British Journal of Pharmacology (2000) 129, 843–852

www.nature.com/bjp

Cardiotoxic effects of fenfluramine hydrochloride on isolated cardiac preparations and ventricular myocytes of guinea-pigs

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1 The cardiotoxic effects of fenfluramine hydrochloride on mechanical and electrical activity were studied in papillary muscles, Purkinje fibres, left atria and ventricular myocytes of guinea-pigs.

2 Force of contraction (f_c) was measured isometrically, action potentials and maximum rate of rise of the action potential (V_{max}) were recorded by means of the intracellular microelectrode technique and the sodium current (I_{Na}) with patch-clamp technique in the cell-attached mode. For kinetic analysis (S)-DPI-201-106-modified Na^+ channels from isolated guinea-pig ventricular heart cells were used.

3 Fenfluramine (1–300 μM) produced negative chronotropic and inotropic effects; additional extracellular Ca^{2+} competitively antagonized the negative inotropic effect.

4 Fenfluramine concentration-dependently reduced V_{max} and showed tonic blockade of sodium channels, shortened the action potential duration in papillary muscles and Purkinje fibres.

5 In cell-attached patches, fenfluramine decreased I_{Na} concentration-dependently (10–100 μM), frequency-independently (0.1–3 Hz; 30 μM). The h_∞ curve was shifted towards hyperpolarizing direction. At 30 μM, fenfluramine blocked the sodium channel at all test potentials to the same degree, and neither changed the threshold and reversal potentials nor the peak of the curve.

6 No effect on single channel availability, but a significant decrease in mean open times and increase in mean closed times was observed.

7 Mean duration of the bursts decreased and number of openings per record increased with increasing drug concentration.

8 It is concluded that the effect on I_{Na} plays an important role in the cardiotoxicity of fenfluramine in addition to primary pulmonary hypertension and valvular disorders.

British Journal of Pharmacology (2000) **129,** 843–852

Keywords: Fenfluramine; sodium current; cardiotoxicity; cardiomyocytes; patch-clamp technique

Abbreviations: APA, action potential amplitude; APD_{20}, APD_{50}, APD_{90}, action potential duration at 20-, 50- and 90% repolarization; f_c, force of contraction; h_∞, fraction of sodium channels in the rested state at equilibrium; I_{Na} sodium current; MRP, membrane resting potential; MDP, maximum diastolic potential; RA, rate of activity; SSDD, slope of slow diastolic depolarization; $\tau_{open(1)}$, fast component of the mean open times; $\tau_{open(2)}$, slow component of the mean open times; $\tau_{closed(1)}$, fast component of the mean closed times; $\tau_{closed(2)}$, slow component of the mean closed times; V_{max}, maximum rate of rise of the action potential

Introduction

Fenfluramine is a fluorinated beta-phenylethylamine, which is known for its anorectic properties (Rowland & Carlton, 1986). Fenfluramine is reported to have been used by 50 million patients worldwide (Voelker, 1994). Twenty-four cases of vascular heart disease in obese woman without history of cardiac disease who had been treated with fenfluramine and phentermine in combination were reported (Connolly *et al*., 1997), but also one case with administration of fenfluramine alone (Graham & Green, 1997) and one with dexfenfluramine alone (Cannistra *et al*., 1997). As Connolly *et al*. (1997) pointed out, the histologic picture they have described is virtually indistinguishable from that of valvular heart disease induced by ergotamine or methysergide, so that the question arises whether serotonin might be involved in the mechanism of cardiac side effects of fenfluramine. Subsequently, reports of valvulopathy associated with fenfluramine or its d-enantiomer dexfenfluramine increased (US Department of Health and Human Services, 1997; Khan *et al*., 1998; Weissman *et al*., 1998; Jick *et al*., 1998). Veltri & Temple (1975) reported 13 lethal cases of fenfluramine poisoning; of these deaths one was attributed to ventricular fibrillation, and three cases to asystole. High doses of fenfluramine directly result in pulmonary hypertension, which secondarily induces ischaemic cardiac injury (Hunsinger & Wright, 1990). Hypotheses have been put forward that implicate serotonin, a pulmonary vasoconstrictor (Herve *et al*., 1995; Rounds & Cutaia, 1998), a direct vasoconstrictor effect through potassium-channel blockade (Michelakis *et al*., 1995; Weir *et al*., 1996), but these hypotheses remain speculative (Abenhaim *et al*., 1996) and enigmatic. This also applies to the loss of normal balance between endogenous pulmonary vasodilators and vasoconstrictors (Rounds & Cutaia, 1998). In order to understand the said cardiotoxicity of fenfluramine, we studied the action of fenfluramine hydrochloride on the action potential of guinea-pig papillary muscles, Purkinje fibres, and left atria, and on the sodium current of ventricular myocytes of guinea-pigs.

Methods

Guinea-pigs of either sex (300–500 g) were killed by a blow to the neck. After excision of the heart, papillary muscles were dissected from the right ventricle for contractility measure-

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ments. The right atria were separated from the ventricles and were further cut into pieces to obtain preparations containing the sino-atrial node.

Contractility experiments

An experimental set-up described by Reiter (1967) was used for the isometric measurement of force of contraction in electrically stimulated papillary muscles using an AE 875 force transducer (Aksjeselkapet Mikro-Elektronikk, Horten, Norway) under a resting tension of 3.92 mN. The preparations (diameter <0.8 mm) were bathed in Krebs-Henseleit solution with the following composition (in mM): NaCl 114.9, KCl 4.73, $CaCl_2$ 3.2, $MgSO_4$ 1.18, $NaHCO_3$ 24.9, KH_2PO_4 1.18 and glucose 10; at pH 7.2–7.4. The bathing solution was continuously gassed with 95% O_2/5% CO_2 at $35\pm1^{\circ}C$. Papillary muscles were electrically driven with an Anapulse Stimulator Model 301-T and an Isolation Unit Model 305-T (WP-Instruments, Hamden, CT, U.S.A.) at a rate of 1 Hz and a pulse duration of 3 ms, 10% above threshold intensity. Signals were recorded with a dual beam oscilloscope Type RM 565 (Tektronix Inc., Beaverton, OR, U.S.A.). Photos from the screen were taken every 5 min (Kymograph Camera Model C45, Grass Instruments Co., Quincy, MA, U.S.A.). Stock solutions of the drug were prepared in distilled water on the day of the experiment and further diluted to the required concentrations. The drug concentration (1–300 μM) was increased cumulatively (duration of exposure: 30 min each). Concentrations of $\leqslant10$ μM refer to the therapeutic drug concentrations (Innes *et al*., 1997). The toxic concentrations range from >10 to 100 μM fenfluramine (Veltri & Temple, 1975).

