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Enkephalins have a direct positive inotropic effect on cultured cardiac myocytes

(opiate receptor/naloxone/chicken embryo heart cells)

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ABSTRACT **Enkephalins have peripheral vascular effects, and enkephalinergic innervation of the heart has been reported. To determine whether enkephalins have direct effects on myocardium, we studied the effects of [D-Ala[2], Met[5]]enkephalinamide and [D-Ala[2], D-Leu[5]]enkephalin on amplitude of contraction (measured with an optical-video system) in spontaneously beating monolayer cultures of chicken embryo ventricular cells, a preparation devoid of intact neural elements. [D-Ala[2], Met[5]]enkephalinamide and [D-Ala[2], D-Leu[5]]enkephalin as well as [Met[5]]- and [Leu[5]]enkephalin increased contractility in a concentration-dependent manner. The enkephalin-induced maximal contractile effects were 28% and 30% above control, with EC_{50} values of 0.53 and 0.17 μM for [D-Ala[2], Met[5]]enkephalinamide and [D-Ala[2], D-Leu[5]]enkephalin, respectively. The positive inotropic effect was antagonized by naloxone but not by propranolol, phentolamine, diphenhydramine, or cimetidine. Naloxone alone had no effect on contractility at a concentration (0.1 μM) that blocked positive inotropic effects of [D-Ala[2], Met[5]]enkephalinamide and [D-Ala[2], D-Leu[5]]enkephalin. To demonstrate the presence of opiate receptors, we studied [^{3}H]naloxone binding in homogenates of cultured chicken embryo ventricular cells. Analysis of binding curves under equilibrium conditions indicated that [^{3}H]naloxone bound specifically to membranes of cultured heart cells with $K_D = 18.5 \pm 5.4$ nM and $B_{max} = 46.8 \pm 11.7$ fmol/mg of protein. We conclude that enkephalins exert a direct positive inotropic effect on cultured heart cells, increasing contractile state via specific opiate receptors.**

The heart, innervated by a dense network of sympathetic and parasympathetic nerve fibers, is a site where opioid peptides could act directly or interact with neurotransmitters. This hypothesis is supported by the observation that morphine reduces the cardiac slowing induced by vagal stimulation (1). Also, an enkephalinergic innervation of the heart, vagal and sympathetic in origin, has been shown in the guinea pig (2, 3). [Leu[5]]Enkephalin has been reported to increase the norepinephrine-stimulated positive chronotropic response and ^{45}Ca uptake in guinea pig (4). Furthermore, circulatory anatomy is such that enkephalins released from adrenal medulla flow directly through the vena cava to the right heart and pulmonary circulation where they could exert direct effects. Their short half-life, however, may limit the concentrations reaching brain centers that could affect cardiovascular responses (5).

There is substantial evidence that opiates and opioid peptides have cardiovascular effects. Morphine has been reported to decrease (6–8) or to increase (9, 10) myocardial contractility. However, the specificity of some of the cardiovascular effects of peripherally injected morphine and enkephalins has been questioned, and their mediation by sympathetic nerve activation (9, 10) and histamine release from mast cells (11) has been reported.

Whether or not circulating or prejunctionally released opioid peptides exert a direct postjunctional effect on the cardiac myocyte is unknown. The existence of direct effects of opioid peptides on the heart cannot be concluded from studies with preparations containing intact nerve endings and catecholamine stores. Thus, a detailed analysis of the direct cardiac effects of enkephalins may best be undertaken in a preparation devoid of intact neural elements. We report here the effects of enkephalins and enkephalin-like peptides Leu-enkephalin, [D-Ala[2], D-Leu[5]]enkephalin, [Met[5]]enkephalin, and [D-Ala[2], Met[5]]enkephalinamide on the contractile function of spontaneously beating, cultured chicken embryo ventricular cells. This preparation, extensively studied in our laboratory (12, 13), obviates diffusion limitations inherent in isolated organ preparations. In addition, the absence of endogenous neuroeffectors simplifies the interpretation of results.

