J Mol Cell Cardiol **29**, 711–720 (1997)

Distinct Components of Morphine Effects on Cardiac Myocytes are Mediated by the κ and δ Opioid Receptors

Catherine Ela[1], Jacob Barg[2,3], Zvi Vogel[2], Yonathan Hasin[4] and Yael Eilam[1]

[1]*Department of Bacteriology, Hebrew University-Hadassah Medical School, Jerusalem, Israel;*
[2]*Department of Neurobiology, The Weizmann Institute, Rehovot, Israel;*
[3]*Ramot Yehuda Therapeutic Community, Beit Shemesh P.O.B. 188, Israel, and*
[4]*Department of Cardiology, Hadassah University Hospital, Jerusalem, Israel*

(Received 17 June 1996, accepted in revised from 9 October 1996)

C. Ela, J. Barg, Z. Vogel, Y. Hasin and Y. Eilam. Distinct Components of Morphine Effects on Cardiac Myocytes are Mediated by the κ and δ Opioid Receptors. *Journal of Molecular and Cellular Cardiology* (1997) **29**, 711–720. Morphine exerts direct effects on cultured cardiac myocytes from neonatal rats. These effects are mediated via the δ and the κ opioid receptors, as μ opioid receptors are not present in neonatal cardiomyocyte cultures. Binding parameters to the δ and κ opioid receptors were determined in membrane preparations from these cultures by heterologous competition to [^{3}H]diprenorphine binding, with [D-Pen^2, D-Pen^5]-enkephalin (DPDPE) and *trans-(dl)*-3,4-dichloro-N-methyl-N- [2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methanesulfonate (U-50,488H) as specific displacers respectively. To define the components of morphine effects mediated via activation of either the δ or the κ opioid receptor alone, cardiac myocytes were exposed to morphine in the presence of specific antagonists to the κ or δ opioid receptor respectively. Activation of the κ opioid receptors by morphine caused a transient increase in Ca^{2+} influx, leading to increase in amplitudes of $[Ca^{2+}]_i$ transients and contraction, with no change in the intracellular pH. Activation of the δ opioid receptors alone by morphine caused a decrease in the amplitude of contraction. This decrease was mediated by a decrease in the intracellular pH leading to reduced responsiveness of the myofilaments to Ca^{2+}. There was no change in Ca^{2+} influx and in the amplitude of $[Ca^{2+}]_i$ transients. The effects mediated through the δ opioid but not through the κ opioid receptors were pertussis toxin sensitive, indicating coupling of the δ opioid receptors to pertussis toxin sensitive GTP-binding proteins. The overall effects of morphine on the neonatal cardiac myocytes were the sum of the effects exerted by morphine when it activated each of the opioid receptors alone.

Key Words: Cardiac myocytes; Morphine; Opioid receptors; Cystolic Ca^{2+}; Contractility; Intracellular pH.

Introduction

It has been recently found in our laboratory that morphine exerts direct effects on cardiac myocytes from neonatal rats. Exposure of cultured ventricular myocytes from neonatal rats to morphine caused an increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) transients, and an increase in Ca^{2+} influx. The increase in $[Ca^{2+}]_i$ transients was not accompanied by an increase in the amplitude of systolic cell motion (ASM), indicating reduced myofibril responsiveness to Ca^{2+}. Intracellular pH measurements revealed that morphine caused acidosis. The effect of morphine on the intracellular pH but not on Ca^{2+} influx was inhibited by pertussis toxin, protein kinase inhibitor K323a, phorbol-ester and ethylisopropyl-amiloride (EIPA), indicating that the pathway is mediated via GTP-binding proteins and by altered activity of protein kinase C (PKC) and the Na^+/H^+ exchanger (Ela *et al.*, 1993).

Please address all correspondance to: Y. Eilam, Dept of Bacteriology, Hebrew University-Hadassah Medical School, Jerusalem, Israel.

0022–2828/97/020711+10 $25.00/0 mc960313

Effects of opioid receptor agonists on cardiac myocytes of various origins have been reported. Enkephalin peptides and their synthetic analogs produced transient positive inotropic effects and increased Ca^{2+} influx in cultured cardiac myocytes from chick embryos. These effects could be blocked by naloxone. The presence of opioid receptors capable of specific binding of [^{3}H]naloxone has been demonstrated in these cells (Laurent *et al.*, 1985; 1986). In isolated cardiac myocytes from adult rats, agonists of the δ and κ opioid receptors caused negative inotropic effects due to decreased $[Ca^{2+}]_i$ transients. These agonists also caused an increase in the level of inositol 1,4,5-trisphosphate (IP_3) (Ventura *et al.*, 1991a; 1992). Recently it has been reported that stimulation of δ opioid receptors in ventricular myocytes from adult rat reduced the L-type Ca^{2+} channel current (Xiao *et al.*, 1993). On the other hand, stimulation of κ opioid receptors in similar cells increased the level of cytosolic Ca^{2+} (Tai *et al.*, 1992, Ventura *et al.*, 1994).

The different results obtained in the three cell types described above raise the question of whether the response of cardiac myocytes to morphine is different from responses to specific δ and κ agonists. Morphine has a higher selectivity for μ receptors, but at the concentrations used (10^{-8}–10^{-6}M) it binds also to δ and κ receptors [μ receptors are not present in cultured cardiac myocytes (Zimlichman *et al.*, 1996)]. It is not yet known whether possible "cross-talk" between these receptors may lead to a different response from that induced by binding to each receptor alone.

Focusing on this question, we have determined, using specific antagonists, which components of the effects of morphine are mediated via the δ or the κ opioid receptors. We have found that the overall effects of morphine on neonatal cardiac myocytes consist of the sum of the effects exerted by morphine via each of the opioid receptors alone.