Electrophysiological experiments

Recording of action potentials from papillary muscles, Purkinje fibres and left atria Guinea-pigs of either sex (300–500 g) were killed by a blow to the neck. After excision of the heart, papillary muscles were dissected from the right ventricle. Purkinje fibres were isolated from both the right and left ventricle. Only spontaneously beating fibres were used for the experiments. For experiments with left atria, the left atrium was excised from the heart. The isolated preparations were fixed in a lucite chamber (volume, 1.5 ml), which was continuously superfused (1.5–2.0 ml min^{-1}) with a gassed (95% O_2/5% CO_2) Tyrode's solution of the following composition (in mM): NaCl 136.9, KCl 5.4, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, $CaCl_2$ 1.8, and glucose 5.55 at pH 7.2–7.4. For experiments with Purkinje fibres, the potassium concentration was reduced to 2.7 mM in order to increase the probability of spontaneous activity. Experiments were performed at $37\pm1^{\circ}C$. Action potentials were differentially recorded by means of the intracellular microelectrode technique and were electronically differentiated for measurement of the maximum rate of rise of the action potential (V_{max}). Action potential measurements were made from single microelectrode impalements during control and in presence of the test compound. Each concentration of the test substance was added to the bathing solution for 45 min to reach a steady state of effects, followed by a wash-out phase of 1 h. For investigation of tonic or use-dependent blockade of V_{max} papillary muscles were continuously stimulated during the control period (30 min) at a rate of 1 Hz. The stimulation was interrupted at the time of drug admission, and was resumed after a period of 1 h. The V_{max} values of the first action potentials after the quiescent period was compared with control values. For recording, two microprobe systems (model M 701; WP Instruments, Hamden, CT, U.S.A.) and a D13 dual-beam storage oscilloscope type 5103 N (Tektronix Inc., Beaverton, OR, U.S.A.) were used. The signal recordings were registered with a digital tape recorder (DTR-1202, Biologic Instruments de Laboratories, Claix, France). Data were stored on magnetic tapes (JVC Corporation, Tokyo, Japan). For evaluation, data were transferred to the computer through an interface (DigiData 1200, Axon Instruments, Foster City, CA, U.S.A.) and analysed using pCLAMP 6 software.

Recording of single channel and macroscopic sodium current Single ventricular myocytes were enzymatically dissociated (Mitra & Morad, 1985). Experiments were performed at room temperature (21–23°C). After isolation the cells were stored in a solution of the following composition (in mM): NaCl 140, $CaCl_2$ 1.8, KCl 5.4, $MgCl_2$ 2, and HEPES 10. The solution was titrated with NaOH to pH 7.4. During the experiments, a bathing solution was used which contained (in mM) potassium aspartate 140, $MgCl_2$ 2, EGTA 10, adenosine triphosphate (ATP) 2, and HEPES 10, and which was titrated with KOH to pH 7.4. Cells were depolarized by this solution to 0 mV. The pipette solution contained (in mM): NaCl 140, HEPES 10, CsCl 10, $MgCl_2$ 2, and $CaCl_2$ 1.8, and was titrated with NaOH to a pH of 7.4. Single channel currents were measured in the cell-attached mode using the patch-clamp technique (Hamill *et al*., 1981).

Borosilicate pipettes (Corning; WPI, Hamden, Connecticut, U.S.A.) with a resistance of 5–8 MΩ coated with Sylgard (Dow Corning, Seneffe, Belgium) were used. Depolarizing steps of 20 ms were delivered from holding potentials between -140 and -60 mV to a test potential of -30 mV by a pulse generator at a frequency of 2 Hz. The same protocol was used during the control period and in the presence of the drug. About 5 min after giga-seal formation control recordings started, followed by the addition of fenfluramine hydrochloride at concentrations of 10, 30 and 100 μM. Steady-state effects of the drug were reached in less than 10 min. In a few experiments reversibility of the drug effect was studied during a wash-out period of 15 min. Frequency-dependent effects were studied under steady-state conditions at different stimulation rates at a holding potential of -100 mV. Voltage-dependence of peak sodium current was studied applying 20 ms depolarizing pulses from a holding potential of -120 mV to test potentials ranging from -60 to $+60$ mV (step voltage protocol). The h_∞-curve was determined by applying depolarizing pulses to -30 mV from holding potentials of -140, -120, -100, -90, -80, -70 and -60 mV at a rate of 2 Hz. Steady-state values during control and in presence of the test substance were compared. The macroscopic current is the ensemble averaged current recorded from multichannel cell-attached patches. The currents were filtered at 10 kHz with a Kemo Variable Filter, VBF/8 (Kemo Limited, Beckenham, Kent, U.K.), and digitized at 20 kHz by using an analogue-to-digital converter (TL-1, Axon Instruments, Foster City, CA, U.S.A.), that was connected with a PC. Each sweep contained 512 samples. In the experiments with (S)-DPI-201-106, currents obtained during a 500 ms depolarizing step were sampled at 5 kHz. Correction of capacity and leak currents was done by digitally subtracting averaged sweeps without channel openings from each trace. Eventual slow drifts in the baseline were corrected by fitting a non-sloping baseline through each sweep. Single channel currents and open probability were obtained from the

distance of the peak and the areas under the amplitude histograms, respectively. Records were idealized by setting the detection threshold to half of the unitary current amplitude. Open and closed time distributions were fitted using a non-linear least-squares method. A histogram of the burst duration was calculated by defining a minimal interburst interval of three times the fast time constant of the closed-time histogram. Data acquisition and analysis were controlled by pCLAMP 5 (Axon Instruments, Foster City, CA, U.S.A.) and ASCD (Droogmans, Laboratorium voor Fysiologie, KU Leuven, Belgium) software.

Drug

Fenfluramine hydrochloride (Sigma, U.S.A.) was used.

Statistical analysis

Quantitative results are represented as mean ± s.e.mean of n experiments. A value of $P<0.05$ was considered as statistically significant (Student's t-test, unpaired observations).