MATERIALS AND METHODS

Tissue Culture. Primary monolayer cultures of beating chicken embryo ventricular cells were prepared as described (12). Briefly, fragments of embryonic chicken ventricules 10 days *in ovo* were dissociated by repeated cycles of trypsinization. The resulting cell suspension (4×10^5 cells per ml) was plated in 100-mm culture dishes. The medium consisted of a bicarbonate-buffered physiological salt solution containing 40% medium 199 (GIBCO), 6% fetal calf serum, and 54% balanced salt solution containing glucose. Final concentrations in the culture medium were 144 mM Na, 4.0 mM K, 0.97 mM Ca, 18 mM HCO_3, 0.8 mM Mg, and 131 mM Cl. Cultures were incubated in humidified 5% CO_2/95% air at 37°C. Spontaneously and synchronously contracting confluent monolayers were present by 3 days in culture, at which time experiments were performed. For contractility and ion-flux experiments, cells were grown on 25-mm circular glass coverslips.

Contractility Measurements. Changes in the position of a cell or attached microsphere border in a monolayer were assessed by the use of an optical-video system as described (12). Briefly, a glass coverslip with attached cells was continuously superfused in a chamber provided with inlet and exit ports. The chamber was placed on the stage of an inverted phase-contrast microscope (Leitz Diavert). The cells were magnified with a ×40 objective, and the image was monitored by a low-light-level television camera (Dage 650) connected to a video motion detector (Colorado Video 633) that monitored a selected raster line segment and provided

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data every 16 msec for an image border moving along that raster line. The medium bathing cells during contractility measurements was culture medium containing 0.6 mM Ca^{2+} without fetal calf serum. The pH of the buffer solution was maintained at 7.4 by continuously gassing the chamber with 95% air/5% CO_2. A constant temperature of 37°C was maintained by enclosing the microscope in a thermostated Lucite box. After a 15-min equilibration period, cells were superfused with solution containing (*i*) no added drug (control), (*ii*) enkephalins at various concentrations, (*iii*) other drugs as specified (see *Results*), or (*iv*) 3.6 mM Ca^{2+}, and changes in the amplitude and velocity of contraction of an individual cell were continuously recorded. At the end of each experiment, after complete wash-out of enkephalin, cells were exposed to 3.6 mM Ca^{2+}. For each cell studied, the inotropic response to enkephalin was expressed as the percentage of the response to 3.6 mM Ca^{2+}, a concentration that elicited a maximal inotropic response (14), to facilitate comparison of results from individual experiments. Contractility measurements were made on one cell per coverslip. All experimental points included results from two or more platings.

[^{3}H]Naloxone Binding to Cultured Heart Cells. Chicken embryo ventricular cells grown in culture dishes were rinsed with a solution containing 4 mM Hepes, 136 mM Na^+, 3.5 mM K^+, 1.05 mM Mg^{2+}, 0.6 mM Ca^{2+} (pH 7.4). Cells were harvested by scraping, then homogenized with a Brinkmann Polytron PT-10 (setting 5 for 10 sec) and washed and centrifuged three times at 40,000 × *g* for 10 min. The pellets were resuspended in buffered solution containing 50 mM Hepes, 0.1% bovine serum albumin, 0.1 mM phenylmethylsulfonyl fluoride (pH 7.4; final protein concentration, 0.5 mg per ml) and were assayed for radioligand binding. Binding of [^{3}H]naloxone (specific activity, 42.7 Ci/mmol; 1 Ci = 37 GBq) took place at 25°C with 0.8 ml of membrane suspension. After a 20-min incubation, the samples were immediately filtered through a Whatman glass-fiber filter (GF/C). The assay tubes were washed three times with 5 ml of ice-cold 25 mM Hepes buffer (pH 7.4). The filters were dried, and 10 ml of Instagel (Packard) was added and assayed for radioactivity at 24% efficiency in a Packard liquid scintillation counter. Specific binding was defined as the difference between binding in the presence and absence of 10 μM naloxone. Values are expressed as the means of three to five replicates, which varied by less than 20%. Protein concentration was determined by the procedure of Lowry *et al.* (15).

[^{3}H]Naloxone Binding to Ventricle and Brain Homogenates. Ventricles and brains were removed from newly hatched chicks and homogenized in the isotonic Hepes solution described above by 20 strokes of a tight-fitting Dounce homogenizer. The homogenate was centrifuged at 1000 × *g* for 10 min and the resulting pellet was discarded; a pellet formed at 48,000 × *g* for 10 min was resuspended in 50 mM Hepes buffer/0.1% bovine serum albumin, pH 7.4 (final concentration, 5 mg of original wet-weight tissue per ml) and assayed for binding as described above.

