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FORMATION OF ADRENOCHROME BY BOVINE CARDIAC SARCOLEMMA

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A sarcolemma preparation from bovine heart was able to promote adrenaline oxidation especially when NADH and NADPH were added. The superoxide anion $O_2^{\cdot-}$ was demonstrated to be involved in the activation of adrenochrome production.

It is well documented that injections of catecholamines in high doses produce cardiac lesions and disturbances of heart function and metabolism (1-2). The studies of Dhalla and coworkers (3-5) showed that the oxidation products of catecholamines such as adrenochrome were able to produce cardiac lesions that were particularly evident at the sarcolemma level. The character of damage appeared to be similar to that seen following the injection of norepinephrine or isoproterenol. Nevertheless, because of the analytical difficulties to detect adrenochrome "in vivo", only a few studies have demonstrated its presence in tissue or plasma (6). Very recently Matthews et al. (7), reported that a substance with a retention time identical to that of adrenochrome was detectable in biological samples by HPLC techniques. This finding has provided evidence for its potential production in vivo. Since the cardiac sarcolemmal membranes are rich in specific receptors interacting with the catecholamines (8), we have investigated whether these membranes may promote the formation of adrenochrome.

Materials and Methods

Bovine cardiac sarcolemma was prepared as described by Reeves and Sutko (9) with the modifications suggested by Lamers and

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Stinis (10). Finely minced cardiac muscle (80g) from the left ventricle was disrupted in ice-cold 0.3M sucrose, 0.5mM EDTA, 10mM MOPS, pH 7.4 and 0.5mM phenyl methane sulfonylfluoride in a Waring Blendor (3 bursts of 10 sec). The muscle was further disintegrated by an Ultra Turrax homogenizer for 10 sec at maximal speed (3 times). The homogenate was incubated with DNAase (30µg/ml) in presence of 1 mM $MgCl_2$ and thereafter the Mg^{2+} ions titrated back with EDTA. The microsomal membranes were harvested by centrifugation at 12400g and further fractionated in a 0.3M/0.6M discontinuous sucrose gradient by centrifuging at 113000g for 3 hrs. The sarcolemmal membranes were collected from the 0.3M/0.6M sucrose interface. After dilution with 3 vol of 0.16M NaCl, 20 mM MOPS, pH 7.4 the vesicles were harvested by centrifugation at 48000g for 30 min. The sedimented membranes were resuspended in the last buffer and stored frozen in small aliquots at -80°C. By this procedure routinely 1.5-2 mg of membrane protein was obtained. (Na^+-K^+)-ATPase activity was measured in presence of 0.3 mg SDS per mg membrane protein as described by Lamers and Stinis (11). The ouabain sensitivity of (Na^+-K^+) ATPase was estimated at 2mM concentration of the drug. Inorganic phosphate formation was measured according to Le Bel et al. (12). 5'-Nucleotidase (5'-AMPase) activity was measured by the procedure of Edwards and Maguire (13). Succinate dehydrogenase activity was measured by the method of King (14). NADH cytochrome c reductase activity was measured according to Sottocasa et al. (15). The formation of adrenochrome was monitored at 480nm at 25°C in a double beam Perkin Elmer spectrophotometer model 559. The assay mixture (volume is 1.0 ml) consisted of 50mM Tris-HCl pH 8.2, 1mM EDTA and 20-30µg of sarcolemmal protein. Adrenaline was added at 1mM final concentration. The concentration of adrenochrome was determined using $E_{480} = 4.02 \times 10^3\ M^{-1}cm^{-1}$. Protein concentration was determined by the method of Bradford (16).

Results

The specific activities (µmol product formed/mg protein.h) of the following enzymes in the sarcolemmal vesicles were: ouabain sensitive (Na^+-K^+) ATPase, 65.8; 5'-nucleotidase, 18.4; NADH-Cyt. c reductase 0.45; succinate dehydrogenase activity was undetectable. The (Na^+-K^+) ATPase activity was found enriched 26 fold in the sarcolemmal fraction as compared with the original homogenate, whereas the specific activity of the mitochondria, NADH-Cyt. c reductase increased only 2 fold. These values indicate a high degree of sarcolemmal purity when compared to other preparations (17,18). Moreover, our sarcolemmal preparation appeared to be essentially free

