Virchows Archiv A Pathol Anat Histopathol (1988) 412:291–299

Virchows Archiv A
Pathological Anatomy and Histopathology

Effects of verapamil and timolol on cellular morphometric changes in cat hearts with regional ischaemia

Gottfried Greve [1], Svein Rotevatn [1], Ketil Berstad [1], Harald Jodalen [1], Ketil Grong [2], and Lodve Stangeland [2]

Department of Anatomy [1] and Surgical Research Laboratory [2], University of Bergen, Årstadveien 19, N-5009 Bergen, Norway

Summary. In twenty-one anaesthetized open chest cats the left anterior descending coronary artery (LAD) was occluded for three hours. Seven cats were pretreated with a bolus injection of Verapamil, followed by a continuous infusion of Verapamil during the ischaemic period. Seven cats were pretreated with a bolus injection of Timolol to a heart rate reduction of 20 beats/min or more and seven cats were given saline. In the latter two groups the cats received a continuous infusion of saline during the period of coronary occlusion. Biopsies were taken from the mid-myocardium of the normal, border and ischaemic zones, as defined by fluorescein staining, and verified by blood flow measurements with radiolabelled microspheres. Standard point counting techniques were used for calculations of fractional volumes of mitochondria, cytoplasm and myofibrils as well as of mitochondrial surface density and surface to volume ratio. We observed a cytoplasmic oedema in the border and ischaemic zones, that was not altered by medical treatment. In the border zone of the control cats there is greater mitochondrial swelling than in the ischaemic zone. This particular swelling is not seen in the treatment groups. However, in the normal and border zones of the verapamil group the mitochondria are smaller when compared with the respective zones in the two other groups, but increases relatively more in size in the border and ischaemic zones. Furthermore, we measured the water content, sarcomere length and per cent heavily damaged cells. These variables were not altered by Verapamil or Timolol in any zone when compared with the respective zones in the control group.

Key words: Lateral border zone – Mitochondrial swelling – Cellular oedema – Calcium channel blockers – Beta adrenoceptor blockers – Regional blood flow

Offprint requests to: G. Greve

Introduction

In the last two decades the cellular composition of the lateral interface between normal and ischaemic tissue during coronary artery occlusion has been disputed. Although the existence of a lateral border zone has been questioned, several reports suggest that cells belonging to the lateral transitional zone possess certain characteristics that are not shared by the cells of the normal and ischaemic zones (Fishbein et al. 1980; Page et al. 1977). Thus, recently we have reported on the presence of a lateral border zone that is characterized by more extensive mitochondrial swelling than in the normal and ischaemic zones (Greve et al. 1988). Cytoplasmic oedema was reported to be less marked in the cells of the border zone than in the cells of the ischaemic zone. Furthermore, Jodalen et al. (1985) have shown that the transitional cells of the infarcted heart are characterized by a significantly higher content of cytoplasmic lipid droplets than the cells of the two remaining zones.

Previous experimental studies have shown an apparent reduction in the infarct size by treatment with beta adrenergic blockers (Rasmussen et al. 1977; Slutsky and Peck 1985; Vik-Mo et al. 1984) and calcium channel blockers (Downey et al. 1985; DeBoer et al. 1980; Reimer and Jennings 1984; Tumas et al. 1985) in hearts subjected to coronary artery occlusion. These results appear to be in part due to reduced percentage of area at risk which evolves to infarction. The beneficial effect of these drugs in ischaemia may occur at a cellular and metabolic level as well as by haemodynamic mech-

anisms. It is, therefore, essential for the understanding of the assumed protective effects of Verapamil and Timolol to study their effects at the border zone.

Both drugs are extensively used in the treatment of myocardial infarction, hypertension and angina pectoris. Verapamil is known to work at a cellular level, whereas the working mechanism of Timolol is less studied. Beta adrenoceptor blockers are reported to reduce mortality and reinfarction in patients surviving acute myocardial infarction (The Norwegian Multicenter Group 1981), and, hence, they are frequently used in secondary prophylaxis after acute myocardial infarction. The beta blockers differ in structure, receptor selectivity and pharmacological properties. They are extensively metabolized in vivo, but to a varying degree (Lange et al. 1983). Despite the extensive use of Timolol, there is a lack of knowledge about its function at the cellular and subcellular level. In our work we have estimated the fractional volumes of mitochondria, myofibrils and remaining cytoplasm, as well as mitochondrial surface density and mitochondrial surface to volume ratio after 3 h of left descending coronary artery occlusion. These variables were studied in the normal, border and ischaemic zones of controls, and of cats treated with either Verapamil or Timolol.

Material and methods

We have described the method used in detail elsewhere (Greve et al. 1988). Twenty-one cats of either sex with an average weight of 3.47 ± 0.19 kg, were anaesthetized by sodium pentobarbital (35 mg/kg b.w.), tracheotomized and ventilated through a positive pressure ventilator with a gas mixture of 60% N_2O and 40% O_2, added 5% CO_2. Adequate ventilation was controlled by arterial blood gas analysis. The chest was opened, and, heart rate (HR), left ventricular systolic pressure (LVSP) and left ventricular end diastolic pressure (LVEDP), as well as cardiac contractility (dP/dt) were continuously recorded from the left ventricle by a pressure transducer. The left atrium was cannulated for injection of radiolabelled microspheres. For collection of reference blood, the abdominal aorta was cannulated via the left femoral artery. A 4-0 silk ligature was loosely placed around the left anterior descending coronary artery (LAD). After stable haemodynamic conditions had been recorded, the cats were randomized into three groups. Seven cats (controls) were given a bolus injection of saline, seven were given a bolus injection (0.25 mg/kg b.w.) of Verapamil (Isoptin, "Knoll AG., Ludwigshafen, FRG" 2.5 mg/ml), and seven given Timolol (Blocadren, "Merck & Co Inc., NJ, USA" 0.25 mg/ml) to a heart rate reduction of 20 beats/min (average dose 140 ± 26 µg/kg b.w.). The bolus injections were followed by a continuous infusion of Verapamil (25 mg/kg b.w./h) in the verapamil group, and saline in the two other groups. When the haemodynamic conditions again had stabilized, the ligature was tightened.