Materials. [D-Ala^2, D-Leu^5]Enkephalin, [Leu^5]enkephalin, [D-Ala^2, Met^5]enkephalinamide, [Met^5]enkephalin, DL-propranolol, isoproterenol, diphenydramine, cimetidine, and phenylmethylsulfonyl fluoride were purchased from Sigma. Naloxone was obtained from DuPont, levallorphan tartrate and dextrorphan tartrate were from Hoffmann–La Roche, and [^{3}H]naloxone was from New England Nuclear.

Statistical analysis of changes in amplitude of contraction was performed by means of the nonparametric test of Kruskall–Wallis at the 3-min plateau time and at the 10th minute. All values are expressed as means ± SEM.

RESULTS

Contractile Responses to Enkephalins. The contractile response to exposure to 10 μM [D-Ala^2, Met^5]enkephalinamide from a typical experiment is shown in Fig. 1. The amplitude of contraction of a cultured ventricular cell perfused with 0.6 mM Ca^{2+} solution is shown, followed by the response to 10 μM [D-Ala^2, Met^5]enkephalinamide. After washout of [D-Ala^2, Met^5]enkephalinamide and return to control conditions, cells were exposed to 3.6 mM Ca. The increase in contractile amplitude induced by 10 μM [D-Ala^2, Met^5]enkephalinamide had a rapid onset, reached a maximum at 2–4 min, and returned gradually to near control within 10–15 min. The maximum increase in contractility in this recording was 27% of the 3.6 mM Ca^{2+} response. Fig. 2 shows the time course of effect of graded concentrations (0.01–100 μM) of [D-Ala^2, D-Leu^5]enkephalin. The kinetics of the contractile response to [D-Ala^2, D-Leu^5]enkephalin were similar to those for [D-Ala^2, Met^5]enkephalinamide. The positive inotropic effect had its threshold at 0.01 μM [D-Ala^2, D-Leu^5]enkephalin and [D-Ala^2, Met^5]enkephalinamide (Fig. 3). The maximum effects achieved at 10 μM [D-Ala^2, D-Leu^5]enkephalin and [D-Ala^2, Met^5]enkephalinamide were 30 ± 2% and 28 ± 3%, respectively, of the contractile response to 3.6 mM Ca^{2+}. Log-logit transformation of the sigmoid concentration-effect curves yielded EC_{50} values of 0.16 ± 0.03 μM and 0.53 ± 0.05 μM for the increases in contractility produced by [D-Ala^2, D-Leu^5]enkephalin and [D-Ala^2, Met^5]enkephalinamide, respectively. No negative inotropic effect was observed with these compounds over the range from 0.01 to 100 μM, nor was there any significant change in the spontaneous beating rate. [Leu^5]Enkephalin and [Met^5]enkephalin at 10 μM increased the amplitude of contraction by 28 ± 3% and 24 ± 3% of the 3.6 mM Ca^{2+} response, respectively. Perfusions with control solutions produced no changes in contractility.

Additional experiments to establish the specificity of the effects observed included exposure of cells to specific antagonists. Naloxone alone at 0.1 μM had no effect on contractility measured in 0.6 mM Ca^{2+} medium. To determine whether naloxone might alter the inotropic response to nonenkephalin inotropic agents in a nonspecific fashion, contractile response to 3.6 mM Ca^{2+} in the absence and presence of 0.1 μM naloxone was determined. Addition of

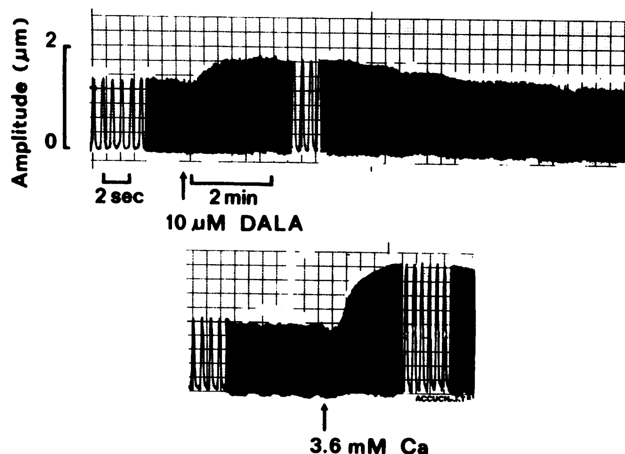

FIG. 1. Time course of the amplitude of contraction of a cultured chicken embryo ventricular cell in response to 10 μM [D-Ala^2, Met^5]enkephalinamide (DALA). At the end of the experiment, after complete washout of [D-Ala^2, Met^5]enkephalinamide, the cell was exposed to 3.6 mM Ca^{2+}. In this recording, the maximum increase in amplitude of contraction induced by 10 μM [D-Ala^2, Met^5]enkephalinamide was 37% above the control level and represents 29% of the 3.6 mM Ca^{2+} response.

