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Opioid receptors in rat cardiac sarcolemma: effect of phenylephrine and isoproterenol

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The present study demonstrates the presence of opioid receptors in the rat cardiac sarcolemma isolated by the hypotonic LiBr-shock procedure. Opioid binding was measured by using [^{3}H]U69 593, [^{3}H](2-D-penicillamine,5-D-penicillamine)enkephalin ([^{3}H]DPDPE) or [^{3}H][D-Ala^2,MePhe^3,Gly-(ol)^5]enkephalin ([^{3}H]DAGO) as selective radioligands for K, δ and μ opioid receptors, respectively. Both the K- and δ-selective ligands exhibited highly specific (75–86%) binding, saturable at a concentration of about 20 nM. No specific binding for the selective agonist DAGO was observed. A marked increase in both [^{3}H]U69593 and [^{3}H]DPDPE binding was observed after incubation of the sarcolemma with the α-adrenoceptor agonist phenylephrine or with the β-adrenoceptor agonist isoproterenol. These stimulatory effects were associated with an increase in the B_{max} values, a decrease in the K_d values, and were completely antagonized by the respective antagonists phentolamine and propranolol.

Introduction

Opioid peptides, such as enkephalins and dynorphin, have been reported to be present in both atria and ventricles of the heart [1–4]. The presence of opioid receptors in the heart has been suggested by several studies [5–7]. Moreover, low binding of opioid agonists and antagonists have been reported in the cardiac muscle from the whole hearts of guinea pigs and rats [8,9]. More recently, Krumis et al. [10] have detected appreciable opioid binding in the rat heart by examining individual chambers of the heart and have also shown inhibition of the opioid binding after hemorrhagic shock.

However, these binding studies were performed on crude heart homogenates which do not localize subcellular fraction of the opioid receptors. Since we have recently shown that opioid peptides interact with the cardiac sarcolemma by modulating the activity of different enzymes of this membrane [11,12] and considering the fact that the sarcolemma is the first membrane that interacts with any drug which influences the myocardial cell, in the present study we decided to evaluate the possibility that opioid receptors of different types may be located on the cardiac sarcolemma. We therefore performed binding studies by using [^{3}H] U69593, [^{3}H]DPDPE or [^{3}H]DAGO as labeled opioid ligands, since they have been shown to bind selectively at K [13], δ [14] and μ [15] opioid receptors, respectively.

To determine whether opioid binding to the sarcolemma may be a dynamically regulated event, we have studied opioid binding in the presence of the α-agonist phenylephrine and the β-agonist isoproterenol. This was because catecholamines activate the endogenous endorphin systems [16,17] and both regulate together the opioidergic system, the central and peripheral cardiovascular system [17–20].

Materials and Methods

The following labeled substances: [^{3}H]U69 593, [^{3}H](2-D-penicillamine,5-D-penicillamine)enkephalin ([^{3}H]DPDPE) and [^{3}H][D-Ala^2,MePhe^4,Gly-(ol)^5]enkephalin ([^{3}H]DAGO) were purchased from NEN-Du Pont de Nemours (F.R.G.); U69593 was obtained from Upjohn (U.S.A.); DPDPE and DAGO were from Peninsula Lab. (U.K.); L-phenylephrine hydrochloride, DL-isoproterenol hydrochloride, DL-propranolol hydrochloride, bestatin and bacitracin were from Sigma

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Chemical Co. (U.S.A.); Phentolamine hydrochloride was a generous gift from Ciba Geigy (Italy).