Results

Contractility

At a rate of 1 Hz, fenfluramine hydrochloride reduced the force of contraction (f_c) in papillary muscles in a concentration-dependent (1–300 μM, $n=3$) (Figure 1), frequency-independent manner (30 μM, $n=3$). The decrease of f_c was significant from a control value of 3.8 ± 0.6 to 2.4 ± 0.2 mN ($P<0.001$, $n=3$) at 30 μM fenfluramine hydrochloride and to 0.8 ± 0.1 mN at 100 μM ($P<0.001$, $n=3$). At 300 μM fenfluramine hydrochloride contractility was completely suppressed. The inhibitory concentration (IC_{50}) for the negative inotropic effect of fenfluramine hydrochloride was graphically calculated to be 52 ± 4 μM (Figure 1). The negative inotropic effect of fenfluramine hydrochloride (10–100 μM) was competitively antagonized by increasing $[Ca^{2+}]_o$ ($n=11$) (Figure 1).

Fenfluramine hydrochloride produced a negative inotropic and chronotropic effect on spontaneously beating sino-atrial nodes; IC_{50} was found to be 134 μM ($n=3$).

Effects on the action potential and spontaneous activity

Fenfluramine hydrochloride concentration-dependently (1–300 μM) decreased V_{max} in papillary muscles, spontaneously beating Purkinje fibres and in left atria (Figure 2, Table 1), without changing the membrane resting potential or maximum diastolic potential. Fenfluramine hydrochloride produced a significant tonic blockade of sodium channels. In the presence of 100 μM fenfluramine hydrochloride, in papillary muscles the V_{max} of the first action potential after a quiescent period of 1 h was significantly decreased from a control value of 210 ± 10 to 155 ± 11 V s^{-1} ($P<0.05$, $n=3$). The effects on V_{max}, action

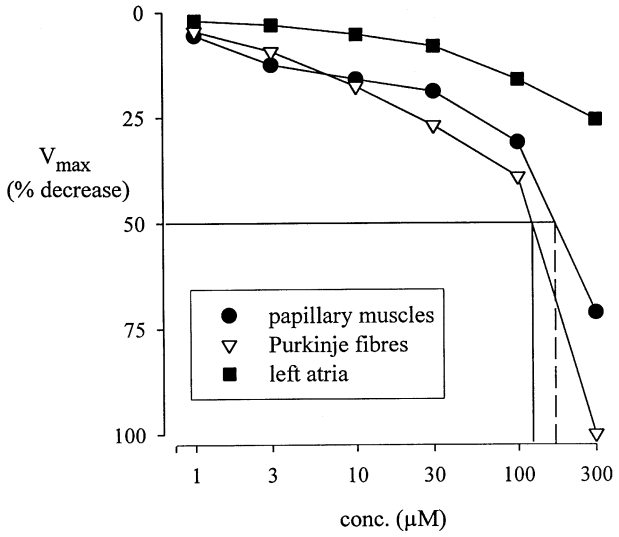

Figure 2 The percentage decrease in V_{max} is plotted against log concentration of fenfluramine hydrochloride. Standard deviation bars are not given in order to facilitate the diagram ($n=30$).

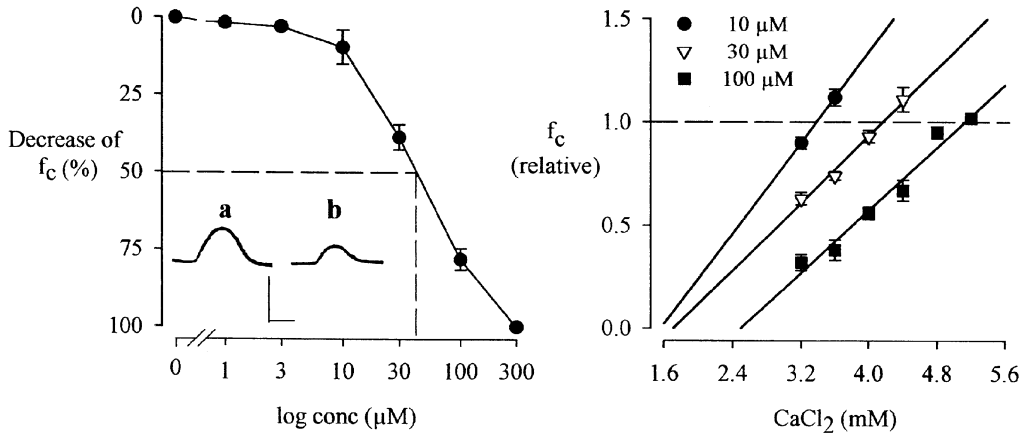

Figure 1 In the left panel the concentration-response curve for the negative inotropic effect of fenfluramine hydrochloride in papillary muscles is shown. The symbols represent mean values ± s.e.mean. The IC_{50}-value was graphically estimated to be 52 ± 4 μM ($n=3$). The inset in the left panel shows original contraction curves during control (left curve) and in presence of 30 μM fenfluramine hydrochloride (right curve). The vertical bar in the inset indicates 3 mN, the horizontal bar 100 ms. In the right panel the abolition of the negative inotropic effect of fenfluramine hydrochloride by $CaCl_2$ is illustrated. The reduction of f_c by fenfluramine hydrochloride is attenuated by increasing the extracellular calcium concentration stepwisely from 3.2 to 5.2 mM $CaCl_2$. Regression lines were calculated to estimate the calcium concentration needed for complete abolition of the negative inotropic effect. Mean values are shown with vertical bars indicating s.e.mean. Control = 1.

potential amplitude and action potential duration were found to be reversible during a washout period of 1 h (Figure 3, Table 1).

In Purkinje fibres fenfluramine hydrochloride (10, 30 and 100 μM) significantly decreased action potential duration at 20, 50 and 90% times to repolarization ($P<0.05$, $n=10$) (Figure 3). No change in slope of slow diastolic depolarization was observed (Table 1). The spontaneous activity was decreased at 100 μM from 52.6 ± 4.9 to 42.2 ± 5.8 beats min^{-1} ($P<0.05$, $n=5$), and ceased after the addition of 300 μM of the drug. Contrary to the lower drug concentrations, the effect of 300 μM fenfluramine hydrochloride was irreversible in Purkinje fibres.

With left atria no significant changes in action potential parameters were observed with 1 to 100 μM fenfluramine hydrochloride (Figure 2); only at 300 μM V_{max} and action potential amplitude were significantly decreased.