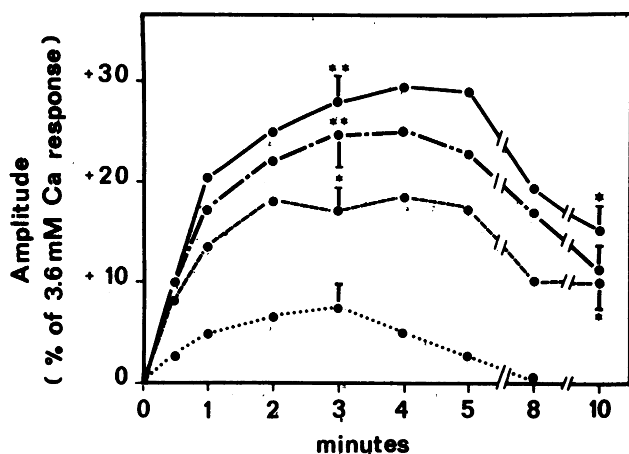

FIG. 2. Time course of the amplitude of contraction of cultured heart cells exposed to graded concentrations of [D-Ala^2, D-Leu^5]enkephalin: 0.01 μM (●·····●), 0.1 μM (●–·–●), 1 μM (●— – –●), and 10 μM (●—●). Each curve is the mean ± SEM of at least five different preparations. Monolayers were superfused continuously with the concentrations above, and the change in the amplitude of contraction induced is expressed as a percentage of the change produced by 3.6 mM Ca^{2+}. * = P = 0.05; ** = P 0.01, both in comparison to control.

naloxone produced a contractile response that was 101 ± 4% (n = 4) of the contractile response in the absence of naloxone. Similarly, the contractile response to 1 μM isoproterenol/0.1 μM naloxone was 103 ± 5% of the response in the absence of naloxone. Thus, naloxone does not appear to have a nonspecific effect on contractility in this preparation. However, when present in the superfusion medium before addition of enkephalins, 0.1 μM naloxone shifted the concentration-effect curve for [D-Ala^2, Met^5]enkephalinamide to the right (Fig. 3). The β-adrenergic antagonist propranolol (1 μM) failed to antagonize 10 μM [D-Ala^2, Met^5]enkephalinamide- or [D-Ala^2, D-Leu^5]enkephalin-induced increases in contractility, while it fully antagonized the effects of 0.1 μM isoproterenol on the same preparation. Diphenhydramine, cimetidine, and phentolamine did not alter contractile responses to 10 μM [D-Ala^2, Met^5]enkephalinamide. For comparison of efficacy, 0.1 μM isoproterenol induced a rapid and sustained increase in contractility, reaching a plateau at 4–6 min, which represented 42 ± 5% of the 3.6 mM Ca^{2+} response.

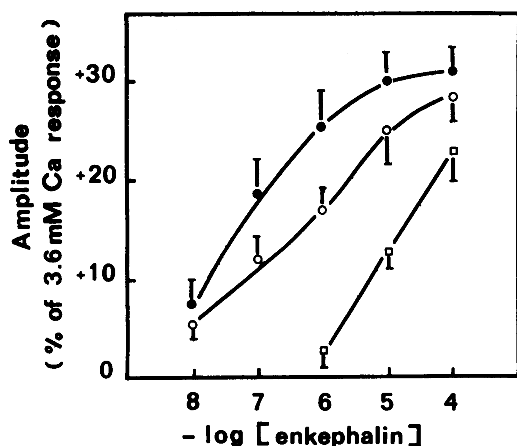

FIG. 3. Concentration-effect curve for increase in amplitude of contraction of cultured heart cells by the enkephalin analogues [D-Ala^2, Met^5]enkephalinamide (●) and [D-Ala^2, D-Leu^5]enkephalin (○). Each point is the mean ± SEM of data from at least five different preparations. The change in the amplitude of contraction induced by either analogue was expressed as a percentage of the change produced by 3.6 mM Ca^{2+}. Naloxone at 0.1 μM shifted the concentration-effect curve for [D-Ala^2, Met^5]enkephalinamide to the right (□).