Material and Methods

Cell Culture

Cultures of ventricular myocytes from neonatal rats were prepared as described previously (Ela *et al.*, 1993). Briefly myocardial cells were isolated from ventricular fragments of the hearts of one day old Sabra rats by serial trypsinization as described (Hallaq *et al*, 1989) and suspended in Ham F10 media containing 20% serum and antibiotics. The cell suspensions were enriched with myocytes by pre-plating on tissue culture plastic Petri dishes for 1 h (during this time, the fibroblasts become attached to the Petri dish). The myocyte-enriched suspension was collected and diluted to 5×10^5 cells/ml. For measurements of contractility and $[Ca^{2+}]_i$, the cells were plated on circular glass coverslips (25 mm). For measurements of pH, the cells were plated on rectangular coverslips (50×12 mm). For measurements of $^{45}Ca^{2+}$ influx, the cells were plated on multi-well plates (12 wells per plate). The cells were maintained in humidified 5% CO_2–95% air atmosphere at 37°C for 4 or 5 days, before performing the experiments.

Membrane preparation and binding assay

Membranes were prepared from cultured cardiac myocytes 4 days after plating. The cultures were washed with phosphate-buffered saline, pH 7.4, the cardiomyocytes were collected in the same buffer and then homogenized in 20 volumes of 50 mM Tris-HCl buffer, pH 7.4 using Polytron homogenizer (Kinematica, Lucerne). The homogenate was centrifuged at $1000 \times \mathbf{g}$ for 10 min and the pellet was discarded. The supernatant was centrifuged at $40\,000 \times \mathbf{g}$ for 20 min and the pellet, containing the crude membranes, was resuspended in Tris buffer with a Dounce homogenizer. Binding assay was carried out as previously described (Zimlichman *et al.*, 1996). In brief, aliquots of crude membranes were incubated with 1 nM [^{3}H]diprenorphine (34 Ci/mmol, Amersham, Buckinghamshire) at 25°C for 60 min. To determine the parameters of δ binding sites, the incubation was done in the presence of a saturating concentration (100 nM) of a specific κ agonist and a range of concentrations of a specific δ agonist as a displacer. To determine the parameters of κ binding sites, the δ agonist was present at a saturating concentration (100 nM) and the κ agonist at a range of concentrations as a displacer. We have used the specific δ and κ agonists [D-Pen^2, D-Pen^5]-enkephalin (DPDPE) and *trans*-(*d*l)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methanesulfonate (U-50,488H), respectively. The amount of protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. B_{max} and K_i values were calculated from three independent experiments (9 points each) with the Inplot 4 computer program (Graph Pad Software, San Diego, CA) and drawn with the Sigmaplot 4.11 computer program (Jandel Scientific, Corta Madera, CA, USA) as previously

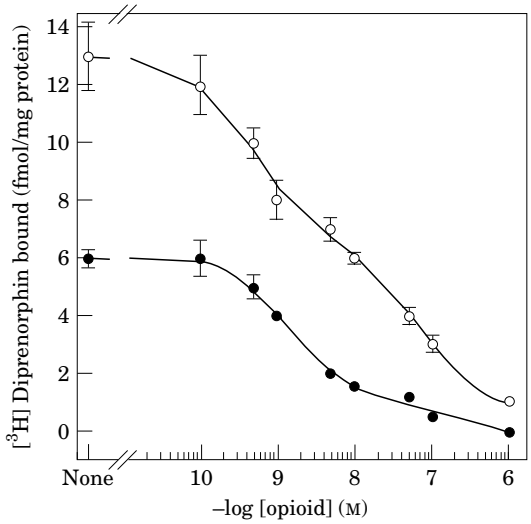

Figure 1. Determination of δ and κ opioid receptor binding parameters by heterologous competition to [^{3}H] diprenorphine binding. Heterologous competition binding assay was performed with membranes of neonatal rat cardiac myocytes. Binding of 1 nM [^{3}H]diprenorphine was measured in the presence of a saturating concentration (100 nM) of a specific κ agonist and a range of concentrations of a specific δ agonist (DPDPE) as a displacer (●), or a saturating concentration (100 nM) of a specific δ agonist and a range of concentrations of a specific κ agonist (U-50,488H) as a displacer (○). Two binding sites fitted best each of the competition curves as detected by the Inplot 4 program. The parameters are shown in Table 1. Data are means ± S.E. of three experiments.

described (Zimlichman *et al.*, 1996). Binding parameters are presented as means ± S.E.

The inplot 4 computer program uses the following mathematical algorithm:

$$Y = B + A - \left[\frac{B(A-B)}{10^{X\log EC_{50\{1\}}}}\right] + \left[\frac{1-(A-B)}{1+10^{X(-\log EC_{50\{2\}})}}\right] \quad (1)$$

Where Y is the total binding, B is the binding at the highest, and A is the binding at the lowest concentration of the competitor, X is the concentration of the competitor and {1} and {2} indicate binding sites 1 and 2. Convergence was reached when two consecutive iterations changed the sum of squares by less than 0.0001% (Fig. 1). B_{max} values were obtained from (1); K_i values were calculated from (2):

$$K_i = \frac{EC_{50}}{(1+[R]/K_d)} \quad (2)$$

Where [R] is the concentration, and K_d is the affinity of the radioactive ligand.

Measurement of the amplitude of cell motion

Changes in the contractile state of the cultured myocytes following the addition of the drugs were determined by measuring the amplitude of systolic cell motion, using a phase contrast video motion detector system (Hallaq *et al.*, 1989). Circular glass coverslips with attached cardiac myocytes were placed in a glass-bottomed cell chamber and were superfused constantly with buffered salt solution (BSS) containing (mM): NaCl, 140; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; glucose, 10; Na_2HPO_4, 1; HEPES, 10, pH 7.4. The cell chamber was mounted on the stage of the phase contrast inverted microscope. The temperature of the solution in the cell chamber was maintained at 37°C. The beating rate was kept constant by field stimulation of the cells via two platinum electrodes based in the superfusion solution. The amplitude of contraction was measured by recording the movement of a microsphere attached to the surface of the cultured cells by the video motion detector system, as previously described (Ela *et al.*, 1994). Drugs, dissolved in BSS, were added by superfusion. Cell motion was recorded continuously before and after drug addition. The amplitude of cell motion is expressed as the percentage of the value in the same cell before drug addition.

