Vol. 127, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1985 Pages 120-128

OPIATE BINDING IN RAT HEARTS: MODULATION OF BINDING AFTER HEMORRHAGIC SHOCK

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Received January 14, 1985

SUMMARY: [^{3}H]Diprenorphine was used to measure binding in sectioned rat hearts. Saturable binding for concentrations up to about 20 nM was obtained in the right atrium and ventricle. Unlabeled diprenorphine displaced bound [^{3}H] diprenorphine most effectively in the right atrium (up to 55%), as compared to less than 27% in the right ventricle and the remaining parts of the heart. Scatchard analysis of the binding in the right atrium revealed cooperative binding. The δ agonist [D-Ala^2,D-Leu^5]enkephalin, the κ agonist ethylketocyclazocine, and levorphanol, but not the μ agonist [D-ala^2,MePhe^4,Gly-(ol)^5]enkephalin or dextrophan competed variably with [^{3}H]diprenorphine for the binding in the right atrium and ventricle. A significant decrease in binding was observed in the right atrium (-66%) and ventricle (-45%) of hearts removed from rats 2 h after hemorrhagic shock; 24 h after shock, recovery of binding was found. This novel observation suggests that the diprenorphine binding sites in the heart may be physiologically active receptors, involved in regulation of peripheral cardiovascular processes.

The heart is known to contain opioid peptides, such as enkephalins. It has been suggested that enkephalins, located in cardiac nerves (1), may modulate the catecholamine-induced activity of the heart by regulating the inward calcium flux (2). The modulation was postulated to occur via activation of presynaptic opiate receptors located on adrenergic nerve terminals of the heart (3).

The presence of opiate receptors in the heart has also been suggested by experimental pharmacologic studies (4). However, only equivocal biochemical data have been reported which support the existence of such receptors. Simantov et al. detected binding of dihydromorphine and the opiate antagonist naloxone to membranes prepared from whole hearts of rats and guinea pigs (5). However, since saturable opiate binding relative to total binding was very small in these

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0006-291X/85 $1.50

studies, 5 to 10% for rats only, as compared to more than 60% for rat brains, their findings were inconclusive. Burnie found evidence of stereospecific opiate binding in cardiac muscle from rat right ventricle using the antagonist [^{3}H]diprenorphine but did not characterize this binding in more detail (6).

Because of the uncertainty that exists with respect to the existence of opiate receptors in the heart, their involvement in cardio-regulatory processes remains therefore only speculative. This study was undertaken to determine the presence of opiate receptors in the heart and to determine their possible role in cardiovascular regulation. We have examined opiate binding in the heart by examining binding in each chamber separately, rather than the whole heart. Since (1) three major types of opiate receptors have been identified, μ, δ and κ, and (2) diprenorphine has been shown to bind to all three types with equal affinity (7), we used [^{3}H]diprenorphine as radioligand.

To determine the physiological significance of diprenorphine binding, we have evaluated binding in animals undergoing hemorrhagic shock, a cardiovascular stress known to activate the endogenous endorphin system (8).

MATERIALS AND METHODS

[^{3}H]Diprenorphine (12 Ci/mmol) was purchased from Amersham. [D-Ala^2, D-Leu^5]enkephalin (DADLE) and [D-Ala^2,MePhe^4, Gly-(ol)^5]enkephalin (DAGO) were purchased from Peninsula Laboratories. Diprenorphine (DPN), levorphanol tartrate (LEV), and dextrophan tartrate (DEX) were a gift from Hoffmann-La Roche; ethylketocyclazocine methanesulfonate (EKC) was a gift from Sterling-Winthrop Research Institute. Bestatin and bacitracin were purchased from Sigma. Sprague Dawley rats weighing 270 to 300 g were used.

Experimental Hemorrhagic Shock. Both femoral arteries were cannulated (PE50) under halothane (2% in oxygen). The arterial lines were then tunneled under the skin of the back and exited at the back of the neck, and the lines were further secured by a spring wire. The animals were allowed to recover for 24-36 h while placed individually in their home cages with food and water ad libitum. On the day of the experiment, one arterial line was attached to a pressure transducer (Narco, RP 1500i) from which blood pressure, heart rate and pulse pressure were continuously recorded (Narcotrace 80 Computerized Dynograph). After 30-60 min of control period recording, the rats were bled (8.5 ml/300 g) through the second arterial line over a 5 min period. Groups of rats were killed by rapid decapitation 2 and 24 h after the bleeding. Intact rats, killed at the same time, served as controls. The rats were either tested individually as in the case of the shock experiments or heart tissue from controls were pooled for the more extensive studies characterizing the diprenorphine binding.