Additional experiments were performed to examine the nature of the attenuation of the enkephalin-induced positive inotropic effect with time. Monolayer cultures were exposed to 10 μM [D-Ala^2, Met^5]enkephalinamide for successive 10-min periods separated by 10-min washout periods. The inotropic response to the second period of [D-Ala^2, Met^5]enkephalinamide exposure was invariably less than the initial response, averaging about 60% of that value. Specificity of this attenuation of enkephalin inotropic effect was documented by the finding that responses to 3.6 mM external Ca^{2+} were indistinguishable before and after enkephalin exposure in each of five such experiments (Fig. 4).

[^{3}H]Naloxone Binding to Cardiac and Brain Preparations. The presence of specific opiate receptors was strongly suggested by the contractile responses to enkephalins and the selective antagonism of these responses by naloxone. To demonstrate directly the presence of opiate receptors, we studied [^{3}H]naloxone binding in membranes of cultured chicken embryo heart cells. Binding was specifically displaceable (about 50–60% of total counts being displaced by unlabeled naloxone) and reached equilibrium within 15–20 min at 25°C (data not shown). Analysis of equilibrium competition binding curves was performed with the nonlinear least squares method of Munson and Rodbard (16) as described (17). Unlabeled naloxone displaced [^{3}H]naloxone (10 nM) in a concentration-dependent manner from binding sites of cultured heart cells. Analysis of competition binding curves indicated that naloxone bound specifically to membranes of chicken embryo ventricular cells with K_d = 25 ± 7.6 nM and B_{max} = 46.8 + 11.7 fmol/mg of protein (Fig. 5). Binding was best described mathematically to a single site rather than to two sites (P < 0.05). To compare data from cultured heart cells with more commonly described systems for opiate binding, we also measured binding to membranes of brain homogenates from hatched chicks. A lower K_d (4.4 ± 2.1 nM) and a higher B_{max} (220 ± 15 fmol/mg of protein) were found (Fig. 5). Membrane preparations from ventricles of newly hatched chicks also showed displaceable [^{3}H]naloxone binding, with an average of 27.1 fmol bound per mg of protein.

Unlabeled [D-Ala^2, Met^5]enkephalinamide, levallorphan, and dextrorphan were able to displace [^{3}H]naloxone binding in cultured heart cells, the opiate dextrorphan being 18 times less potent than its active isomer levallorphan. Graphical analysis revealed IC_{50} values of 0.30 ± 0.15 and 0.54 ± 0.35 μM for levallorphan and dextrorphan, respectively.

DISCUSSION

The principal findings from these studies are the direct positive inotropic effect of enkephalin peptides and their analogs [D-Ala^2, Met^5]enkephalinamide and [D-Ala^2, D-Leu^5]enkephalin on cultured heart cells and the existence in these cells of opiate receptors capable of binding [^{3}H]naloxone in a manner specifically displaceable in the order naloxone, levallorphan, [D-Ala^2, Met^5]enkephalinamide, and dextrorphan. The cultured heart cell preparation has been studied extensively in our laboratory and has proved to be a useful model for the examination of basic mechanisms of inotropic action of cardiac glycosides (18) and β-adrenergic agonists (19) and of mechanisms underlying the control of β-adrenergic and muscarinic cholinergic receptors (19, 20). The absence of neural elements from the cultured heart cell preparation facilitates the interpretation of experiments such as those reported here. A direct inotropic effect on the myocyte can be inferred from the absence of endogenous neuroeffectors, the presence in the myocytes of opiate

