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Short-term Stimulation by Propranolol and Verapamil of Cardiac Cellular Autophagy

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M. Bahro and U. Pfeifer. Short-term Stimulation by Propranolol and Verapamil of Cardiac Cellular Autophagy. *Journal of Molecular and Cellular Cardiology* (1987) **19**, 1169–1178. The influence of propranolol and verapamil, i.e. two cardiodepressant drugs differing in their pharmacological actions, on cellular autophagy in the left ventricular myocardium of the rat was investigated. In the first experimental series 10 animals were given propranolol subcutaneously (3 mg/kg body weight). Ten controls received physiological saline. In the second series 8 animals were treated with verapamil subcutaneously (9 mg/kg body weight) and 8 controls with physiological saline. Two to 4 h after the injections in the first series and 1 3/4 to 3 h in the second series retrograde perfusion fixation was carried out via the abdominal aorta with a paraformaldehyde-glutaraldehyde mixture. Myocardial tissue from the left anterior wall was processed for electron microscopy and was morphometrically evaluated for volume fraction and numerical density of early stages of autophagic vacuoles (AVs). Propranolol and verapamil significantly increased the AV volume fraction 4.3- and 2.7-fold, respectively. The numerical density was increased by the two drugs, although to a lesser degree (2.3-fold, and 1.7-fold, respectively). As verapamil affects neither the beta-adrenoreceptors nor the intracellular levels of the second messenger cAMP, the only common denominator for the stimulation of cellular autophagy seems to be the cardiodepressive effect of the two drugs. The data suggest that the rise in cellular autophagy is an early regulatory step in the adaptation of heart muscle mass to reduced work load.

Key Words: Autophagic vacuoles; Propranolol; Verapamil; Morphometry; Electron microscopy; Protein degradation.

Introduction

Cellular autophagy is known as a biological phenomenon that regulates the degradation of cytoplasmic components [*9, 34*]. Small parts of cytoplasm are segregated by membranes forming the so-called autophagic vacuoles (AVs) which are the site of lysosomal digestion of the segregated material into low molecular split products [*23*].

By combining ultrastructural and morphometric methods, it has been shown in earlier investigations that AVs are regularly encountered in the myocardium of untreated rats, and that their volume fraction and numerical density is subject to circadian variations [*38*]. Isoproterenol, which has a positive inotropic effect via stimulation of beta-adrenoreceptors, has been shown to lower significantly the AV volume fraction in myocardium within short-term intervals [*5, 39*].

In order to arrive at more general conclusions concerning the inverse relationship between cardiac work on the one hand, and intracellular degradation by autophagy on the other, the effects of two different cardiodepressant agents on cardiac cellular autophagy have been examined in the present study. Propranolol has been used as an agent that blocks beta-receptors, thus being the pharmacological antagonist of isoproterenol. The second substance tested was verapamil, a calcium channel blocker, which inhibits cardiac work without affecting the beta-receptors.

Material and Methods

1. Experimental conditions and tissue preparations

Thirty six adult male Sprague–Dawley rats (Charles River Wiga, Sulzfeld, Germany) were housed for about 3 weeks in the animal room under the following conditions: 22°C

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room temperature, artificial light from 6.00 a.m. to 6.00 p.m. and darkness from 6.00 p.m. to 6.00 a.m. The animals were fed with a standard diet (Altromin®; Altromin, Lage, Germany) and received tap water *ad libitum*.

The investigation comprised two experimental series. In the first experiment 10 rats were given propranolol hydrochloride (Rhein–Pharma, Plankstadt, Germany) at a dose of 3 mg/kg body weight s.c., and 10 control animals received physiological saline. In the second series 8 rats were treated with verapamil hydrochloride (Knoll, Ludwigshafen, Germany) at a dose of 9 mg/kg body weight s.c., whereas 8 control animals received physiological saline. The injections were given at 5.00 p.m., i.e. 1 h before light-dark change. The mean body weight of the animals was 325 (S.D. 20) g in the propranolol series and 300 (S.D. 25) g in the verapamil series.

Two to 4 h after the injections in the propranolol series, and 1 3/4 to 3 h in the verapamil series, respectively, retrograde perfusion fixation was performed via the abdominal aorta [*16*] under chloralhydrate anaesthesia (Merck, Darmstadt, Germany). The pressure of perfusion was about 100 cm water column. After the vascular system had been washed out with a Ringer solution containing 4% Dextran 35 and 0.5% Procain, the fixative was perfused. The latter was a modification of Karnovsky's fixative [*20*] and contained 3.2% paraformaldehyde, 4% glutaraldehyde and 4% Dextran 35.

