The G_K protein alpha subunit appears to inhibit the 'pacemaker' current I_f, thus partly explaining the decrease of heart rate associated with ACh stimulation (Di Francesco, 1995). The beta-gamma subunit assembly activates I_{KACh} (Trautwein & Dudel, 1958; Logothetis *et al*., 1987).

The I_{KACh} channels are heterotetramers composed of GIRK1 and GIRK4 pore-forming subunits (Krapivinsky *et al*., 1995; Corey *et al*., 1998). The precise physiological role of the current remains a matter of debate. Targeted gene disruption of GIRK4, is one of the few demonstrations that implicate I_{KACh} in the parasympathetic control of the murine heart rate (Wickman *et al*., 1998). I_{KACh} predominates in the atria of mammalian hearts, including human and guinea-pig, where it is activated by ACh with comparable characteristics (Koumi & Wasserstrom, 1994). An intense vagal stimulation of the whole heart often results in a complete AV block. Even if I_{KACh} plays an important role in the resting potential and the repolarization of atrial cells (Kaibara *et al*., 1990), the precise mechanisms by which the AV block occurs still are still a matter of speculation (Accili *et al*., 1998; Wickman *et al*., 1998). A reduction of inward Ca^{2+} currents (I_{Ca}) as well as an increase in outward K^+ conductance may contribute to this phenomenon (Loffelholz & Pappano, 1985).

Since a considerable increase of the refractory period of the atrioventricular (AV) node has been ascribed to such a vagal stimulation (Alanis *et al*., 1958; 1959), we aimed to block I_{KACh} during an ACh challenge, in order to evaluate the implication of this current in ACh-induced cardiac effects. Peptides purified from bee venom (Habermann, 1972) have been very useful for studying the structure and physiological function of potassium channels (Mockzydlowski *et al*., 1988; Pongs, 1992). Tertiapin is a bee venom peptide (Gauldie *et al*., 1976) devoid of neurotoxicity, that was recently shown to potently block GIRK1/4 current (Jin & Lu, 1998). Because of the electrophysiological similarities of human and guinea-pig I_{KACh} (Koumi & Wasserstrom, 1994) we have used models of

*Author for correspondence; E-mail: ipmc@ipmc.cnrs.fr

guinea-pig and rabbit hearts sequentially challenged with ACh to evaluate the effects of tertiapin on ACh-induced negative dromotropic and chronotropic effects.

Methods

Langendorff preparations

Two-month-old guinea-pigs (145–155 g) and 3-month-old female NZ rabbits were studied. Experiments were conducted in accordance with the guidelines of the University of Nice (France) and the American Heart Association's position statement on use of animals in research. The Langendorff preparations and EKG measurements have been described previously (Drici *et al*., 1996; 1998b). Briefly, after guinea-pigs and rabbits were anaesthetized with sodium pentobarbital (Sanofi-France, 50 mg kg^{-1}, IP), the hearts were excised, mounted in a modified Langendorff apparatus and perfused with Tyrode's solution at 37°C, until equilibrium was reached (15 ± 5 min). Preliminary experiments ($n=4$) were performed to determine the ACh concentration necessary to induce a complete heart block (0.5 μM) and the stability of the model over 2 h.

Complete AV blocks were induced in unpaced guinea-pig hearts ($n=8$) by means of a perfusion of Tyrode's solution containing 0.5 μM of ACh. In further experiments ($n=4$) a pair of stimulating electrodes was placed 1.5 mm apart in the apex of the right atrial appendage. Pacing was achieved by applying electrical square pulses of 2 ms duration and twice the electric threshold at a rate of 5 Hz (cycle length 200 ms).

Measurements of EKG parameters

Two standard bipolar leads were recorded and the RR, PP, PR and QT intervals were measured. In unpaced hearts, the PR interval was measured from the beginning of the surface P wave to that of the R wave. The QT interval was measured from the beginning of the Q wave (or from the base of the R wave if not possible) to the end of the T wave, defined as the point at which it returns to the isoelectric line. The QT intervals were corrected with Bazett's formula ($Qtc(ms) = QT/RR(s)^{1/2}$) that allows the comparison of QT duration at slightly different heart rates, which was the case in our experiments (standard deviations of RR intervals measured at baseline and end of experiments being of 16 and 15 ms respectively). For each heart, a set of three consecutive RR cardiac cycle length–QT interval pairs was obtained from the EKG recordings.

Acetylcholine challenge and perfusion of tertiapin

Experiments were conducted as follows. After a period of equilibrium, a control ACh challenge was performed, until a complete AV block occurred within 1 min (Figure 1A,B). Tyrode's was switched immediately upon appearance of the conduction and the wash-out normalized the EKG within 1 min (Figure 1C). Then, increasing concentrations of tertiapin at 10, 30, 100, 300 and 1000 nM were perfused for successive