Measurement of $[Ca^{2+}]_i$ transients

Measurement of $[Ca^{2+}]_i$ transients was done in cells loaded with the fluorescent Ca^{2+} indicator indo-1. Coverslips with attached cultured cells were incubated for 45 min at room temperature in BSS containing indo-1 acetoxymethylester (indo-1/AM) (5 μM) and probenecid (3 mM), then washed and incubated again in BSS containing probenecid (3 mM) for 30 min before measurement. The coverslip with the loaded cardiac myocytes was then placed in the cell chamber which was mounted on the stage of the inverted microscope. The cell chamber was superfused with BSS, maintained at 37°C and the cells in the chamber were field stimulated as described above. The drugs were dissolved in BSS and added by superfusion.

Simultaneous measurements of calcium transients and cell motion were made as described by Peeters *et al.* (1987) using an FM-1000 dual wavelength fluorescence microphotometer (Rincon Inc., San Paulo, CA, USA). The FM-1000 system was connected to the inverted microscope and to the video motion detector system as described previously (Ela *et al.*, 1993). The amplitude of systolic

cell motion (ASM) was recorded simultaneously with the recording of the indo-1 fluorescence ratio at 410/480 nm. Before measurements, autofluorescence of unloaded cultures was determined and this value was subtracted from the fluorescence of the indo-1 loaded cultures before the computation of the ratio. Measurements were done on groups of a few attached cells which contracted synchronously, before and at different time intervals after drug addition. Each measurement continued for approximately 5 s; between measurements the cells were kept in the dark to minimize indo-1 bleaching.

It has been suggested that in rat cardiac myocytes loaded with indo-1, calibration of $[Ca^{2+}]_i$ is not precise due to subcellular compartmentalization of indo-1. Because the fluorescence ratio was shown to be a monotonic function of $[Ca^{2+}]_i$, we have presented indo-1 signals in uncalibrated form as suggested by Spurgeon *et al.* (1990).

Measurement of $^{45}Ca^{2+}$ influx rates

Cardiac myocytes, plated in 12-well multiwell plates, were preincubated in BSS for 1 h at 37°C, then placed in a shaker at 37°C. The BSS solution was then exchanged with fresh BSS at 37°C to which the examined drugs were subsequently added. Before drug addition and at different time intervals thereafter, $^{45}Ca^{2+}$ was added (4 μCi/well). The influx of Ca^{2+} was terminated 15 s later by the removal of the medium and washing the cells in the well with 3 portions of 2 ml BSS at 0°C. Subsequently, 0.4 ml of NaOH (0.2 N) was added to each well, and the plates were incubated at 37°C for 30 min. Aliquots were then taken from each well for protein determination by the method of Lowry *et al.* (1951) and for measurement of the radioactivity in toluene triton scintillation fluid.

Measurement of intracellular pH

Intracellular pH was measured using the pH-sensitive fluorescent dye 2′,7′-bis(carboxyethyl-5(6)-carboxy-fluorescein) (BCECF). Cardiac myocytes attached to rectangular coverslips were incubated in BSS containing 20 μM BCECF-acetomethoxy ester (BCECF/AM) for 45 min, then washed with BSS to remove extracellular dye. The coverslip with the loaded cells was inserted diagonally into a cuvette containing 3 ml BSS at 37°C which was placed in a temperature-controlled chamber of a fluorescence spectrophotometer. The pH was measured as described previously (Ela *et al.*, 1993). Fluorescence emission at 535 nm was recorded as a function of time, before and after drug addition, in cells excited at 495 nm. Excitation at 450 nm was done before and after the measurements. Calibration was done as described previously (Ela *et al.*, 1993).

Results

Binding parameters of δ and κ opioid receptor agonists to the δ and κ opioid receptors were determined in crude membrane preparations from cultured neonatal rat cardiomyocytes. It was found previously that μ opioid receptors are not present in cardiac myocyte membranes from cultured neonatal rats (Zimlichman *et al.*, 1996) and from adult rats (Ventura *et al.*, 1989). Determination of δ and κ opioid receptor binding parameters was performed by heterologous competition to [^{3}H]diprenorphine binding, with DPDPE and U-50,488H as specific displacers respectively (Fig. 1). Two binding sites fitted best each of the competition curves as detected by the Inplot 4 program. The parameters are shown in Table 1.

Morphine acts on ventricular myocytes from neonatal rats by two distinct pathways distinguishable by their sensitivity to pertussis toxin: (1) Increased Ca^{2+} influx leading to increased $[Ca^{2+}]_i$ transients; (2) Decreased intracellular pH leading to reduced myofibril responsiveness to Ca^{2+} (Ela *et al.*, 1993).

Further experiments were designed to determine which of the opioid receptors mediated each component of the effect of morphine. Experiments were done in the presence of naltrindole, a selective antagonist of δ opioid receptors (Portoghese *et al.*, 1988), to determine the components mediated through the κ opioid receptors, and in the presence of norbinaltorphimine dihydrochloride (nor-BNI), a selective antagonist of the κ opioid receptors (Portoghese *et al.*, 1987), to determine the components mediated though the δ opioid receptors.

Exposure of ventricular myocytes to nor-BNI alone did not affect the rate of Ca^{2+} influx, but the presence of nor-BNI together with morphine abolished the morphine-induced increase in Ca^{2+} influx (Fig. 2). On the other hand, the δ antagonist naltrindole did not affect the morphine-induced increase in Ca^{2+} influx. An increase to 356% of control level was observed in the presence of morphine and naltrindole. Thus, the morphine-induced increase in Ca^{2+} influx is mediated via the κ opioid receptors.

Preincubation of ventricular myocytes for 10 min with naltrindole (1 nM) alone did not affect the intracellular pH. However, the subsequent exposure

Table 1. Binding parameters of δ and κ opioid receptor agonists to the δ and κ opioid receptors in membranes from cultured neonatal rat cardiomyocytes.

