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COMPARISON OF THE EFFICIENCY OF NA+/CA2+ EXCHANGER OR NA+/H+ EXCHANGER INHIBITION AND THEIR COMBINATION IN REDUCING CORONARY REPERFUSION-INDUCED ARRHYTHMIAS

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During ischaemia/reperfusion, the rise in $[Na^+]_i$, induced by simultaneous depression of the Na^+/K^+-ATPase and activation of the Na^+/H^+ exchanger (NHE), shifts the Na^+/Ca^{2+} exchanger (NCX) into reverse transport mode, resulting in Ca^{2+}_i overload, which is a critical factor in enhancing the liability to cardiac arrhythmias. The inhibition of NHE, and recently NCX has been suggested to effectively protect the heart from reperfusion-induced arrhythmias. In this study, we investigated and compared the efficacy of individual or the simultaneous inhibition of the NHE and NCX against reperfusion-induced arrhythmias in Langendorff-perfused rat hearts by applying a commonly used regional ischaemia-reperfusion protocol. The NHE and NCX were inhibited by cariporide and SEA0400 or the novel, more selective ORM-10103, respectively. Arrhythmia diagrams calculated for the reperfusion period were analysed for the incidence and duration of extrasystoles (ESs), ventricular tachycardia (VT) and ventricular fibrillation (VF). NHE inhibition by cariporide was highly efficient in reducing the recorded reperfusion-induced arrhythmias. Following the application of SEA0400 or ORM-10103, the number and duration of arrhythmic periods were efficiently or moderately decreased. While both NCX inhibitors effectively reduced ESs, the most frequently triggered arrhythmias, they exerted limited or no effect on VTs and VFs. Of the NCX inhibitors, ORM-10103 was more effective. Surprisingly, the simultaneous inhibition of the NCX and NHE failed to significantly improve the antiarrhythmic efficacy reached by NCX blockade alone. In conclusion, although principal simultaneous NHE+NCX inhibition should be highly effective against all types of the recorded reperfusion-induced arrhythmias, NCX inhibitors, alone or in combination with cariporide, seem to be moderately suitable to provide satisfactory cardioprotection - at least in the present arrhythmia model. Since ORM-10103 and SEA0400 are known to effectively inhibit after-depolarisations, it is suggested that their efficacy and that of other NCX inhibitors may be higher and more pronounced in the predominantly Ca^{2+}_i-dependent triggered arrhythmias.

K e y   w o r d s : *isolated heart, ischaemia-reperfusion injury, arrhythmia, Na+/Ca2+ exchanger, Na+/H+ exchanger, cariporide, extrasystole, ventricular tachycardia*

INTRODUCTION

The crucial role of severe disturbances in intracellular ionic homeostasis (including changes in $[Na^+]_i$, $[K^+]_i$, $[H^+]_i$, $[Ca^{2+}]_i$) in the initiation and progression of potentially lethal cardiac arrhythmias has been demonstrated in a wide range of experimental and clinical studies (1). These arrhythmias have poor prognosis and a high rate of mortality, primarily due to their complex and not fully understood pathomechanisms and the consequent sub-optimal medical treatment (2).

An important component of the underlying mechanisms is an increase in the activity of sarcolemmal Na^+/H^+ (NHE) and Na^+/Ca^{2+} (NCX) exchangers (3, 4). During myocardial ischaemia the aerobic energy metabolism of the heart is compromised. A pathological consequence of the anaerobic shift is intracellular acidosis. Simultaneous inactivation of the Na^+/K^+-ATPase (NKA) and activation of the NHE leads to gradual $[Na^+]_i$ accumulation, which, in turn, shifts the NCX into

reverse transport mode, generating a marked elevation in $[Ca^{2+}]_i$ (5). During the early phase of reperfusion, the NHE becomes fully activated and $[Na^+]_i$ accumulation is further accelerated. The reverse transport activity of NCX is also highly enhanced and severe $[Ca^{2+}]_i$ overload, the most important factor in generation of post-ischaemic arrhythmias and hypercontracture, may build up (6).

A reasonable defensive strategy to limit the ischaemia/reperfusion-induced $[Na^+]_i$ accumulation and subsequent $[Ca^{2+}]_i$ overload seems to be a pharmacological inhibition of one or both exchangers. However, since their normal activity is vital, only a partial and temporal blockade may be safely introduced. In preclinical studies, NHE inhibition has been shown to be highly effective, especially when applied before ischaemia (7-10). Based on the promising results of these studies, NHE inhibition has been suggested as a straightforward therapeutic strategy against ischaemia-reperfusion-induced cardiac injuries. Human results from phase2/phase3 clinical

trials (GUARDIAN, EXPEDITION, ESCAMI), however, produced disappointing results, reporting only limited efficacy of this strategy in the majority of cases (11).

Theoretically, NCX inhibitors administered shortly after the onset of a heart attack might reduce the probability of arrhythmia generation by limiting damage in the border zone. In a range of preclinical studies, especially under moderate ischaemia, reverse mode inhibition of NCX has been found to suppress both $[Na^+]_i$ and $[Ca^{2+}]_i$ overload, hypercontracture, enzyme release and cell damage (12). Consequently, the inhibition of NCX may have substantial therapeutic potential in the prevention of cardiac ischaemia/reperfusion-induced arrhythmias and injuries (13). Evaluation of the experimental data on NCX inhibition was, however, seriously hampered by the lack of selective and highly effective NCX inhibitors. This may explain why only NHE inhibitors are used to some extent in clinical practice. NCX inhibitors have never been tested in clinical trials.

In summary, there is convincing evidence suggesting that in clinical practice, NHE inhibition, *per se,* fails to prevent or substantially reduce ischaemia/reperfusion-induced arrhythmogenesis (14). On the other hand, reverse mode NCX inhibition displayed promising cardioprotective effects in animal studies (15). The present work was based on the assumption, that since the enhanced NHE activity is only one - although probably the most important - factor resulting in cardiac arrhythmogenesis due to the subsequent $[Ca^{2+}]_i$ overload, the inhibition of NHE itself, does not seem to be the optimal treatment to reduce the incidence and severity of cardiac arrhythmias. One may also speculate that since the NHE and NCX seem to play a joint key role in the induction of ischaemia/reperfusion-induced $[Ca^{2+}]_i$ overload, the deteriorative effects might be better attenuated by simultaneously inhibiting both the NHE and NCX. Therefore, the primary goal of the present study was to evaluate and compare the putative protective effects of individual or combined inhibition of NHE and NCX against post-ischaemic arrhythmias, at least partially caused by the reperfusion-induced $[Ca^{2+}]_i$-overload. For this purpose, a well-known, well-characterised NHE inhibitor, cariporide (16), and two NCX inhibitors, the fairly selective SEA0400 (17) and the novel, more selective ORM-10103 (18), were used.

METHODS AND MATERIALS

All experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals (USA NIH publication No. 86-23 revised 1996) and fully conformed to Directive 2010/63/EU of the European Parliament. Experimental protocols were approved by the Ethics Committee for the Protection of Animals in Research of the University of Szeged, Szeged, Hungary (permit No. I-74-9/2009).