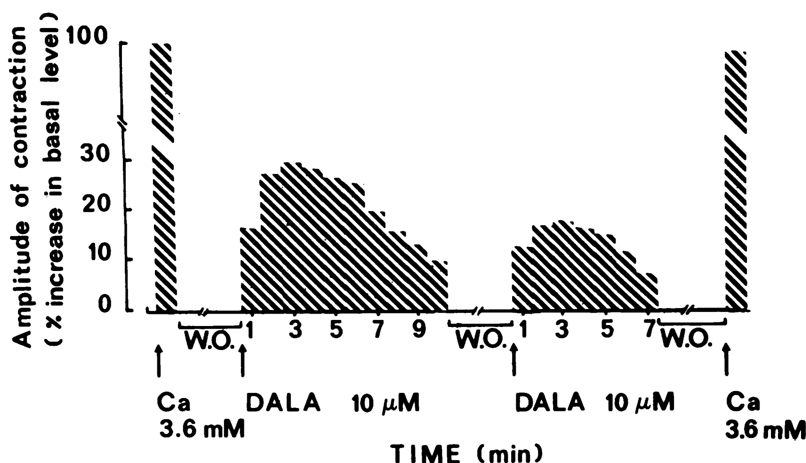

FIG. 4. Reduced contractile response of a cultured heart cell during sequential exposure to 10 μM [D-Ala^2, Met^5]enkephalinamide (DALA). The contractile response to 3.6 mM Ca^{2+} (shown at the 3-min plateau value) was similar at the beginning and at the end of the experiment. After washout (W.O.) of the 3.6 mM Ca^{2+} and return to baseline state, the cell was exposed to 10 μM [D-Ala^2, Met^5]enkephalinamide (maximum increase in amplitude = +29%) for 10 min. Then, after a 10-min washout period, the cell was reexposed to the same [D-Ala^2, Met^5]enkephalinamide concentration. The maximal increase in contractile amplitude was attenuated to +18% during the second exposure.

receptors with appropriate stereospecific binding properties, and blockade of the enkephalin-induced inotropic response with the specific opiate antagonist naloxone (but not antagonists specific for α- or β-adrenergic or histaminergic receptors). Those findings are of particular interest in the light of previous reports suggesting the mediation of opiate effects on the heart by β-adrenergic and histamine-related mechanisms (9–11). The positive inotropic effect is clearly not limited to agents with a D-alanine residue at position 2 (21), since both [Leu^5]- and [Met^5]enkephalin elicited inotropic responses comparable to [D-Ala^2, D-Leu^5]enkephalin and [D-Ala^2, Met^5]-enkephalinamide.

The attenuation of the positive inotropic response to enkephalins with time is of interest and may represent a specific desensitization mechanism analogous to those described for β-adrenergic and muscarinic cholinergic responses described in cultured heart cells (19, 20) and other intact cell preparations (22). Since there was no concomitant attenuation of the contractile response to 3.6 mM Ca^{2+}, nonspecific deterioration of the preparation during enkephalin exposure can be excluded as a mechanism of apparent desensitization. It is also unlikely that this attenuation of effect results from breakdown of enkephalin peptides, since the analogs [D-Ala^2, Met^5]enkephalinamide and [D-Ala^2, D-Leu^5]enkephalin are resistant to enzymatic hydrolysis (23, 24) and the bathing media were continuously renewed from a reservoir containing no hydrolytic enzyme activity. [D-Ala^2, D-Leu^5]enkephalin has the additional advantage of higher selectivity at the δ receptor than does [Leu^5]enkephalin.

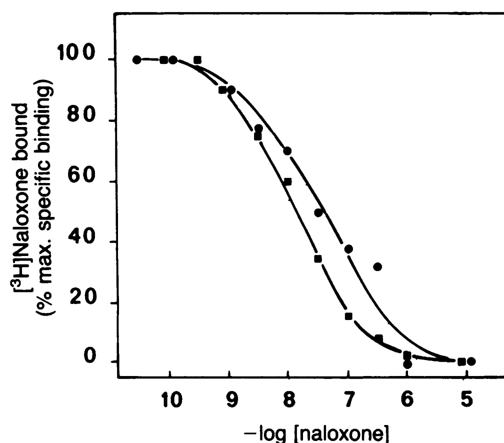

FIG. 5. Displacement of [^{3}H]naloxone binding to preparations from chicken heart and brain by unlabeled naloxone. [^{3}H]Naloxone (10 nM) binding was assayed in the presence of graded concentrations (0.1 nM to 10 μM) of unlabeled naloxone, either in homogenates of cultured chicken embryo heart cells (●) or in homogenates of hatched chick brain (■). Values are expressed as the mean of three replicates.