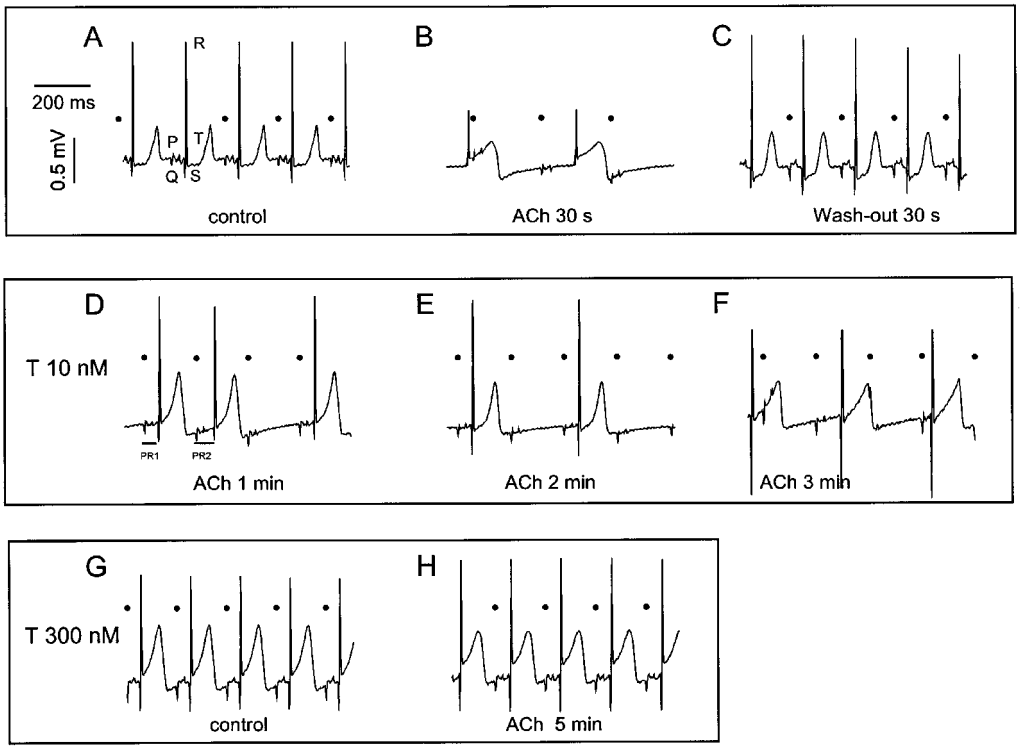

Figure 1 A representative experiment from a guinea-pig isolated heart. The P wave, QRS deflection and T wave are indicated. Closed circles indicate P waves. (A) Normal rhythm. RR interval: 242 ms, PR: 56 ms, QT: 152 ms. (B) EKG during ACh challenge. Within 20 s ACh induces a 3rd degree AV block. PP interval: 310 ms, RR interval: 486 ms. (C) Upon switching to Tyrode's, the block disappears within 1 min. (D) Tertiapin (T: 10 nM with ACh) within 1 min, rapid onset of a Mobitz type I AV block (Wenckebach phenomenon, bar = PR interval. PR1: 62 ms, PR2: 80 ms. (E) At 2 min, a Mobitz type II block occurs: not all P waves are followed by QRS (2 : 1), but those that are have an unvarying PR interval (70 ms). (F) At 3 min, a high-degree, rapidly followed by a complete AV block. RR: 408–416 ms, PP intervals: 236–244 ms. (G) T (300 nM), inhibits the occurrence of a complete block, as seen in (H) (after 5 min of perfusion with ACh). Control conditions: RR 224 ms (PR 60 ms); ACh: RR 230 ms (PR 64 ms). Tertiapin dose-dependently prevents the occurrence of conduction decrements ($P<0.01$, $n=8$).

periods of 5 min each, being followed by the same ACh challenge mixed with the appropriate concentration of tertiapin. In case of no occurrence of block within 1 min, that tertiapin concentration was deemed efficient. Hence, the challenge was still maintained either for 5 min (at most) or up to the occurrence of a third degree AV block. At the end of experiments a wash-out period was applied prior to an ultimate ACh challenge. Conduction problems were graded as follows: (i) first-degree block (ii) second-degree heart block (Mobitz type I) in presence of Wenckebach periods (Figure 1D), (iii) second-degree heart block (Mobitz type II) with constant PR intervals and unexpectedly dropped atrial complexes (Figure 1E) and (iv) third-degree atrioventricular block with a complete dissociation between P waves and QRS complexes (Figure 1F). In case of complete block, the RR and PP interval duration were measured independently.

In order to evaluate the effects of tertiapin on the sinus rhythm, PP intervals were measured in unpaced hearts during control conditions and at the end of each ACh challenge. In paced hearts, the experiments were identical, but for the measurements related to spontaneous sinus rhythm. In rabbit hearts, 5 μmol/l of ACh induced a second degree AV block.

Effect of tertiapin on native I_{KACh} *and on GIRK1/4 current recorded in* Xenopus *oocytes*

Myocyte isolation Cell isolation was performed according to the method of Mitra and Morad (Isenberg & Klockner, 1982; Mitra & Morad, 1986). Myocytes were stored at $+4^{\circ}$C in a modified KB medium until use. Cells were perfused with 1 μM ACh to stimulate I_{KACh} before applying increasing concentrations of tertiapin (3 to 300 nM).

Oocyte preparation cRNA synthesis of GIRK1/4 and M2 receptor, injections and measurement of GIRK currents in *Xenopus* oocytes have been previously detailed (Guillemare *et al*., 1992; Lesage *et al*., 1995). Currents were stimulated by a perfusion 1.5 μM ACh, prior to application of tertiapin (300 nM).

Evaluation of the specificity of tertiapin In order to determine the effects of tertiapin (300 nM) on ionic currents, it was tested on each of the two components of the delayed rectifier, I_{Kr} and I_{Ks}, by means of HERG and KvLQT1/IsK expression in COS cells. Native I_{to1}, I_{sus}, I_{K1} and I_{KATP} of rat ventricular cardiomyocytes were also challenged by 300 nM of tertiapin. Due to the importance of calcium and sodium currents in the process of cardiac automatism and conduction, the effect of tertiapin (300 nM) was evaluated on native I_{Na} and $I_{Ca(L)}$. HERG and KvLQT1/IsK were expressed in COS cells as published previously (Barhanin *et al*., 1996; Chouabe *et al*., 1998; Fink *et al*., 1996; Jurman *et al*., 1994) and currents were recorded within 2 days following transfection.

Electrophysiological measurements

Electrophysiological measurements on COS cells and cardiomyocytes were performed using the whole-cell configuration of the patch clamp technique. The pipette solution used for COS cells was (in mM): KCl 150, $MgCl_2$ 0.5, EGTA 5, HEPES-KOH 10 at pH 7.2. The pipette solution used for myocytes was (in mM): KCl 140, EGTA 10, $MgCl_2$ 2, HEPES 10, at pH 7.2. The extra-cellular solution composition for K^+ currents recordings in COS cells was (in mM): KCl 5, NaCl 150, $CaCl_2$ 1, $MgCl_2$ 3, HEPES-NaOH 10, pH 7.4. The extra-cellular solution used for myocytes was (in mM): KCl 5, NaCl 140, $CaCl_2$ 1.8, $MgSO_4$ 2, HEPES 10, at pH 7.2. $CoCl_2$ (5 mM), $BaCl_2$ (5 mM), or glibenclamide (10 μM) were added when necessary to block $I_{Ca(L)}$, I_{K1} and I_{KATP} respectively. In all cases, the holding potential was -80 mV. For measurement of HERG and KvLQT1/IsK currents in COS cells incremental depolarizing steps (20 mV, 2 or 3 s) were applied up to $+60$ mV followed by a step to -40 mV to elicit tail-currents. Native I_{to} was elicited by a pulse to $+60$ mV (0.2 Hz), preceded by a step from -80 to -40 mV (50 ms) to inactivate I_{Na}. I_{K1} was elicited by 10 mV-voltage steps (100 ms) from -30 mV to -120 mV (0.2 Hz). I_{KATP} was recorded with the latter protocol, after stimulation by cromakalim (50 μM). Tertiapin effects on I_{KACh} were determined at -120 mV with the same protocol. The native I_{CaL} was measured in sodium-free solution ($BaCl_2$ 2.5 mM, NMDG 130 mM, $MgCl_2$ 1 mM, HEPES/KOH 10 mM) by 10 mV incremental voltage steps (500 ms) from -40 mV to $+50$ mV. Native I_{Na} was elicited in low sodium solution (NaCl 40 mM, NMDG 100 mM, Co^{2+} 1 mM, Mg^{2+} 2 mM, HEPES/KOH 10 mM) by 10 mV incremental voltage steps of 50 ms duration from -70 mV to $+60$ mV. Both currents were challenged by a maximum concentration of 300 nM of tertiapin ($n=5$ cells for each current).