Agonist	$K_{i,1}$	$K_{i,2}$	$B_{max,1}$	$B_{max,2}$	B_{max} total
	(nM)		(fmol/mg protein)		
U-50,488H	0.22 ± 0.33	32 ± 5	11.0 ± 3.0	13.2 ± 2.2	24.0 ± 5.2
DPDPE	0.73 ± 0.07	140 ± 13	2.4 ± 0.3	10.5 ± 1.5	12.9 ± 2.8

Determination of δ and κ opioid receptor binding parameters was performed by heterologous competition to [^{3}H]diprenorphine binding, with DPDPE and U-50,488H as specific displacers respectively (see Materials and Methods and Fig. 1). The values were determined by the Inplot 4 program.

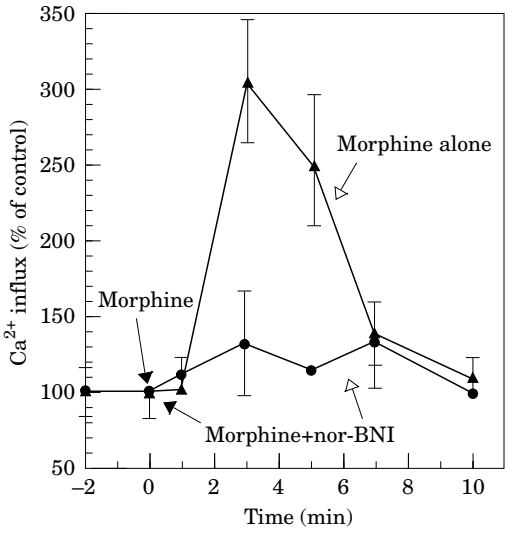

Figure 2. Effects of morphine, in the presence and absence of nor-BNI, on the rates of $^{45}Ca^{2+}$ influx into neonatal cardiac myocytes. The cultures were incubated in BSS at 37°C. The arrows indicate the addition of morphine (1 μM) (▲), or morphine + nor-BNI (1 nM) (●). At the indicated times, $^{45}CaCl_2$ (4 μCi/well) was added for 15 s, followed by washing the cells and determination of the radioactivity within the cells as described in Materials and Methods. Data are means ± S.E. of six measurements.

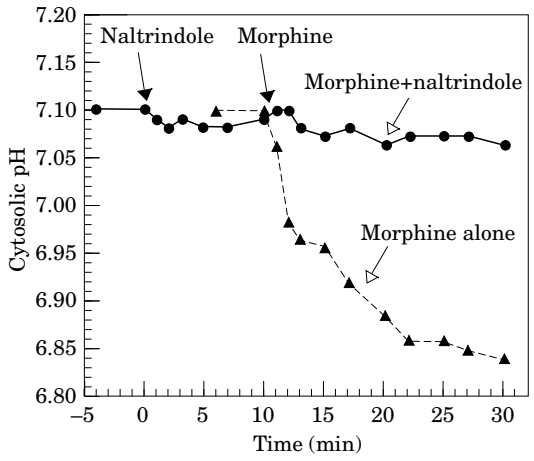

Figure 3. Effects of morphine in the presence and absence of naltrindole on the intracellular pH in the neonatal cardiac myocytes. The cardiac myocytes, loaded with BCECF, were incubated in BSS at 37°C. The arrows indicate the addition of morphine alone (1 μM) (--▲--), or naltrindole alone (1 nM) at 0 time followed by the addition of morphine (1 μM) + naltrindole (1 nM) at 10 min (—●—). Intracellular pH was determined as described in Materials and Methods. A representative experiment is shown. Similar results were obtained in five experiments.

to morphine (1 μM) in the presence of naltrindole abolished the morphine-induced decrease in intracellular pH (Fig. 3). On the other hand, the intracellular pH decreased to pH 6.93 in the presence of morphine and nor-BNI. We conclude that this component of the effect of morphine is mediated via the δ opioid receptors.

An increase in $[Ca^{2+}]_i$ transients would be expected to cause a positive inotropic effect. Indeed such an effect was observed in myocytes exposed to morphine in the presence of naltrindole, when only κ receptors should be active. Preincubation for 10 min with naltrindole alone (1 nM) caused a small increase in ASM (by 7%, Fig. 4). Addition of morphine (1 μM) together with naltrindole (1 nM) (which was added 10 min earlier) caused a further increase in ASM reaching 115% of the control level (Fig. 4). On the other hand, the δ receptor-mediated decrease in intracellular pH which leads to a decrease in myofibril responsiveness to Ca^{2+} (Fabiato and Fabiato, 1978) would be expected to cause a negative inotropic effect. Such an effect was indeed observed in myocytes exposed to morphine in the presence of nor-BNI, when only the δ receptors would be activated. Preincubation of the cultures with nor-BNI (1 nM or 100 nM) for 10 min caused a small increase in ASM (7% of control level). Subsequent addition of morphine together with nor-BNI caused a marked decrease in ASM reaching 78% of the control level (Fig. 5). Exposure of the ventricular myocytes to morphine (1 μM) without the antagonists did not induce any appreciable change in the ASM (Figs 4 and 5, and Ela *et al.*,

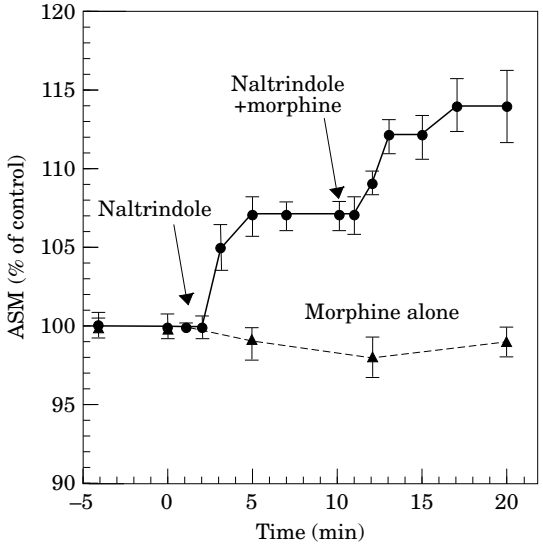

Figure 4. Effects of morphine and naltrindole on the amplitudes of contraction (ASM) of neonatal cardiac myocytes. The cardiac myocytes were incubated in BSS at 37°C. The arrows indicate the addition of morphine alone (1 μM) at 0 time (--▲--), or naltrindole alone (1 nM) at 0 time followed by the addition of morphine (1 μM) + naltrindole (1 nM) at 10 min (●). Contractility was measured as described in Materials and Methods. Data are means ± S.E. of six measurements.