Heart sarcolemma was isolated by the hypotonic shock-LiBr treatment as described previously [21,22]. Male Wistar rats (250–300 g) were decapitated, their hearts were quickly removed and placed in ice-cold 10 mM Tris-HCl buffer (pH 7.4). The ventricles were washed thoroughly, cut into small pieces, and then homogenized in an Ultra Turrax homogenizer (velocity setting 8 for 15 s) in 10 volumes of 10 mM Tris-HCl buffer (pH 7.4), containing 1 mM EDTA. The homogenate was filtered through four layers of cheesecloth and centrifuged at $1000 \times g$ for 10 min. The sediment was suspended in 10 vol. of 10 mM Tris-HCl buffer (pH 7.4), stirred for 30 min and centrifuged at $1000 \times g$ for 10 min. This procedure was repeated two more times, first by suspending the sediment in 10 mM Tris-HCl (pH 8.0), and then in 10 mM Tris-HCl buffer (pH 7.4). The resulting sediment was extracted for 45 min with 0.4 M LiBr in 20 vol. of 10 mM Tris-HCl (pH 7.4) and centrifuged at $1000 \times g$ for 10 min. The sediment was again washed and stirred for 20 min in 10 mM Tris-HCl (pH 7.4), and then centrifuged at $1000 \times g$ for 10 min. The sediment was further purified by extracting for 30 min in 20 vol. of 0.6 M KCl containing 10 mM Tris-HCl buffer (pH 8.0) and centrifuged at $1000 \times g$ for 10 min. This sediment was resuspended in 20 vol. of 10 mM Tris-HCl (pH 7.4) and again centrifuged at $1000 \times g$ for 10 min. The final pellet was suspended in a medium containing 250 mM sucrose, 30 mM imidazole and 120 mM NaCl at pH 7.4. All procedures were carried out at 4°C; freshly prepared membranes were used in this study.

Enzyme assays were performed as follows: Na^+/K^+-ATPase activity was studied as described by Lamers and Stinis [23]; ouabain-sensitive Na^+/K^+-ATPase activity was taken as the activity inhibitable by 1 mM ouabain. The extent of ATP hydrolysis was followed by measuring the release of inorganic phosphate, according to the method of Le Bel et al. [24]. The 5′-nucleotidase activity was measured by the procedure of Edwards and Maguire [25]. Succinate dehydrogenase, rotenone-insensitive NADH–cytochrome-*c* reductase and rotenone-insensitive NADPH–cytochrome-*c* reductase activities were measured as indicated by Sottocasa et al. [26]; K^+-EDTA ATPase activity was determined by the method described by Scholte [27]. Proteins were determined by following the method of Lowry et al. [28] using bovine serum albumin as a standard.

The opioid binding was performed by incubating 250 μg of the sarcolemmal membrane proteins in 0.5 ml of 50 mM Tris-HCl buffer (pH 7.4) in the presence of the indicated amounts of each selective labeled ligand and 0.5 μM bestatin plus bacitracin (50 μg/ml).

The binding reactions were allowed to run for the indicated times at 25°C and stopped by diluting 5-times with ice-cold Tris-HCl buffer (pH 7.4). The incubation media were then filtered over vacuum on Whatman GF/B glass fiber filters followed by three wash with 10 ml of ice-cold Tris-HCl buffer. In all the experiments, the specific binding of [^{3}H]U69 593, [^{3}H]DPDPE or [^{3}H]DAGO was measured as the difference between binding in the presence and absence of 10 μM of the corresponding unlabeled ligand.

The effects produced on the opioid binding by the α-agonist phenylephrine or by the β-agonist isoproterenol were studied as indicated in the legends of the figures, by incubating the sarcolemma in the absence or presence of the respective antagonists phentolamine or propranolol.

Statistical analysis was performed by calculating the variance (ANOVA), and considering a p value less than 0.05 as significant.

Results

Table I shows the activity of marker enzymes of selected subcellular cardiac fractions. It is evident that, in the sarcolemma, both the ouabain-sensitive Na^+/K^+-ATPase and 5′-nucleotidase activities were about 10-fold higher than in the original homogenate. In the sarcolemmal fraction, the activity of K^+-EDTA ATPase and rotenone-insensitive NADPH–cytochrome-*c* reductase were undetectable. Both succinate dehydrogenase and rotenone-insensitive NADH–cytochrome-*c* reductase activities were extremely low in the sarcolemma when compared to the activity in the homogenate.

Fig. 1 shows the specific binding of increasing concentrations of [^{3}H]U69 593 and [^{3}H]DPDPE in the cardiac sarcolemma. Both these selective ligands had saturable binding at a concentration of about 20 nM. Specific binding ranged between 75% and 86% of the

TABLE I

Specific enzymatic activities in the homogenate and sarcolemmal membranes of rat heart muscle

Enzymatic activities (μmoles product formed/mg protein per h) are expressed as means ± S.E. of duplicate determinations from four different sarcolemmal preparations.

