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ASSOCIATION OF ADENYLATE CYCLASE INHIBITION BY NaF WITH LOSS OF A FACTOR IN RAT HEART SARCOLEMMA[1]

D.F. MICHIEL, J.M.J. LAMERS, V. PANAGIA[2] and N.S. DHALLA

Experimental Cardiology Section and Departments of Physiology and Oral Biology[2], University of Manitoba, Winnipeg, Canada R3E OW3

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Heart sarcolemma from different animals was isolated for studying the effect of NaF on membrane-bound adenylate cyclase activity. Unlike dog and rabbit heart, a depression of adenylate cyclase by NaF was observed in sarcolemma from rat heart. There was a progressive attenuation of the NaF ability to stimulate the enzyme at different steps of the sarcolemmal isolation procedure. The activation by epinephrine in the presence of Gpp(NH)p also decreased progressively but unlike NaF, this agent did not show an inhibition of the enzyme. The inhibitory action of NaF was not reversed upon the treatment of heart membranes with deoxycholate or by Ca^{2+}. Lubrol extract (supernatant) of a particulate fraction from rat heart, which showed NaF activation, returned the stimulatory response of the sarcolemmal adenylate cyclase to NaF. These results suggest that some regulatory factor is required for the stimulation of adenylate cyclase by NaF in myocardium and rat heart is susceptible for the loss of such a factor during the sarcolemmal isolation by the hypotonic shock-LiBr treatment method.

Hormone-sensitive adenylate cyclase is a multifactorial transduction system which mediates hormone effects on target cells. It consists of at least three individual protein components: hormone receptor, guanine nucleotide-binding regulatory unit and catalytic unit (1,2). Recent observations indicate that there are probably two functionally distinct guanine nucleotide-binding moieties, one mediating hormonal stimulation and the other hormonal inhibition of the enzyme (3,4). This enzyme complex can be affected not only by hormones but also by non-hormonal effectors like stable GTP analogues, fluoride ion and forskolin (1,5). Considerable lack of knowledge exists about the molecular actions of NaF on the adenylate cyclase system, particularly in cardiac sarcolemma (for review, see Refs. 1,6,7). The present study

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[2] To whom the reprint requests should be addressed.

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was therefore undertaken to examine the interaction of NaF with adenylate cyclase in purified sarcolemmal membrane from myocardium of dog, rabbit and rat.

METHODS AND MATERIALS

Purified sarcolemmal membranes were isolated from dog, rabbit and rat ventricles by the hypotonic shock-LiBr treatment method as already described (8). We have previously demonstrated that the membrane preparation obtained by this procedure is enriched with sarcolemma containing glycocalyx and possesses minimal contamination by other subcellular components (8-10). The adenylate cyclase activity was assayed at 37°C for 10 min according to the method of Drummond and Duncan (11), except that the ATP regenerating system used was creatine kinase/creatine phosphate and 2 mM cyclic AMP was added to the assay medium in order to inhibit the breakdown of cyclic [^{14}C] AMP. The enzyme activity was also measured in the presence of 8 mM sodium fluoride, 100 µM l-epinephrine bitartrate or 40 µM guanyl-5'-yl imidodiphosphate [Gpp (NH)p], a hydrolysis resistant analog of GTP.

For lubrol treatment, rat heart washed particles obtained just before the LiBr treatment step were isolated according to the procedure described previously (8). This particulate fraction was centrifuged at 3000 x g and resuspended in a Potter Elvehjem homogenizer with 0.7% (w/v) Lubrol 12A9 in 10 mM Tris-HCl (pH 7.5) containing 2 mM dithiothreitol to a final concentration of 4 mg membrane protein/ml and incubated for 30 min at 4°C. The suspension was separated into supernatant and pellet fractions by centrifugation at 50000 x g for 30 min. The washed treated pellet was then resuspended in 10 mM Tris-HCl (pH 7.5) or in the lubrol extract (supernatant) for reconstitution. This procedure is similar to the solubilization procedure of Welton et al. (12). Protein was assayed by the method of Lowry et al. (13). Fresh membrane preparations were used in this study for each experiment.

Sodium salt of deoxycholate acid and Lubrol 12A9 were obtained from Sigma Chemical Co., St. Louis, U.S.A. and ICI Ltd., Montreal, Canada, respectively. [8-^{14}C] ATP (specific activity: 44.2 mCi/mmol) was purchased from New England Nuclear, Lachine, Canada. All other reagents were of analytical grade.

RESULTS

The basal adenylate cyclase activity of sarcolemmal membranes was found to be higher in rat than in the other two species under study (Table 1). On the other hand, rat membranes exhibited lower epinephrine stimulation and an inhibition of the cyclase activity by NaF. Lack of stimulation by fluoride, but not inhibition, has been reported in rat heart sarcolemma by Engelhard et al. (15). The inhibitory response to fluoride appeared to occur only in rat since dog and rabbit sarcolemma retained fluoride stimulation of adenylate cyclase (Table 1). For this reason, adenylate cyclase activity was examined at different steps of isolation and purification of sarcolemma from rat heart (Table 2). A progressive attenuation of the fluoride ability to stimulate the enzyme was observed, that changed into an inhibition of the sarcolemmal enzyme activity. In this and in the following series of experi-

Table 1
Adenylate cyclase activity in dog, rabbit and rat heart sarcolemma isolated by the hypotonic shock-LiBr treatment method.