The fixation was carried out in the early hours of the dark period because the volume fraction of AVs is strongly affected by circadian variations and tends towards a minimum after the onset of darkness and of food intake [*35*, *38*]. In order to examine if the drugs administered interfere with the actual intake of food, the stomach together with its contents was weighed after resection from the carcass. In the first series, the average weight of the stomachs of the animals treated with propranolol was 4.9 g which was about 20% less than the average weight (6.25 g) in the control group. On the other hand, in the second series, the average weight of the stomachs of the animals treated with verapamil (4.8 g) was about 10% over the control group (4.4 g). These differences could not be proved to be statistically significant.

After fixation, samples of myocardial tissue were taken in the following way: slices of about 1 mm thickness were cut from the left ventricle perpendicularly to the heart axis. Trapezoid specimens from the anterior wall were cut in a way that the basis represented the epicardial layer. After overnight fixation in the glutaraldehyde mixture they were put into the Ultroprocessor (LKB, Munich, Germany) where the following steps were run automatically: after having been washed with phosphate buffer the tissue samples were post-fixed in 2% phosphate buffered osmium tetroxide for 1.5 h, washed with phosphate buffer, and dehydrated in a graded series of ethanols. Finally, they were infiltrated with epon via propylenoxide.

The specimens were embedded in flat moulds in a way that the epicardial layer corresponded to the prospective section plane. Areas of about 0.3 × 0.3 μm thickness were viewed under the light microscope. Silver-grey thin sections were put on rhodium-copper grids (300 mesh) and stained for added contrast with lead citrate.

2. *Electron microscopic and morphometric methods*

The sections were investigated with an EM 10 (Zeiss, Oberkochen, Germany) at an acceleration voltage of 60 kV. The AVs were distinguished according to morphological criteria already described in previous papers [*5*, *32*, *38*]. In short, AVs containing mitochondria (AV_{Mito}) and those containing portions of ER, t-system, Golgi vesicles, glycogen granules, and ground substance (AV_{ERGS}) were recorded. Because of their rare occurence the AVs were counted and measured directly in the electron microscope. The square openings of the copper-rhodium grids were used as test fields when completely covered by the specimen (Fig. 1). Six test fields per animal were systematically evaluated for AVs at a primary magnification of × 7900 using in addition a binocular lens of 9-fold magnification. The surface areas of the square openings were calculated from coordinated values obtained from the digital counter of the driving mechanism of the specimen stage [*33*].