Data and statistical analysis

Results are shown as mean $\pm$ s.e. The prevention of a complete (or a high degree) AV block upon the ACh-challenge was the main endpoint for the efficacy of tertiapin. The secondary endpoint was the severity of the conduction problems and their time of onset (up to 5 min) during the ACh challenge. Depending on the degree of conduction decrement, blocks were classified as 3rd degree AV block, Mobitz type II AV block, Mobitz type I AV block, 1st degree AV block. Normal PR interval was defined as measured during control conditions. The relationship between the concentration of tertiapin and the occurrence of Ach-induced complete AV block was explored by logistic regression. The relationship between increasing concentrations of tertiapin and the overall degree of severity of the conduction problems was explored by a general linear model (Logistic and General Linear Model procedures, SAS 6.12, SAS Institute Inc., Cary, NC, U.S.A.). Continuous variables, such as RR and PP or QT intervals, and their change from baseline, were analysed by Mann-Whitney-Wilcoxon rank-sum test or one way analysis of variance. The Bonferroni/Dunn correction was used to adjust for multiple comparisons whenever appropriate (ANOVA, Statview 4.5, and SuperAnova 1.11, Abacus Corp., CA, U.S.A.). $P<0.05$ was considered statistically significant.

Drugs

Tertiapin was purified according to Gauldie *et al*. (1976) from the venom of *Apis mellifera* (gift of Charles Mraz, Beekeeper, Middlebury, VT, U.S.A.). Acetylcholine chloride (Sigma Chemical Co, St Louis, MO, U.S.A.) was dissolved in deionized water. Tertiapin and acetylcholine were further diluted in the Tyrode solution for the Langendorff experiments. For cardiomyocyte dissociation, collagenase type II (Worthington) was purchased from ATGC Biotechnologie (Orléans, France) and hyaluronidase (type IV-S) from Sigma Chemical Co. (St Louis, MO, U.S.A.). Glibenclamide and Chromanol 293B were a generous gift from Hoechst Laboratories (Germany) and cromakalim from SKF-Beecham Laboratories (U.K.). Fresh solutions were prepared on the day of each experiment.

Results

ACh induces reversible atrioventricular block in the guinea-pig isolated heart

Preliminary experiments showed that the perfusion of 0.5 μM of ACh steadily and reproducibly induced a third degree AV block within 1 min of perfusion in guinea-pig isolated hearts. ACh concentrations ranging from 0.3 to 0.4 μM inconstantly induced a complete block whereas concentrations of 0.1 and 0.2 μM only resulted in various degrees of conduction decrement but no complete block. The complete block induced by 0.5 μM ACh (eight cases out of eight, Figure 1A, B) was always reversible within 1 min upon wash-out with Tyrode's solution (Figure 1C). The average cycle length (RR interval) at baseline was 226 ± 6 ms. The average PR interval was 52 ± 4 ms. The T wave was well defined in all cases and the average QT was 144 ± 3 ms, yielding a corrected QT interval (QTc) of 302 ± 5 ms.

Tertiapin dose-dependently prevents ACh-induced AV blocks and decrease in heart rate

Tertiapin prevented the occurrence of a complete block in all hearts and in a dose-dependent manner ($P=0.02$, $n=8$) (Figure 1D–H for representative traces). Little effect was observed at a concentration of 10 nM, except in one heart (out of four tested), in which a Mobitz II AV block occurred within the first min instead of a complete block. It evolved to a third degree AV block after 5 min. At a concentration of 30 nM, tertiapin prevented the occurrence of a complete heart block in three cases out of four, in which the perfusion was maintained for 5 min. The challenge with ACh and tertiapin produced a Mobitz II AV block after 2 min and two Mobitz I AV blocks (representative traces: Figure 1D,E). At the end of the 5 min, one type I block evolved to type II and one type II block evolved to complete heart block (Figure 1F). It took an average time of 3 ± 1 min of ACh infusion with tertiapin for high degree conduction problems to occur. At 100 nM, tertiapin prevented the occurrence of a complete block within the first min in three cases out of five, one of them remaining in sinus rhythm throughout the 5 min challenge. In two other cases, a Mobitz II block occurred after 2.5 min. Tertiapin at 300 nM prevented a complete block during the first minute of ACh challenge in eight cases out of eight (Figure 1G,H). At this concentration, whereas six hearts were in normal sinus rhythm within the first minute, two hearts presented a 2nd degree AV block (one Mobitz type I and one Mobitz type II block). After 5 min, the Mobitz I block went to sinus rhythm whereas the Mobitz II block evolved to a complete block. A concentration of 1 μM of tertiapin was required to prevent a complete block in that last heart (Figure 2).

In seven cases out of eight, the unpaced Langendorff heart preparations were washed-out (2–15 min) with control Tyrode's solution, after the last infusion of tertiapin. All hearts were then re-challenged with the same concentration of ACh (0.5 μM). ACh induced in each case a complete heart block within 1 min, as observed in control conditions. When considering the decrease in severity and the delay observed for the conduction problems to occur during tertiapin challenges, there was a clear dose-effect relationship between the concentration of the peptide and the resulting prevention of ACh effects on AV conduction (F test, $P<0.01$, $n=8$). In paced hearts, to avoid any unwanted cardiac tissue deterioration, the sinus node was maintained and an overdriving pacing was applied. As for unpaced hearts, a concentration of 0.5 μM ACh was necessary to induce a complete block, that occurred in each of the four hearts tested. The blocks were prevented by tertiapin 100 nM in one heart, and 300 nM in the remaining three hearts. Ten or 30 nM of tertiapin did not prevent a complete block within the first min, even though the delay necessary for the AV block to occur from the beginning of the infusion increased from 5–10 s (control, $n=4$), to $\sim$ 25 s ($n=2$) at 10 nM, 40 s at 30 nM ($n=2$) and $\sim$ 55 s at 100 nM ($n=3$). Similarly to the unpaced hearts ACh 0.5 μM induced a complete AV block