Enzyme activities	Homogenate	Sarcolemma	Enrichment
Na^+/K^+-ATPase (ouabain-sensitive)	1.70 ± 0.09	22.7 ± 0.64	13.3
5′-Nucleotidase	0.84 ± 0.10	7.4 ± 0.30	8.8
K^+-EDTA ATPase	3.60 ± 0.10	n.d.	–
NADPH–cyt-*c* reductase (rotenone-insensitive)	0.017 ± 0.01	n.d.	–
NADH–cyt-*c* reductase (rotenone-insensitive)	0.26 ± 0.19	0.48 ± 0.11	1.8
Succinate dehydrogenase	7.80 ± 0.47	0.78 ± 0.16	–

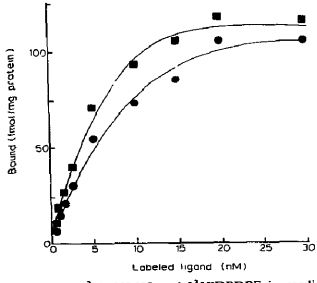

Fig. 1. Binding of [^{3}H]U69593 and [^{3}H]DPDPE in cardiac sarcolemmal membranes. ■, [^{3}H]U69593; ●, [^{3}H]DPDPE. Each point is the mean of duplicate determinations from four different sarcolemmal preparations.

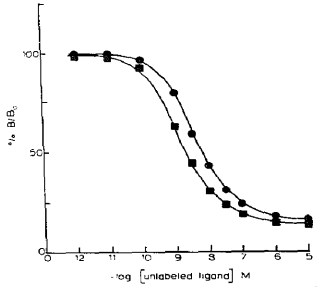

Fig. 3. Competition of unlabeled U69593 and DPDPE with [^{3}H]69593 and [^{3}H]DPDPE binding to the cardiac sarcolemma. ■, [^{3}H]U69593; ●, [^{3}H]DPDPE. Each point is the mean of duplicate determinations from four different sarcolemmal preparations.

total bound. No specific binding for the μ-selective agonist DAGO was observed at the concentrations of 0.5–30 nM (data not shown). The Scatchard plots of the specific [^{3}H]U69593 (Fig. 5A) and [^{3}H]DPDPE (Fig. 5B) binding were linear and characterized by a single dissociation constant in the nM range. In Fig. 2, it is evident that the specific binding of both [^{3}H]U69593 and [^{3}H]DPDPE rapidly increased during the first 15 min of the incubation period and reached a plateau at 30 min. The binding was constant until 60 min and then slightly decreased during the following 30 min. U69593 of DPDPE competed in a dose-dependent manner with the corresponding labeled agonist for the binding to the cardiac sarcolemma (Fig. 3). The high degree of specificity of the opioid binding was confirmed in these competitive inhibition studies, since more than 70% of the total [^{3}H]U69593 or [^{3}H]DPDPE binding was displaced by 10 nM of the cold agonist. The incubation of the sarcolemmal membranes in the presence of increasing concentrations of the α-agonist phenylephrine or of the β-agonist isoproterenol produced a dramatic and dose-dependent stimulation in the specific binding of both the K- and δ-selective agonist (Fig. 4). This effect was already significant at a concentration of 0.1 nM and reached a plateau at 1 μM. The stimulatory action elicited by the two adrenergic compounds was associated with an increase in the B_{max} values, together with a

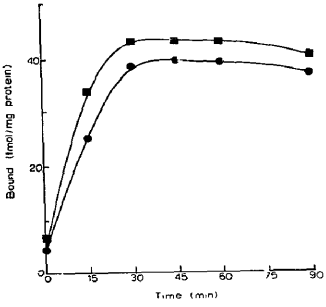

Fig. 2. Time-course of [^{3}H]U69593 and [^{3}H]DPDPE binding in the cardiac sarcolemma. ■, [^{3}H]U69593; ●, [^{3}H]DPDPE. Each point is the mean of duplicate determinations from four different sarcolemmal preparations.