Species and Fraction	Basal	8 mM NaF	100 μM Epinephrine
	pmol cyclic AMP/min/mg		
Dog Sarcolemma	171 ± 4	710 ± 27 (+315)	260 ± 5 (+52)
Rabbit Sarcolemma	144 ± 3	507 ± 5 (+252)	184 ± 4 (+28)
Rat Sarcolemma	386 ± 17	316 ± 21 (-18)	443 ± 20 (+15)

Results are expressed as the mean ± S.E.M. of 3 experiments. Values in brackets refer to percent stimulation or inhibition.

ments, GTP-free ATP was employed as a substrate for assaying adenylate cyclase, since sufficient GTP may be present in commercial ATP to cause undesired activation (16). Results in Table 2 show indeed that epinephrine stimulation was not evident in washed particles and sarcolemmal fractions unless a guanyl nucleotide was present. As illustrated in Fig. 1A, deoxycholate treatment of the rat sarcolemmal membranes at different concentrations for increasing membrane permeability and accessibility of effectors to their active sites (14) failed to show NaF stimulation. The use of EDTA in the sarcolemma iso-

Table 2
Adenylate cyclase activity assayed with GTP-free at different steps of the sarcolemmal isolation from rat heart.

Fraction	Basal Activity (pmol/min/mg)	Percent Change in Adenylate Cyclase Activity			
		NaF	Epi	Gpp(NH)p	Gpp(NH)p + Epi
Homogenate	42 ± 8	137 ± 12	106 ± 40	71 ± 51	271 ± 37
Washed particles Before LiBr	103 ± 13	68 ± 7	4 ± 6	50 ± 27	71 ± 22
Washed particles After LiBr	200 ± 16	24 ± 16	9 ± 14	3 ± 8	28 ± 14
Sarcolemma	368 ± 30	-18 ± 10	2 ± 13	4 ± 10	23 ± 12

Results are expressed as the mean ± S.E.M. of 3 to 5 experiments. The adenylate cyclase assay was performed in the presence of GTP-free ATP as a substrate. NaF, l-epinephrine (Epi) and Gpp(NH)p were present in concentration of 8 mM, 100 μM and 40 μM, respectively.

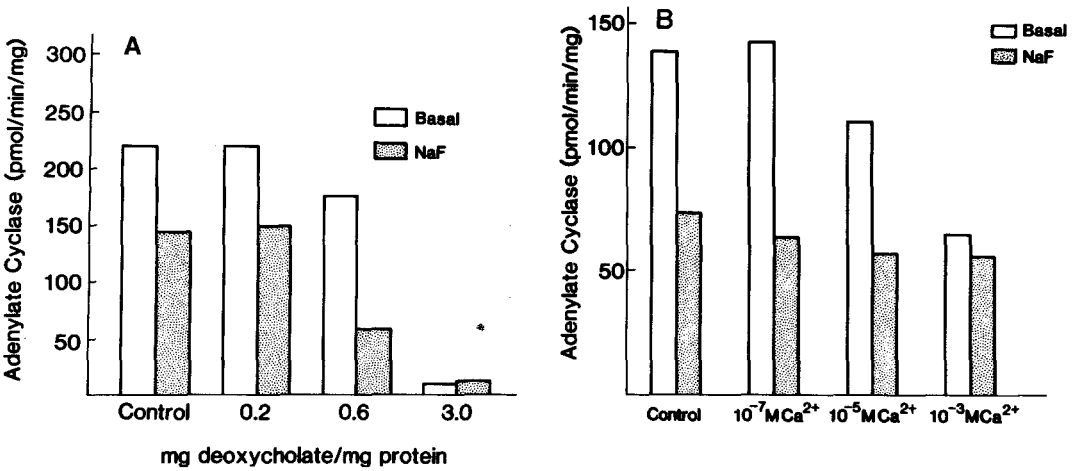

Fig. 1. Effect of deoxycholate treatment and calcium on adenylate cyclase activity of rat heart sarcolemma. A. Membranes (2 mg) were preincubated at the indicated deoxycholate to protein ratios in 1 ml of 50 mM Tris-HCl (pH 7.5) containing 20 mM KCl for 10 min at 30°C (14). The reaction was terminated by 20-fold dilution with Tris buffer, followed by centrifugation at 5000 g for 10 min. Resuspended washed pellet was employed for the study of adenylate cyclase activity. B. Plasma membranes were preincubated at the indicated Ca^{2+} concentrations for 20 min at 30°C. The adenylate cyclase assay was performed at 30°C.