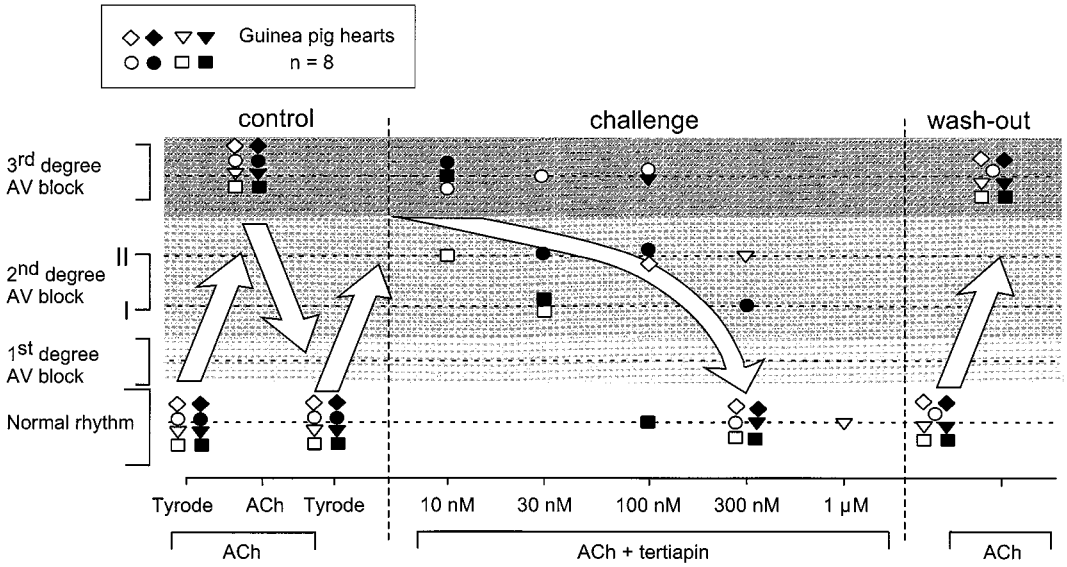

Figure 2 Preventive effect of tertiapin on ACh-induced decremental conduction in guinea-pig isolated hearts. Control: a third degree AV block occurs within 1 min of perfusion with 0.5 μM ACh. The block is lifted upon wash-out with Tyrode within 1 min (arrow). Tertiapin dose-dependently prevents complete AV blocks ($n=8$, $P=0.02$). At 100 nM, ACh induced a 3rd degree AV block in two hearts, and a Mobitz type II block in three hearts. At 300 nM, six hearts out of seven were in normal rhythm during the challenge. Seven out of eight hearts could be re-challenged with ACh that re-induced a 3rd degree AV block. The upper area in gray indicates complete AV block, the lower indicates a normal sinus rhythm on the EKG.

within one min in all hearts that were re-challenged, after a wash-out period of 15 min. The prevention of blocks does not seem associated with muscarinic receptor desensitization since (i) repeated challenges constantly induced blocks, (ii) each ACh challenge was preceded by an infusion of the peptide for 5 min with no ACh and (iii) the reoccurrence of the block after wash-out was comparable to that observed in control conditions.

In control experiments with unpaced hearts, ACh decreased the heart rate by $39 \pm 16\%$, from a PP interval of 226 ± 6 ms at baseline to 316 ± 42 ms during the ACh challenge. Tertiapin dose-dependently blunted such a response, with ACh-induced decrements of $27 \pm 19\%$ at 10 nM, $7 \pm 4\%$ at 30 nM, $2 \pm 2\%$ at 100 nM and $3 \pm 3\%$ at 300 nM ($P = 0.01$). At that concentration, PP intervals, with tertiapin only, were of 228 ± 5 ms as compared as 235 ± 5 ms during the ACh challenge with tertiapin (NS).

Tertiapin does not modify other standard EKG parameters

In unpaced hearts, tertiapin did not significantly change the PP intervals, from 226 ± 6 ms in control conditions to 228 ± 5 ms at the end of the perfusion of 300 nM, which was the concentration that prevented the occurrence of a severe block in about all hearts. The PR interval did not significantly change either. It varied from 52 ± 4 ms (control values) to 50 ± 4 ms (at 300 nM). The mean QRS interval duration remained at 12.2 ± 0.4 ms, which does not favour any important effect of the peptide *per se*, on sodium channels in this non-paced model. Tertiapin tended to shorten the mean duration of the ventricular repolarization, from an average QTc of 302 ms in control conditions to 288 ms (NS) at the end of the perfusion of tertiapin 300 nM.

Effect of tertiapin on ACh challenge in isolated rabbit hearts

The effect of tertiapin was reproducible in isolated rabbit hearts ($n = 4$). They were challenged with 5 μM ACh. EKG parameters were measured after an equilibrium period of 12 ± 2 min. The average RR intervals were 269 ± 29 ms, the PR intervals were 55 ± 5 ms and the QTc values were 133 ± 11 ms. The ACh challenge steadily induced a second degree AV block (Mobitz type II) within one min. This block regressed upon washing out. During the AV block, the RR interval was 678 ± 150 ms, with PP intervals of 339 ± 75 ms. Five minutes of tertiapin infusion (300 nM) barely changed the RR, PR or QTc values that were respectively 272 ± 16, 48 ± 8 and 152 ± 10 ms, as compared to control conditions. During the challenge with ACh and tertiapin, no AV block occurred, even though the heart rate decreased by about 25%, with RR intervals at 342 ± 38 ms and corresponding QTc of 292 ± 28 ms.

Specificity of channel inhibition by tertiapin

We confirmed that tertiapin was effectively blocking GIRK1/4 current and I_{KACh}. GIRK1/4 inward currents were recorded after applying ACh (1.5 μM) on *Xenopus* oocytes co-injected with GIRK1 and GIRK4 and with the M2 receptor cRNAs (Duprat *et al*., 1995). As previously described (Jin & Lu, 1998), this current was totally blocked by tertiapin 300 nM ($n = 4$, Figure 3A). The same effects were observed using 150 μM ACh showing that tertiapin efficacy is independent of ACh concentration.

Tertiapin also reversibly blocked ACh-enhanced I_{KACh} in atrial cardiomyocytes (Figure 3B) with an IC_{50} of 29.7 ± 3.8 nM and a Hill coefficient of 1 (Figure 3C).

The specificity of 300 nM of tertiapin was evaluated by challenging native currents of isolated cardiomyocytes and cloned K^+ channels expressed in COS transfected cells. In cardiomyocytes, a transient (I_{to1}) and a sustained component (I_{sus}) characterized the I_{to} current (Figure 3D). Neither of them was significantly affected by tertiapin ($n = 5$, 300 nM). None of those currents: I_{Kr}, I_{Ks} (Figure 3E), or I_{KATP} was sensitive to tertiapin ($5 < n < 8$ for each current), even though they were inhibited by their respective blockers when available, i.e. E4031 (1 μM) for I_{Kr}; chromanol 293B (10 μM) for I_{Ks}; glibenclamide (10 μM) for I_{KATP}. I_{K1} was not modified by tertiapin (IRK1 was previously shown to be insensitive to tertiapin (Jin & Lu, 1998)). We verified that tertiapin up to 300 nM did not block the other inward rectifier currents IRK2 or IRK3 recorded in *Xenopus* oocytes ($n = 3$ in each condition). As predicted by the lack of effect of tertiapin on QRS interval measured in Langendorff preparations, tertiapin had no effect on native $I_{Ca(L)}$ or I_{Na} ($n = 5$, Figure 3F, G). Tertiapin appears therefore to be a selective blocker of I_{KACh}.