Radioligand Binding Assay. The hearts were removed immediately after decapitation, washed in ice-cold 0.32 M sucrose, 50 mM Tris-HCL, pH 7.4, and separated into the right and left atria and the right and left ventricles. Each part was homogenized using a Brinkmann Polytron (15 sec, setting 10) in at least 40 vol of 50 mM ice-cold Tris-HCL. The pellets were resuspended in Tris-HCL to

6 mg (original wet weight) per ml. Aliquots (approximately 0.25 mg of protein) of this suspension were assayed for diprenorphine binding. Total volume of the binding mixture was 2 ml and contained, in addition to the indicated amounts of ^{3}H-DPN and unlabeled ligands, 0.5 μM bestatin and bacitracin (50 μg/ml). The binding mixture was allowed to incubate for the indicated time at 25° before filtration (Whatman GF/B glass fiber filters) over vacuum, which was followed by rapid washes using 10 ml Tris-HCL. The protein concentration was determined by the method of Lowery et al. (9) using bovine serum albumin as a standard. Binding data of controls and animals undergoing hemorrhagic shock were compared by the Two-sample Unpaired t-Test. A p value < 0.05 was considered significant.

RESULTS

Characterization of [^{3}H]Diprenorphine Binding

Specific binding of ^{3}H-DPN, measured as the difference between binding in the presence and absence of 10 μM of unlabeled DPN, was measured to crude membranes prepared from the tissue of each heart chamber. Saturable binding for concentrations of ^{3}H-DPN up to about 20 nM was observed in the right atrium and ventricle. However, the fraction of total binding defined as specific binding was greatest in the right atrium. This fraction varied from 35 to 55% with different membrane preparations for the right atrium, as compared to less than 27% for the right ventricle (or the left chambers). Specific binding as a function of concentration of ^{3}H-DPN is shown for the right atrium in Fig. 1. The concave-downward curvature of the Scatchard plot of the ^{3}H-DPN binding data suggested cooperative binding (Fig. 1, Upper Inset). In this case K_D varies with amount bound. When the ^{3}H-DPN binding data for concentrations of ^{3}H-DPN greater than 10 nM were replotted by the "Wilkinson inversion" (10, 11) (Fig. 1, Lower Inset), the K_D value derived from the intersect of the line with the abscissa was 6.3×10^{-9} M (correlation coefficient 0.94), determined by linear regression analysis. If binding data measured at about 7 nM were included in the plot and analysis, the K_D value was 0.9×10^{-9} M (correlation coefficient 0.75). The respective values for the maximal binding capacity, derived from the slope of the Wilkinson plot, were 8×10^{-11} M and 6×10^{-11} M. Fig. 1 is based on two experiments using freshly dissected heart tissue. Three additional studies of concentration dependence of ^{3}H-DPN binding were performed using frozen heart tissue. Although specific binding decreased by about 40% for frozen tissue as compared to fresh, cooperativity of binding was confirmed in these studies.