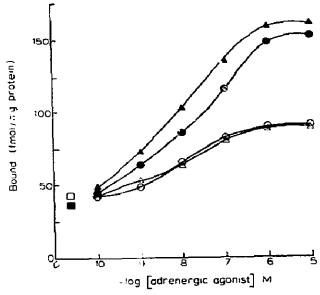

Fig. 4. Modulation of sarcolemmal opioid binding by phenylephrine and isoproterenol. □, [^{3}H]U69593; ○, [^{3}H]U69593 + phenylephrine; △, [^{3}H]U69593 + isoproterenol; ■, [^{3}H]DPDPE; ●, [^{3}H]DPDPE + phenylephrine; ▲, [^{3}H]DPDPE + isoproterenol. Each point is the mean of duplicate determinations from four different sarcolemmal preparations.

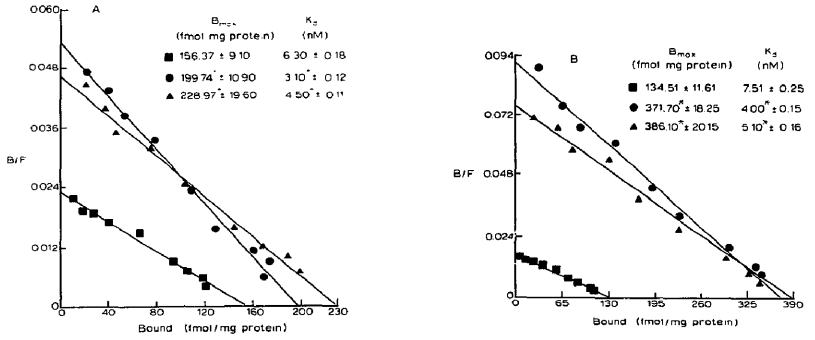

Fig. 5. Effect of phenylephrine and isoproterenol on the binding parameters of [^{3}H]U69 593 (A) and [^{3}H]DPDPE (B) in the cardiac sarcolemma. ■, Control; ●, +1 μM phenylephrine; ▲, +1 μM isoproterenol. Data are the means ± S.E. of duplicate determinations from four different sarcolemmal preparations. * Significantly different from the control.

significant decrease in the K_d values (Figs. 5A and 5B). The effects produced by phenylephrine or isoproterenol were completely antagonized by preincubation of the sarcolemma with the respective antagonists phentolamine or propranolol (Table II).

Discussion

The sarcolemmal fraction used in these experiments was obtained by a hypotonic shock-LiBr treatment. This kind of membrane preparation has been reported to possess a high content of 'cell surface material' [29,30], shown to play an important role in vitro expression of functional characteristics of the sarcolemma [31–33] which is absent in most preparations obtained by a sucrose density gradient method [34,35]. In agreement with other studies which utilized sarcolemmal membranes prepared by the LiBr method [29,30], our sarcolemmal preparation exhibited a high degree of purity, since the activity of the marker enzymes ouabain-sensitive Na^+/K^+-ATPase and 5′-nucleotidase was about 10-times higher in the sarcolemma than in the original homogenate. Lack of contamination by myofibrils and sarcoplasmic reticular membranes was indicated by measurements of the activities of the respective marker enzymes K^+-EDTA ATPase and rotenone-insensitive NADPH–cytochrome-*c* reductase, which were both undetectable in the sarcolemmal fraction. Contamination due to inner and outer mitochondrial membranes in the sarcolemmal fraction was minimal as indicated by the very low activities observed of marker enzymes succinate dehydrogenase and rotenone-insensitive NADH–cytochrome-*c* reductase.

TABLE II

Effect of phentolamine and propanolol on the adrenergic-stimulated opioid binding in the cardiac sarcolemma

Adrenergic agonists and antagonists were used at a concentration of 1 μM. Data are the means ± S.E. of duplicate determinations from four different sarcolemmal preparations.