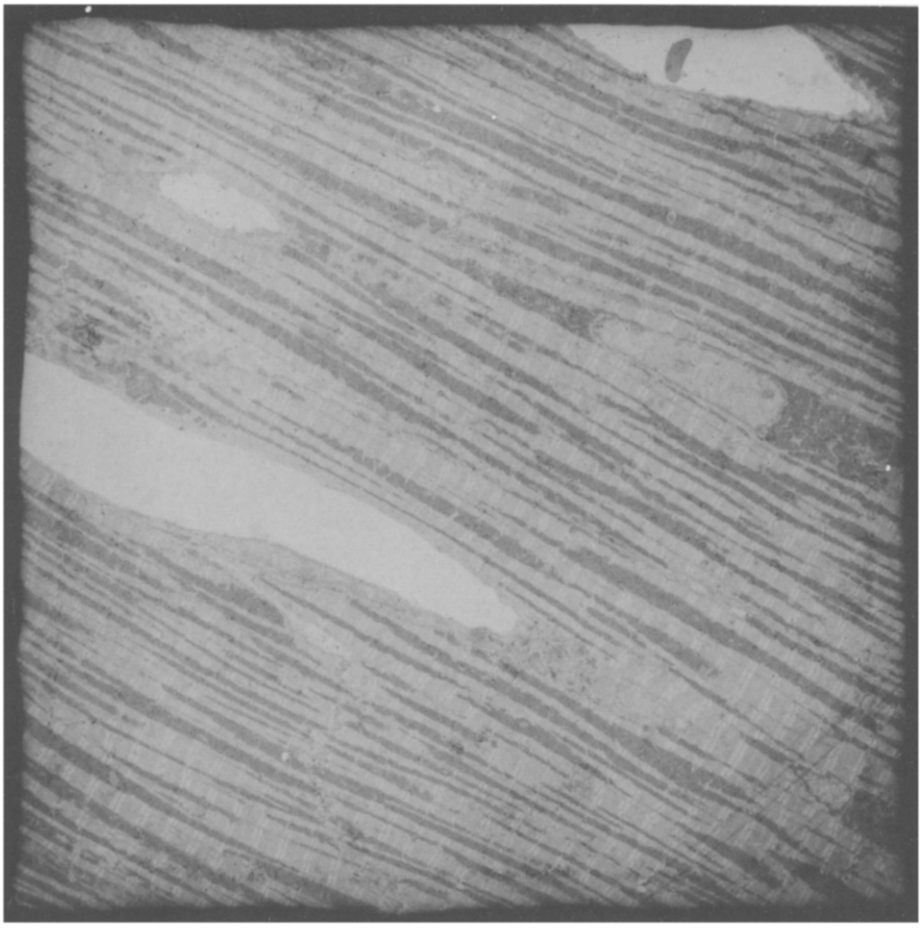

FIGURE 1. Square opening of the rhodium-copper grid viewed under the electron microscope, covered with myocardial tissue. × 2040.

Two diameters of each AV-profile encountered were determined by means of a crossed mm-scale engraved onto the fluorescent screen of the electron microscope. As has been shown earlier on [6] the product of these two diameters is in close linear correlation to the AV-profile area as determined by point counting at a high point density. In practice, the AV-profile area could be calculated by dividing the diameter product by the slope of the regression line. The fractional area of sectioned cytoplasm of cardiomyocytes was determined by point counting micrographs at a magnification of × 2080 using a square lattice (15 mm distance between the lines). In the propranolol series the fractional cytoplasmic area was 76% (S.E.M. ± 0.957), in the verapamil series it was 75% (S.E.M. ± 1.247). The total area of cytoplasm evaluated was 4×10^5 μm^2 in the propranolol series, and 3.2×10^5 μm^2 in the verapamil series. The total number of AV-profiles encountered in these evaluations was 335. The volume fraction of AVs (V_{AV}/V_{Cyto}) was calculated for each animal by relating the total area of AV-profiles to the total area of sectioned cytoplasm.

In order to assess whether changes in the AV volume fraction are due mainly to changes in the single AV volume, or to changes in AV number per unit volume, numerical densities were estimated according to the Weibel and Gomez formula [46]. In view of the variations in shape of the AV profiles (Figs 2 and 3) the figures obtained have to be regarded as biased. Since, however, the

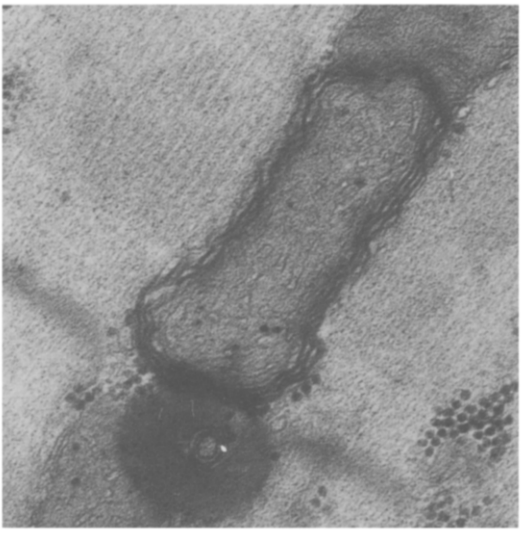

FIGURE 2. AV with mitochondrial content, limited by a triple osmiophilic-layered membrane. × 51 800.

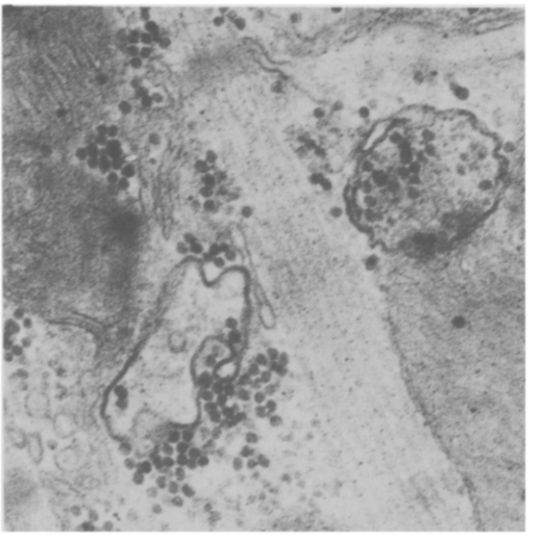

FIGURE 3. Two AVs containing glycogen particles, vesicular profiles and ground substance. × 57 500.

bias could be assumed to be the same in control and experimental animals the compromise seemed to be tolerable with respect to the underlying question.