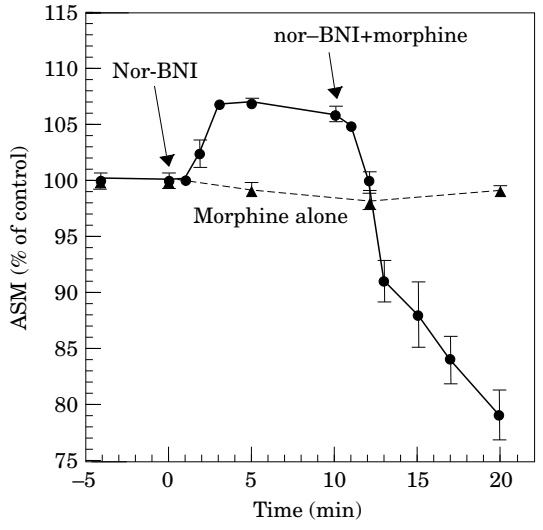

Figure 5. Effects of morphine and nor-BNI on the amplitudes of contraction (ASM) of neonatal cardiac myocytes. Cardiac myocytes were incubated in BSS at 37°C. The arrows indicate the addition of morphine alone (1 μM) at 0 time (--▲--), or nor-BNI alone (1 nM) at 0 time followed by the addition of morphine (1 μM) + nor-BNI (1 nM) at 10 min (●). Contractility was measured as described in Materials and Methods. Data are means ± S.E. of six measurements.

1993). It appears that the overall effect of morphine on the ASM (no change) is the sum of the positive and negative effects mediated via the κ and δ opioid receptors, respectively. It is not clear why nor-BNI alone and naltrindole alone caused small positive inotropic effects (by 7%). The effect of naltrindole may be caused by antagonizing endogenous opioid peptides which are produced in cultured ventricular myocytes (Springhorn and Claycomb, 1989).

It has been found previously that preincubation with pertussis toxin abolished the morphine-induced decrease in intracellular pH (Ela *et al.*, 1993 and Fig. 3). We examined therefore the effect of morphine on ASM and on $[Ca^{2+}]_i$ transients after preincubation with pertussis toxin. Similar to the results obtained in the presence of naltrindole but to a larger extent, a positive inotropic effect was induced by morphine, reaching 135% of the control level, in cells preincubated with pertussis toxin (Fig. 6). On the other hand, preincubation with pertussis toxin did not affect the increase in $[Ca^{2+}]_i$ transients induced by morphine (Fig. 6).

Discussion

It was found in the present study that [^{3}H]dipronorphine, an analogue of morphine, showed specific binding to membranes of cultured cardiac myocytes from neonatal rats and could be displaced by δ and κ opioid receptor agonists. Analysis of the displacement curves suggested the presence of δ and κ opioid receptors with two subclasses of binding sites for each receptor type (K_i values below nanomolar and at the nanomolar range). The presence of δ and κ opioid receptors on the sarcolemma of isolated cardiac myocytes from adult rats was found previously in binding studies with labeled δ and κ agonists (Ventura *et al.*, 1989). In the study of Ventura *et al.*, (1989) a single class of binding sites was detected for each receptor, with K_d values in the low nanomolar range. On the other hand, Jin *et al.* (1995) reported in membrane homogenate from adult rat hearts the presence of two subclasses of κ-binding sites, similar to κ_{1a} and κ_{1b} reported by Rothman *et al.* (1990). The differences in binding parameters between the results of the various experiments may originate from the differences in the type of cells (neonatal *v* adult), in the methods of membrane preparation or in the experimental procedures.

Consistent with the binding results it was found in the present study that the effect of morphine on neonatal rat cardiac myocytes is composed of two