lation procedure from guinea pig heart has been shown to reduce fluoride activation of adenylate cyclase (17). Since EDTA was present in the homogenization medium of hypotonic shock-LiBr method, it was possible that the loss of fluoride sensitivity was due to the removal of calcium during isolation. Thus, the effect of NaF was tested in the presence of different concentrations of Ca^{2+} but stimulation of adenylate cyclase by NaF was not evident (Fig. 1B). Instead, basal adenylate cyclase activity was inhibited by Ca^{2+} as already reported (11). Since other investigators (18,19) have solubilized a protein factor responsible for regulating fluoride responsiveness, Lubrol 12A9, a nonionic detergent, was employed to extract the washed particles fraction from rat heart which showed about 70% fluoride activation. After lubrol treatment, the pellet exhibited low basal adenylate cyclase that was insensitive to NaF (Table 3, Exp. A). Readdition of the lubrol supernatant to the pellet cause no change in the basal activity but did cause a 7-fold increase in activity when assayed in the presence of NaF. Table 3 (Exp. B) also shows the effects of addition of the lubrol extract from the washed particles on rat heart sarco-

Table 3
Treatment of rat heart washed particles with Lubrol 12A9 and effects of lubrol supernatant on washed particles and sarcolemmal adenylate cyclase activity.

Fraction or Addition	Adenylate cyclase (pmol cAMP/10 min assay)	
	Basal	NaF
Experiment A. Fraction		
Untreated washed particles	53 ± 10	96 ± 4
Supernatant after Lubrol treatment	1 ± 1	6 ± 3
Pellet after Lubrol treatment	6 ± 2	5 ± 2
Treated pellet and Lubrol supernatant	7 ± 1	42 ± 4
Experiment B. Addition		
None	398 ± 26	350 ± 28
0.7% Lubrol	84 ± 12	104 ± 18
Supernatant after Lubrol treatment	93 ± 11	158 ± 14

Experiment A. Rat heart washed particles were treated with 0.7% Lubrol 12A9 as indicated in METHODS AND MATERIALS. The washed treated pellet was resuspended in 10 mM Tris-HCl (pH 7.5) or in the supernatant for reconstitution. Experiment B. A fixed amount (100 µg protein) of untreated sarcolemma was incubated during the assay in the absence or presence of 25 µl 0.7% Lubrol 12A9 or Lubrol supernatant obtained upon incubation of rat heart washed particles with 0.7% Lubrol 12A9. Values shown are mean ± S.E.M. of 3 separate observations.

lemma. Presence of 0.7% Lubrol 12A9 in the assay medium caused a decrease in the sarcolemmal adenylate cyclase activity and the fluoride inhibition displayed by the native sarcolemma was not evident. Addition of lubrol extract caused a similar decrease in the sarcolemmal basal enzyme activity due to presence of the same lubrol concentration; however, the adenylate cyclase regained its characteristic stimulatory response to NaF.

DISCUSSION

The results of this study show a depression of adenylate cyclase activity by NaF in the sarcolemmal fraction obtained from the rat heart. Inhibitory responses to fluoride have also been observed in solubilized brain adenylate cyclase (20), particulate fat cell preparations (21) and rat liver plasma

membrane (22). Moreover, during rat heart sarcolemmal isolation, there was a progressive attenuation of the ability of NaF to stimulate the enzyme suggesting the loss of a factor for fluoride stimulation. Although such a factor appeared to be solubilized by lubrol from the heart particulate fraction, preliminary attempts to purify this factor by ammonium sulfate precipitation, dialysis and chromatography on Sephadex columns were unsuccessful (data not shown).

Fluoride ion has been used for years as a nonphysiological activator of membrane-bound adenylate cyclase and the GTP-binding regulatory component of the enzyme is considered to be a site of activation by NaF (2). Thus, the abnormal inhibitory response to fluoride, which has been observed in rat heart sarcolemma, is not easily interpretable. However, one possible explanation is that NaF may induce both stimulation and inhibition of adenylate cyclase, the former being predominant and dependent upon a factor which is selectively lost during the isolation procedure employed in this study. The experiments reported here would indicate that some regulatory factor is required for the activation of adenylate cyclase by NaF in myocardium, and the rat heart is quite susceptible for its loss in comparison to other species. Indeed, incubation of native sarcolemma from rat heart at 30°C for 10 min in a medium of moderate ionic strength (50 mM Tris-HCl, pH 7.5, and 20 mM KCl) yielded pellet exhibiting the same degree of hormonal activation as the native sarcolemma and an increased inhibition by fluoride (37%, average of 3 experiments). Thus it appears that the unknown factor may be peripheral protein which is loosely bound to the membrane through electrostatic interactions. Interestingly, the hormonal activation, which is exerted through the GTP-binding regulatory protein (2), was observable even in the absence of the factor putatively required for fluoride activation. It is therefore probable that such a factor may differ from the regulatory GTP-binding protein in myocardium. Hebdon et al. (23) and Ross et al. (24) have also suggested that there may be a distinct regulatory protein involved in fluoride stimulation. It is also conceivable that the factor evidenced in this study may participate

in the fluoride activation of myocardial adenylate cyclase through an interaction with the GTP-binding regulatory protein, although the characteristics of this interaction in situ as well as the significance of the fluoride inhibition for the adenylate cyclase function remain obscure.

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