The effect of opiate agonists upon a receptor–effector system is usually inhibitory (25–27). Enkephalins inhibit the release of acetylcholine from myenteric nerves and inhibit spontaneous contractions in the guinea pig ileum (28, 29). In other systems such as isolated gastric smooth muscle cells, however, enkephalins have been reported to exert a direct positive contractile effect that is selectively antagonized by naloxone (30). Enkephalins, which inhibit the release of norepinephrine at the level of the neuroeffector junction in several autonomically innervated tissues (28, 31), are stored with catecholamines in sympathetic nerve terminals in the guinea pig heart (3). It is possible that under physiologic or stress conditions, enkephalins may exert a direct postjunctional excitatory action on the cardiac cell.

Radioligand binding experiments were performed to investigate the involvement of opiate receptors in the mediation of the contractile response to enkephalins. Levels of [^{3}H]naloxone specific binding were appreciably larger in chicken embryo heart cells than those reported for rat or guinea pig heart (32). Forty to 50% specific binding of [^{3}H]naloxone in our cultured heart cell experiments, while not optimal for detailed characterization of receptor properties, permitted estimation of K_d and B_{max}. [^{3}H]Naloxone binding to heart membranes was not limited to early developmental stages, since it occurred also in hatched chick heart membrane preparations. The absence of intact neural elements in chicken embryo heart cells grown in tissue culture indicates that [^{3}H]naloxone bound specifically to myocytes. In brain, we found K_d and B_{max} values similar to those reported for other species (32, 33). In cultured heart cells, K_d was a factor of 5 greater and B_{max} was a factor of 5 less than values observed in homogenates of chick brain, indicating a lower affinity and a smaller number of naloxone binding sites in the heart. For comparison, in our intact cultured heart cell preparation, receptors for other agonists are present in both lower (19) and higher (20) density. For example, isoproterenol binds to intact cells with K_d = 0.26 μM and B_{max} = 10.3

fmol/mg of protein (19); carbachol binds with $K_d = 0.39$ and 82 μM and $B_{max} = 140$ fmol/mg of protein (20). Naloxone binding sites (46.8 fmol/mg of protein) are considerably more sparse than sarcolemmal Na^+/K^+-ATPase complexes as judged by [^{3}H]ouabain binding (6 pmol/mg of protein) (34). Unlabeled [D-Ala^2, Met^5]enkephalinamide, levallorphan, and dextrorphan were found to compete for naloxone binding sites. Stereospecificity is suggested by the higher IC_{50} for the relatively inactive opiate dextrorphan relative to its more active isomer levallorphan. However, these data must be viewed cautiously. In brain, dextrorphan is 1000 to 10,000 times less potent than levallorphan in displacing [^{3}H]-naloxone (32).

The cellular mechanism by which enkephalin peptides exert a positive inotropic effect on cardiac myocytes requires further study. While inhibitory effects of opiate agonists on adenylate cyclase have been reported for brain preparations (25), this has not been explored in heart. The absence of a positive chronotropic effect on cultured chicken embryo ventricle myocytes cannot be viewed as arguing against changes in intracellular cyclic AMP levels because, in the preparation studied, isoproterenol exerts a potent positive inotropic effect with no accompanying change in beating rate (19).

In conclusion, our results indicate that enkephalin peptides have a direct effect on cultured heart cells, increasing contractility via specific opiate receptors. The physiological implications of these findings are uncertain. Plasma concentrations of enkephalins are not known in embryonic chicken, but data obtained in other species (35) indicate that the circulating concentrations of enkephalins are 1 to 2 orders of magnitude less than the EC_{50} values calculated for [D-Ala^2, D-Leu^5]enkephalin and [D-Ala^2, Met^5]enkephalinamide. However, the concentration of enkephalins at the cardiac myocyte surface *in vivo* might be enhanced by (*i*) stressful conditions, increasing the release of enkephalins from adrenal medulla and autonomic nerve terminals (36); (*ii*) the proximity of the right heart to the adrenal veins; and (*iii*) the reported inhibition of the enzymatic degradation of [Met^5]enkephalin by catecholamines, leading to the rapid accumulation of enkephalins in the synaptic cleft (37). The net physiological effect of enkephalins on the heart may be the resultant of direct excitation and indirect neurally mediated inhibitory effects, the former inducing a positive inotropic effect and the latter a decrease in contractility through a decrease in norepinephrine release.

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