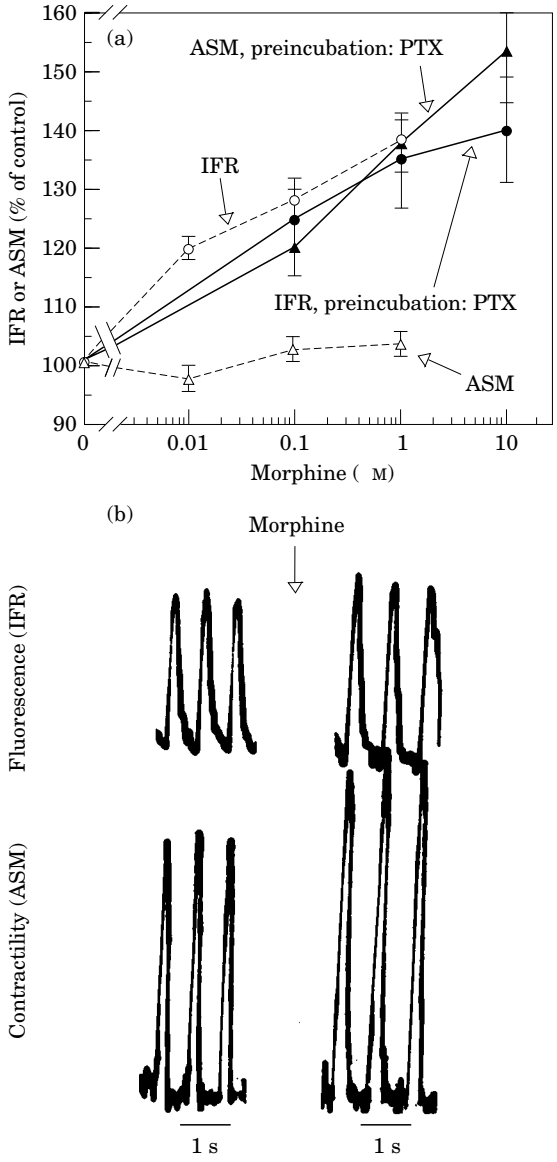

Figure 6. Effects of morphine on contractility and $[Ca^{2+}]_i$-transients after preincubation with pertussis toxin. (a) Morphine, at the indicated concentrations, was added to cultures of neonatal cardiac myocytes which were preincubated overnight with pertussis toxin (10 μg/ml) (full symbols), and to cultures of the same preparation preincubated without pertussis toxin (open symbols, broken lines). Amplitudes of contraction (ASM) (▲,△) and amplitudes of $[Ca^{2+}]_i$ transients (IFR) (●,○) were measured simultaneously before, and 10 min after the addition of morphine, as described in Materials and Methods. Data are means ± S.E. of six measurements. (b) Representative original traces of indo-1 fluoresence ratio (IFR) and amplitude of contraction (ASM) recorded simultaneously from neonatal cardiac myocyte cultures which were pre-incubated overnight with pertussis toxin. The recording was done before and 10 min after the addition of morphine (0.1 μM).

effects mediated through the δ and κ opioid receptors. In the presence of a specific antagonist to the δ opioid receptors, morphine caused, via the κ receptors, a transient increase in Ca^{2+} influx, leading to increased $[Ca^{2+}]_i$ transients, and increased amplitude of contraction, with no effect on the intracellular pH. In the presence of a specific antagonist to the κ opioid receptor the effects of morphine were mediated through the δ opioid receptors and consisted of a decrease in the amplitude of contraction, caused by a decrease in the intracellular pH leading to reduced responsiveness of the myofilaments to Ca^{2+} (Fabiato and Fabiato, 1978). There was no change in Ca^{2+} influx and in the amplitude of $[Ca^{2+}]_i$ transients. Preincubation with pertussis toxin led to effects similar to those obtained in the presence of naltrindole: no decrease in intracellular pH and a positive inotropic effect in response to morphine. On the other hand, preincubation with pertussis toxin did not affect the increase in $[Ca^{2+}]_i$ transients induced by morphine via the κ opioid receptors. Our results indicate, therefore, that the δ opioid receptors in cardiac myocytes, but not the κ opioid receptors, are coupled to pertussis toxin sensitive GTP-binding proteins. The overall effects of morphine on neonatal cardiac myocytes consisted of the sum of the effects exerted by morphine via each of the opioid receptors alone.

The direct effects of specific δ and κ opioid receptor agonists on the functions of cardiac myocytes have been previously studied in isolated cardiac myocytes from adult rats (Ventura *et al.*, 1992). In these cells the κ opioid receptor agonist U-50,488H caused a transient increase in contraction which was followed by a sustained decrease. These changes were accompanied by alkalosis and increased responsiveness of the myofilaments to Ca^{2+}. The δ opioid receptor agonist DPDPE caused a sustained decrease in contractility without an initial increase. Changes similar to these in the contractile amplitudes were observed in $[Ca^{2+}]_i$ transients. Marked and sustained increase in inositol 1,4,5-trisphosphate (IP_3) was observed following κ and δ opioid receptor stimulation. It was suggested that the negative inotropic responses to the κ and δ opioid receptor agonists were mediated by sarcoplasmic reticulum Ca^{2+} depletion (Ventura *et al.*, 1992).

The differences between the responses to opioid receptor stimulation in neonatal and adult rat cardiac myocytes may result from the differences in the developmental stage of the cardiomyocytes. In this regard, developmental differences in the responses to opioid receptor stimulation have been reported in nerve cells from chicks and from rats.

(Sakellaridis and Vernadakis, 1986; Barg *et al.*, 1989). Neonatal ventricular myocytes are immature cells with less developed sarcoplasmic reticulum than adult cells. Studies on the effects of ryanodine and nicardipine on the inotropic response of cardiac muscles of neonatal and adult rats indicated that contraction of adult rat myocardium is highly dependent on Ca^{2+} release from the sarcoplasmic reticulum, while that of the neonatal rat is more dependent on trans-sarcolemma Ca^{2+} influx (Tanaka and Shigenobu, 1989). Comparison of the effects of thapsigargin on the amplitude of systolic motion in neonatal and adult cardiomyocytes has shown that inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase by thapsigargin in adult cells decreased the amplitude of contraction by 76.3%, whereas complete inhibition of the function of the sarcoplasmic reticulum in neonatal myocytes reduced the amplitude by only 34% of control (Ela *et al.*, 1994; Novakova *et al.*, 1995). These differences may help to explain the different response to morphine in neonatal and adult rat cardiac myocytes. Because in neonatal cardiac myocytes contraction depends mainly on the trans-sarcolemma Ca^{2+} influx, effects of δ and κ receptor ligands on sarcoplasmic reticulum Ca^{2+} release may not be observed, or alternatively, do not exist in neonatal cardiac myocytes.

Differences in responses to activation of other receptors in cardiomyocytes from neonatal rats and adult rabbits and rats have been reported recently (Kohmoto *et al.*, 1993). Exposure of cultured neonatal rat ventricular myocytes to endothelin-1 (10 nM), angiotensin II (10 nM) or 12-O-tetradecanoylphorbol-13-acetate (TPA) (80 nM) decreased both the amplitude of cell contraction and the $[Ca^{2+}]_i$ transients. The same ligands induced marked increases in the amplitude of contraction with no change in $[Ca^{2+}]_i$ transients in adult rabbit cardiomyocytes. A similar increase in contraction amplitude induced by endothelin-1 has been observed in adult rat ventricular myocytes (Kramer *et al.*, 1991). The mechanisms mediating these differences have not yet been elucidated. Differences were also found in the responses of ventricular myocytes from neonatal and adult rats to the activation of σ receptors (Novakova *et al.*, 1995).