Discussion

Muscarinic stimulation can modulate phosphoinositide turnover (Roffel *et al*., 1994; Felder, 1995) as well as activate phospholipases, tyrosine kinases and calcium influx (Gilman, 1987; Cauldfield, 1993; Reuveny *et al*., 1994). 'Muscarinic K^+ channels' have also been implicated in relaying acetylcholine stimulation (Noma & Trautwein, 1978; Logothetis *et al*., 1987). The native G-protein-regulated I_{KACh}, an heterotetramer composed of GIRK1 and GIRK4 subunits (Hartzell, 1980; 1981; Dascal *al*., 1993; Kubo *et al*., 1993; Reuveny *et al*., 1994) is a member of G-protein gated family of inward rectifier channels coupled to various receptors like the muscarinic M2 receptor (Kurachi *et al*., 1992; Lesage *et al*., 1995; Wickman & Clapham, 1995). Parasympathetic stimulation of muscarinic receptors induce a negative chronotropic action in sinoatrial and AV nodes (Loffelholz & Pappano, 1985). Even though a reduction of calcium influx partly accounts for these effects (Loffelholz & Pappano, 1985), I_{KACh} is an important end-target of ACh (Hartzell, 1980; 1981; Deal *et al*., 1996) especially in human and guinea-pig heart (Koumi *et al*., 1994; Koumi & Wasserstrom, 1994).

The main results of this study are that tertiapin prevents ACh-induced negative dromotropic and chronotropic effects in mammalian isolated hearts. These physiological effects are strictly dependent on the concentration of tertiapin used for the challenge. Moderate concentrations of tertiapin (below the IC_{50} of 30 nM for blocking native I_{KACh}) mainly prevent ACh negative chronotropic effects whereas higher concentrations (30 to 300 nM) prevent severe conduction decrements. Since the submission of this manuscript, other authors have confirmed the specific blockade of native I_{KACh} by tertiapin in rabbit isolated cardiomyocytes (Kitamura *et al*., 2000).

Tertiapin prevents ACh negative dromotropic effects

That tertiapin prevents ACh-induced complete heart blocks in isolated heart probably occurs largely, through I_{KACh} inhibition. Indeed (i) tertiapin blocks the GIRK1/4 current (Dascal *et al*., 1993; Jin & Lu, 1998; Kubo *et al*., 1993; Reuveny *et al*., 1994) as well as native I_{KACh} (this work), with

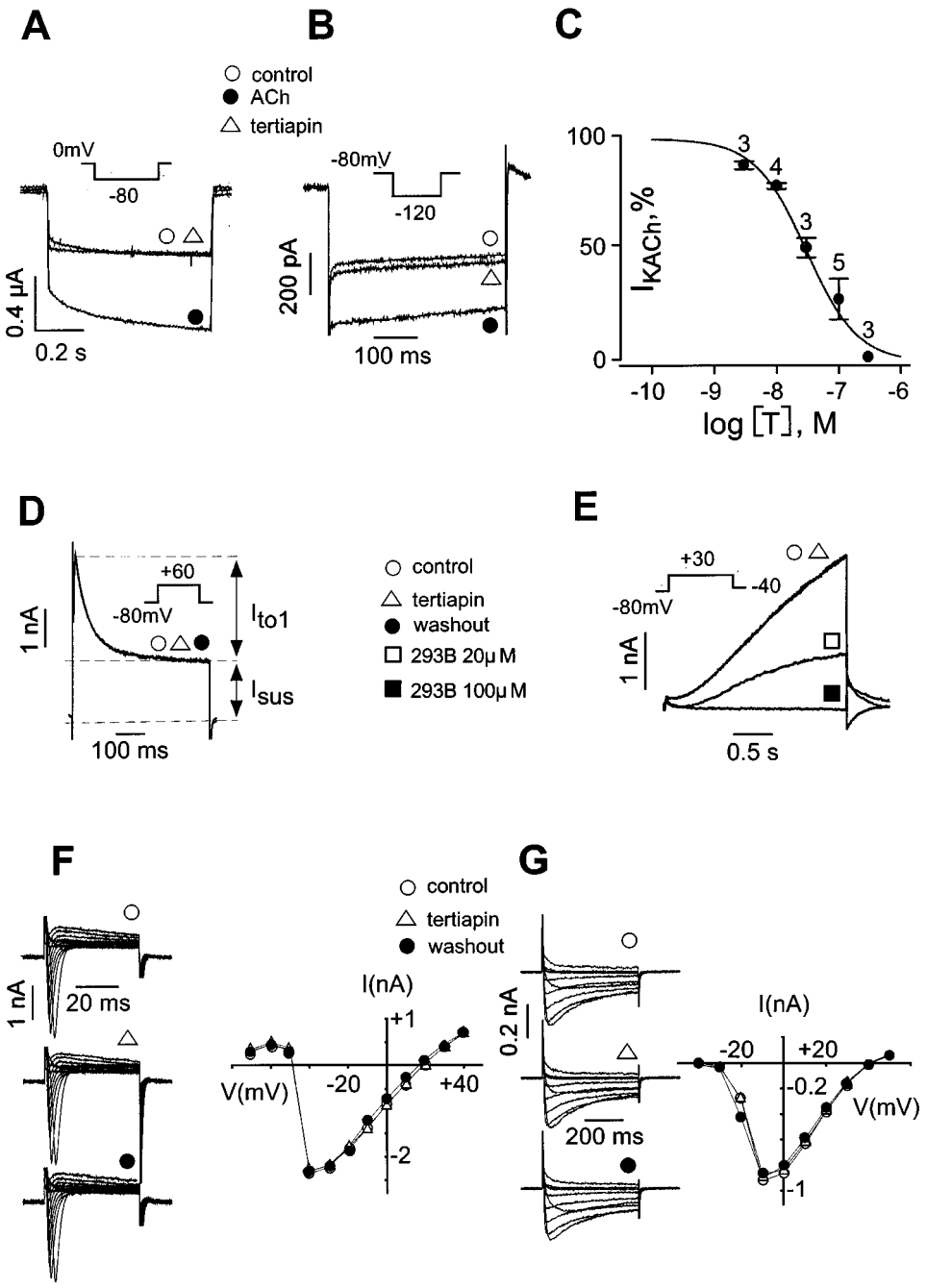

Figure 3 Specificity of tertiapin on I_{KACh}. (A) Inhibition of I_{KACh} current expressed in *Xenopus* oocytes injected with GIRK 1 and 4 and M2 receptor cRNAs. I_{KACh} stimulated by a perfusion of 1.5 μM ACh from baseline were blocked by 300 nM tertiapin ($n=4$). (B) Inhibition of native I_{KACh} by tertiapin recorded by the whole-cell patch clamp technique in rat atrial cardiomyocytes. I_{KACh} stimulated by a perfusion of 1 μM ACh, from control, was blocked by tertiapin 100 nM. The blockade produced by tertiapin was totally reversible within 2 min of wash-out with standard solution. (C) Tertiapin (10 nM – 1 μM) blocks native I_{KACh} with an IC_{50} of 29.7 ± 3.8 nM and a Hill coefficient of 1 ($n=3$ to 6 cells for each concentration). (D) Representative trace illustrating the lack of effect of tertiapin on rat cardiomyocyte I_{to} (pulse protocol inset). Control, tertiapin 300 nM, and wash-out traces are superimposed. (E) Lack of effect of tertiapin on a representative trace of I_{Ks} current recorded in COS cells transfected with KvLQT1/IsK plasmids. The control time-dependent current was insensitive to tertiapin 300 nM ($n=5$), but dose-dependently inhibited by chromanol 293B. (F) Representative traces illustrating the lack of effect of tertiapin on I_{Na}. Currents were evoked by 10 mV (50 ms) incremental pulses from -70 mV to $+60$ mV from a holding potential of -80 mV. A maximum concentration of 300 nM of tertiapin had no effects on I_{Na} ($n=5$). (G) Representative traces illustrating the lack of effect of tertiapin on $I_{Ca(L)}$. Currents were evoked by 10 mV (500 ms) incremental pulses from -40 mV to $+50$ mV from a holding potential of -80 mV. A maximum concentration of 300 nM of tertiapin had no effects on $I_{Ca(L)}$ ($n=5$).