Effects on sodium current (I_{Na})

Effects on macroscopic sodium current (I_{Na}) The mean current of multichannel patches in the cell-attached configuration was used to study the effects of fenfluramine hydrochloride on the macroscopic sodium current of ventricular myocytes. The effect of fenfluramine hydrochloride on the macroscopic sodium current (I_{Na}) was studied in multichannel cell-attached patches at concentrations of 10, 30 and 100 μM. Fenfluramine hydrochloride was added to the bathing solution in all experiments. The drug concentration-dependently decreased the peak I_{Na}. At a stimulation rate of 2 Hz and a holding potential of -100 mV the time integral of current was insignificantly decreased from 12.6 ± 2.89 to 11.4 ± 2.9 pA $\times$ ms at 10 μM ($n=3$), from 19.6 ± 5.5 to 10.7 ± 3.6 pA $\times$ ms at 30 μM ($n=4$) and from 7.7 ± 1.7 to 0.5 ± 0.1 pA $\times$ ms at 10 μM ($P<0.01$, $n=3$) fenfluramine hydrochloride, respectively. The

Table 1 Effects of 10 μM fenfluramine hydrochloride on action potential parameters of papillary muscles ($n=5$) and Purkinje fibres

	Papillary muscle			*Purkinje fibre*		
Parameters	*Control*	*10 μM F.*	*Wash-out*	*Control*	*10 μM F.*	*Wash-out*
MRP/MDP (mV)	-90 ± 2	-91 ± 1	-91 ± 1	-91 ± 6	-90 ± 3	-90 ± 4
APA (mV)	119 ± 4	114 ± 3	117 ± 3	129 ± 9	127 ± 12	130 ± 8
V_{max} (V s^{-1})	256 ± 13	208 ± 5**	262 ± 10	338 ± 39	278 ± 10**	341 ± 25
APD_{20} (ms)	77 ± 19	69 ± 27	73 ± 10	83 ± 24	60 ± 23	82 ± 22
APD_{50} (ms)	131 ± 22	118 ± 58	128 ± 26	181 ± 46	141 ± 38	183 ± 45
APD_{90} (ms)	163 ± 26	148 ± 61	160 ± 46	276 ± 17	243 ± 12	275 ± 16
SSDD (mV s^{-1})	–	–	–	14 ± 4	14 ± 4	14 ± 4
RA (beats min^{-1})	–	–	–	52 ± 9	49 ± 7	52 ± 8

**$P<0.01$ MRP/MDP: membrane resting potential/maximum diastolic potential; APA: action potential amplitude; V_{max}: maximum rate of rise of the action potential; APD_{20}, APD_{50}, APD_{90}: action potential duration at 20-, 50- and 90% repolarization; SSDD: slope of slow diastolic depolarization; RA: rate of activity.

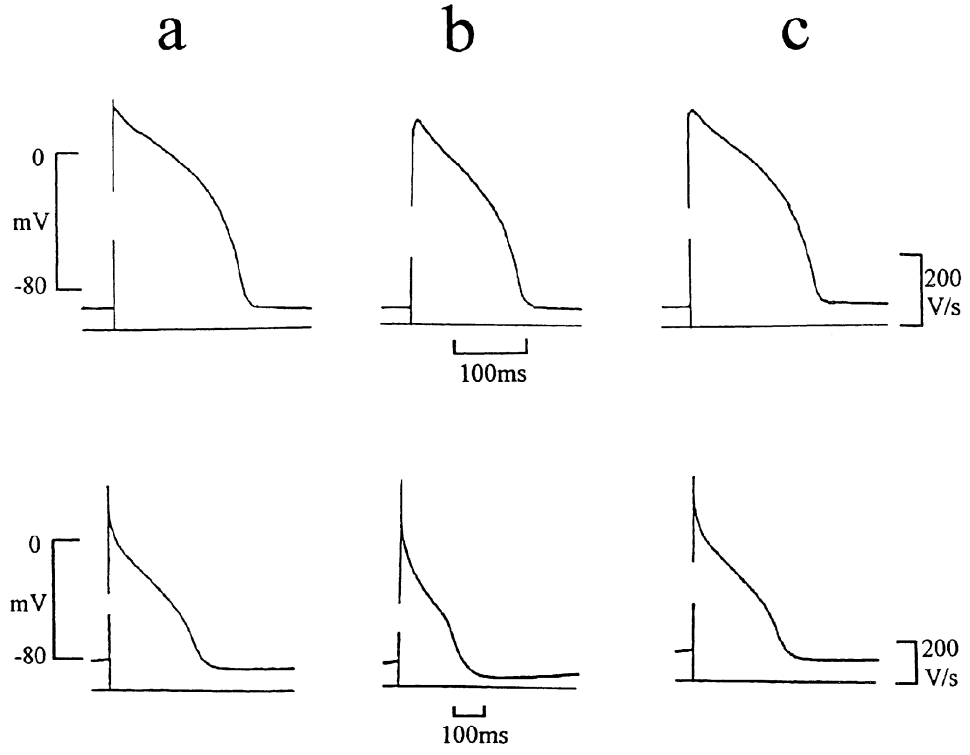

Figure 3 Original recordings from papillary muscle (upper row) and Purkinje fibre (lower row). Action potentials and V_{max} are shown during control (a), with 10 μM fenfluramine hydrochloride (b) and after wash-out (c).

decrease in peak I_{Na} was reversible during a washout period of 15 min only at 10 and 30 μM. The possibility of a frequency-dependent effect was studied in four cell-attached patches at 30 μM fenfluramine hydrochloride. Depolarizing pulses of 20 ms were applied from holding potential of -100 mV to a test potential of -30 mV at intervals of 333 ms, 500 ms, 1 s and 10 s until a steady state was reached. Under control conditions, a change in the stimulus interval caused no significant effect on I_{Na}. Fenfluramine hydrochloride (30 μM) was found to exert no significant frequency-dependent effect (Figure 4).

The membrane potential required for half-maximal steady-state inactivation ($E_{0.5}$) of the cardiac sodium channels (peak I_{Na}, 2 Hz) was shifted by fenfluramine hydrochloride (30 μM, $n=4$) along the voltage axis towards hyperpolarizing direction. The membrane potential at which h_∞ equals 0.5 was shifted from -91 ± 3 mV in control to -97 ± 4 mV by 30 μM fenfluramine hydrochloride ($n=4$, Figure 5).

The current-voltage relation was studied with 30 μM fenfluramine hydrochloride by applying depolarizing pulses from a holding potential of -120 mV to test potentials ranging from -60 to $+60$ mV. I_{Na} elicited from a holding potential of -120 mV started to activate at potentials positive to -60 mV, and was maximal at a test potential of -30 mV. I_{Na} was decreased by 30 μM fenfluramine hydrochloride but the shape of the IV-curve and the reversal potential were not altered ($n=4$, Figure 5).