An additional cause for the difference between the results of the two groups may be the differences in the experimental procedures, since the results of Ventura *et al.* (1991b; 1992) were obtained at 23°C and the results of the present study were obtained at 37°C.

Our results show that activation of κ receptors in neonatal rat cardiac myocytes increased the influx of Ca^{2+} and the amplitude of $[Ca^{2+}]_i$ transients. This result is consistent with the finding that κ agonists cause arrhythmia in perfused hearts (Wong and Lee, 1987; Wong *et al.*, 1990) and increase the level of cytosolic free Ca^{2+} in isolated rat ventricular myocytes (Tai *et al.*, 1992; Ventura *et al.*, 1994). The mechanism of the response to κ receptor activation is not known. It was previously reported that exposure of adult rat cardiomyocytes to the κ agonist U-50,488H causes a marked increase in inositol 1,4,5-trisphosphate (IP_3) and in inositol 1,3,4,5-tetrakisphosphate (IP_4) formation (Ventura *et al.*, 1991a). It has been found in various types of cells that IP_4 (Shirakawa and Miyazaki, 1995) and IP_3 (Mochizuki-Oda *et al.*, 1994) increase the rates of Ca^{2+} influx across cell membranes (see also Berridge, 1993), either directly or indirectly via a diffusible messenger activated by depletion of IP_3-Ca^{2+} stores (Randriamampita and Tsien, 1993; Parekh *et al.*, 1993). If similar mechanisms operate in cardiac myocytes, the observed increase in Ca^{2+} influx in neonatal cardiac myocytes following κ opioid receptor activation may be mediated through a similar pathway. The increase in the amplitude of $[Ca^{2+}]_i$ transients caused by κ receptor activation, appears to be mediated by the increase in Ca^{2+} influx (present study) together with mobilization of Ca^{2+} from intracellular stores by IP_3 (Ventura *et al.*, 1992). However, in neonatal cardiomyocytes the contribution of the second component to the amplitude of $[Ca^{2+}]_i$ transients is relatively smaller than in adult cardiac myocytes (Tanaka and Shigenobu, 1989).

Activation of the δ opioid receptors led to a negative inotropic response in cardiomyocytes from both adult and neonatal rats but the mechanisms mediating these effects appear to be different. In adult cardiomyocytes activation of δ receptors led to depletion of sarcoplasmic reticulum Ca^{2+} (Ventura *et al.*, 1992), whereas in neonatal cardiac myocytes this activation led to a decrease in intracellular pH and consequently to reduced responsiveness of the myofilaments to Ca^{2+}. This response was mediated *via* pertussis toxin sensitive GTP-binding proteins, protein kinase C and the Na^+/H^+ exchanger (Ela *et al.*, 1993 and the present study). A different response was reported in chick embryo cardiomyocytes, in which δ receptor agonists caused an increase in contractility mediated by an increase in the level of cAMP (Laurent *et al.*, 1985; 1986). It appears that the responses and the signal transduction pathways coupled to δ opioid receptor activation in cardiac myocytes are different in various species and may depend on the developmental stage.

In conclusion in the present study we show that morphine activates both κ and δ opioid receptors in cardiac myocytes from neonatal rats. Activation of each of the opioid receptors alone leads to specific and different effects which are mediated via a different signal transduction pathway. The overall effect of morphine on cardiac myocytes from neonatal rats appears to be the sum of the effects mediated by the δ and κ opioid receptors with no evidence for "cross-talk" between the signals from the two receptors.

Acknowledgement

This study was supported by a grant (to YE) from the Chief Scientist, The Ministry of Health, Israel. This study is a part of a Ph.D. thesis to be submitted by Ms C. Ela to the senate of the Hebrew University, Jerusalem.

References

Barg J, Levy R, Simantov R, 1989. Paradoxical and subtype-specific effects of opiate antagonists on the expression of opioid receptors in rat brain cultures. *J Neurosci Res* **22:** 322–330.

Berridge MJ, 1993. Inositol trisphosphate and calcium signalling. *Nature* **361:** 315–325.

Ela C, Hasin Y, Eilam Y, 1993. Opioid effects on contractility, Ca^{2+}-transients and intracellular pH in cultured cardiac myocytes. *J Mol Cell Cardiol* **26:** 599–613.

Ela C, Barg J, Vogel Z, Hasin Y, Eilam Y, 1994. *Sigma* receptor ligands modulate contractility, Ca^{2+} influx and beating rate in cultured cardiac myocytes, *J Pharmacol Exp Ther* **269:** 1300–1309.

Fabiato A, Fabiato F, 1978. Effects of pH on the myofilaments and the sarcoplasmic reticulum on skinned cells from cardiac and skeletal muscles. *J Physiol (Lond)* **276:** 233–255

Hallaq H, Hasin Y, Fixler R, Eilam Y, 1989. Effect of ouabain on the concentration of free cytosolic Ca^{2+} and on contractility in cultured rat cardiac myocytes. *J Pharmacol Exp Ther* **248:** 716–721.

Jin W-Q, Tai KK, Chan TKY, Wong TM,1995. Further characterization of [^{3}H]U69593 binding sites in the rat heart. *J Mol Cell Cardiol* **27:** 1507–1511.

Kohmoto O, Ikenouchi H, Hirata Y, Momomura S-I, Serizawa T, Barry WH, 1993. Variable effects of endothelin-1 on $[Ca^{2+}]_i$ transients, pH_i, and contraction in ventricular myocytes. *Am J Physiol* **265:** H793–H800.

Kramer BK, Smith TW, Kelly RA, 1991. Endothelin and increased contractility in adult rat ventricular myocytes: role of intracellular alkalosis induced by activation of the protein kinase C-dependent Na^+–H^+ exchanger. *Circ Res* **68:** 269–279.

Laurent S, Marsh JD, Smith TW, 1985. Enkephalins have a direct positive inotropic effect on cultured cardiac myocytes. *Proc Natl Acad Sci USA* **82:** 5930–5934.