Preparation of isolated hearts

120 male Sprague-Dawley rats (250–350 g) were anaesthetised with sodium thiopental (0.1 g/kg, i.p.) and injected with heparin sodium (500 IU i.v.). Hearts were rapidly excised, mounted *via* the aorta on a Langendorff apparatus and perfused retrograde with warm (37°C), modified Krebs-Henseleit bicarbonate (KHB) buffer at a constant pressure (80 mmHg). The KHB solution contained (in mmol/l): $NaHCO_3$ 25; KCl 4.3; NaCl 118.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; Na-pyruvate 5; glucose 11; $CaCl_2$ 2, having a pH of 7.4±0.05 when gassed with carbogene (95% O_2 + 5% CO_2). For the induction of regional ischaemia, a 6/0 braided silk suture was placed around the left anterior descending coronary artery (LAD). A water-filled latex balloon was inserted into the left ventricular cavity and inflated

to obtain a control state end-diastolic pressure (LVEDP) of 4–8 mmHg. Coronary flow (CF), left ventricular pressure (LVP), perfusion pressure, and the electrocardiogram (ECG) were simultaneously recorded using the Haemosys software (Experimetria, Hungary). Left ventricular contractility (±dP/dt$_{max}$) and heart rate (HR) were calculated off-line from the recorded LVP curve.

Experimental protocol

All hearts were allowed to stabilise for 10 min. The perfusion of drugs or vehicle started 5 minutes before occlusion of the left anterior descending coronary artery (LAD) and were maintained throughout the protocol. Hearts were randomly assigned into six experimental groups: the control (CON) group was perfused with KHB solution containing solely the vehicle (dimethyl sulphoxide (DMSO) in 0.01% final concentration). Three heart groups were perfused with KHB solution containing only a single inhibitor: 5 μM cariporide (CAR); 1 μM SEA0400 (SEA) or 1 μM ORM-10103 (ORM). Two experimental groups were treated with a combination of the NHE and NCX inhibitors: 5 μM cariporide + 1 μM SEA0400 (SEA+CAR) or with 5 μM cariporide + 1 μM ORM-10103 (ORM+CAR). Ischaemia-reperfusion-induced arrhythmias were evoked by pulling on the suture around the artery for 10 minutes. Successful occlusion was confirmed by a significant reduction in coronary flow. To ensure the homogeneity of ischaemic damage and to exclude possible bias due to surgical artefacts, hearts with a more than 65% decrease in coronary flow rate were excluded from the study. Furthermore, hearts developing severe arrhythmias during the ischaemic period were also excluded. The 10 min period of regional ischaemia was followed by 30 min reperfusion initiated *via* releasing the suture. Successful reperfusion was confirmed by an immediate increase in coronary flow. LVP and ECG were recorded and analysed off-line for duration and incidence of the sinus rhythm periods (SR), SR periods with multiple extrasystoles (ES), periods of ventricular tachycardia (VT), defined by the incidence of four or more consecutive ventricular premature beats. The onset of ventricular fibrillation (VF) was verified by the permanent lack of individual QRS deflections.

Drugs

SEA0400 and cariporide were synthesised in the Department of Pharmaceutical Chemistry, University of Szeged. ORM-10103 was a gift from Orion Pharma (Espoo, Finland). The inhibitory effect of both inhibitors is bidirectional with nearly symmetrical concentration dependency for the reverse and forward transport modes. All three drugs were dissolved in dimethyl-sulphoxide (DMSO) and diluted in KHB until establishing the final concentration. The DMSO concentrations in the stock solutions and in the final perfusion solution were 5% and 0.01%, respectively. At this final concentration, the effect of DMSO on the heart is generally considered negligible.

Data processing and statistical analysis

Major haemodynamic parameters (CF, HR, LVSP, LVEDP, DP and ±dP/dt$_{max}$) were either directly measured or calculated from the pressure recording (LVP curve) four times during the experiment: at the end of the equilibration (control), pre-treatment, ischaemic and reperfusion periods, respectively. All haemodynamic values calculated for the reperfusion phase and summarised in *Table 1* were obtained from hearts devoid of sustained arrhythmias or were evaluated during a period of sinus rhythm between two arrhythmic periods.

The severity of reperfusion-induced arrhythmias observed in the LVP and ECG recordings was judged by evaluating three crucial arrhythmia-related variables: the type of arrhythmic activity (i.e. extrasystoles (ES), ventricular tachycardia (VT) and ventricular fibrillation (VF)) and the incidence and duration of time periods with that arrhythmia type. Furthermore, total duration of the regular sinus rhythm periods (SR) was also calculated. It should be emphasised that although transient and sustained ventricular fibrillatory periods were both observed during the 30 min reperfusion period, since we were primarily interested in the efficiency of the applied pharmacological treatments against ventricular arrhythmias generated during the early phase of reperfusion, these two forms of VF were not separated during evaluation of the individual arrhythmia diagrams.

Since in many of the hearts subjected to the regional ischaemia/reperfusion protocol the ventricular fibrillation, if started, became sustained - probably, but not necessarily reflecting ischaemia-reperfusion-induced irreversible shifts in

Table 1. Major hemodynamic variables: coronary flow (CF); heart rate (HR); left ventricular systolic pressure (LVSP); left ventricular end-diastolic pressure (LVEDP); developed pressure (DP); and contractility ($\pm dp/dt_{max}$) at the end of the control, pretreatment, ischemia and reperfusion periods. The n number for the first three columns: CON=19; CAR=9; SEA=13; ORM=12; SEA+CAR=12; ORM+CAR=13; for the reperfusion column: CON=8; CAR=9; SEA=6; ORM=7; SEA+CAR=6; ORM+CAR=5. Significance signs * and represent $P<0.05$ compared to the control group for the ischemia (identical n numbers) and reperfusion (different n numbers) groups, respectively (repeated measure ANOVA).