Three hours after coronary artery occlusion and three minutes before sacrifice, approximately $1-2 \times 10^6$ radiolabelled microspheres were injected into the left atrium for measurements of regional blood flow. The reference blood sample was collected from the abdominal aorta by a constant rate extraction pump in 2 min, starting 10 s before and terminating fifty seconds after the injection of microspheres which lasted for sixty seconds. Immediately prior to sacrifice, 10% fluorescein was injected for visualization of normally perfused tissue. Immediately thereafter the cats were killed by infusion of 50 ml ice cold modified Karnovsky's fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 0.1 M sucrose and 1.25 mM $CaCl_2$, pH = 7.4, vehicle osmolarity = 300 milliosmols) into the left ventricle during partial occlusion of aorta. The hearts were rapidly excised from the animal and submerged in an ice cold modified Karnovsky's fixative.

Eight transmural specimens were collected under ultraviolet illumination from the left ventricle of all hearts; two from the non-fluorescent ischaemic zone, two from the fluorescent normal zone and four from the lateral margin of the ischaemic zone. Each of the specimens was divided in two subsamples, and biopsies for ultrastructural studies were collected from the midwall portion of the cut surface between the paired subsamples. The four specimens from the lateral margin of the ischaemic zone were divided exactly along the fluorescein demarcation line. The two with the largest difference in regional blood flow values in adjacent subsamples, were selected for electron microscopy of this particular zone. The latter biopsies extended at a maximum 1 mm from the fluorescein line in either direction. The remaining subsamples together with the reference blood samples were counted for γ-emission, and, the regional myocardial blood flow and cardiac output calculated. After counting, all subsamples were dried at 73° C for 3 days, reweighted, and, the water content calculated.

The biopsies collected for electron microscopy were postfixed in 1% OsO_4 in cacodylate buffer, and stained en bloc with uranyl acetate before embedding in Epon. Ultrathin sections stained for fifteen minutes in lead citrate were examined in the electron microscope. Eight micrographs (18×24 cm) of a magnification of 9600 were taken from each zone in each cat. Fractional volumes of the mitochondria ($V_{V\,mit}$), myofibrils ($V_{V\,myo}$) and the remaining cytoplasm ($V_{V\,cyt}$), were measured by standard point counting techniques according to the Delesse principle. We used a grid lattice with a distance of 25 mm between the lines i.e. all micrographs were covered by a total number of seventy line intersections. Data on mitochondrial surface area were obtained by counting intercepts of the outer mitochondrial membrane with the lines of the superimposed grid lattice, correcting for magnification and for the distance between the lines in the grid lattice. Surface density, i.e. the ratio of mitochondrial surface to total cytoplasmic volume and mitochondrial surface to volume ratio were calculated as previously described (Greve et al. 1988).

To minimize the effect of a possible anisotropy, all morphometric measurements were performed with the lines in the grid lattice both at 0° and 15° angles to the edge of the micrographs. The mean of the two values were used in the further calculations. The morphometric data were tested for differences between the three groups, between the three zones in each group, between the seven cats in each group and between the eight micrographs obtained from every zone of all cats. The statistical analysis was carried out by a two way four level nested analysis of variance using the program BMDP8V, and Scheffe's multiple comparison method to compare the results in each zone in each group.

Percentage of heavily damaged cells were calculated in the same micrographs as used for the morphometric analysis. Cells with sarcolemmal, and nuclear membrane fragmentation, were regarded as heavily injured cells (Kloner et al. 1977; Kloner

Table 1. Haemodynamic registration in 7 control cats, and 7 verapamil and 7 timolol treated cats. Mean values ± SEM

	Preocclusion values		3 h postocclusion	Statistics[a]
	Baseline	After drug		
Heart rate (beats/min)				
Control	169 ± 12		190 ± 12	NS
Verapamil	154 ± 14	144 ± 12	155 ± 9	NS
Timolol	169 ± 9	145 ± 7	149 ± 4	$u_1 \# u_2 = u_3$
Left ventricular systolic pressure (mmHg)				
Control	114 ± 6		111 ± 6	NS
Verapamil	123 ± 8	115 ± 5	120 ± 5	NS
Timolol	112 ± 7	117 ± 11	118 ± 11	NS
Left ventricular end-diastolic pressure (mmHg)				
Control	5.4 ± 0.5		8.7 ± 1.4	$u_1 \# u_3$
Verapamil	4.6 ± 0.7	5.3 ± 0.8	9.6 ± 1.0	$u_1 = u_2 \# u_3$
Timolol	4.6 ± 0.6	5.4 ± 0.8	10.4 ± 1.2	$u_1 = u_2 \# u_3$
dP/dt[b] (mmHg/s)				
Control	3125 ±290		3607 ±243	NS
Verapamil	3250 ±437	2518 ±304	2786 ±221	$u_1 \# u_2 = u_3$
Timolol	2893 ±398	2375 ±404	2268 ±312	$u_1 \# u_2 = u_3$
Cardiac output (ml/min)				
Control			194 ± 19	
Verapamil			198 ± 16	
Timolol			213 ± 22	