	B_{max} (fmol/protein)	K_d (nM)
[^{3}H]U69593 (A)	156.37 ± 9.10	6.30 ± 0.18
(A) + phenylephrine + phentolamine	160.05 ± 12.01	6.11 ± 0.16
(A) + isoproterenol + propranolol	159.12 ± 18.51	6.02 ± 0.21
[^{3}H]DPDPE (B)	134.51 ± 11.61	7.51 ± 0.25
(B) + phenylephrine + phentolamine	135.43 ± 10.98	7.12 ± 0.30
(B) + isoproterenol + propranol	140.12 ± 11.70	7.35 ± 0.40

The present study demonstrates the presence of K and δ opioid receptors in the cardiac sarcolemma of ventricular origin. The failure to detect any specific binding with the μ-selective agonist [^{3}H]DAGO indicates that the heart sarcolemma lacks μ sites. This observation agrees with the results obtained by Krumis et al. [10] in heart homogenates. The linearity of the Scatchard plot of [^{3}H]U69 593 or [^{3}H]DPDPE binding data is consistent with labelling of a single type of site and is in agreement with the high degree of selectivity of these radioligands [13,36]. The calculated parameter for the sarcolemmal opioid binding are comparable to those observed for the selective ligands in the brain [36,37].

The present study demonstrates that opioid binding in the cardiac sarcolemma is modulable by the α-agonist phenylephrine and by the β-agonist isoproterenol. Both these stimulatory effects were accompanied by an in-

crease in B_{max} values and by a significant decrease in K_d values for the opioid binding. These data suggest that, under basal conditions, most of the sarcolemmal opioid receptors are scarcely accessible to their ligands and may by dynamically regulated by catecholamines which appear to increase both the number and specific affinity of K and δ opioid receptors in the sarcolemmal membranes.

These findings agree with the general concept that the cardiac sarcolemma is a dynamic excitable structure [38,39] and that the number of receptors at the cell surface is not fixed [40]. Moreover, the fact that the α- and β-antagonists prevented the effects induced by phenylephrine and isoproterenol on opioid binding clearly indicates the specificity of their action at the level of the respective α and β sarcolemmal receptors.

A number of experimental evidence suggests a close interaction between adrenergic and opiate receptor systems both at the level of the central and peripheral nervous system. It is interesting to note that β-endorphin like substances are released from the brain-stem of spontaneously hypertensive rats in the presence of clonidine, a central adrenoceptor agonist [41]. Moreover, Williams et al. [42] recently confirmed these findings also in normotensive animals, showing that the injection of clonidine into the cerebral aqueduct of cats eliminated the increase in blood pressure during fatiguing muscular contractions. This effect was antagonized by the specific opioid antagonist naloxone and by yohimbine, an α-adrenoceptor blocker. Furthermore, a link between adrenergic and opiate receptor systems has also been demonstrated in the peripheral nervous system. Enkephalins have been reported to be contained in sympathetic nerve fibers, sympathetic ganglia and preganglionic sympathetic neurons [19,43,44] and the co-storage of catecholamines and enkephalins in many of these peripheral nervous structures has been demonstrated by immunohistochemical and biochemical methods [44–46]. Moreover, multiple experimental evidence indicates that an increase in sympathoadrenergic outflow, occurring in different experimentally-induced shock or stress models, significantly enhances the cardio-depressant effects elicited by the opioids [47–50].

The data reported in the present study indicate the existence of specific δ and K opioid receptors located on the cardiac sarcolemma, which can be stimulated by catecholamine interaction with α and β-adrenoceptors. At the present, the mechanism(s) for adrenergic stimulation of opioid binding in the sarcolemma, as well as its possible implications for the heart function remain to be established. However, the fact that α- and β-receptors are implicated suggests that both the cAMP- and Ca^{2+}-dependent mediators could be involved in the stimulation of opioid binding even if we cannot exclude that the adrenergic activation changes some properties of the sarcolemmal membrane, for example its reactivity with the opioids. Another interesting aspect is that the presence of mRNA for preproenkephalin A in the heart tissue is more abundant than in the brain [51] suggesting that the myocardial cell has the potential for the local synthesis of opioids. The fact that in the heart of cardiomyopathic hamsters, the mRNA for preproenkephalin A progressively increases with the development of the cardiomyopathy [52] provides evidence that alterations in the intracellular levels of enkephalin precursors may be associated with the development of cardiac hypertrophy and failure. We therefore suggest that sarcolemmal opioid receptors may have a role in the regulation of the myocardial function, especially under a condition of adrenergic stimulation. Further experiments are in progress to study the possible biochemical implications of the interaction of opioids at the sarcolemmal level.

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