	Control	Pretreat	Ischemia	Reperfusion
CF (ml/min)				
CON	17 ± 0.9	17 ± 1.0	9 ± 0.9 *	15 ± 1.4
CAR	19 ± 1.5	18 ± 1.5	13 ± 1.8 *	16 ± 2.3
SEA	16 ± 0.8	16 ± 1.1	10 ± 1.4 *	14 ± 1.3
ORM	16 ± 1.1	17 ± 1.1	9 ± 0.9 *	15 ± 1.5
SEA + CAR	16 ± 1.4	15 ± 1.3	9 ± 1.1 *	13 ± 1.6
ORM + CAR	20 ± 0.9	20 ± 1.1	11 ± 1.3 *	16 ± 1.3 §
HR (beats/min)				
CON	313 ± 11	295 ± 10	280 ± 12 *	277 ± 12 §
CAR	325 ± 13	320 ± 11	310 ± 12	280 ± 14 §
SEA	297 ± 10	301 ± 11	294 ± 12	281 ± 20 §
ORM	311 ± 12	308 ± 12	302 ± 12	303 ± 19 §
SEA + CAR	303 ± 15	288 ± 21	287 ± 18	308 ± 28 §
ORM + CAR	343 ± 09	310 ± 13	309 ± 11	279 ± 07 §
LVSP (mmHg)				
CON	101 ± 3	102 ± 3	55 ± 6 *	73 ± 07 §
CAR	101 ± 3	98 ± 2	55 ± 7 *	88 ± 02 §
SEA	92 ± 3	90 ± 4	47 ± 6 *	74 ± 05 §
ORM	96 ± 3	94 ± 7	37 ± 5 *	61 ± 16 §
SEA + CAR	92 ± 4	94 ± 5	47 ± 6 *	55 ± 12 §
ORM + CAR	95 ± 3	98 ± 3	39 ± 4 *	90 ± 07 §
LVEDP (mmHg)				
CON	5.0 ± 1.4	5.0 ± 1.6	5.6 ± 1.6	18.0 ± 4.8 §
CAR	7.4 ± 1.5	7.8 ± 1.5	8.1 ± 1.2	15.0 ± 3.9 §
SEA	5.9 ± 0.7	5.4 ± 1.1	6.3 ± 1.3	11.2 ± 6.3 §
ORM	5.8 ± 0.5	4.3 ± 0.8	5.3 ± 0.9	5.1 ± 0.3 §
SEA + CAR	5.1 ± 1.1	7.4 ± 2.3	7.4 ± 2.5	15.7 ± 7.4 §
ORM + CAR	5.5 ± 0.9	4.3 ± 0.9	4.0 ± 0.8	21.1 ± 8.1 §
DP (mmHg)				
CON	96 ± 3	97 ± 3	55 ± 5 *	55 ± 06 §
CAR	93 ± 4	90 ± 4	47 ± 7 *	72 ± 06 §
SEA	86 ± 3	85 ± 5	41 ± 6 *	63 ± 08 §
ORM	90 ± 3	90 ± 6	32 ± 4 *	61 ± 12 §
SEA + CAR	85 ± 4	86 ± 6	39 ± 6 *	39 ± 11 §
ORM + CAR	89 ± 3	94 ± 3	35 ± 4 *	69 ± 08 §
+dp/dt$_{max}$ (mmHg/s)				
CON	2678 ± 124	2721 ± 140	1510 ± 147 *	1587 ± 155 §
CAR	2388 ± 199	2422 ± 186	1344 ± 181 *	2222 ± 238 §
SEA	2315 ± 176	2361 ± 209	1238 ± 138 *	1583 ± 186 §
ORM	2691 ± 172	2791 ± 259	1166 ± 113 *	1714 ± 295 §
SEA + CAR	2666 ± 193	2733 ± 170	1533 ± 170 *	1600 ± 358 §
ORM + CAR	2191 ± 096	2208 ± 100	975 ± 088 *	1640 ± 208 §
-dp/dt$_{max}$ (mmHg/s)				
CON	-1942 ± 070	-1963 ± 075	-994 ± 094 *	-1150 ± 125 §
CAR	-1722 ± 151	-1733 ± 113	-966 ± 148 *	-1422 ± 098 §
SEA	-1784 ± 093	-1723 ± 127	-938 ± 104 *	-1266 ± 120 §
ORM	-2108 ± 113	-2175 ± 171	-891 ± 079 *	-1400 ± 296 §
SEA + CAR	-1822 ± 111	-1822 ± 145	-911 ± 116 *	-1166 ± 322 §
ORM + CAR	-1825 ± 093	-1891 ± 096	-808 ± 067	-1480 ± 131 §

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electrophysiological properties of the heart - the raw values obtained for the incidence and duration of the ES and VT periods were highly dependent on the total length of the VF-free periods. Therefore, in order to provide more realistic information, the total duration of these periods has been normalised for the total duration of the VF-free periods. Furthermore, in order to avoid dividing by zero or close to zero values in cases of very low VF duration (occurring primarily in cariporide treated hearts), nominal durations of the VF periods were assigned to score levels changing in 5 min intervals between 1 and 7 (i.e. VF of 0–5 min corresponded to level 1, 5–10 min to level 2, etc.).

Usually (absolute) incidence means the number of occurrences (i.e. counts); therefore, it should be dimensionless. In our analysis, relative incidence values were used, i.e. the absolute count numbers were normalised to the duration of the VF-free periods, and therefore expressed as a ratio (the occurrence divided by the total duration of VF-free periods during reperfusion). Hence, the dimension of this variable became frequency (1/min).
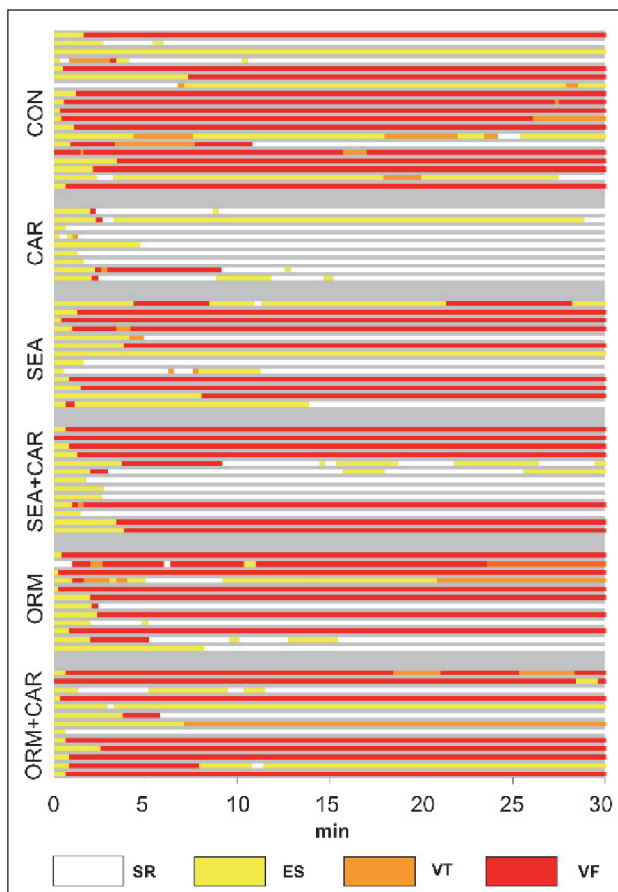

Fig. 1. Integrated arrhythmia diagram calculated from all hearts. X axis: reperfusion time; Y axis: arrhythmia diagrams of 78 individual hearts. The hearts were divided into 6 groups: CON (n=19): control (only vehicle: 0.01mM DMSO); CAR (n=9): 5 μM cariporide, SEA (n=12): 1 μM SEA0400; ORM (n=12): 1 μM ORM-10103; SEA+CAR (n=13): 1 μM SEA0400 + 5 μM cariporide; ORM+CAR (n=13): 1 μM ORM-10103 + 5 μM cariporide. Each of the horizontal lines represents a single heart. Time periods with the various arrhythmia stages are shown in colour: white: regular sinus rhythm (SR); yellow: multiple extrasystoles (ES); brown: ventricular tachycardia (VT) and red: ventricular fibrillation (VF).

Statistical analysis

Statistical calculations were performed using Statistica 9.0 (StatSoft Inc. USA). All data are expressed as means ± S.E.M. Statistical analysis was performed using parametric or nonparametric (Kruskal-Wallis) ANOVA, depending on the homogeneity or inhomogeneity of the variances between individual groups. All statistical calculations were performed either relative to the control group (one-way ANOVA) in case of evaluating the differences between groups with no treatment or various pharmacological treatments in the same experimental state (i.e. intergroup differences) or relative to the corresponding control (non-ischaemic, non-treated) baseline when evaluating the effect of interventions – i.e. the application of inhibitor(s), ischaemia or reperfusion (repeated measures ANOVA). Statistically or marginally significant differences are marked in figures by * ($P<0.05$), or # ($P<0.1$), respectively.