[a] Statistics by ANOVA for repeated measurements. u_{1-3} are the mean values from one treatment group from left to right. The sign # means statistical differences whereas the signs = and NS mean not significant

[b] dP/dt is the first derivative of left ventricular pressure

and Braunwald 1980). The length of the sarcomeres was measured in strictly longitudinally orientated myofibrils in micrographs at the same magnification. A total number of 2100 sarcomeres were studied. Percentage water content was calculated from wet weight minus dry weight. The latter obtained by drying the samples used for counting γ-emission at 73° C. The data on sarcomere length and water content were analyzed by a two way three level analysis of variance, and the Scheffe's multiple comparison method was used to compare the results.

All results from haemodynamic, blood flow measurements, water content and morphometry are given as mean values ± SEM. Significant differences have been noted for p-values of 0.05 or less.

Results

Haemodynamics

In the Timolol treated cats the average body weight was somewhat higher (4.17 ± 0.039 kg) than in the other two groups (control 3.18 ± 0.09 kg; verapamil 3.04 ± 0.23 kg). There was no significant difference between the three groups in pretreatment values of heart rate (HR), left ventricular end diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP) and contractility (dP/dt) (Table 1). Timolol treatment reduced heart rate from 169 ± 9 to 145 ± 7 beats/min and dP/dt from 2893 ± 398 to 2375 ± 404 mmHg/s, whereas Verapamil reduced dP/dt from 3250 ± 437 to 2518 ± 304 mmHg/s.

Following LAD ligation, LVEDP increased (control 5.4 ± 0.5 to 8.7 ± 1.4, timolol 5.4 ± 0.8 to 10.4 ± 1.2 and verapamil 5.3 ± 0.8 to 9.6 ± 1.0 mmHg) whereas HR and LVSP remained stable in all three groups during the time of LAD occlusion. dP/dt dropped suddenly after LAD occlusion, but increased gradually during the ischaemic period (results not shown). Three hours after occlusion it was not significantly different from the preocclusion values in any group. No difference in cardiac output was observed between the three groups 3 h after occlusion (Table 1).

In all three groups there was a significant higher water content in the ischaemic zone and in the ischaemic part of the border zone, than in the normal zone and in the normally perfused part of the border zone (Table 2). These results indicate the presence of a cellular oedema in the ischaemic myocardium. In all three groups the blood flow was significantly reduced in the ischaemic part of

Table 2. Regional water content (%) in control hearts and hearts treated with either timolol or verapamil. Mean values ± SEM

	Water content $\frac{(\text{wet weight}-\text{dry weight})*100}{\text{wet weight}}$		
	Control ($N=7$)	Timolol ($N=7$)	Verapamil ($N=7$)
Normal	76.06±0.88	75.87±0.46	75.52±0.55
Border normal	77.10±0.40	76.61±0.40	76.20±0.38
Border ischaemic	78.63±0.49*	78.12±0.55*	78.30±0.57*
Ischaemic	79.16±0.44*	78.55±0.44*	78.65±0.72*

* Significant different from the normal and border normal values. Statistics by two way two level ANOVA. Significant differences noted for P-values <0.05

Table 3. Regional blood flow values (ml/min per g) in control cats and cats treated with timolol or verapamil. Mean values ± SEM

	Control ($N=7$)	Timolol ($N=7$)	Verapamil ($N=7$)
Normal	1.72±0.28	1.47±0.16	1.68±0.20
Border normal	1.74±0.22	1.43±0.12	1.56±0.18
Border ischaemic	0.20±0.08*	0.06±0.02*	0.08±0.03*
Ischaemic	0.06±0.02*	0.03±0.01*	0.03±0.02*

* Significant different from the normal and border normal values. Statistics by two way two level ANOVA. Significant differences noted for P-values <0.05

the border zone and in the central ischaemic zone as compared to the normal zone and normal part of the border zone (Table 3). There were no significant difference in blood flow between the corresponding zones in the three groups.

Morphometry

We observed no significant difference in sarcomere length between any zones or groups (Table 4). The sarcomere lengths in the normal zone of the control group (2.07±0.05 μm), the timolol group (1.9±0.09 μm) and the verapamil group (2.1±0.11 μm) correspond well with the sarcomere length reported in normal cat myocardium by Fawcett and McNutt (1969).

I. The normal zone in the three treatment groups. Verapamil and Timolol had no significant effect on the fractional volumes of mitochondria, myofibrils or remaining cytoplasm in the normal zone (Table 5), even though in the Verapamil treated hearts we observed a slightly decreased $V_{V\,cyt}$ (3.93±0.44%) and an increased $V_{V\,mit}$ (29.28±0.60%) and $V_{V\,myo}$ (66.78±0.70%) as compared to control values (8.44±0.50, 27.86±0.66 and 63.65±0.65%, respectively). The surface density of mitochondria is significantly higher in the verapamil group (1.93±0.04 μm²/μm³) when compared with the timolol group (1.36±0.02 μm²/μm³) and the controls (1.56±0.02 μm²/μm³) (Table 6). The mitochondrial surface to volume ratio is also higher in the verapamil group (6.80±0.17 μm²/μm³) as compared to the two remaining groups (5.23±0.10 μm²/μm³ and 5.74±0.11 μm²/μm³ in the timolol group and control group respectively) (Table 7). These results indicate an increased number of mitochondrial section profiles in each micrograph in combination with a reduced mitochondrial size in the normal zone of the verapamil group (Fig. 1).