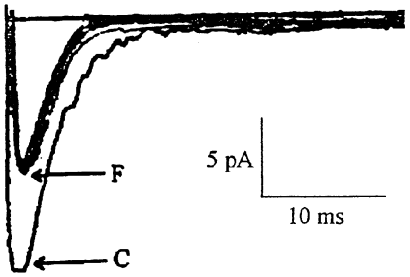

Figure 4 Effect of fenfluramine hydrochloride on the sodium channel current. Superimposed averaged currents of 202 consecutive sweeps each are shown during control (C) and in the presence of 30 μM fenfluramine hydrochloride (F). The absence of any frequency-dependent effect in presence of 30 μM fenfluramine hydrochloride is illustrated. The stimulation interval was 10 s, 1 s, 500 ms and 333 ms, respectively. Under control conditions, a change in the stimulus interval caused no significant effect on single-sodium-channel current (I_{Na}). The cell-attached patch was clamped from a holding potential of -100 mV to a test potential of -30 mV for 20 ms.

Effects of single-channel I_{Na} of (S)-DPI-201-106-modified sodium channels To study the influence of fenfluramine hydrochloride on single-channel kinetics, experiments were performed in the presence of (S)-DPI-201-106 ($n=5$) in the bathing solution. The effect of fenfluramine hydrochloride was studied at concentrations of 10 and 30 μM in cell-attached patches. Five μM (S)-DPI-201-106 were used to slow fast inactivation of the sodium current to facilitate kinetic analysis.

From a holding potential of -120 or -100 mV depolarizing pulses of 500 ms to a test potential of -30 mV were applied at a driving rate of 1 Hz in the presence of 5 μM (S)-DPI-201-106 in the pipette. Ten and 30 μM fenfluramine hydrochloride were added to the bathing solution, cumulatively (Figure 6).

Fenfluramine hydrochloride showed no effect on single channel availability since no change in number of nulls was observed. Open state probability decreased significantly from 0.42 ± 0.03 to 0.38 ± 0.05 at 10 μM ($P<0.05$, $n=5$) and to 0.15 ± 0.03 at 30 μM ($P<0.05$, $n=5$), respectively. The drug at 10 and 30 μM produced a significant decrease in the fast ($\tau_{open(1)}$) component of the mean open times (control: $\tau_1=4.1\pm0.5$ ms; 10 μM: $\tau_1=3.3\pm0.2$ ms; $P<0.05$, $n=5$;

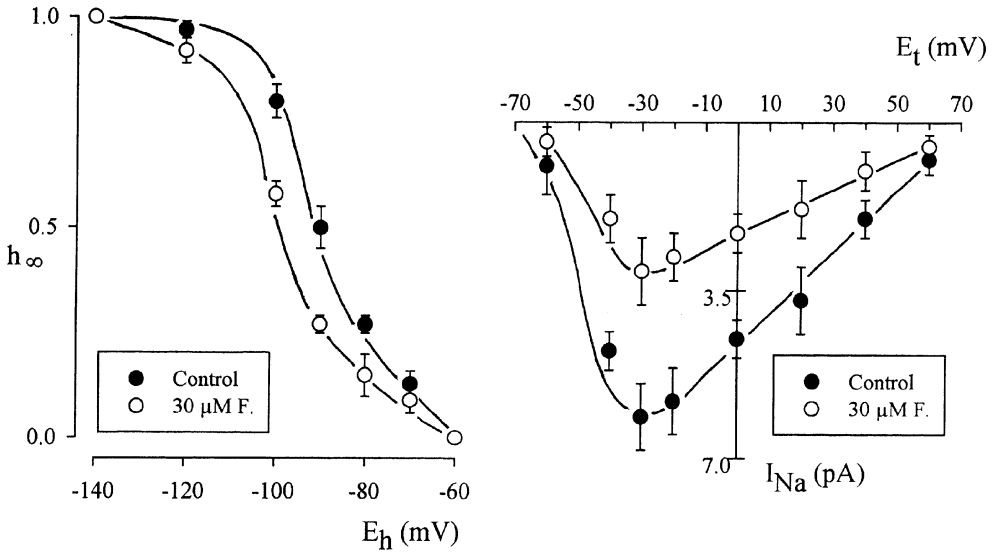

Figure 5 Effect of fenfluramine hydrochloride on the steady-state inactivation curve and current-voltage relationship. Left panel: In four cell-attached multichannel patches 20 ms depolarizing pulses were applied from different holding potentials between -140 and -60 mV to a test potential of -30 mV at a constant stimulation rate of 2 Hz. The inactivation curve for the peak of the ensemble averaged current is shown. Each symbol represents the arithmetic mean of four experiments $\pm$ s.e.mean. Right panel: In cell-attached patches 20 ms depolarizing pulses ranging from -60 mV to $+60$ mV were applied from a holding potential of -120 mV at a rate of 2 Hz. The relative decrease of the sodium current by 30 μM fenfluramine hydrochloride was independent of the test potential. Each symbol represents the arithmetic mean of four experiments $\pm$ s.e.mean. The E_t represents the test potential in mV, while I_{Na} represent the sodium current in pA.