no significant effects on other tested currents (voltage-sensitive Ca^{2+}, Na^+ and K^+ currents and other inward rectifier currents) and (ii) the preventive effects of tertiapin begin for concentrations as low as 10 and 30 nM (preventing complete blocks as well as most of the negative chronotropic action). These concentrations are in the range of the dose-response curve that we have observed for the effect of tertiapin on native I_{KACh} (IC_{50}: 30 nM). In challenges involving paced guinea-pig isolated hearts the occurrence of the block – hence its prevention – could be precisely measured: tertiapin from 10 nM on, dose-dependently increased its delay of appearance within the first minute.

Tertiapin prevents ACh negative chronotropic effects

Tertiapin blunts the ACh-induced decrease in heart rate. The involvement of I_{KACh} in the overall modulation of the murine heart rate activity has been thoroughly analyzed in GIRK4 knockout mice (Wickman *et al*., 1998; Kovoor *et al*., 1999). In that model, a reflex negative chronotropic response was obtained by a methoxamine-induced rise in blood pressure. Invalidation of I_{KACh} in that model determined that this current was mediating 65% of the bradycardic response (Wickman *et al*., 1998). In our model, which is directly stimulated by ACh, we observed results of a similar magnitude: the spontaneous cycle length of isolated hearts increased by $\sim$ 40% with ACh, half of this effect being antagonized by 10 nM of tertiapin. However I_{KACh} was considered not critical for AV conduction in mice (Wickman *et al*., 1998; Kovoor *et al*., 1999). Different elements can justify the discrepancy between these findings and our results. As suggested by Kovoor *et al*. (1999), it is possible also that other K^+ channels compensate to maintain normal electrophysiological properties in the AV node of GIRK4 knockout engineered mice. Second, as for other K^+ channels, mice and guinea-pigs are obviously different (Drici *et al*., 1998a; Diochot *et al*., 1999). Species differences as well as channel densities that vary in different cardiac regions of different species may then account for such a discrepancy (Koumi & Wasserstrom, 1994).

Tertiapin exerts its preventive effects at the AV junction

The atrioventricular node was described as the only electrical connexion between the atria and the ventricles of mammalian hearts (Tawara, 1906); its function though, has remained incompletely elucidated. Even if a variety of mechanisms and structures can affect cardiac negative dromotropic effects (Rankin & Workman, 1999), the preventive effect of complete blocks observed with tertiapin is likely to be exerted at the level of the AV-junction. In our study, ACh progressively decreased the conduction up to a complete AV block characterized by a regular and stable ventricular escape rhythm and normal QRS complexes which do not favour an infra-nodal origin. Furthermore, ACh was shown to induce dose-dependently a negative dromotropic effect in guinea-pig hearts, characterized by changes in AV conduction delay (Ceballos & Rubio, 1998). That decremental conduction occurred in the AV node and not in the atrium-to-AV node segment nor in the His-bundle-to-ventricle circuit, both of which remaining unchanged during ACh challenges (Ceballos & Rubio, 1998).

Limits of the study, clinical relevance and implications for the role of I_{KACh}

The present results should be considered as a contribution to experimental electrophysiology, even if human and guinea-pig I_{KACh} share similar biophysical characteristics (Koumi & Wasserstrom, 1994). In a setting of hypervagotony or in early inferior myocardial infarction in man (Tans *et al*., 1980), intense vagal stimulation may effectively impair the AV conduction up to require temporary electrical stimulation (Tans *et al*., 1980). Were the role of I_{KACh} confirmed in human, specific blockers of I_{KACh} would be of beneficial assistance in such emergency settings. These data may also explain the unwanted 1 : 1 conduction phenomenon associated with atrial flutter treated by some class IC antiarrhythmic drugs (Heldal & Orning, 1989; Roden, 1998). Similarly to tertiapin, both flecainide and cibenzoline have previously been shown to block native I_{KACh} (Inomata *et al*., 1991; 1993; Wu *et al*., 1994). These drugs sometimes induce a paradoxical increase of the ventricular rate of atrial flutters (Mehta *et al*., 1988; Crozier, 1992) which could result from the inhibition of I_{KACh}. This may help to design safer new antiarrhythmics.

In conclusion, tertiapin dose-dependently prevents acetylcholine-induced complete heart block and negative chronotropic effect in guinea-pig isolated hearts. Its potency in doing so is compatible with the blockade exerted on native I_{KACh}, which appears therefore as the most relevant mechanism for such a phenomenon.

We gratefully thank Mr Clément François for very useful discussions, Valérie Lopez for their skilful help in the planning of this manuscript. This work was supported by the Centre National de la Recherche Scientifique (CNRS), the Association Française contre les Myopathies (AFM) and the Ministère de la Défense Nationale (Grant DRET 96/096).

References

ACCILI, E.A., REDAELLI, G. & DI FRANCESCO, D. (1998). Two distinct pathways of muscarinic current responses in rabbit sino-atrial node myocytes. *Pflügers Arch.,* **437,** 164 – 167.

ALANIS, J., GONZALES, H. & LOPEZ, E. (1958). The electrical activity of the bundle of His. *J. Physiol (Lond.),* **142,** 127 – 140.

ALANIS, J., LOPEZ, E. & MANDOCKI, J. (1959). Propagation of impulses through the atrioventricular node. *Am. J. Physiol.,* **197,** 1171 – 1174.

BARHANIN, J., LESAGE, F., GUILLEMARE, E., FINK, M., LAZDUNSKI, M. & ROMEY, G. (1996). KvLQT1 and IsK (minK) proteins associate to form the IKs cardiac potassium current. *Nature,* **384,** 78 – 80.

CAULDFIELD, M.P. (1993). Muscarinic receptors – characterization, coupling and function. *Pharmacol. Ther.,* **58,** 319 – 379.

CEBALLOS, G. & RUBIO, R. (1998). Endothelium-mediated negative dromotropic effects of intravascular acetylcholine. *Eur. J. Pharmacol.,* **362,** 157 – 166.

CHOUABE, C., DRICI, M.D., ROMEY, G., BARHANIN, J. & LAZDUNSKI, M. (1998). HERG and KvLQT1/IsK, the cardiac K^+ channels involved in long QT syndromes, are targets for calcium channel blockers. *Mol. Pharmacol.,* **54,** 695 – 703.