Table 4. Sarcomer length (μm) in different zones in control cats and cats treated with timolol or verapamil. Mean values ± SEM

		Normal	Border	Ischaemic
Control	($N=7$)	2.07±0.05	2.12±0.05	2.08±0.05
Timolol	($N=7$)	1.90±0.03	1.95±0.08	2.09±0.04
Verapamil	($N=7$)	2.10±0.11	1.99±0.05	2.00±0.06

II. Verapamil treated hearts versus controls. In the controls there is a larger increase in $V_{V\,mit}$ of the border zone (31.01±0.66%) than of the ischaemic zone (27.45±0.82%), whereas we observed no significant difference in $V_{V\,mit}$ between the normal, border (29.90±0.79%) and ischaemic zones (29.90±0.82%) of the verapamil group (Table 5). There is no significant difference in $V_{V\,mit}$ between controls and Verapamil treated hearts in any zone.

In the Verapamil treated hearts we observed an increased $V_{V\,cyt}$ (12.61±0.99%) and reduced $V_{V\,myo}$ (57.35±1.10%) in the ischaemic zone, which indicate a cytoplasmic oedema. Intermediate values are indicated in the border zone (8.93±1.17% and 60.96±1.19%, respectively), even though they are not significantly different from those observed in the ischaemic zone. A similar oedema is observed in the controls, and no difference in $V_{V\,cyt}$ and $V_{V\,myo}$ is observed in any zone between the two groups. This was verified by the absence of significant differences in water content of the corresponding zones between the two groups.

The mitochondrial surface density of the border zone and of the normal zone is higher in the verapamil hearts (1.68±0.08 μm²/μm³) than in the control hearts (1.40±0.03 μm²/μm³). No such difference occurred in the ischaemic zone

Table 5. Fractional volumes (%) of main cellular compartments in the normal, border and ischaemic zones of control cats, and of cats treated with either timolol or verapamil. Mean values ± SEM

	Zones			Statistics[a]
	Normal	Border	Ischaemic	
A Fractional volumes of mitochondria				
Con ($N=7$)	27.86±0.66	31.01±0.66	27.45±0.82	$u_1 \# u_2 \# u_3$ $u_1 = u_3$
Tim ($N=7$)	26.97±0.79	26.15±0.75	27.34±0.75	NS
Ver ($N=7$)	29.28±0.60	29.90±0.79	29.90±0.82	NS
	NS	$y_1 \# y_2 \# y_3$	NS	
		$y_1 = y_3$		
B Fractional volumes of myofibrils				
Con ($N=7$)	63.65±0.65	57.85±0.85	57.79±0.98	$u_1 \# u_2 = u_3$ $u_1 \# u_3$
Tim ($N=7$)	65.78±0.97	62.89±0.87	56.61±0.78	$u_1 = u_2 \# u_3$ $u_1 \# u_3$
Ver ($N=7$)	66.78±0.70	60.96±1.19	57.36±1.10	$u_1 \# u_2 = u_3$ $u_1 \# u_3$
	NS	NS	NS	
C Fractional volumes of remaining cytoplasm				
Con ($N=7$)	8.44±0.50	11.22±0.70	14.66±0.98	$u_1 = u_2 = u_3$ $u_1 \# u_3$
Tim ($N=7$)	7.24±0.62	10.95±0.70	15.90±0.66	$u_1 = u_2 \# u_3$ $u_1 \# u_3$
Ver ($N=7$)	3.93±0.44	8.93±1.17	12.61±0.99	$u_1 \# u_2 = u_3$ $u_1 \# u_3$
	NS	NS	NS	

[a] Statistics by two ways four level analysis of variance. u_{1-3} are mean values in the 3 zones in each treatment group from left to right. y_{1-3} are mean values in one zone in the three treatment groups from top to bottom. The sign # means significant differences whereas the sign = and NS mean not significant

Table 6. Mitochondrial surface density ($\mu m^2/\mu m^3$) in control cats and in cats treated with either timolol or verapamil. Mean values ± SEM

	Zones			Statistics[a]
	Normal	Border	Ischaemic	
Con ($N=7$)	1.56±0.02	1.40±0.03	1.30±0.04	$u_1 \# u_2 = u_3$ $u_1 \# u_3$
Tim ($N=7$)	1.36±0.02	1.16±0.04	1.19±0.02	$u_1 \# u_2 = u_3$ $u_1 \# u_3$
Ver ($N=7$)	1.93±0.04	1.68±0.08	1.48±0.04	$u_1 \# u_2 = u_3$ $u_1 \# u_3$
	$y_1 = y_2 \# y_3$	$y_1 = y_2 \# y_3$	$y_1 = y_2 \# y_3$	
	$y_1 \# y_3$	$y_1 \# y_3$	$y_1 = y_3$	

[a] Statistics by two ways four level analysis of variance. u_{1-3} are mean values in the 3 zones in each treatment group from left to right. y_{1-3} are mean values in one zone in the three treatment groups from top to bottom. The sign # means significant differences whereas the sign = and NS mean not significant

Table 7. Mitochondrial surface to volume ratio ($\mu m^2/\mu m^3$) in the normal, border and ischaemic zones of control cats and of cats treated with either timolol or verapamil. Mean values ± SEM