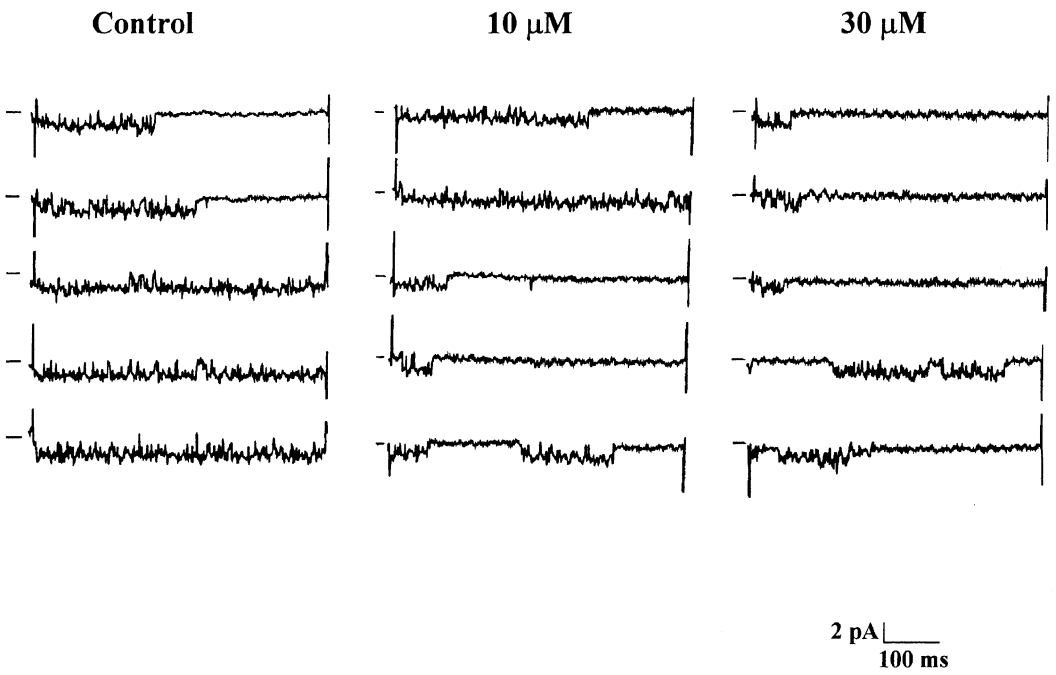

Figure 6 Five consecutive sweeps of original recordings from a cell-attached patch containing one DPI-modified sodium channel (5 μM (S)-DPI-201-106) are shown during control and in the presence of 10 and 30 μM fenfluramine hydrochloride in the bathing solution. Depolarizing pulses of 500 ms were applied from a holding potential of -100 mV to a test potential of -30 mV at a rate of 1 Hz. The mark at the beginning of each trace indicates the baseline, downward deflections represent channel openings.

30 μM: 2.6 ± 1.2 ms; $P<0.05$, $n=5$), but the decrease in the slow component ($\tau_{open(2)}$) of the mean open times was significant only at 30 μM (control: $\tau_2 = 142.0 \pm 33.7$ ms; 10 μM: $\tau_2 = 131.5 \pm 39.3$ ms; 30 μM: $\tau_2 = 90.5 \pm 14.8$ ms; $P<0.05$, $n=5$) (Figure 7). A concentration-dependent (10 and 30 μM) increase in both the fast ($\tau_{closed(1)}$) (control: $\tau_1 = 1.7 \pm 0.2$ ms; 10 μM: $\tau_1 = 2.7 \pm 1.0$ ms; 30 μM: $\tau_1 = 3.9 \pm 1.2$ ms; $P<0.05$, $n=5$) and the slow component ($\tau_{closed(2)}$) (control: $\tau_2 = 34.4 \pm 8.0$ ms; 10 μM: $\tau_2 = 43.9 \pm 18.4$ ms; 30 μM: $\tau_2 = 80.5 \pm 21.5$ ms ($P<0.05$, $n=5$) (Figure 7) was observed.

Burst analysis showed a significant decrease of the burst duration at both tested drug concentrations (control: 93.8 ± 5.1 ms; 10 μM: 63.0 ± 4.5 ms; $P<0.05$; 30 μM: 52.8 ± 10.3 ms; $P<0.05$) (Figure 8). This effect was attended by a significant increase in the number of bursts per record (2.0 ± 0.5 under control to 4.4 ± 0.2 ($P<0.05$, $n=5$) at 10 μM and to 5.7 ± 1.1 ($P<0.05$, $n=5$) at 30 μM) with a significant change in the number of openings per record (5.6 ± 0.4 under control to 9.1 ± 0.6 ($P<0.01$, $n=5$) at 10 μM and to 12.4 ± 1.1 ($P<0.01$, $n=5$) at 30 μM fenfluramine hydrochloride).

Single channel conductivity was insignificantly changed from a control value of 20.8 ± 4.8 to 17.4 ± 3.0 pS ($n=8$; from five DPI-modified and three unmodified single sodium channel patches) by 30 μM fenfluramine hydrochloride (Figure 9).

Discussion

Although fenfluramine is structurally related to amphetamine, these two compounds not only differ in their central pharmacological properties (e.g. Costa *et al*., 1971; Mullen *et al*., 1977), but also in isolated heart muscle preparations the electro-mechanical and -physiological effects of fenfluramine are clearly different from those of amphetamine (Fitzgerald & Reid, 1994).

In papillary muscles and in spontaneously beating Purkinje fibres of guinea-pigs, fenfluramine hydrochloride significantly shortened the action potential duration. From these data, it is possible to deduce that fenfluramine hydrochloride does not block I_K, but these data may suggest a possible Ca^{2+} channel blocking activity of fenfluramine hydrochloride. This assumption is supported by the negative inotropic effect in papillary muscles that could be antagonized by increased calcium concentrations, and the negative chronotropic effects in spontaneously beating sino-atrial nodes.

In papillary muscles of guinea-pigs, fenfluramine hydrochloride produced a concentration-dependent decrease in action potential amplitude and V_{max} without decrease in membrane resting potential. The decrease in V_{max} and action potential amplitude in various heart muscle preparations will lead us to expect a decrease in the conduction velocity by fenfluramine. Depression of conduction may result in the development of areas of unidirectional block and re-entry. The effects of fenfluramine hydrochloride on both V_{max} and action potential amplitude without change in resting potential indicate a blocking action of fenfluramine hydrochloride on the sodium channel. Further, fenfluramine hydrochloride at a concentration of 100 μM reduced V_{max} of the first action potential after a quiescent period of 1 h by 26.2% in guinea-pig papillary muscles, i.e. fenfluramine hydrochloride exerts a significant tonic block or apparent tonic block of sodium channels. However, the actual percentage of this block exerted by fenfluramine hydrochloride cannot be decided due to the following factors: firstly, the non-linear relationship between V_{max} and the open sodium channel (Cohen *et al*., 1984), and secondly, quantitatively, about 10% of the total open channel time occurs before V_{max}, and an even greater percentage before the peak of the sodium current. Any blockade of channels occurring during this time would be measured as apparent rested block (see Hondeghem & Katzung, 1984).