The volume fraction and the numerical density were calculated separately for each type of AVs. From the values obtained for each animal the mean values and the standard error of the means (S.E.M.) were calculated. Levels of significance between measurements in control and experimental animals were assessed by the U-test.

Results

1. Morphology of AVs

The AVs represent cytoplasmic structures that are bordered by a single, double, or sometimes even a multiple membrane. Until now it is not yet clear whether these membranes are formed *de novo* [*31*] or are derived from pre-existing cytoplasmic membranes [*1*].

In cardiomyocytes two types of AVs can be distinguished: those that include mitochondria (AV_{Mito}) (Fig. 2) and those that contain other components of cardiomyocyte cytoplasm, as sarcoplasmic reticulum, T-tubuli, Golgi vesicles, glycogen particles, ribosomes, and ground substance (AV_{ERGS}) (Fig. 3). A combination of both types is possible. In that case the diameter product of the mitochondrial profile was subtracted from the diameter product of the total AV profile and the two values were treated separately in the calculation of the volume fractions of the different types of AVs.

By definition, only those AVs were evaluated that were enveloped by a membrane surrounding a minimum of three quarters of the AV's circumference. Furthermore, when the segregated cytoplasm could not clearly be identified because of lytic changes, the profiles were excluded from the evaluation (Fig. 4). Myofibrils could not be detected among the contents of AVs.

2. Quantitative results

Propranolol

After the injection of propranolol the volume fraction of total AVs significantly increased 4.3-fold from 2.8×10^{-5} (control group) to 11.9×10^{-5} (experimental animals) [Fig. 5, Table 1(a)]. The volume fraction of AV_{Mito} showed even a 5.2-fold increase, whereas AV_{ERGS} were increased only 3-fold. The numerical density of total AVs increased 2.3-fold from 6.2×10^5 mm^{-3} to 14.6×10^5 mm^{-3} [Fig. 6, Table 1(b)]. In this case, too, the AV_{Mito} showed a greater increase, 4-fold, than AV_{ERGS}, 2-fold.

Verapamil

The administration of verapamil led to a significant 2.7-fold increase of the total AV

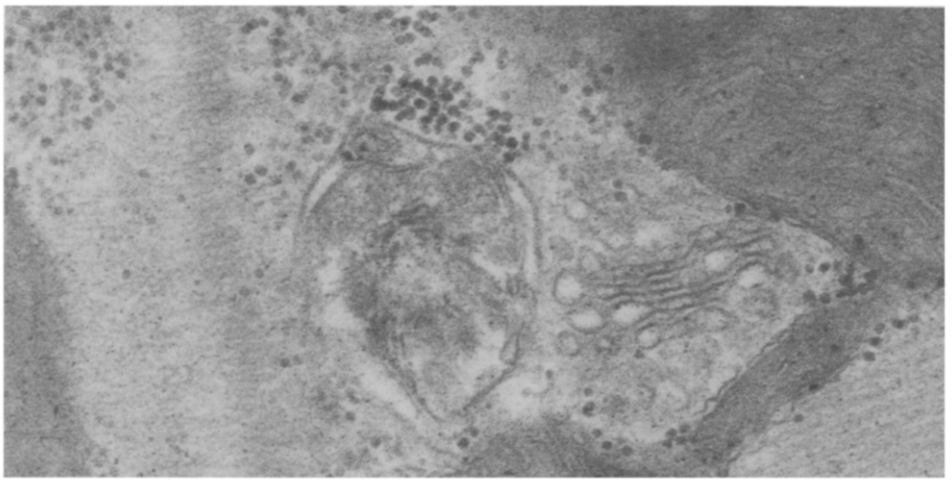

FIGURE 4. The content of this AV is no longer clearly identifiable because of lytic changes. This AV was excluded from evaluation. × 72 000.

volume fraction from 6.6×10^{-5} (control animals) to 17.8×10^{-5} (experimental group) [Fig. 5, Table 2(a)]. In this series, too, the AV_{Mito}-fraction increased more (3-fold) than the AV_{ERGS}-fraction (1.7-fold). The numerical density of total AVs significantly increased from 10.2×10^{5} mm^{-3} to 16.9×10^{5} mm^{-3} [Fig. 6, Table 2(b)]. This increase was 1.7-fold and therefore less than the increase of the volume fraction. The segregated mitochondria showed a greater increase (4.3-fold) than AV_{ERGS} (1.3-fold).