RESULTS

Haemodynamic parameters

Major haemodynamic parameters (CF, HR, LVSP, LVEDP, DP, and ± dP/dt$_{max}$), measured or calculated for all six experimental groups, in all four phases (control, pre-treatment, ischaemia, and reperfusion) are summarised in *Table 1*. Pre-treatment of the hearts with the vehicle (DMSO), an NCX or NHE inhibitor, or their combination had no apparent effect on any of the haemodynamic parameters. Compared to control values, however, ischaemia induced a large, significant decrease in CF, LVSP, DP and ± dP/dt$_{max}$, while the HR and LVEDP were practically unchanged. During the reperfusion phase, the CF recovered, while the LVSP, DP and ± dP/dt$_{max}$ remained significantly lower than in the control state. In contrast, LVEDP became significantly enhanced in all experimental groups. Nonetheless, is should be emphasised that the n numbers for the reperfusion data are substantially lower than for all other states. Importantly and interestingly, using one-way ANOVA, no significant intergroup differences could be found in values obtained from the control and test groups during any experiment phase.

Arrhythmia diagrams

The raw results of the arrhythmia analysis for all experimental groups are summarised in the graphical arrhythmia diagram shown in *Fig. 1*. Following a relatively short (10 min) period of regional ischaemia, the most common type of reperfusion-induced arrhythmias evoked in control (CON) rat hearts (treated solely with the vehicle, DMSO) was VF. The antiarrhythmic efficacy of the selective NHE inhibitor, cariporide, at least in this simple model, was excellent. Compared to the very high incidence of VFs present in the control group, cariporide treatment drastically reduced the incidence and even more the duration of reperfusion-induced arrhythmias. The antiarrhythmic efficacy of the two NCX inhibitors, SEA0400 and ORM-10103, either alone or in combination with cariporide, can hardly be quantitatively estimated by simply looking at the arrhythmia diagram. Nonetheless, it is obvious that none of these treatments has been as effective as cariporide treatment alone. In order to better reveal the relatively moderate but possibly important differences between various treatments, especially for the two types of less severe reperfusion induced arrhythmias (ES and VT), a more detailed, quantitative analysis has been carried out (see below).
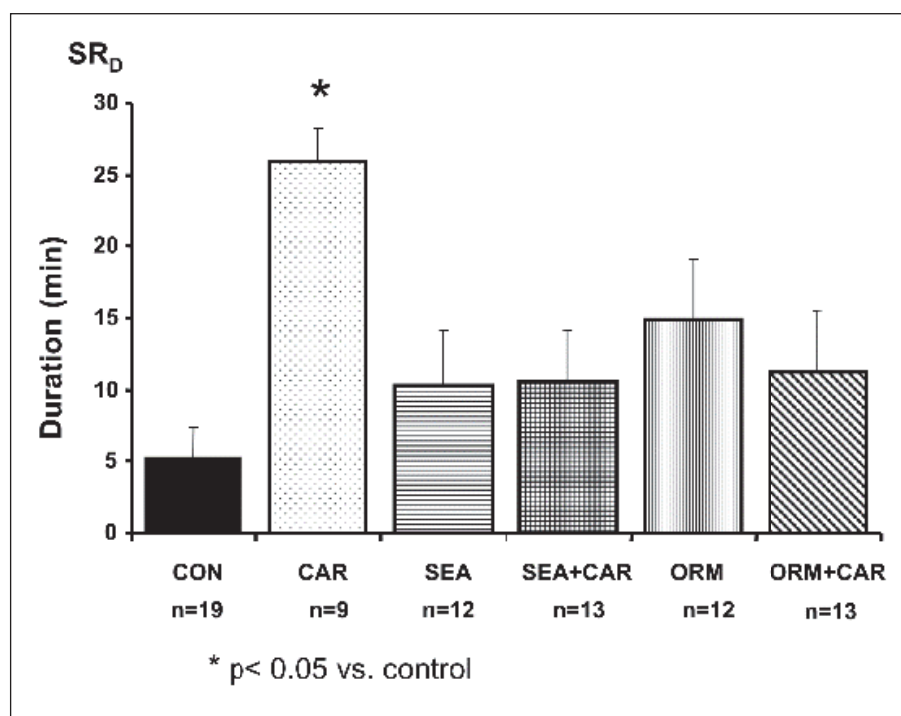
Fig. 2. Total duration of periods with regular sinus rhythm (SR$_D$: min) during the 30 min reperfusion phase in all groups. The efficacy of individual inhibitors and inhibitor combinations has been evaluated against the control hearts. * P<0.05, compared to the control group.

Quantitative analysis of the incidence and duration during reperfusion of the sinus rhythm (SR) and the three major types of arrhythmia (ES, VT and VF)

Average durations of periods with regular sinus rhythm (SR$_D$) during the 30 min reperfusion phase are summarised in *Fig. 2*. The control value (5.26±2.14 min) was calculated from the unified pool of control rats collected throughout the study. All further differences were evaluated relative to this control value.

Cariporide, when used alone, significantly increased the overall duration of the SR periods during reperfusion (25.8±2.4 min). All other pharmacological treatments were apparently beneficial, but neither was found to be significant. While in three of the four groups with an NCX inhibitor, the effect was similar (10.26±3.89; 10.57±3.61 and 11.25±4.16 min for SEA, SEA+CAR and ORM+CAR groups, respectively), further to cariporide, the second largest improvement was achieved in the group treated with ORM alone (14.86±4.24 min).

The incidence and average duration of the VF periods, calculated for all groups, are shown in *Fig. 3*. In control hearts, both the average duration (VF$_D$) (19.39±3.01 min) and incidence (VF$_I$) (1.35±0.24 min^{-1}) of VF was high. Furthermore, 11 out of the 19 hearts utilised in the control group were in the state of sustained fibrillation at the end of the reperfusion phase.

Compared to the control hearts, cariporide, if used alone, significantly decreased the incidence (0.55±0.24 min^{-1}) and particularly the duration (0.25±0.21 min) of the fibrillatory periods. At the end of reperfusion in this group, none of the 9 hearts was fibrillating. Unlike cariporide, however, the NCX inhibitors, although exerting a moderate beneficial effect, were apparently unable to significantly reduce the incidence or duration of the VF periods. Neither SEA0400 (I: 1.08±0.37 min^{-1}; D: 17.86±4.07 min) nor ORM-10103 (I: 0.80±0.13 min^{-1}; D: 11.57±4.29 min) could markedly decrease the incidence or average duration of the VF periods. From this aspect, the overall anti-VF efficacy of ORM-10103 was slightly more promising. While no apparent difference could be observed in the number

of hearts with sustained fibrillation (6/12 (ORM-10103) compared to 7/13 (SEA0400), the duration of the VF periods was moderately lower in the ORM-10103-treated group compared to the SEA0400-treated group.

Surprisingly, if cariporide was administered together with an NCX inhibitor, it failed to improve the moderate anti-VF protection reached by application of the NCX inhibitor alone (SEA+CAR, I: 1.07±0.36 min^{-1}; D: 15.67±4.10 min; ORM+CAR, I: 1.00±0.29 min^{-1}; D: 11.25±3.85 min). These results suggest that instead of providing enhanced cardioprotection, the simultaneous application of inhibitors of the two transporters results in a "loss of action" effect of cariporide.

The incidence and duration values calculated for periods with multiple extrasystoles (ESs) are shown in *Fig. 4*. As explained in METHODS, in order to more realistically compare the incidence and duration of the ES (and VT) intervals during the reperfusion phase, the calculated raw values for each heart were normalised to the duration of the VF-free periods. With this step, these variables became independent of the incidence and duration of the VF periods.