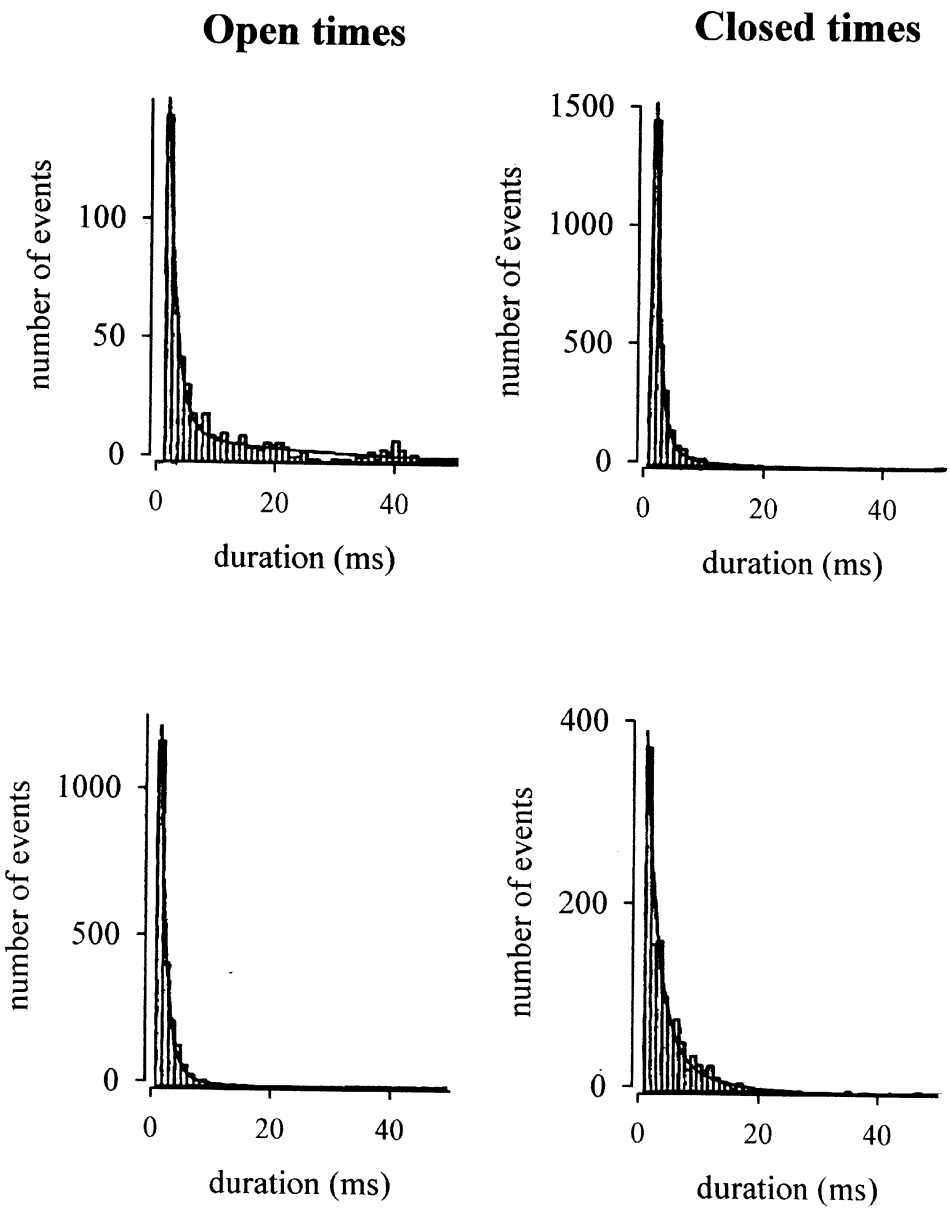

Figure 7 The histograms of mean open (left panel) and mean closed times (right panel) are shown during control (top) and in the presence of 30 μM fenfluramine hydrochloride (bottom) from the experiment illustrated in Figure 6. Mean open time was decreased by fenfluramine hydrochloride, while mean closed time was increased.

Changes in V_{max} are regarded as a qualitative measure of the effect of an agent on the sodium current (Grant *et al*., 1984). Therefore the effect of fenfluramine hydrochloride on I_{Na} was studied directly. Fenfluramine hydrochloride reduced I_{Na} in multi-channel patches of ventricular myocytes. Fenfluramine hydrochloride reduced the Na^+ current in a voltage-dependent and frequency-independent way. The inactivation curve was shifted in the hyperpolarizing direction. These results suggest that fenfluramine hydrochloride apparently has an affinity to the inactivated state of the sodium channel. In the interpretation of pharmacological studies it is important to keep in mind the time-dependent voltage shift of inactivation kinetics. These changes need to be accounted for in the interpretation of possible drug effects. Previous work (e.g. Cachelin *et al*., 1983; Fernandez *et al*., 1984; Makielski *et al*., 1987; Kimitsuki *et al*., 1990) has shown that the steady-state inactivation curve shifts in the hyperpolarizing direction with time. So, the shift of the h_∞ curve could be interpreted as time-dependent shift and not as a drug-induced effect. But, in our experiments with fenfluramine, the binding of the drug to the inactivated state slightly increased with increasing duration of depolarizing steps (20 vs 500 ms). This also suggests that there is an affinity for binding to the inactivated state of the channel. So far, our results with V_{max} and I_{Na} from multi-channel patches show a block of the sodium channels in the closed and inactivated state.

Furthermore, experiments at the single-channel level revealed open channel block showing that fenfluramine reduced I_{Na} in unmodified and DPI-modified sodium channels in cell-attached patches by affecting single-channel kinetics without changing single channel availability.

With a pK_a of 10.0 (Dollery, 1991) more than 99% of fenfluramine molecules are present in their charged form under the experimental conditions used. It is assumed that this feature of a drug strongly limits free diffusibility through the lipid membrane (Hille, 1977). Drugs with a