Time intervals

When plotting the total AV volume fraction against the time interval after the injections (Figs 7 and 8), no significant correlation could be found in the two experimental series. However, a trend towards decreasing values in the course of time can be seen from the figures. This may be explained, in part, by the physiological circadian rhythm of cellular autophagy [*38*], and, possibly, in the experimental animals, by a time depending loss of effectiveness of the drugs administered.

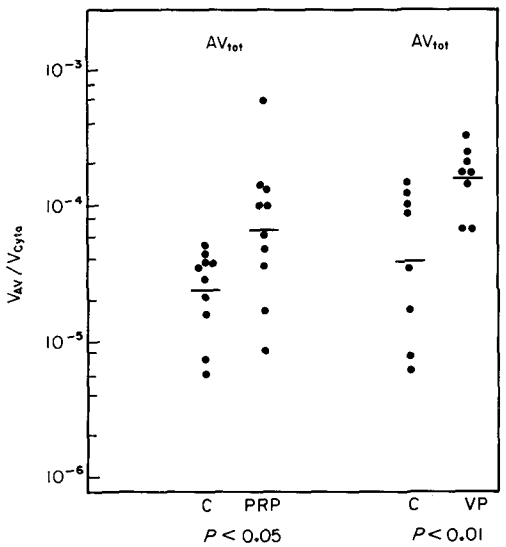

FIGURE 5. The volume fraction V_{AV}/V_{cyto} of total AVs (AV_{tot}) in animals treated with propranolol (PRP) or verapamil (VP) and in control animals (C).

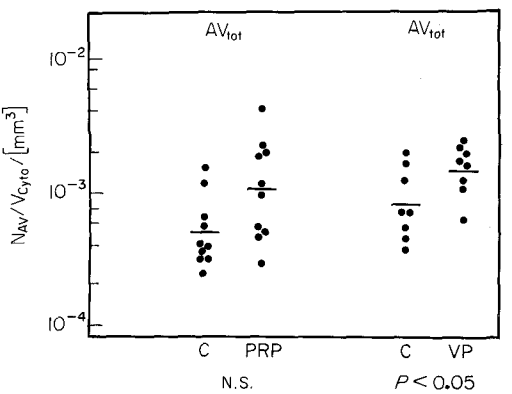

FIGURE 6. The numerical densities N_{AV}/V_{cyto} of total AVs (AV_{tot}) in animals treated with propranolol (PRP) or verapamil (VP) and in control animals (C).

TABLE 1. The volume fractions V_{AV}/V_{cyto}(a) and the numerical densities N_{AV}/V_{cyto} (b) of AV_{ERGS}, AV_{Mito} and of total AVs (AV_{tot}) in the cytoplasm of heart muscle cells are shown in controls (C) and propranolol-treated animals (PRP).

(a)

	$V_{AV}/V_{cyto}[10^{-5}]$			
AV-content	C	PRP	%-change	*U*-test
ERGS	1.21 ± 0.29	3.65 ± 1.00	202	$P < 0.05$
Mito	1.57 ± 0.50	8.23 ± 5.11	425	N.S.
tot	2.78 ± 0.47	11.89 ± 5.11	328	$P < 0.05$

(b)

	$N_{AV}/V_{cyto}[10^5/mm^3]$			
AV-content	C	PRP	%-change	*U*-test
ERGS	5.10 ± 0.47	10.20 ± 2.19	100	N.S.
Mito	1.12 ± 0.25	4.49 ± 3.15	301	N.S.
tot	6.22 ± 1.42	14.59 ± 3.78	134	N.S.

The %-change is calculated as PRP × 100/C − 100. Mean values and S.E.M. are given.