COREY, S., KRAPIVINSKY, G., KRAPIVINSKY, L. & CLAPHAM, D.E. (1998). Number and stoichiometry of subunits in the native atrial G-protein- gated K^+ channel, IKACh. *J. Biol. Chem.,* **273,** 5271 – 5278.

CROZIER, I. (1992). Flecainide in the Wolff-Parkinson-White syndrome. *Am. J. Cardiol.,* **70,** 26A – 32A.

DASCAL, N., SCHREIBMAYER, W., LIM, N.F., WANG, W.Z., CHAVKIN, C., DIMAGNO, L., LABARCA, C., KIEFFER, B.L., GAVERIAUXRUFF, C., TROLLINGER, D., LESTER, H.A. & DAVIDSON, N. (1993). Atrial G-Protein-Activated K^+-Channel - expression cloning and molecular properties. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 10235 – 10239.

DEAL, K.K., ENGLAND, S.K. & TAMKUN, M.M. (1996). Molecular physiology of cardiac potassium channels. *Physiol. Rev.,* **76,** 49 – 67.

DI FRANCESCO, D. (1995). Cardiac pacemaker: 15 years of new interpretation. *Acta Cardiol.,* **50,** 414 – 427.

DI FRANCESCO, D., DUCOURET, P. & ROBINSON, R. (1989). Muscarinic modulation of cardiac rate at low acetylcholine concentrations. *Science,* **243,** 669 – 671.

DIOCHOT, S., DRICI, M.D., MOINIER, D., FINK, M. & LAZDUNSKI, M. (1999). Effects of phrixotoxins on the Kv4 family of potassium channels and implications for the role of Ito1 in cardiac electrogenesis. *Br. J. Pharmacol.,* **126,** 251–263.

DRICI, M.D., ARRIGHI, I., CHOUABE, C., MANN, J.R., LAZDUNSKI, M., ROMEY, G. & BARHANIN, J. (1998a). Involvement of IsK associated K^+ channel in heart rate control of repolarization in a murine engineered model of Jervell and Lange-Nielsen syndrome. *Circ. Res.,* **83,** 95–102.

DRICI, M.D., BURKLOW, T.R., HARIDASSE, V., GLAZER, R.I. & WOOSLEY, R.L. (1996). Sex hormones prolong the QT interval and downregulate potassium channel expression in the rabbit heart. *Circulation,* **94,** 1471–1474.

DRICI, M.D., WANG, W.X., LIU, X.K., WOOSLEY, R.L. & FLOCKHART, D.A. (1998b). Prolongation of QT interval in isolated feline hearts by antipsychotic drugs. *J. Clin. Psychopharmacol.,* **18,** 477–481.

DUPRAT, F., LESAGE, F., GUILLEMARE, E., FINK, M., HUGNOT, J.P., BIGAY, J., LAZDUNSKI, M., ROMEY, G. & BARHANIN, J. (1995). Heterologous multimeric assembly is essential for K^+ channel activity of neuronal and cardiac G-protein-activated inward rectifiers. *Biochem. Biophys. Res. Commun.,* **212,** 657–663.

FELDER, C.C. (1995). Muscarinic acetylcholine receptors: signal transduction through multiple effectors. *FASEB J.,* **9,** 619–625.

FINK, M., DUPRAT, F., LESAGE, F., REYES, R., ROMEY, G., HEURTEAUX, C. & LAZDUNSKI, M. (1996). Cloning, functional expression and brain localisation of a novel unconventional outward rectifier K^+ channel. *EMBO J.,* **15,** 6854–6862.

FISCHMEISTER, R. & HARTZELL, H.C. (1986). Mechanism of action of acetylcholine on calcium current in single cells from frog ventricle. *J. Physiol.,* **376,** 183–202.

GAULDIE, J., HANSON, J.M., RUMJANEK, F.D., SHIPOLINI, R.A. & VERNON, C.A. (1976). The peptide components of bee venom. *Eur. J. Biochem.,* **61,** 369–376.

GILMAN, A.G. (1987). G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.,* **56,** 615–649.

GOYAL, R.K. (1989). Muscarinic receptor subtypes. Physiology and clinical implications. *N. Engl. J. Med.,* **321,** 1022–1029.

GUILLEMARE, E., HONORE, E., PRADIER, L., LESAGE, F., SCHWEITZ, H., ATTALI, B., BARHANIN, J. & LAZDUNSKI, M. (1992). Effects of the level of messenger RNA expression on biophysical properties, sensitivity to neurotoxins, and regulation of the brain delayed-rectifier K^+ channel Kv1.2. *Biochemistry,* **31,** 12463–12468.

HABERMANN, E. (1972). Bee and wasp venoms. *Science,* **177,** 314–322.

HARTZELL, H.C. (1980). Distribution of muscarinic acetylcholine receptors and presynaptic nerve terminals in amphibian heart. *J. Cell Biol.,* **86,** 6–20.

HARTZELL, H.C. (1981). Mechanisms of slow postsynaptic potentials. *Nature,* **291,** 539–544.

HARTZELL, H.C. & SIMMONS, M.A. (1987). Comparison of effects of acetylcholine on calcium and potassium currents in frog atrium and ventricle. *J. Physiol,* **389,** 411–422.

HELDAL, M. & ORNING, O.M. (1989). A trial flutter with 1:1 AV conduction during intravenous flecainide treatment. *Tidsskr. Nor. Laegeforen.,* **109,** 2309–2310.

HELLER BROWN, J. & TAYLOR, P. (1996). Muscarinic receptor agonists and antagonists. In *Goodman & Gilman's The Pharmacological Basis of Therapeutics*. 9th edn. ed. Hardman, J.G., Goodman Gilman, A. & Limbird, L.E. pp. 141–160. New York: McGraw-Hill.

INOMATA, N., ISHIHARA, T. & AKAIKE, N. (1991). Mechanisms of the anticholinergic effect of SUN 1165 in comparison with flecainide, disopyramide and quinidine in single atrial myocytes isolated from guinea-pig. *Br. J. Pharmacol.,* **104,** 1007–1011.

INOMATA, N., OHNO, T., ISHIHARA, T. & AKAIKE, N. (1993). Antiarrhythmic agents act differently on the activation phase of the ACh-response in guinea-pig atrial myocytes. *Br. J. Pharmacol,* **108,** 111–115.

ISENBERG, G. & KLOCKNER, U. (1982). Calcium tolerant ventricular myocytes prepared by preincubation in a "KB medium". *Pflugers Arch,* **395,** 6–18.

JIN, W. & LU, Z. (1998). A novel high-affinity inhibitor for inward-rectifier K^+ channels. *Biochemistry,* **37,** 13291–13299.

JURMAN, M.E., BOLAND, L.M. & YELLEN, G. (1994). Visual identification of individual transfected cells for electrophysiology using antibody-coated beads. *BioTechniques,* **17,** 876–881.