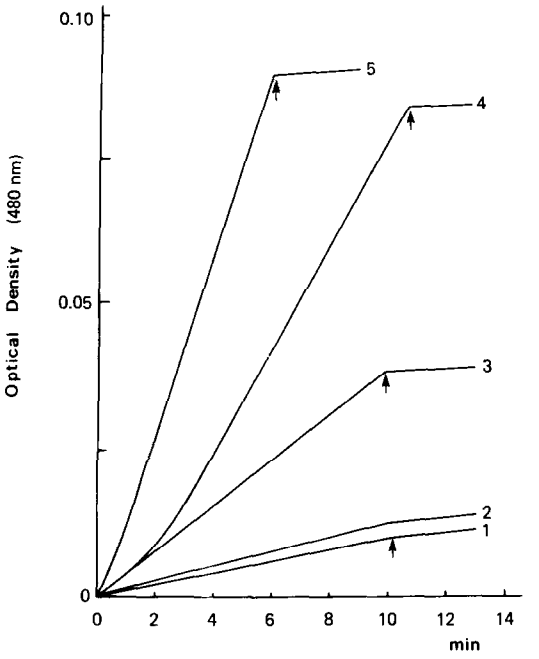

Fig. 1 Formation rate of adrenochrome in presence of bovine sarcolemmal membranes.
The production of adrenochrome was followed as described in Materials and Methods.
Trace 1, Incubation mixture; Trace 2, Incubation mixture plus denatured sarcolemma (100°C per 5 min); Trace 3, Incubation mixture plus sarcolemma; Trace 4, as trace 3 plus 0.4mM NADH; Trace 5, as trace 3 plus 0.4mM NADPH.
The arrows indicate the addition of superoxide dismutase (2μM).

of mitochondria as shown by the activity of the marker NADH--Cyt. c reductase.

One the several representative experiments which demonstrates the time-rate formation of adrenochrome from adrenaline is shown in fig.1. The oxidation of adrenaline in the assay mixture (trace 1) was increased by the presence of the sarcolemmal membranes (trace 3). In this condition the addition of NADH (trace 4) or of NADPH (trace 5) progressively enhanced the rate of adrenochrome production. The arrows in Fig.1 show that superoxide dismutase inhibited by more than 90% the adrenaline oxidation and this effect was particularly evident when the sarcolemmal membranes were present in the incubation

Table 1. Effect of various scavengers on adrenochrome formation by cardiac sarcolemma

	nmol/min-mg prot
None	169.6 ± 4.2
Superoxide dismutase (2μM)	14.4 ± 0.4
Catalase (2μM)	97.2 ± 1.6
Mannitol (5mM)	165.4 ± 1.8
α-tocopherol phosphate (600μM)	42.8 ± 0.4
Ascorbate (100μM)	26.8 ± 0.9
Deferoxamine (100μM)	143.6 ± 2.1

The scavengers were added to the incubation mixture 3 min before the addition of NADPH.
The values are means ± SD of 3 samples from 3 hearts.

mixture. Moreover, the sarcolemmal membranes denaturated by heating lost their ability to promote adrenochrome production (Trace 2).

The results indicated in Table 1 show that the formation of adrenochrome by sarcolemmal membranes induced by NADPH was strongly reduced by superoxide dismutase (91.6%), by ascorbate (84.2%) and by α-tocopherol phosphate (74.8%). Catalase produced a less evident inhibitory effect (42.7%), while mannitol and deferoxamine did not considerably modify the rate of adrenaline oxidation. The oxidation of adrenaline could not be induced by succinate + antimycin nor was it affected by rotenone when initiated by NADH or NADPH (not shown); these controls reject the possibility that a small contamination of mitochondria (compare Table 1) is the source of $O_2^{\cdot-}$ radicals.

Discussion

From this study it follows that the formation of adrenochrome from adrenaline is stimulated by bovine heart sarcolemmal membranes. The rate of this reaction is progressively more

evident when the membranes were incubated with NADH or NADPH respectively. The exact mechanism(s) is not known, but the fact that the reaction is inhibited by the enzyme superoxide dismutase or by compounds such as ascorbate or α-tocopherol, which are effective scavengers of the superoxide ion $O_2^{\cdot-}$ (19), strongly suggests the participation of these radicals during adrenaline oxidation. Moreover, since catalase only had a partial inhibitory effect and mannitol no effect, it is likely that H_2O_2 or $OH^{\cdot}$ radicals did not play a prevalent role in the formation of adrenochrome by heart sarcolemma. In addition, this reaction seems to require the presence of non-denaturated sarcolemmal membranes suggesting an involvement of its enzymatic activities. In connection with this conclusion there is also the result which demonstrates that the formation of the $O_2^{\cdot-}$ inducing adrenaline oxidation does not result from iron contamination,since the potent iron chelator deferoxamine did not modify the rate of adrenochrome formation. We would like therefore to suggest that in the sarcolemmal membranes there is an enzymatic activity that in consequence of its autoxidation may produce $O_2^{\cdot-}$ radicals and stimulate the formation of adrenochrome. In particular,our data support the hypothesis that a dehydrogenase is a component of this enzymatic complex, since NADPH is stimulating the oxidation of adrenaline dramatically. The presence of dehydrogenase activity in plasma membranes has been described for several tissues (see review (20)). Recently Cseh and Lerant (21) have identified a NADH oxidase in cardiac sarcolemmal membranes. This finding may therefore be important because it is known that several types of dehydrogenases can produce $O_2^{\cdot-}$ radicals (19).

In conclusion this research shows that adrenaline may be formed by sarcolemmal membranes by a $O_2^{\cdot-}$ dependent reaction stimulated by NADH or by NADPH,with the last cofactor the more active.This conclusion can give additional evidence in clarifying the mechanism by which high levels of catecholamines are toxic for heart cells.

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