	Zones			Statistics[a]
	Normal	Border	Ischaemic	
Con ($N=7$)	5.74±0.11	4.65±0.11	5.02±0.12	$u_1 \# u_2 \# u_3$ $u_1 \# u_3$
Tim ($N=7$)	5.23±0.10	4.73±0.14	4.51±0.11	NS
Ver ($N=7$)	6.80±0.17	5.64±0.18	5.11±0.12	$u_1 \# u_2 = u_3$ $u_1 \# u_3$
	$y_1 = y_2 \# y_3$	$y_1 = y_2 \# y_3$	NS	
	$y_1 \# y_3$	$y_1 \# y_3$		

[a] Statistics by two ways four level analysis of variance. u_{1-3} are mean values in the 3 zones in each treatment group from left to right. y_{1-3} are mean values in one zone in the three treatment groups from top to bottom. The sign # means significant differences whereas the sign = and NS mean not significant

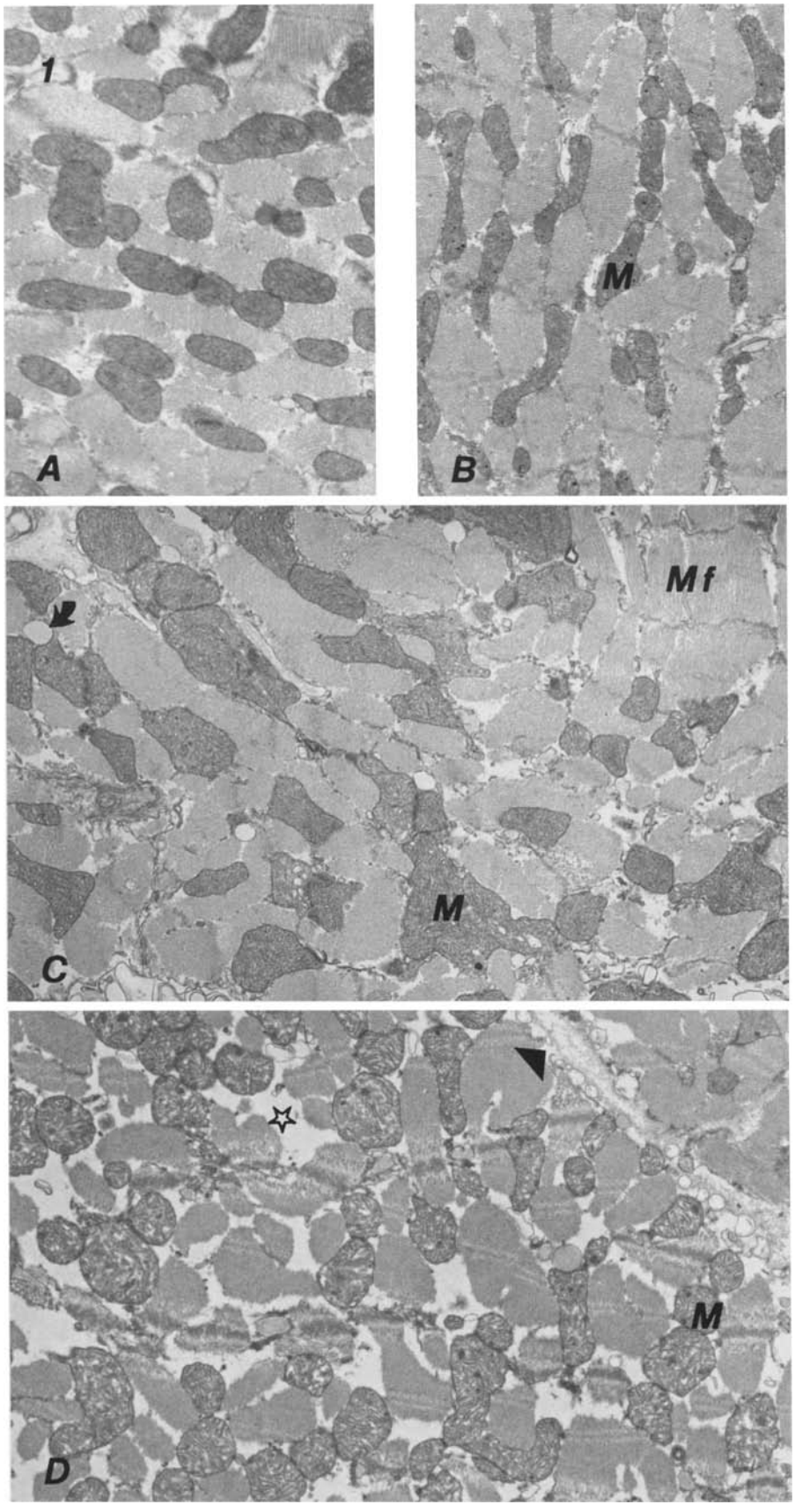

Fig. 1. In the normal zone of verapamil treated hearts (**B**) the mitochondria are smaller than in the normal zone of controls (**A**). In the border zone of verapamil treated hearts (**C**) lipid droplets (*arrow*) could be observed. The mitochondria (*M*) are larger than in the normal zone. The sarcomere length of myofibrils (*Mf*) is unchanged. In the ischaemic zone of verapamil treated hearts the mitochondrial (*M*) size is enhanced, and, there is a pronounced cytoplasmic oedema (*asterisks*). Fragmentation of the sarcolemma (*arrow head*). Magnification ×9600