KAIBARA, M., NAKAJIMA, T., IRISAWA, H. & GILES, W. (1990). Regulation of spontaneous opening of muscarinic K^+ channel in rabbit atrium. *J. Physiol. (London),* **433,** 589–613.

KITAMURA, H., YOKOYAMA, M., AKITA, H., MATSUSHITA, K., KURACHI, Y. & YAMADA, M. (2000). Tertiapin potently and selectively blocks muscarinic K^+ channels in rabbit cardiac myocytes. *J.Pharm.Exp.Ther.,* **293,** 196–205.

KOUMI, S., ARENTZEN, C.E., BACKER, C.L. & WASSERSTROM, J.A. (1994). Alterations in muscarinic K^+ channel response to acetylcholine and to G protein-mediated activation in atrial myocytes isolated from failing human hearts. *Circulation,* **90,** 2213–2224.

KOUMI, S.I. & WASSERSTROM, J.A. (1994). Acetylcholine-sensitive muscarinic K^+ channels in mammalian ventricular myocytes. *Am. J. Physiol.,* **266,** H1812–H1821.

KOVOOR, P., WICKMAN, K., MAGUIRE, C.T., GEHRMANN, J., BERUL., C.I. & CLAPHAM, D.E. (1999). Role of I_{KACh} in cardiac electrophysiology and arrhythmias : evaluation using a GIRK4 knockout mouse model. *Circulation,* **100,** I-768 (Abstract form).

KRAPIVINSKY, G., GORDON, E.A., WICKMAN, K., VELIMIROVIC, B., KRAPIVINSKY, L. & CLAPHAM, D.E. (1995). The G-protein-gated atrial K^+ channel IKACh is a heteromultimer of two inwardly rectifying K^+-channel proteins. *Nature,* **374,** 135–141.

KUBO, Y., REUVENY, E., SLESINGER, P.A., JAN, Y.N. & JAN, L.Y. (1993). Primary structure and functional expression of a rat G-protein-coupled muscarinic potassium channel. *Nature,* **364,** 802–806.

KURACHI, Y., NAKAJIMA, T., ITO, H., TAKIKAWA, R. & SUGIMOTO, T. (1992). On the regulation of cardiac K channel by GTP-binding proteins-basic mechanism and clinical aspect of acetylcholine and adenosine-increase of cardiac potassium conductance. *Jpn. Circ. J.,* **56** (Suppl.5): 1362–1366.

LESAGE, F., GUILLEMARE, E., FINK, M., DUPRAT, F., HEURTEAUX, C., FOSSET, M., ROMEY, G., BARHANIN, J. & LAZDUNSKI, M. (1995). Molecular properties of neuronal G-protein-activated inwardly rectifying K^+ channels. *J. Biol. Chem.,* **270,** 28660–28667.

LOEWI, O. (1921). Über humorale Übertragbarkeit der Herznervenwirkung. *Pflügers Arch.,* **189,** 239–242.

LOEWI, O. & NAVRATIL, E. (1926). Über humorale Übertragbarkeit der Herznervenwirkung, X. Mitteilung. Über das Schicksal des Vagusstoffs. *Pflügers Arch.,* **214,** 678–688.

LOFFELHOLZ, K. & PAPPANO, A.J. (1985). The parasympathetic neuroeffector junction of the heart. *Pharmacol. Rev.,* **37,** 1–24.

LOGOTHETIS, D.E., KURACHI, Y., GALPER, J., NEER, E.J. & CLAPHAM, D.E. (1987). The beta gamma subunits of GTP-binding proteins activate the muscarinic K^+ channel in heart. *Nature,* **325,** 321–326.

MEHTA, D., CAMM, A.J. & WARD, D.E. (1988). Clinical electrophysiologic effects of flecainide acetate. *Cardiovasc. Drugs Ther.,* **1,** 599–603.

MITRA, R. & MORAD, M. (1986). A uniform enzymatic method for the dissociation of myocytes from heart and stomach of vertebrates. *Am. J. Physiol.,* **249,** H1056–H1060.

MOCKZYDLOWSKI, E., LUCCHESI, K. & RAVIDRAN, A. (1988). An emerging pharmacology of peptide toxins targeted against potassium channels. *J. Membr. Biol.,* **105,** 95–111.

NOMA, A. & TRAUTWEIN, W. (1978). Relaxation of the ACh-induced potassium current in the rabbit sinoatrial node cell. *Pflügers Arch.,* **377,** 193–200.

PONGS, O. (1992). Structural basis of voltage-gated K^+ channel pharmacology. *Trends Physiol. Sci.,* **13,** 359–365.

RANKIN, A.C. & WORKMAN, A.J. (1999). Rate control in atrial fibrillation: role of atrial inputs to the AV node. *Cardiovasc. Res.,* **44,** 249–251.

REUVENY, E., SLESINGER, P.A., INGLESE, J., MORALES, J.M., INIGUEZ-LLUHI, J.A., LEFKOWITZ, R.J., BOURNE, H.R., JAN, Y.N. & JAN, L.Y. (1994). Activation of the cloned muscarinic potassium channel by G protein beta gamma subunits. *Nature,* **370,** 143–146.

RODEN, D.M. (1998). Mechanism and management of proarrhythmia. *Am. J. Cardiol.,* **82,** 491–571.

ROFFEL, A.D., MEURS, H. & ZAAGSMA, J. (1994). Muscarinic acetylcholine receptors and control of smooth muscle tone. *Trends Pharmacol. Sci.,* **15,** 407.

TANS, A.C., LIE, I. & DURRER, D. (1980). Clinical setting and prognostic significance of high degree atrioventricular block in acute inferior myocardial infarction : a study of 144 patients. *Am. Heart J.,* **99,** 4–8.

TAWARA, S. (1906). *Das Reizleitungs System des Herzens*. Germany: Fisher.

TRAUTWEIN, W. & DUDEL, J. (1958). Zum Mechanismus der Membranwirkung des Acetylcholines as der Herzmusklefaser. *Pflügers Arch.,* **266,** 324 – 334.

WICKMAN, K. & CLAPHAM, D.E. (1995). Ion channel regulation by G proteins. *Physiol. Rev.,* **75,** 865 – 885.

WICKMAN, K., NEMEC, J., GENDLER, S.J. & CLAPHAM, D.E. (1998). Abnormal heart rate regulation in GIRK4 knockout mice. *Neuron,* **20,** 103 – 114.

WU, S.N., NAKAJIMA, T., YAMASHITA, T., HAMADA, E., HAZAMA, H., IWASAWA, K., OMATA, M. & KURACHI, Y. (1994). Molecular mechanism of cibenzoline-induced anticholinergic action in single atrial myocytes: comparison with effect of disopyramide. *J. Cardiovasc. Pharmacol.,* **23,** 618 – 623.

YAMADA, M., INANOBE, A. & KURACHI, Y. (1998). G Protein regulation of potassium channels. *Pharmacol. Rev.,* **50,** 723 – 757.

(Received April 28, 2000
Revised July 10, 2000
Accepted July 19, 2000)