In control hearts, both the incidence (ES$_I$) (0.11±0.02 min^{-1}) and duration (ES$_D$) (0.14±0.04 min) of the ES episodes was high. Cariporide, if applied alone, significantly decreased the incidence (0.03 ± 0.007 min^{-1}) and markedly reduced the average duration (0.066±0.05 min) of these episodes. The incidence and especially the duration of the ES episodes were also significantly reduced by both NCX inhibitors (ES$_I$: SEA: 0.05±0.02 min^{-1}; ORM: 0.05±0.02 min^{-1}; ES$_D$: SEA: 0.02±0.01 min; ORM: 0.02±0.007 min^{-1}). As expected, in the latter case, the antiarrhythmic efficacy of the NCX inhibitors was apparently greater than that of NHE inhibition. In contrast, if applied in combination with an NCX inhibitor, cariporide not only failed to improve, but virtually eliminated the beneficial effect of NCX inhibition on the duration and incidence of the ES episodes (SEA+CAR: 0.11±0.05 min and 0.09±0.03 min^{-1}; ORM+CAR: 0.17±0.09 min and 0.09±0.02 min^{-1}, respectively).
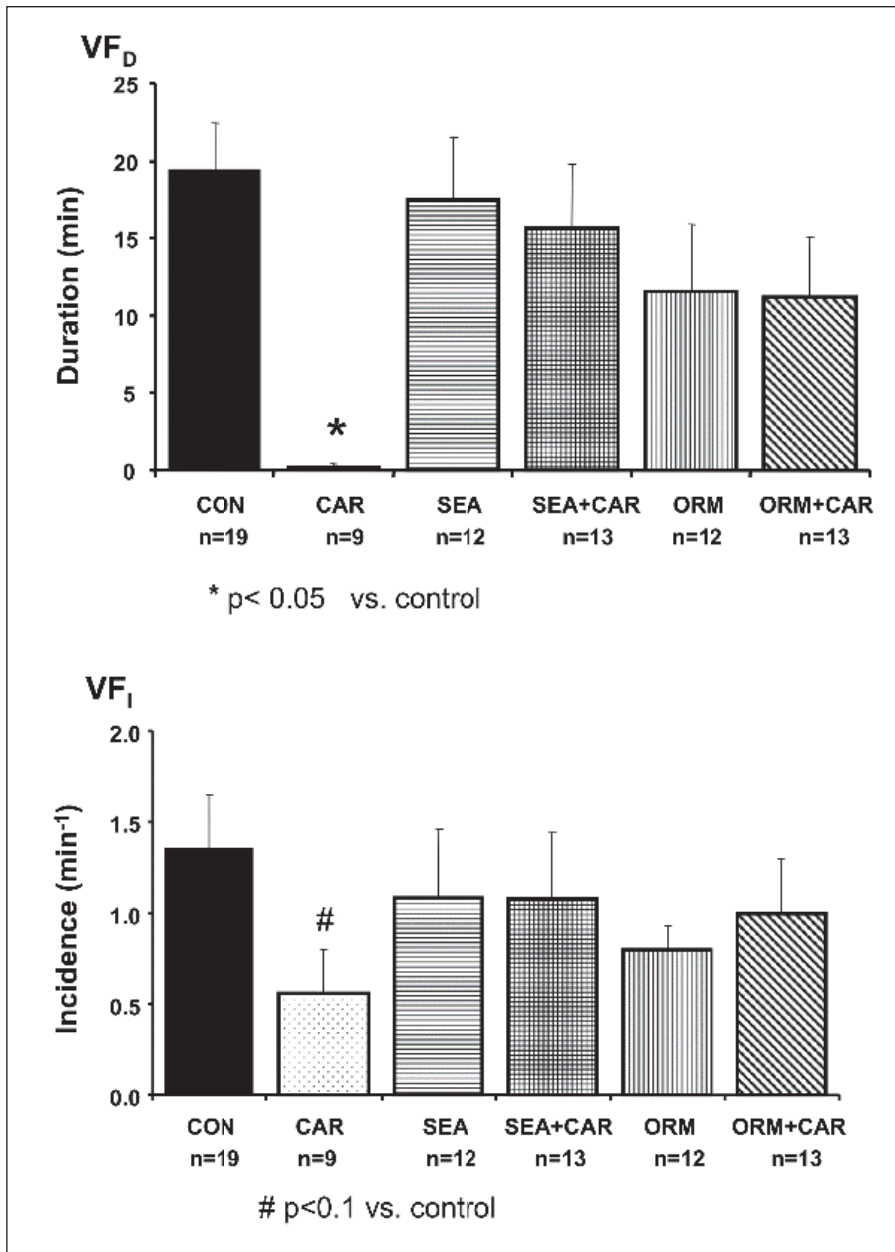
Fig. 3. Total duration (VF$_D$: min) and incidence (VF$_I$: min^{-1}) of periods with ventricular fibrillation (VF) during the 30 min reperfusion period. Two levels of significance, has been calculated: *P<0.05, #P<0.1, compared to the control group.

Normalised incidence and average duration values obtained for the ventricular tachycardia (VT) periods generated during reperfusion are summarised in *Fig. 5*. As in other arrhythmia types, in control hearts, both the incidence (0.17±0.04 min^{-1}) and average duration (0.14±0.05 min) of the periods with VT were high. Cariporide treatment provided the most effective anti-VT protection by dramatically decreasing both the incidence and duration of the post-ischaemic VT periods (VT$_I$: 0.01±0.002 min^{-1}; VT$_D$: 0.005±0.001 min). However, unlike the ES episodes, while both NCX inhibitors exerted a moderate beneficial effect, neither could significantly reduce the incidence or duration of the VT periods (SEA: 0.16±0.04 min^{-1} and 0.13±0.05 min; ORM: 0.10±0.03 min^{-1} and 0.09±0.07 min, respectively). Furthermore, the simultaneous application of cariporide and an NCX inhibitor resulted in the almost complete abolishment of the protection provided by cariporide treatment alone (SEA+CAR: 0.13±0.06 min^{-1} and 0.05±0.05 min; ORM+CAR: 0.09±0.07 min^{-1} and 0.10±0.05 min).