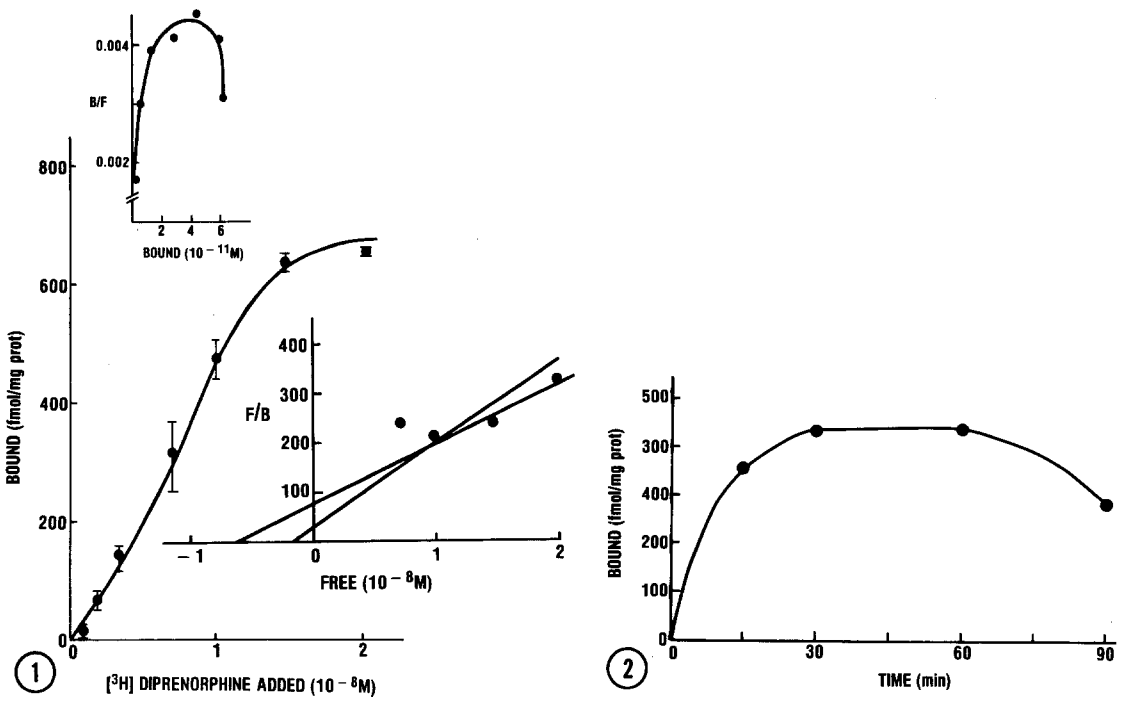

Fig. 1. Concentration dependence of the specific binding of diprenorphine in the right atrium: Scatchard plot (Upper Inset) of the binding data analyzed by the "Wilkinson inversion" plot (Lower Inset). Membranes prepared from pooled atria of control rats were incubated for 1 h as described under Methods with increasing concentrations of [^{3}H]diprenorphine. Nonspecific binding was defined using 10 μM of unlabeled diprenorphine.

Fig. 2. Time course of the specific binding of diprenorphine in the right atrium. Membranes prepared from pooled atria of control rats were incubated with 7.5 nM of [^{3}H]diprenorphine for the indicated times.

Specific binding of ^{3}H-DPN in the right atrium increased rapidly for the first 15 min and reached a plateau by 30 min (Fig. 2). The binding remained constant up to 60 min. For prolonged incubations of 90 and 120 min (not shown), binding decreased markedly. Binding of ^{3}H-DPN in the two right chambers was inhibited by unlabeled DPN in a dose-dependent manner (Fig. 3). The relatively high degree of inhibition using 10 μM of unlabeled DPN (up to 55%) was confirmed for the right atrium in these competitive inhibition studies. A high degree of inhibition of the ^{3}H-DPN binding was also observed with 10 μM of the prototypical δ ligand DADLE, whereas the κ agonist EKC inhibited only a minor fraction of the ^{3}H-DPN binding at this concentration (Fig. 3, Upper Panel). In the right ventricle, the ^{3}H-DPN binding was variably inhibited by EKC and DADLE at