Table 8. Per cent injured cells[a] in controls and verapamil or timolol treated hearts. Mean values

	Control	Verapamil	Timolol
Normal	1	2	3
Border	27	29	26
Ischaemic	53	57	46

[a] Cells with sarcolemmal or nuclear membrane fragmentation are regarded as severely damaged cells

(1.48 ± 0.04 $\mu m^2/\mu m^3$ and 1.3 ± 0.04 $\mu m^2/\mu m^3$ respectively) (Table 6). In both groups the surface density appears more extensively reduced in the ischaemic than in the border zone, even though these differences are not significant (Table 6). The mitochondrial surface to volume ratio is smallest in the border zone of the controls (4.65 ± 0.11 $\mu m^2/\mu m^3$), whereas this ratio is more extensively but not significantly more reduced in the ischaemic zone (5.11 ± 0.12 $\mu m^2/\mu m^3$) than in the border zone (5.64 ± 0.18 $\mu m^2/\mu m^3$) of the verapamil group (Table 7).

These observations indicate that the smallest mitochondria are found in the normal zone of the Verapamil treated hearts. At the same time there is a relatively more extensive mitochondrial swelling in the border and ischaemic zones of the verapamil hearts than in the controls (Fig. 1).

III. The timolol group versus the controls. There is no significant difference in fractional volumes between the timolol group and the controls (Table 5), except for a smaller $V_{V\,mit}$ in the border zone of the timolol group ($26.15 \pm 0.75\%$) than in the controls ($31.01 \pm 0.66\%$). Likewise there is no change of $V_{V\,mit}$ between the normal zone ($26.97 \pm 0.79\%$), the border zone and the ischaemic zone ($27.34 \pm 0.75\%$) of the timolol group. As for the control group there is a more extensive cytoplasmic oedema in the ischaemic than in the border zone of the Timolol treated hearts, expressed as increased $V_{V\,cyt}$ ($15.90 \pm 0.66\%$ and $10.95 \pm 0.70\%$, respectively) and decreased $V_{V\,myo}$ ($56.61 \pm 0.78\%$ and $62.89 \pm 0.87\%$, respectively). These differences are, however, not significant between the two latter zones and do not differ from the control values in the corresponding zones.

The surface density is reduced to the same extent in both the ischaemic (1.16 ± 0.04 $\mu m^2/\mu m^3$) and border zone (1.19 ± 0.02 $\mu m^2/\mu m^3$) as compared with the normal zone (1.36 ± 0.02 $\mu m^2/\mu m^3$) of the Timolol treated hearts, whereas no significant difference in mitochondrial surface to volume occurs in any zone. Quantification of severely damaged cells showed no significant effects of Verapamil or Timolol treatment on the number of severely damaged cells in any zone (Table 8).

Discussion

A beneficial effect of both beta blockers and calcium antagonists on the infarct size in hearts subjected to temporary coronary occlusion, has been reported by several authors (Downey et al. 1985; Slutsky and Peck 1985; Vik-Mo et al. 1984; DeBoer et al. 1980; Reimer and Jennings 1984; Tumas et al. 1985; Nayler et al. 1978). Reduced infarct size has been suggested to implicate a reduction of the area at risk evolving irreversible damages. The effects of Verapamil and Timolol on the border zone may, therefore, be of significance for the understanding of the assumed protective mechanisms of these drugs.

In this study we report an elevated $V_{V\,mit}$ in the border zone of the control group. No such enhancement of fractional volume of the mitochondria was seen in the two treatment groups. This indicates that any change in the mitochondrial volumes in the border and ischaemic zones would be proportional to alterations in the total cytoplasmic volume of these two groups. There is, however, no significant difference in the border zone of controls and verapamil treated hearts, whereas verapamil treated hearts have a little larger $V_{V\,mit}$ even in the normal zone. The other fractional volumes ($V_{V\,myo}$ and $V_{V\,cyt}$) were not significantly altered by drug treatment in any zone.

More pronounced differences between the groups appeared after studying the mitochondrial surface density and the surface to volume ratio of the mitochondria. The mitochondrial surface to volume ratio is largest in the normal zone of the verapamil group when compared with the other two groups, but appears to decrease relatively more in the border and ischaemic zones. In the ischaemic zone there is no significant difference between the three groups. The maximal decrease in surface to volume ratio observed in the border zone of control hearts, is not seen in the two treatment groups. No significant difference in mitochondrial surface to volume ratio is observed between the timolol and the control groups in any zone. Irrespective of treatment, as compared with the normally perfused myocardium, surface density and mitochondrial surface to volume ratio were reduced in the ischaemic zone. Intermediate values were observed in the border zones except for a smaller mitochondrial surface to volume ratio

in the border than in the ischaemic zone in the control group (Table 7). This indicates a significant mitochondrial swelling in the latter two zones.

The differences in the mitochondrial surface to volume ratios between the corresponding zones in the treatment groups, are at least as large as the differences between the various zones within a group. Thus the mitochondrial swelling seen in this study, must be a complex result of both drug treatment and ischaemia.

The mitochondrial surface density is significantly highest in the verapamil group of all zones. In all groups, however, there is a significant decrease in mitochondrial surface density in the ischaemic and border zones.