DISCUSSION

In the present study, the efficacy of two NCX inhibitors, the fairly selective SEA0400 and the novel, more selective ORM-10103, against reperfusion-induced arrhythmias were analysed in detail. Experimental data on the antiarrhythmic effects of SEA0400 are sparse and contradictory, although mostly positive (19-22), while similar data for ORM-10103 have become available only very recently (18, 23, 24). SEA0400 has a moderate inhibitory effect on the L-type Ca^{2+} channels (17), while ORM-10103 has a minor effect on I$_{Kr}$; neither of these pleiotropic effects could substantially modulate the observed antiarrhythmic efficacy of the NCX inhibition. Both inhibitors were applied alone or in combination with a well-characterised selective NHE inhibitor, cariporide. Cariporide is proposed to be highly selective and to not influence any of the important ion channels (25-27). It was assumed that the antiarrhythmic efficacy of a combination of NCX and an NHE inhibitor would be enhanced.
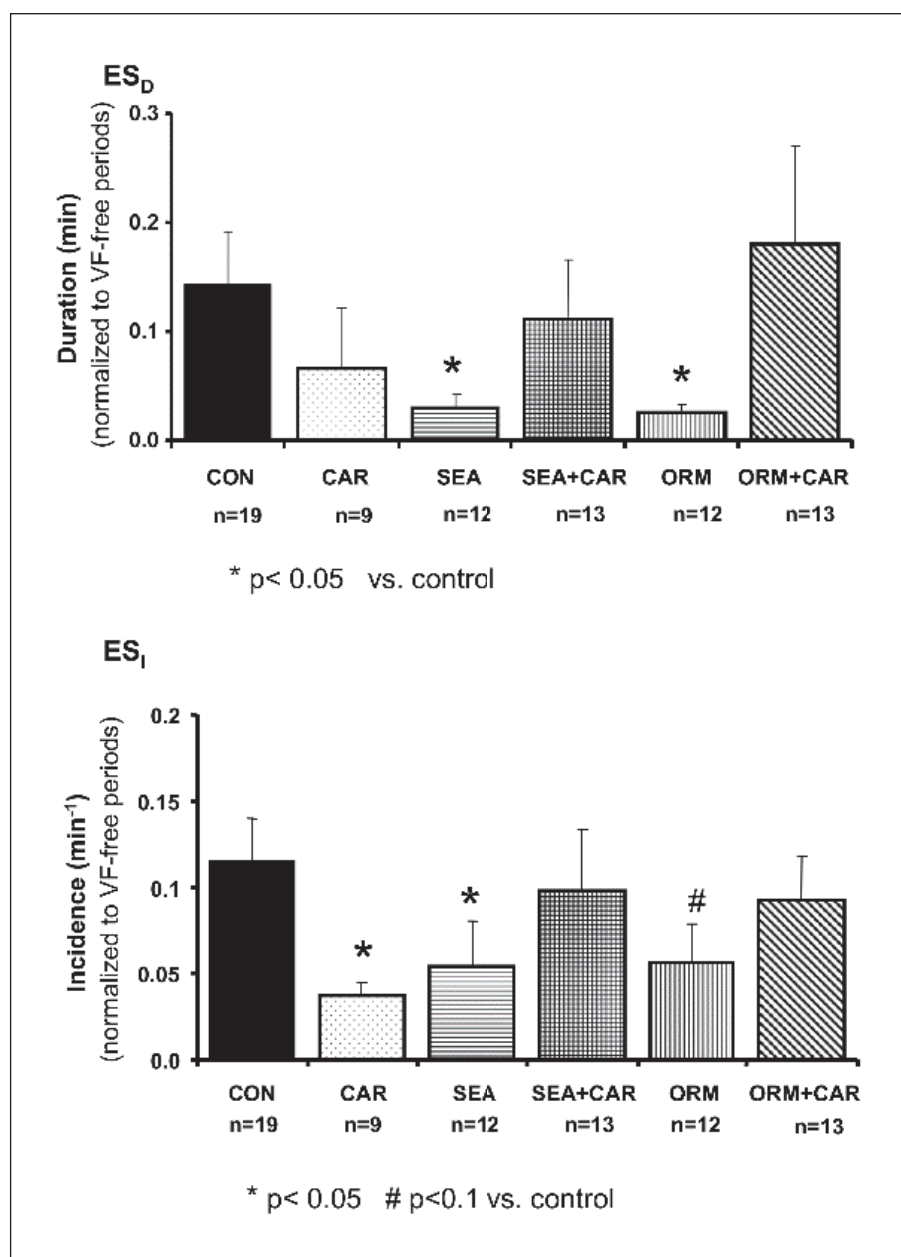
Fig. 4. Relative duration (ES_D: min) and incidence (ES_I: min^{-1}) of episodes with multiple extrasystoles (ESs) during the reperfusion phase. In order to better characterise the sensitivity of the heart to reperfusion-induced VT, the raw duration (min) and incidence (min^{-1}) values for each heart were normalised to the total duration of the VF-free periods obtained for the same heart (see the METHODS section for details). Two levels of significance were calculated: $*P<0.05$, $\#P<0.1$, compared to the control group.

Lethal ventricular arrhythmias are among the most common reasons of high mortality in patients with acute ischaemia-reperfusion injury. Severe perturbations in intracellular Na^+ and Ca^{2+} homeostasis leading to gradually elevated $[Na^+]_i$ and subsequent $[Ca^{2+}]_i$ overload are frequently observed in the background of the increased incidence of post-ischaemic ventricular arrhythmias (6). Recent experimental data demonstrate that these pathological processes are tightly linked to the abnormal activities of two vital sarcolemmal ion transporters, the Na^+/H^+ exchanger and Na^+/Ca^{2+} exchanger, key effectors of pH_i and $[Ca^{2+}]_i$ regulation, respectively (3, 4). In the majority of related experimental studies, the inhibition of one of these transporters could reduce the incidence and/or severity of ventricular arrhythmias (9, 10, 21, 28, 29). Therefore, restoring the normal levels of their activities *via* pharmacological intervention is generally considered a highly feasible therapeutic strategy to prevent, or at least substantially reduce, reperfusion-induced arrhythmogenesis.

NHE inhibition, as a cardioprotective, antiarrhythmic strategy, has also been evaluated in a few clinical trials; however, the routine use of NHE inhibition in human clinical pharmacology is compromised by the fact that, in contrast to the highly promising experimental results, relevant human data obtained from these clinical trials are controversial (11).