Laurent S, Marsh JD, Smith TW, 1986. Enkephalins increase cyclic adenosine monophosphate content, Ca^{2+} uptake and contractile state in cultured chick embryo heart cells. *J Clin Invest* **77:** 1436–1440.

Lowry DA, Rosenbrough NJ, Farr AL, Randall RJ, 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265–275.

Mochizuki-Oda N, Nakajima Y, Nakanishi S, Ito S, 1994. Characterization of substance P receptor-mediated calcium influx in cDNA transfected Chinese hamster ovary cells, a posible role of inositol 1,4,5-trisphosphate in calcium influx. *J Biol Sci* **269:** 9651–9658.

Novakova M, Ela C, Barg J Vogel Z, Hasin Y, Eilam Y, 1995. Inotropic action of σ receptor ligands in isolated cardiac myocytes from adult rats. *Eur J Pharmacol* **286:** 19–30.

Parekh AB, Terlau H, Stuhmer W, 1993. Depletion of $InsP_3$ stores activates a Ca^{2+} and K^+ current by means of a phosphatase and a diffusable messenger. *Nature* **364:** 814–818.

Peeters GA, Hlady V, Bridge JHB, Barry H, 1987. Simultaneous measurement of calcium transients and motion in cultured ventricular cells. *Am J Physiol* **253** (*Heart Circ Physiol* **22**), H1400–H1408.

Portoghese PS, Lipkowski AW, Takemori AE, 1987 Binaltorphimine and nor-binaltorphimine, potent and selective κ opioid receptor antagonists. *Life Sci* **40:** 1287–1292.

Portoghese PS, Sultana M, Takemori AE, 1988. Naltrindole, a highly selective and potent non-peptide δ opioid receptor antagonist. *Eur J Pharmacol* **146:** 185–186.

Randriamampita C, Tsien RY, 1993. Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. *Nature* **364:** 809–814.

Rothman RB, Bykov V, deCosta BR, Jacobson AE, Rice KC, Brady LS, 1990. Interaction of endogenous opioid peptides and other drugs with four kappa opioid binding sites in guinea pig brain. *Peptides* **11:** 311–331.

Sakellaridis N, Vernadakis A, 1986. An unconventional response of adenylate cyclase to morphine and naloxone in chicken during early development. *Proc Natl Acad Sci USA* **83:** 2738–2742.

Shirakawa H, Miyazaki S, 1995. Evidence for inositol tetrakisphosphate-activated Ca^{2+} influx pathway refilling inositol trisphosphate-sensitive Ca^{2+} stores in hamster eggs. *Cell Calcium* **17:** 1–13.

Springhorn JP, Claycomb WC, 1989. Proenkephaline mRNA expression in developing rat heart and in cultured ventricular cardiac muscle cells. *Biochem J* **258:** 73–78.

Spurgeon HA, Stern MD, Baartz G, Raffaeli S, Hansford RG, Talo A, Lakatta EG, Capogrossi MC, 1990. Simultaneous measurement of Ca^{2+}, contraction and potential in cardiac myocytes. *Am J Physiol* **258**: (*Heart Circ Physiol* **27**): H574–H586.

Tai KK, Bian CF, Wong TM, 1992. κ-Opioid receptor stimulation increases intracellular free calcium in isolated rat ventricular myocytes. *Life Sci* **51:** 909–913.

Tanaka H, Shigenobu K, 1989. Effect of ryanodine on neonatal and adult rat heart: Developmental increase in sarcoplasmic reticulum function, *J Mol Cell Cardiol* **21:** 1305–1313.

Ventura C, Bastagli L, Bernardi P, Caldarera CM, Guarrnieri C, 1989. Opioid receptors in rat cardiac sarcolemma: effects of phenylephrine and isoproterenol. *Biochim Biophys Acta* **987:** 69–74.

Ventura C, Capogrossi MC, Spurgeon HA, Lakatta EG, 1991b. κ-Opioid peptide receptor stimulation increases cytosolic pH and myofilament responsiveness to Ca^{2+} in cardiac myocytes. *Am J Physiol* **261** (Heart Circ. Physiol. 30): H1671–H1674.

Ventura C, Guarnieri C, Stefanelli C, Cirielli C, Lakatta EG, Capogrossi MC, 1991a. Comparison between alpha-adrenergic and κ-opioidergic mediated inositol (1,4,5)P_3 / inositol (1,3,4,5)P_4 formation in adult cultured rat ventricular cardiomyocytes. *Biochem Biophys Res Commun* **179:** 972–978.

Ventura C, Spurgeon H, Lakatta EG, Guarnieri C, Capogrossi MC, 1992. κ and δ opioid receptor stimulation affects cardiac myocyte function and Ca^{2+} release from an intracellular pool in myocytes and neurons. *Circ Res* **70**: 66–81.

Ventura C, Guarnieri C, Vaona I, Campana G, Pintus G, Spampinato S, 1994. Dynorphine gene expression and release in myocardial cells. *J Biol Chem* **269**: 5384–5396.

Wong TM, Lee AYS, 1987. Chronic morphine treatment reduces the incidence of ventricular arrhythmias in isolated rat heart induced by $dynorphin_{1-13}$ or myocardial ischemia and reperfusion. *Neurosci Lett* **77:** 61–65.

Wong TM, Lee AYS, Tai KK, 1990. Effects of drugs interacting with opioid receptors during normal perfusion or ischemia and reperfusion in isolated rat heart - an attempt to identify cardiac opioid receptor subtype(s) involved in arrhythmias. *J Mol Cell Cardiol* **22:** 1167–1175.

Xiao R-P, Spurgeon HA, Capogrossi MC, Lakatta EG, 1993. Stimulation of opioid receptors on cardiac ventricular myocytes reduces L type Ca^{2+} channel current. *J Mol Cell Cardiol* **25:** 661–666.

Zimlichman R, Gefel D, Eliahou H, Matas Z, Rozen B, Gass S, Ela C, Eilam Y, Vogel Z, Barg J, 1996. Expression of opioid receptors during heart ontogeny in normotensive and hypertensive rats. *Circulation* **93:** 1020–1025.