Discussion

As it has already been mentioned in the introduction the causal relationship between autophagic segregation of cytoplasmic components on the one hand and degradation of intracellular proteins on the other hand is evident. Systematic investigations combining biochemical and morphological methods have shown that cellular autophagy is an ultrastructural expression of the degradation of intracellular proteins [*19*, *23*, *36*, *41*]. Since the half-life of AVs seems to be a more or less constant parameter [*29*] the volume fraction of AVs can be regarded as a relative measure of intracellular protein degradation. This is true, however, only for the non-myofibrillar components. Myofibrils have never been detected as contents of AVs [*5*, *6*, *38*, *44*]. It is discussed that myofibrils first are degraded on extralysosomal pathways and, later, at a stage which can no longer be resolved by electron microscopy, they are degraded within lysosomes [*18*, *25*].

TABLE 2. The volume fractions V_{AV}/V_{cyto} (a) and the numerical densities N_{AV}/V_{cyto} (b) of AV_{ERGS}, AV_{Mito} and of total AVs (AV_{tot}) in the cytoplasm of heart muscle cells are shown in controls and verapamil-treated animals (VP).

(a)

	$V_{AV}/V_{cyto}[10^{-5}]$			
AV-content	C	VP	%-change	*U*-test
ERGS	1.42 ± 0.51	2.47 ± 0.59	74	$P < 0.05$
Mito	5.18 ± 1.32	15.36 ± 2.86	197	$P < 0.01$
tot	6.61 ± 2.00	17.84 ± 3.14	170	$P < 0.01$

(b)

	$N_{AV}/V_{cyto}[10^5/mm^3]$			
AV-content	C	VP	%-change	*U*-test
ERGS	9.17 ± 2.26	12.31 ± 2.19	34	N.S.
Mito	1.07 ± 0.31	4.58 ± 0.86	327	$P < 0.001$
tot	10.24 ± 2.26	16.89 ± 2.23	65	$P < 0.05$

The %-change is calculated as VP × 100/C − 100. Mean values and S.E.M. are given.

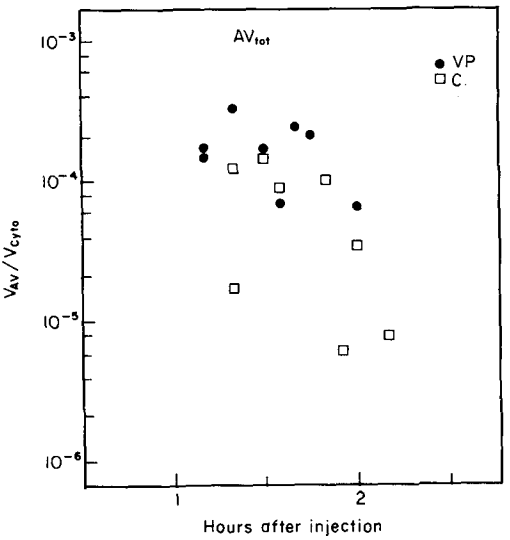

FIGURE 7. The volume fractions V_{AV}/V_{cyto} of all AVs (AV_{tot}) plotted against the time after injection in controls (C) and propranolol-treated animals (PRP).

The present investigation shows that the volume fraction of autophagic vacuoles (AVs) increases significantly in cardiomyocytes after treatment with propranolol. Opposite effects, i.e. inhibition of autophagy, have been reported after surgical induction of cardiac hypertrophy by supravalvular aortic constriction [*6*] and, in short-term studies, after giving isoproterenol [*5*, *39*]. This drug has a positive inotropic effect and, if applied during a longer period, leads to cardiac hypertrophy [*10*, *43*]. It is tempting to attribute the increase in segregated cytoplasmic components after treatment with propranolol to the decrease in cardiac output by about 30% as observed in normotensive rats under the influence of this drug [*4*, *8*].

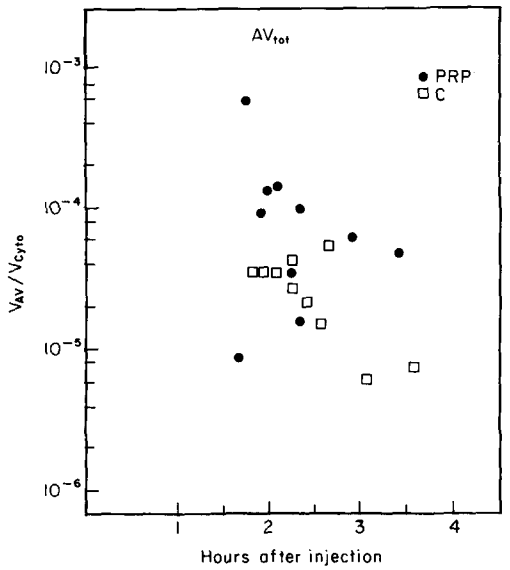

FIGURE 8. The volume fractions V_{AV}/V_{cyto} of all AVs (AV_{tot}) plotted against the time after injection in controls (C) and verapamil-treated animals (VP).