Since the data obtained from human trials are not fully satisfactory, in seems reasonable to assume that combining NHE inhibition with other protective interventions may substantially improve its antiarrhythmic efficacy. NCX inhibition is, no doubt, one of the logical choices, however, this approach was never explored in details. Indeed, in contrast to the NHE, NCX - in spite of its obvious significance in pathomechanisms leading to ischaemia/reperfusion induced arrhythmogenesis - is only emerging as a possible target for pharmacological intervention (30). One reason for this apparent delay may be, that while well established, selective NHE inhibitors have since long been readily available, until recently experimental efforts to evaluate
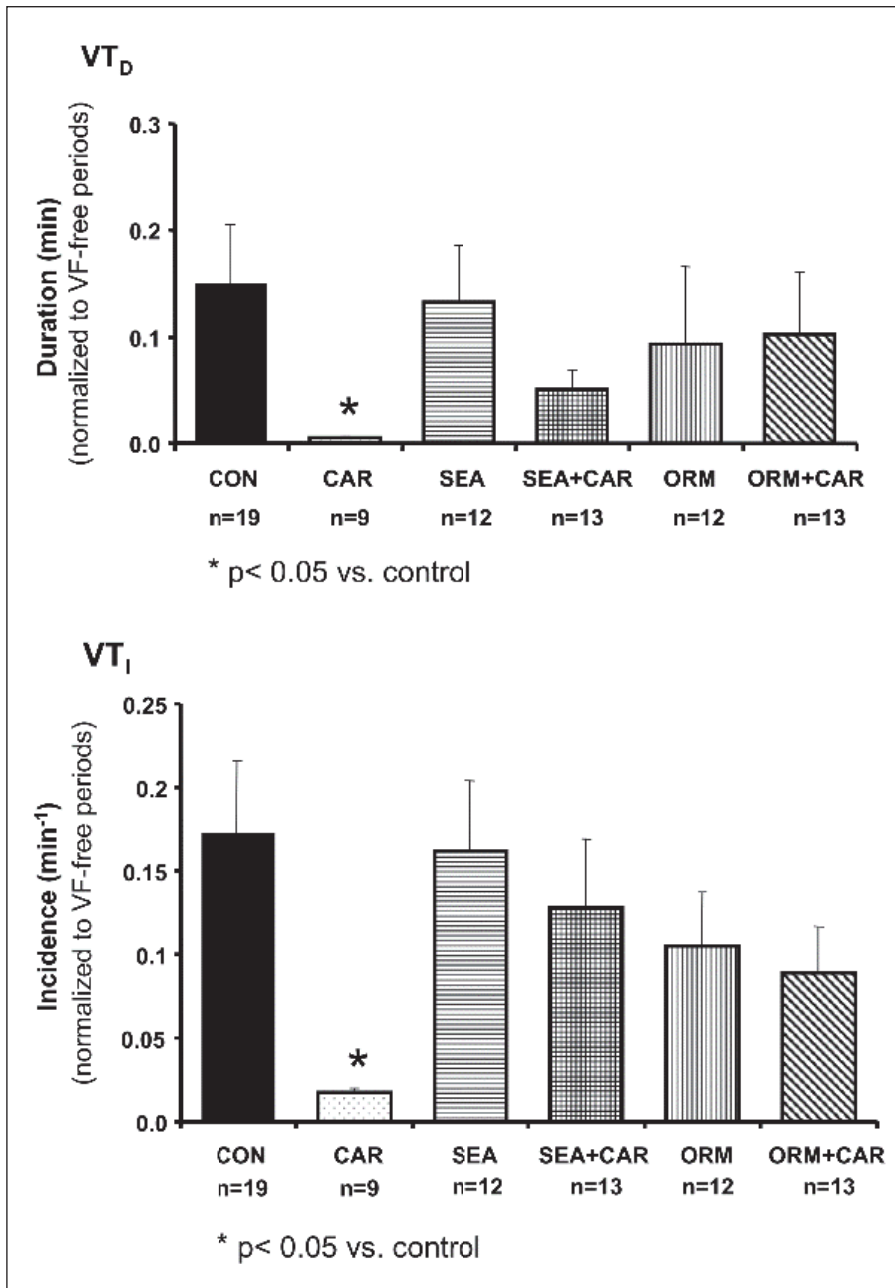
Fig. 5. Relative duration (VT$_D$: min) and incidence (VT$_I$: min^{-1}) of episodes with ventricular tachycardia (VT) during the reperfusion phase. Like in Fig. 4, the raw duration (min) and incidence (min^{-1}) values for each heart were normalised to the total duration of the VF-free periods obtained for the same heart; *P<0.05, compared to the control group.

the therapeutic value of NCX inhibition were hampered by the absence of effective, specific inhibitors, therefore often led to unclear results (17). Another reason for the lack of adequate information on the consequences of pharmacological targeting of NCX in cardiac diseases lies in its complex behavior and poorly understood physiological regulation - while its transport activities into both reverse and forward directions are essential for normal cardiac function, its abnormal activity into any transport direction may initiate and maintain severe cardiac diseases (4, 31).

For experimental model a widely used arrhythmia protocol with rather short (10 min) regional ischaemia, induced by LAD occlusion, followed by a short (30 min) but complete reperfusion has been selected. An important benefit of this model is that it provides a rapid experimental procedure to study the antiarrhythmic efficacy of a drug in a highly reproducible manner.

From the aspect of survival and full recovery to normal sinus rhythm the critical arrhythmia type in this model is VF. Without intervention the incidence of VF in control hearts is usually very high, close to 100% (28), as also has been observed in the present study (Figs. 1 and 3). Furthermore, though transient (reversible) VF periods could be often observed, in the majority of cases with VF, if initiated, became sustained; it did not terminate spontaneously and the heart was unable to return to normal SR (32). Further common arrhythmia types, ESs and VT were also often observed in the present experiments.

The antiarrhythmic efficacy of NHE inhibition has been evaluated in a number of studies. Pre-ischaemic administration of cariporide significantly attenuated ischaemia/reperfusion induced arrhythmogenesis in a variety of in vitro and in vivo models (26) while its post-ischaemic administration, though reduced the incidence of VF and subsequent cardiac death, the effect on mortality was not significant (28, 33, 34). Our results obtained by the pre-ischaemic application of cariporide reflect the high antiarrhythmic efficacy of the treatment, and strongly support the conclusions of similar studies (7-10). As shown in Figs. 2-5, cariporide significantly increased the duration of the

regular sinus rhythm and - with the exception of ES_D - all arrhythmia-related variables were significantly suppressed by the compound. The high antiarrhythmic efficacy is especially obvious in the case of VT and VF, since both arrhythmia types were almost completely eliminated following its application.

The background for the observed high efficacy of cariporide against VF and VT is not fully clarified. Data from previous studies indicate that during early reperfusion increased NHE activity promotes transient shortening of the APD, which renders the heart susceptible to re-entrant arrhythmias, therefore NHE inhibition should significantly reduce the susceptibility of the heart to VF and VT (35). Furthermore, NHE inhibition has been shown to improve action potential (AP) propagation and reduce the ischaemia-induced increase in AP dispersion, most probably *via* markedly delaying cell-to-cell electrical uncoupling (36). Since increased AP dispersion is a critical factor in the initiation and maintenance of VT and VF, improved AP conduction and subsequently decreased dispersion may be an important component in the strong anti-VT and -VF effect of cariporide.

In the present study, the antiarrhythmic efficacy of SEA0400 was largely subject to the given arrhythmia type. Its application effectively reduced the incidence and duration of triggered arrhythmias (ESs) (*Fig. 4*), but its efficacy in lengthening the arrhythmia-free SR periods, preventing initiation and reducing the duration of VTs and VFs was apparently limited (*Figs. 3* and *5*).

Compared to SEA0400, the structure of the novel ORM-10103 is markedly different and its selectivity is enhanced (18). Regarding its antiarrhythmic effects, when applied alone, ORM-10103 resembles SEA0400, but its overall efficacy against triggered arrhythmias (ESs) seems to be even somewhat higher (*Fig. 4*). Like SEA0400, it moderately increased the length of arrhythmia-free periods (slightly more than SEA0400) (*Fig. 2*), but unlike SEA0400, it also tended to reduce the incidence and duration of the VFs and VTs (*Figs. 3* and *5*). It must be mentioned, however, that although all of these effects could be regularly observed, in most cases, for the large standard deviations, the differences were not significant when using traditional statistical procedures.

The reasons for the strong arrhythmia-type dependence of the efficacy of NCX inhibition against reperfusion-induced arrhythmias are probably complex and may reflect intimate interactions among a few factors with opposite consequences. The excellent protection against triggered arrhythmias may be due to strong inhibition of the reverse NCX transport activity, leading to a significant reduction in reperfusion-induced $Ca^{2+}{}_i$ load (37-39), while the lack of a similarly beneficial effect of SEA0400 on VFs and VTs suggests that to prevent the induction and maintenance of these arrhythmias, in addition to an effective reduction of the $Ca^{2+}{}_i$ overload, a similarly effective suppression of the re-entry activity is also essential (22).