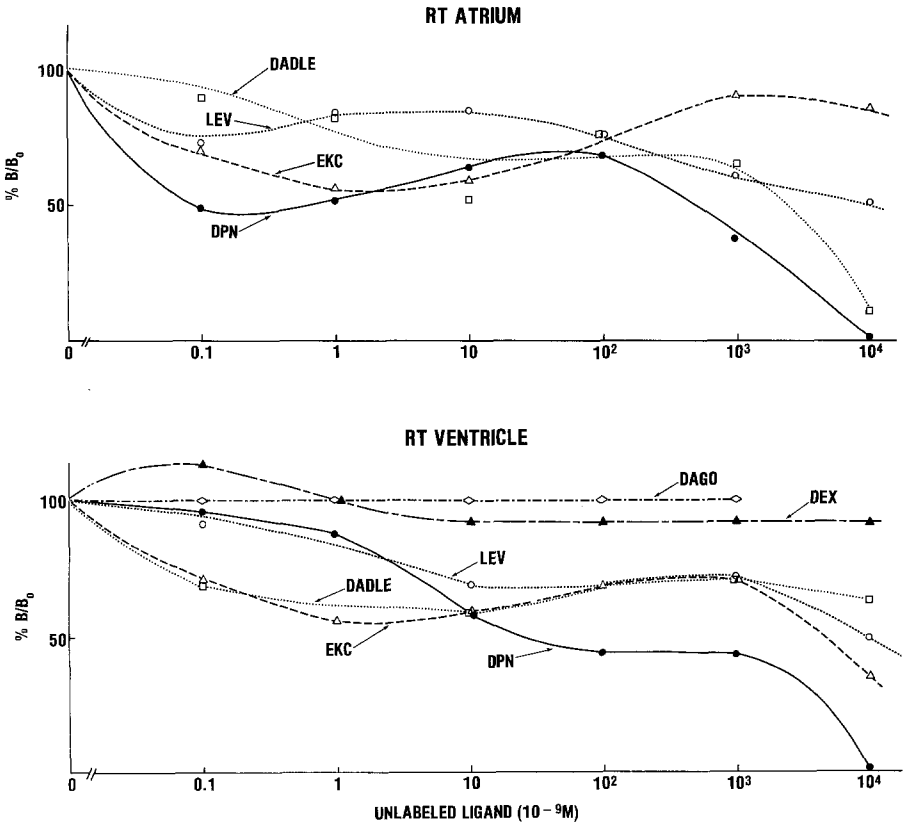

Fig. 3. Competitive inhibition studies of the binding of diprenorphine: Right atrium (Upper Panel) and right ventricle (Lower Panel). Membranes prepared from pooled atria of control rats were incubated with 7.5 nM of [^{3}H]diprenorphine for 1 h with increasing concentrations of an unlabeled ligand. B and B_o are the specific binding in the presence and absence of the unlabeled ligand, respectively.

10 μM, whereas at concentrations less than 10 μM, EKC and DADLE appeared equipotent (Fig. 3, Lower Panel). Inhibition of ^{3}H-DPN binding by EKC and DADLE but not the μ agonist DAGO strongly suggests that the ^{3}H-DPN binding sites are of the δ and κ type in the right chambers. Stereospecificity of ^{3}H-DPN binding was established from selective inhibition by levorphanol but not by dextrorphan. The ^{3}H-DPN binding data for the right atrium using DAGO were as shown for the right ventricle. When dextrorphan was used as a potential inhibitor, the ^{3}H-DPN binding in the right atrium decreased by less than 12% at 10^{-5} M of added dextrorphan. Fig. 3, based on studies using freshly dissected heart tissue, is representative of other studies; five studies of this type were performed.

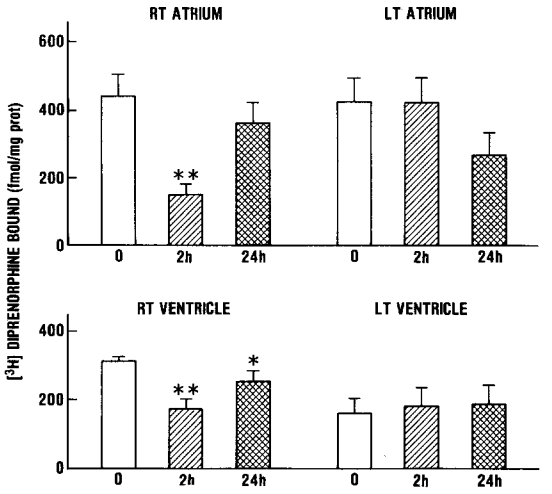

Fig. 4. Effect of hemorrhagic shock on the specific binding of diprenorphine to separate heart chambers. The values represent the mean ± S.E. of measurements of the specific binding to membranes prepared individually from five shock animals. Binding was measured after 1 h incubation with 7.5 nM of [^{3}H]diprenorphine. Nonspecific binding was defined using 10 μM of unlabeled diprenorphine; ** $p < 0.005$ and * $p < 0.05$ compared to control.