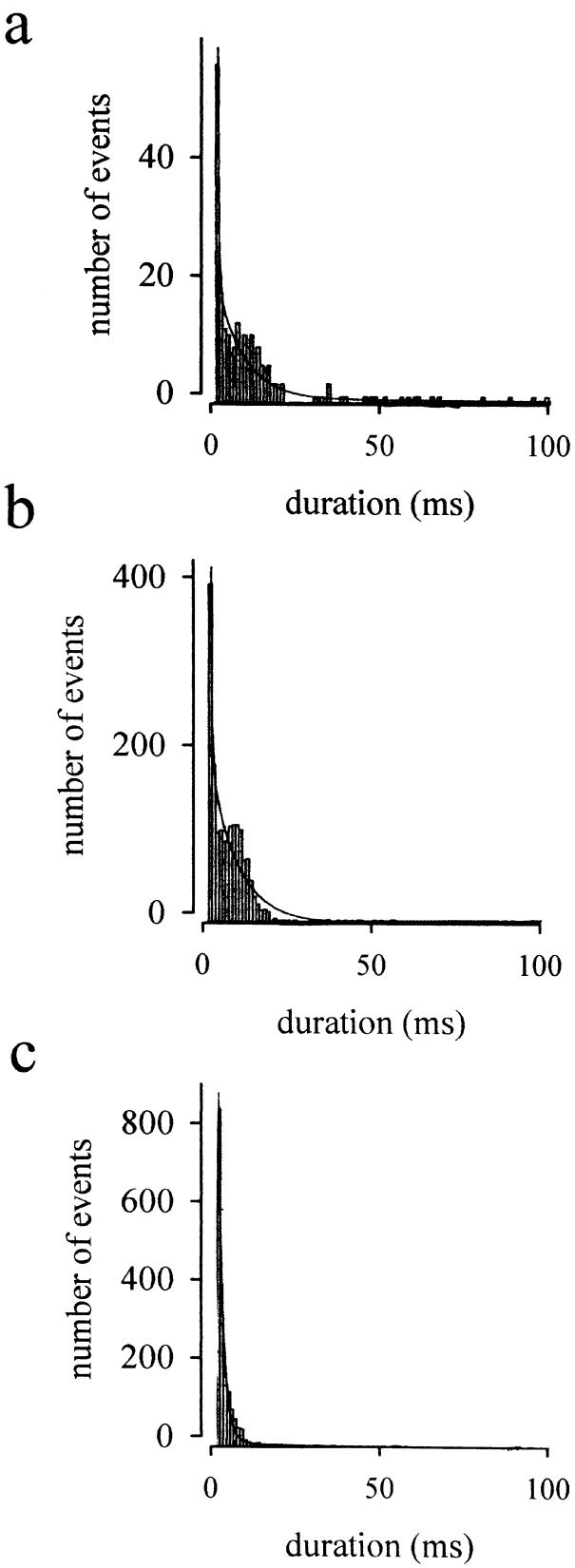

Figure 8 The histograms of burst duration are shown during control (a), in the presence of 10 μM (b) and 30 μM fenfluramine hydrochloride (c), respectively. A dose-dependent decrease in burst duration was observed.

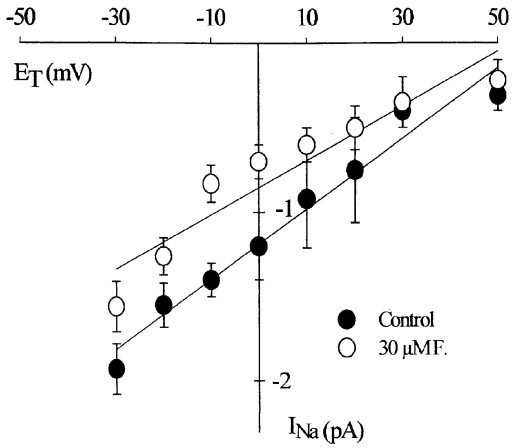

Figure 9 Single channel current-voltage relationship at different test potentials: The test potential (E_T) in mV is plotted against the amplitude of I_{Na} in pA. The vertical lines represent s.e.mean. Regression lines were calculated to estimate the mean conductance of the sodium channel (control: $\gamma = 20.8 \pm 4.8$ pS; 30 μM fenfluramine hydrochloride: $\gamma = 17.4 \pm 3.0$ pS; $n = 8$).

higher pK_a value show activated channel block like quinidine (Heistracher, 1971), disopyramide (Gruber & Carmeliet, 1989), penticainide (Carmeliet, 1988), QX-314 (Yeh & Tanguy, 1985) and transcainide (Gingrich *et al*., 1993; Zamponi & French, 1994). Although the effects of fenfluramine hydrochloride on macroscopic I_{Na} fail to show open state block, experiments with single channels give enough evidence to presume that the drug preferably binds to the open state. So that tonic blockade of V_{max} and frequency-independent effect on macroscopic I_{Na} can be interpreted as apparent rested block or very fast open channel block.

When the drug interacts with the sodium channel in its open state, and when the blocking agent has a high dissociation rate from the channel, flickering occurs. Flickering is caused by repeatedly occurring, fast transitions between the open, conducting state and a nonconducting state of the bursting sodium channel. Such behaviour is reflected by a decrease in mean open time and an increase of the number of openings per burst as shown for example with penticainide (Gruber *et al*., 1991). With fenfluramine we observed a significant decrease in mean open time and an increase in both bursts per record and number of openings per record.

If the open state shows the highest affinity, the block is an activated, open-state block (Wang *et al*., 1996). Depending on the rate of binding and unbinding, the single channel behaviour and the whole-cell current are changed differently. Drugs with very fast on/very fast off kinetics show burst-like activity, which cannot be resolved (due to limited bandwidth of the recording apparatus) and appears as a decrease in channel amplitude (Carmeliet & Mubagwa, 1998). At the macroscopic level, current amplitude is only slightly decreased and slowed. For drugs with fast on/fast off kinetics the drug effect appears as an induction of burst-like activity without change in channel amplitude. The mean duration of a burst is prolonged if the blocked channel cannot inactivate, but can be reduced if the blocked channel inactivates (Carmeliet & Mubagwa, 1998) as seen with fenfluramine. At the macroscopic level, the amplitude of the current is decreased (Carmeliet & Mubagwa, 1998). In our experiments with fenfluramine hydrochloride we observed a non-significant decrease in current amplitude which may be due to limited bandwidth of the recording apparatus and a burst-like activity induced by the drug. This might lead us to presume that the drug

has very fast on/very fast off kinetics in producing open-state block of the sodium channels. This would also lead us to expect that at the macroscopic level current amplitude is only slightly decreased or the rate of inactivation is slowed. By following the results of the kinetic analysis with fenfluramine hydrochloride closely, a different picture emerged, where at the single channel level, events that lead to flickering are produced, and at the macroscopic level the amplitude of the current is decreased. Hence, from the above-mentioned results, fenfluramine hydrochloride may produce open-state block with fast on/fast off kinetics with an additional blockade of sodium channels in the inactivated state.

From our results, we conclude that fenfluramine hydrochloride binds to the sodium channel predominantly in the open state with fast on/fast off kinetics, which also plays an important role in the cardiotoxicity, in addition to primary pulmonary hypertension and valvular disorders.

S. Rajamani was supported by Austrian Academic Exchange Service (EZA-894).

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(Received August 2, 1999
Revised October 5, 1999
Accepted November 26, 1999)