In the present experimental model, VF had by far the largest contribution to total "arrhythmia time"; thus, its influence on the outcome of a pharmacological trial is predominant. Regarding its origin, VF is considered a re-entry type and not triggered-type arrhythmia; the key factor in its induction and maintenance is increased AP dispersion and ischaemia/reperfusion-induced spatial and/or temporal heterogeneities in the resting membrane potential. Consequently, since NCX inhibition is less suitable to protect the heart against substrate-induced arrhythmias, it is not surprising that the efficacy of SEA0400 and ORM-10103 against VF was also limited in our model. Furthermore, since ischaemia is short and incomplete in this arrhythmia model, the elevation of $[Ca^{2+}]_i$ during ischaemia and upon reperfusion is probably moderate, but heterogeneous. Therefore, activation of the reverse NCX transport mode is probably also moderate but heterogeneous, and the effect of NCX inhibition on the ischaemia/reperfusion-induced $[Ca^{2+}]_i$ overload in

cardiomyocytes is uneven, unlike how it would be in the case of a severe, long-lasting ischaemia. Nonetheless, both NCX inhibitors substantially reduced the overall incidence of Ca^{2+}-dependent (triggered) arrhythmias, probably originating from locally injured regions. This finding is in line with most of the previous studies reporting the marked efficiency of NCX inhibition in reducing the formation of early and delayed after-depolarisations (18, 20). Indeed, the significantly higher efficiency of NCX inhibition may be expected in conditions where the rise in $[Ca^{2+}]_I$ is substantially larger and the Ca^{2+} overload is more pronounced (e.g. following more extensive, complete or long-lasting ischaemia).

An unexpected finding of the present study was that cariporide, when applied simultaneously with any of the NCX inhibitors, failed to enhance the antiarrhythmic protection provided by the NCX inhibitor alone. While the effect of SEA+CAR was practically the same as the effect of SEA alone during the SR periods, the simultaneous application of ORM+CAR apparently decreased this parameter (*Fig. 2*). Qualitatively, the results obtained for both NCX inhibitors and all three arrhythmia types were similar: the simultaneous application of an NHE and an NCX inhibitor did not improve and eventually worsened the moderate antiarrhythmic efficacy of the NCX inhibitor alone.

While the results obtained with single inhibitors are logical and comprehensive, the outcome of the experiments with simultaneous NHE and NCX inhibition first seems highly surprising. Indeed, one would expect that the antiarrhythmic efficacy of simultaneous inhibition should exceed or, at least, meet the efficacy reached alone by any of the contributors. The fact that cariporide alone had significantly higher antiarrhythmic efficacy than in combination with an NCX blocker suggests that NCX inhibition was an important limiting factor in these groups. The reason for this apparent restrain is not clear at present and needs further investigation.

A logical, but largely hypothetical explanation may be as follows. In these experiments, the antiarrhythmic effect of cariporide could not be further improved by a synergic effect of NCX inhibition, since both NHE and reverse NCX inhibition could prevent the $[Na^+]_i$-induced rise in $[Ca^{2+}]_i$. Indeed, this assumption is supported by the almost identical effects of combined NCX+CAR and NCX alone inhibition. The decreased efficiency of cariporide is most probably caused by inactivation of its beneficial effect on the arrhythmogenic substrate. In other words, in the combined groups, the NCX inhibitors apparently exerted a unique anti-antiarrhythmic effect *via* releasing the suppressing effect of cariporide treatment and restoring the susceptibility of the heart to re-entrant arrhythmias. This indirect proarrhythmic effect is obviously not present if cariporide is administered alone.

The observed "loss of effect" of cariporide may be related to the significant inhibition of the forward transport mode of NCX by SEA0400 or ORM-10103. Indeed, one may speculate that the beneficial anti-VF effect of cariporide (applied alone) should manifest in decreasing the spatial and/or temporal heterogeneities below the critical level required to evoke fibrillation. Simultaneous inhibition of the forward transport mode of NCX may exert an opposite effect by increasing these heterogeneities *via* inhibiting Ca^{2+} extrusion needed for restoration of the normal Ca^{2+} cycle of the cell. This effect may be less important in cardiomyocytes affected by a lower level of ischaemia and subsequent $[Ca^{2+}]_i$ load compared to those with more significant rise in $[Ca^{2+}]_i$. On the other hand, NHE inhibition may remain latent in normal, non-ischaemic myocardium, since NHE is mostly inactive at normal pH_i, while NCX inhibition undoubtedly affects Ca^{2+} handling and AP kinetics probably differentially in the ischaemic and non-

ischaemic myocardium. Therefore, the consequence of the secondary, forward NCX transport mode inhibition may be a concomitant increase in heterogeneities (eventually shifting those above the critical level), neutralising the beneficial blocking effect of cariporide on VFs. To evaluate the validity of this hypothesis, however, further studies are needed.

Conclusion and perspectives

In principle, inhibition of either the NCX or the NHE and especially their combined inhibition should effectively protect the heart against reperfusion-induced harmful arrhythmias, since both mechanisms target and block the same $[Ca^{2+}]_i$ overload pathway, at different points. The results of this study, however, are much less straightforward and do not fully support this presumption. The NHE blocker cariporide, if administered alone, by also decreasing the incidence and duration of the re-entrant arrhythmias, provided wider antiarrhythmic efficacy than when applied simultaneously with an NCX inhibitor, in which case the probability of VF induction did not differ significantly from the control. On the other hand, all three inhibitors, alone or in combination, were found to be effective against triggered arrhythmias. Therefore, we concluded that NCX inhibition alone, or in combination with NHE blockade, appears to be a less than optimal therapeutic strategy against the full range of reperfusion-induced arrhythmias. A possible reason for the limited efficacy may be that the currently available selective NCX inhibitors with their equally effective and long-lasting bidirectional blockade of the NCX activity are not fully suitable for this purpose. On the other hand, these NCX inhibitors proved to be highly effective against EAD- and DAD-induced arrhythmias (20). To better explore the suggested therapeutic potential of combined NCX+NHE inhibition, further studies based on novel NCX inhibitors with different inhibitory characteristics are needed.

Limitations

An important limitation of the present study is that all three inhibitors were used at a single concentration. While it is obvious that a concentration-dependent relationship on the efficacy of a drug or drug combination could be obtained by using several concentrations, applying a single, optimally selected concentration may still satisfactorily characterise its efficacy. In a number of similar studies (40-46), a single drug concentration has been utilised to characterise the effect of an inhibitor on ischaemia-reperfusion injury. In these studies, a concentration-dependent relationship of the inhibitory effect has not been explored. Therefore, we are convinced that our present study will be similarly informative. It should be emphasised, however, that the selection of an optimal concentration is, indeed, critical in order to obtain results that can be considered representative. Therefore, the drug concentrations used here were selected with extreme care - especially in the case of NCX inhibitors (e.g. for ORM-10103 EC_{50} <0.2 μM).

A second limitation of the present study is that the evaluation of a haemodynamic parameter during the reperfusion phase may be severely hampered or even prevented by the presence of an abnormal heart rhythm (VT or VF), since the correct determination of pressure-related parameters during abnormal heart rhythm periods is practically impossible. Therefore, all haemodynamic values calculated for the reperfusion phase and presented in *Table 1* were obtained from hearts devoid of sustained arrhythmias or were evaluated during a period of sinus rhythm between arrhythmic periods. Consequently, one should be aware that

while still providing useful information, this analysis may result in substantial bias, as well, and these data should only be considered qualitative.

A third important limitation is that the raw values obtained for the incidence and duration of the ES and VT periods may strongly depend on the length of VF-free periods. In order to provide more realistic information, the summed duration of these periods has been normalised for the summed duration of the VF-free periods. Although these time-normalised values are not completely independent of each other, they still appear to better approximate the duration and incidence of these arrhythmia types in the VF-free heart.

Conflict of interest: None declared.

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