Changes in the Binding of [^{3}H]Diprenorphine in Rat Hearts After Hemorrhagic Shock

Fig. 4 shows the effect of shock on the specific binding of ^{3}H-DPN to the four chambers of freshly dissected hearts measured 2 and 24 h after experimental shock. Significant decreases in binding were observed at 2 h in the right atrium ($t = 4.0$, $p < 0.005$) and the right ventricle ($t = 4.9$, $p < 0.05$). At 24 h, binding in the right atrium was restored to pre-shock values, whereas binding in the right ventricle was only partly restored ($t = 2.0$, $p < 0.05$). Insignificant changes occurred on the left side. Similar changes in binding were obtained using frozen hearts from control and shock animals.

Effect of Hemorrhage on Cardiovascular Parameter of Conscious Rats

Mean arterial blood pressure (MAP) of conscious rats prior to hemorrhage was 114 ± 7 mm Hg. MAP at the end of the bleeding, 2 and 24 h after the bleeding was 42 ± 6, 67 ± 6 and 95 ± 4 mm Hg, respectively.

DISCUSSION

These studies are the first to show (1) time and concentration dependent binding of diprenorphine to rat hearts and (2) modulation of diprenorphin

binding after a specific cardiovascular stress, i.e., hypovolemic hypotension. Previous studies of binding to membranes from whole hearts using preferentially μ specific ligands, such as dihydromorphine and naloxone, failed to show any appreciable amounts of saturable binding (5). The low amounts of binding measured previously could be due to cross-reactivity with δ sites. Since the present studies showed no inhibition of ^{3}H-DPN binding by the μ selective ligand DAGO, the heart appears to lack μ sites. An alternate explanation might be that the number of μ sites in the heart is below the detectable level for this assay under the present experimental conditions. Finally, the failure by other investigators to measure appreciable opiate binding may in part be due to the use of membranes prepared from whole hearts. The highest density of opiate receptors appears to be in the right atrium, which represents only a fraction of the entire tissue mass of the heart.

The observation that the heart, and in particular the right atrium, contains enkephalins (1), and the reported modulatory action of enkephalins on the beating rate of isolated rat atria (2), support our conclusion that the diprenorphine binding sites in the right atrium are of the δ or enkephalin preferring subtype. It is known from in vitro studies that the number of δ sites decrease or are "down-regulated" after continuous exposure to enkephalins (12). Shock represents a cardiovascular stress which activates the opioid system in the brain (13) and enhances the release of enkephalins from the adrenal medulla into circulation (14). Since opiate receptor blockade by treatment with the opiate antagonist naloxone has been shown to produce hemodynamic improvement in shock (8) and increase heart rate after bleeding, it is tempting to speculate that the regional loss in diprenorphine binding observed in animals 2 h after shock represents an endogenously controlled opiate receptor blockade occurring subsequent to increased plasma levels of enkephalins. At 24 h after shock, when hemodynamic improvement has occurred, the acute stress has subsided and circulating enkephalins have probably been reduced, opiate receptor binding in the heart is again restored.

The inhibitory effect of EKC on ^{3}H-DPN binding observed in these studies suggests also the presence of κ sites in the heart. Indirect studies for the existence of peripheral κ sites located in the heart, as well as of their possible involvement in regulation of peripheral cardiovascular processes are available. Thus, in studies in which EKC was administered intravenously to decerebrated dogs (15) or anesthetized rats (16), heart rate and blood pressure were found to decrease.

Due to the scarcity of tissue from individual rat atria, no attempts were made in this study to resolve which receptor type changed in shock. The paucity of concentration points entering into the Wilkinson plot and the estimation of K_D is also due to this scarcity.

In the displacement curves shown in Fig. 3, the "low dose" hook effect, indicative of cooperative interactions, is apparent (17). According to De Lean and Rodbard (17), this hook effect is most marked when both the radioligand and the receptor concentrations are small. As these concentrations increase, the hook may flatten. In this study, low receptor concentration was used to allow for rapid filtration of the binding reaction mixtures.

The results of this study characterize, for the first time, the asymmetric opiate binding and response to a specific cardiovascular stress in the heart.

ACKNOWLEDGEMENT

This work was supported by the Uniformed Services University of the Health Sciences protocol #RO9215 to Solveig A. Krumins and #RO9211 to Giora Feuerstein. The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the view of the Department of Defence or the Uniformed Services University of the Health Sciences. The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. [NIH] 78-23, 1978).

The authors wish to thank Brenda McKelvin and Karen R. Wolff for their technical assistance.

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