Biochemical data concerning the effects of beta-blocking agents are so far contradictory. While Davies *et al.* [*7*] failed to detect any change in structure, synthesis, or function of cardiac proteins under the influence of the beta-antagonistic agent atenolol, other authors confirmed an inhibitory action of beta-blockers on several synthetic processes. It was shown that isoproterenol enhanced the synthesis of proteins and of adenine nucleotides in myocardium and that these effects could be blocked immediately and completely by propranolol [*48*]. Correspondingly, opposite effects of isoproterenol and propranolol on the synthesis of alanine and glutamine were described in the skeletal muscle [*17*].

It is still a matter of debate whether isoproterenol affects the degradative pathway, too. Some investigators have emphasized that this drug changes only protein synthesis, but not protein degradation [*11*, *40*], while others provide evidence that isoproterenol inhibits the catabolism of proteins [*3*, *21*, *24*, *28*, *47*]. Quantitative morphological studies have confirmed the inhibitory action of isoproterenol on cellular autophagy in cardiac muscle [*5*, *39*]. The present results indicate that the effects of beta-blockers are opposite to those of isoproterenol, as concerns synthetic pathways, but also degradation by autophagy. Similar results have been obtained from studying the isolated perfused rat heart [*21*]. All these data are in line with the concept of coordinated regulation of protein synthesis and degradation [*2*].

In the second series, after treatment with verapamil, we also found a statistically significant increase in the volume fraction of AVs compared with the control group. This drug, too, has been shown to decrease cardiac output in normotensive rats to a similar degree as propranolol, i.e. by about 30% [*14*].

However, its principle of action is different from that of beta-blockers. While the latter inhibit the formation of intracellular cyclic 3',5'-adenosine monophosphate (cAMP) [*13*], i.e. the second messenger of isoproterenol, verapamil has no influence on cAMP-levels [*45*], but rather acts at specific calcium binding sites on the surface of the sarcolemma as well as in the slow calcium channels [*15*].

One has to conclude then that cellular autophagy is stimulated when cardiac output is decreased, independently of the level at which transmission of the impulses is blocked. Intracellular cAMP levels, therefore, do not seem to be critical in the regulation of cellular autophagy. This is evident also from the fact that, contrary to the inhibitory effect of signals rising cAMP in cardiac muscle, cAMP is known to stimulate autophagy in liver [*42*] as well as in kidney [*37*]. As for myocardium, a close functional relationship has to be assumed, therefore, between work performance on the one hand and anticatabolic signals on the other. Precise knowledge how these two parameters depend from each other is still missing. The possibility is discussed that the sarcomere length might take influence on the degradation of cytoplasmic components via an unknown principle of control [*21*].

Since protein synthesis and degradation are the key factors in the regulation of myocardial mass, the question rises whether the observed stimulation of autophagy by cardiodepressant drugs could serve as a mechanism of regression of cardiac hypertrophy. Whilst, in the case of beta-blockers, Malik and Geha [*22*] did not see any reduction in heart muscle mass by these drugs in a case of hypertrophy that had been elicited by aortic constriction, Östman-Smith [*27*] came to opposite results in the same experimental model. Cardiac hypertrophy as the consequence of renal hypertension could be well reduced by a beta-blocking substance [*12*], whereas the augmented heart muscle mass in spontaneously hypertensive rats was not affected by beta-antagonists [*30*]. As concerns calcium antagonists Narita *et al.* [*26*] reported that left ventricular wet weight was significantly decreased in diltiazem treated spontaneously hypertensive rats so that cardiac hypertrophy induced by hypertension could be suppressed.

In summary, the following conclusions can be drawn: whereas stimulation of cardiac work is accompanied by an anticatabolic reaction which shifts the balance towards hypertrophy, an early shift towards a reduction of myocardial mass is mediated by cardiodepressant substances, be it beta-blocking drugs or calcium antagonists. Therefore, autophagic segregation and degradation of cytoplasmic components can be considered to represent an important short-term available reaction in the regulation of myocardial mass.

Acknowledgements

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