Rather opposed to this are the results from the quantitative assessments of the number of severely injured cells and water content which were found to be influenced only by ischaemia and not by Verapamil or Timolol treatment. Furthermore, neither the increased $V_{V\,cyt}$ nor the decreased $V_{V\,myo}$ seen in ischaemia, are significantly affected by drug treatment. We therefore conclude that mitochondrial swelling is an earlier sign of ischaemic injury than cytoplasmic oedema. The lack of a simple correlation between the degree of mitochondrial oedema and the extent of ischaemic injury is noteworthy since mitochondrial swelling is usually considered to be a common finding in ischaemic injury (Kloner and Braunwald 1980; Jennings and Ganote 1976).

Several protective effects on the ischaemic myocardium have been suggested for Verapamil (Kloner et al. 1982). In our study, Verapamil did not alter the haemodynamic variables or the regional blood flow neither before nor after LAD occlusion as compared with control values. This in accordance with the work of Reimer and Jennings (1984). Calcium channel blockers are presumed to inhibit mitochondrial Ca^{2+} overload induced by both Na^+ (Matlib and Schwartz 1983; Vághy et al. 1982; Wolkowicz et al. 1983) and inorganic phosphate (Matlib et al. 1983) during ischaemia. Further, some authors have shown that Verapamil prevents hypoxic induced malfunction of the mitochondrial respiration, i.e. respiratory control index and state III respiration (Nayler et al. 1978; Sugiyama et al. 1983). Verapamil induced protection against the ischaemic induced mitochondrial swelling is indicated here since we found a larger mitochondrial surface to volume ratio in the border zone of the Verapamil treated animals than in the controls. However, as the effect of Verapamil is even more pronounced in the normal zone, we rather suggest that Verapamil primarily effects the normal mitochondrial volume regulation.

Timolol is a nonselective beta adrenergic antagonist with both $beta_1$ and $beta_2$ blocking properties and has low intrinsic beta stimulating effect (Mouillé et al. 1976). Its protective effect is by some authors regarded merely as a beta adrenergic blockade (Lefer et al. 1977). In our study Timolol reduced dP/dt and heart rate, which imply a reduced cardiac work load and by this a reduced energy and O_2 demand. In the same model Timolol has been reported by Grong et al. (1981) not to improve the regional myocardial blood flow in any zone after LAD occlusion, except in the subendocardial part of the border zone. In hearts under controlled haemodynamic conditions, however, the latter authors have found that Timolol also increases blood flow in the normally perfused myocardium as well (Grong et al. 1982).

Timolol is less hydrophobic than propranolol for example (Quinn and Crutcher 1984), and interacts less readily with bilayer membranes. At therapeutic doses Timolol has no known membrane stabilizing effects (Conolly et al. 1976; Frishman 1982). Yet, in concentrations higher than in vivo use, beta-blockers are reported to impair mitochondrial function (Quinn and Crutcher 1984). Kloner et al. (1978) reported that propranolol reduced mitochondrial swelling induced by ischaemia in the rat heart. In our study we found no significant mitochondrial swelling in the border or in the ischaemic zones of the Timolol treated group. Furthermore, neither the mitochondrial surface density nor the surface to volume ratio of Timolol treated tissue differed from the control values in the border or ischaemic zones. This discrepancy might be explained by the differences between the two beta-blockers used. Another explanation might be that Kloner et al. (1978) did not measure the mitochondrial volume fraction, surface density or surface to volume ratio.

It should also be noted that no variables under study showed any difference between the groups in the ischaemic zone, whereas such differences were present both in the normal and border zones. This may suggest that cellular injury in the central ischaemic tissue three hours after LAD occlusion is severe and, therefore, only insignificantly affected by drug treatment.

In conclusion, therefore, mitochondrial swelling is an early sign of ischaemic injury that appears prior to cytoplasmic oedema and the signs of irreversible ischaemic injury (Fig. 1). Additionally, Verapamil induces changes in the normal mitochondrial volume regulation, whereby the size of

the mitochondria is being reduced in both the normal and border zones. Neither Verapamil nor Timolol have any effect on the cytoplasmic oedema or mitochondrial swelling in the ischaemic zone of the ischaemic heart.

Acknowledgments. We express our appreciation to Prof. I. Heuch for his advice on statistical method and to E.K. Frotjold, E. Soltvedt, L.H. Andreassen and T. Fiskeseth for their skilled technical assistance. This work was supported by grants from the Norwegian Research Council on Cardiovascular Disease.

References

Conolly ME, Kersting F, Dollery CT (1976) The clinical pharmacology of beta-adrenoceptor-blocking drugs. Prog Cardiovasc Dis 19(3):203–235

DeBoer LWV, Strauss HW, Kloner RA, Rude RE, Davis RF, Maroko PR, Braunwald E (1980) Autoradiographic method for measuring the ischemic myocardium at risk: Effects of verapamil on infarct size after experimental coronary artery occlusion. Proc Natl Acad Sci USA 77(10):6119–6123

Downey JM, Hearse DJ, Yoshida S, Maxwell MP, Yellon DM (1985) Verapamil and nifedipine limit infarct size in the dog. Adv Myocardiol 6:529–543

Fawcett DW, McNutt NS (1969) The ultrastructure of the cat myocardium. I Ventricular papillary muscle. J Cell Biol 42:1–45

Fishbein MC, Hare CA, Gissen SA, Spadaro J, Maclean D, Maroko PR (1980) Identification and quantification of histochemical border zones during the evolution of myocardial infarction in the rat. Cardiovasc Res 14:41–49

Frishman WH (1981) β-adrenoceptor antagonists: New drugs and new indications. N Engl J Med 305(9):500–509

Greve G, Rotevatn S, Grong K, Stangeland L (1988) Cellular morphometric changes in cat hearts subjected to 3 hours of regional ischaemia. Virchows Arch A [Pathol Anat] 412:205–213

Grong K, Stangeland L, Andersen KS, Lekven J (1981) Effect of timolol on blood flow distribution in the myocardium during acute regional ischaemia in cats. Cardiovasc Res 15:430–435

Grong K, Stangeland L, Andersen KS, Lekven J (1982) Effects of timolol on blood flow distribution in the feline myocardium with acute regional ischaemia during controlled haemodynamic conditions. Cardiovasc Res 16:269–275

Jennings RB, Ganote CE (1976) Mitochondrial structure and function in acute myocardial ischemic injury. Circ Res 38 (5 Suppl 1):I80–I91

Jodalen H, Stangeland L, Grong K, Vik-Mo H, Lekven J (1985) Lipid accumulation in the myocardium during acute regional ischaemia in cats. J Mol Cell Cardiol 17:973–980

Kloner RA, Braunwald E (1980) Observations on experimental myocardial ischemia. Cardiovasc Res 14:371–395

Kloner RA, DeBoer LWV, Carlson N, Braunwald E (1982) The effect of verapamil on myocardial ultrastructure during and following release of coronary artery occlusion. Exp Mol Pathol 36:277–286

Kloner RA, Fishbein MC, Braunwald E, Maroko PR (1978) Effect of propranolol on mitochondrial morphology during acute myocardial ischemia. Am J Cardiol 41:880–886

Kloner RA, Fishbein MC, Maclean D, Braunwald E, Maroko PR (1977) Effect of hyaluronidase during the early phase of acute myocardial ischemia: An ultrastructural and morphometric analysis. Am J Cardiol 40:43–49

Lange R, Kloner RA, Braunwald E (1983) First ultra-short-acting beta-adrenergic blocking agent: Its effect on size and segmental wall dynamics of reperfused myocardial infarcts in dogs. Am J Cardiol 51:1759–1767

Lefer AM, Cohn JR, Osman GH Jr (1977) Protective action of timolol in acute myocardial ischemia. Eur J Pharmacol 41:379–385

Matlib MA, Schwartz A (1983) Selective effects of diltiazem, a benzothiazepine calcium channel blocker, and diazepam, and other benzodiazepiner on the Na^+/Ca^{2+} exchange carrier system of heart and brain mitochondria. Life Sci 32(25):2837–2842

Matlib MA, Vághy PL, Epps DE, Schwartz A (1983) Actions of certain calcium channel blockers and calmodulin antagonists on inorganic phosphate-induced swelling and inhibition of oxidative phosphorylation of heart mitochondria. Biochem Pharmacol 32(17):2622–2625

Mouillé P, Schmitt H, Cheymol G, Gautier E (1976) Cardiovascular and β-adrenergic blocking effects of timolol. Eur J Pharmacol 35:235–243

Nayler WG, Fassold E, Yepez C (1978) Pharmacological protection of mitochondrial function in hypoxic heart muscle: Effect of verapamil, propranolol and methylprednisolone. Cardiovasc Res 12:152–161

Page E, Polimeni PI (1977) Ultrastructural changes in the ischemic zone bordering experimental infarcts in rat left ventricles. Am J Pathol 87:81–104

Quinn PJ, Crutcher EC (1984) The action of β-adrenoceptor antagonists on rat heart mitochondrial function in vitro: A comparison of propranolol, timolol and atenolol. Cardiovasc Res 18:212–219

Rasmussen MM, Reimer KA, Kloner RA, Jennings RB (1977) Infarct size reduction by propranolol before and after coronary ligation in dogs. Circulation 56(5):794–798

Reimer KA, Jennings RB (1984) Verapamil in two reperfusion models of myocardial infarction. Temporary protection of severely ischemic myocardium without limitation of ultimate infarct size. Lab Invest 51(6):655–666

Slutsky RA, Peck WW (1985) Effects of beta-adrenergic blockade on the natural progression of myocardial infarct size and compensatory hypertrophy. J Am Coll Cardiol 5(5):1132–1137

Sugiyama S, Miyazaki Y, Kotaka K, Ozawa T (1983) The effects of verapamil on mitochondrial dysfunction associated with coronary reperfusion. Jpn Circ J 47(7):830–836

The Norwegian Multicenter Study Group (1981) Timolol-induced reduction in mortality and reinfarction in patients surviving acute myocardial infarction. N Engl J Med 304(14):801–807

Tumas J, Deth R, Kloner RA (1985) Effects of Nisoldipine: A new calcium antagonist, on myocardial infarct size and cardiac dynamics following acute myocardial infarction. J Cardiovasc Pharmacol 7:361–367

Vághy PL, Johnson JD, Matlib MA, Wang T, Schwartz A (1982) Selective inhibition of Na^+-induced Ca^{2+} release from heart mitochondria by diltiazem and certain other Ca^{2+} antagonist drugs. J Biol Chem 257(11):6000–6002

Vik-Mo H, Maroko PR, Ribeira LGT (1984) Comparative effects of propranolol, timolol and metoprolol on myocardial infarct size after experimental coronary artery occlusion. J Am Col Cardiol 4:735–741

Wolkowicz PE, Michael LH, Lewis RM, McMillin-Wood J (1983) Sodium-calcium exchange in dog heart mitochondria: Effects of ischemia and verapamil. Am J Physiol 244 (Heart Circ Physiol 13):H644–H651

Accepted November 25, 1987