Opioid peptides are coreleased with other neurotransmitters from sympathetic and parasympathetic nerve endings and presynaptic terminals. They act on somatomotor nerve terminals and may influence heart rate and blood pressure through presynaptic modulation of neurotransmitter release.

We investigated the effects of \( \mu \), \( \delta \), and \( \kappa \) opioid receptor stimulation on the contractile properties and cytosolic \( Ca^{2+} \) (\( [Ca]_c \)) of adult rat left ventricular myocytes. Cells were field-stimulated at 1 Hz in 1.5 mM bathing \( Ca^{2+} \) at 23°C. The \( \mu \)-agonist \([\text{d-Ala}^2,\text{N-Me-Phe}^4,\text{Gly}^\text{\beta}-\text{ol}]\)-enkephalin (10\(^{-5}\) M) had no effect on the twitch. The \( \delta \)-agonists methionine enkephalin and leucine enkephalin (10\(^{-10}\) to 10\(^{-6}\) M) and the \( \kappa \)-agonist \((\text{trans-(dl)})\cdot3,4\)-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclo-hexyl]-benzeneacetamide)methanesulfonate hydrate (U-50,488H; 10\(^{-7}\) to 2\(\times\)10\(^{-5}\) M) had a concentration-dependent negative inotropic action. The sustained decrease in twitch amplitude due to U-50,488H was preceded by a transient increase in contraction. The effects of \( \delta \)- and \( \kappa \)-receptor stimulation were antagonized by naloxone and \((-\)-(3-furyl-methyl)-\(\alpha\)-normetazocine methanesulfonate, respectively. In myocytes loaded with the \( Ca^{2+} \) probe indo-1, the effects of leucine enkephalin (10\(^{-8}\) M) and U-50,488H (10\(^{-5}\) M) on the twitch were associated with similar directional changes in the \( Ca \) transient. Myofilament responsiveness to \( Ca^{2+} \) was assessed by the relation between twitch amplitude and systolic \( Ca^{2+} \) transient. Leucine enkephalin (10\(^{-8}\) M) had no effect, whereas U-50,488H (10\(^{-5}\) M) increased myofilament responsiveness to \( Ca^{2+} \). We subsequently tested the hypothesis that \( \delta \) and \( \kappa \) opioid receptor stimulation may cause sarcoplasmic reticulum \( Ca^{2+} \) depletion. The sarcoplasmic reticulum \( Ca^{2+} \) content in myocytes and in a caffeine-sensitive intracellular \( Ca^{2+} \) store in neurons was probed in the absence of electrical stimulation via the rapid addition of a high concentration of caffeine from a patch pipette above the cell. U-50,488H and leucine enkephalin slowly increased \( Ca \), or caused \( Ca \) oscillations and eventually abolished the caffeine-triggered \( Ca \) transient. These effects occurred in both myocytes and neuroblastoma-2a cells. In cardiac myocyte suspensions U-50,488H and leucine enkephalin both caused a rapid and sustained increase in inositol 1,4,5-trisphosphate. Thus, \( \delta \) and \( \kappa \) but not \( \mu \) opioids have a negative inotropic action due to a decreased \( Ca \) transient. The decreased twitch amplitude due to \( \kappa \)-receptor stimulation is preceded by a transient increase in contractility, and it occurs despite an enhanced myofilament responsiveness to \( Ca^{2+} \). The effects of \( \delta \) and \( \kappa \) opioids appear coupled to phosphatidylinositol turnover and, at least in part, may be due to sarcoplasmic reticulum \( Ca^{2+} \) depletion. \( Ca^{2+} \) release and depletion of an intracellular store site occur in both myocytes and neurons and may represent a general mechanism for the effects of opioids. (Circulation Research 1992;70:66–81)
reach the heart either via the systemic circulation, 10,11 when released from the adrenal medulla, 12-15 or more directly, when coreleased with other neurotransmitters from the nerve endings distributed within the cardiac tissue. 16-21 Recently, large amounts of mRNA for preproenkephalin A have been identified in the rat ventricle, 22 in cultured rat ventricular myocytes, 23 and in the hamster heart, 24 which raises the possibility that the cardiac myocyte may be capable of synthesizing opioid peptides. These reports suggest that the myocardium may be exposed to opioid receptor agonists in vivo, and from binding studies there is evidence that opioid receptors are present in rat cardiac sarcolemma. 25 However, the effect of opioid peptides on myocardial function is still poorly defined. 26 In multicellular preparations, which possess nerve terminals, opioids have been reported to have no inotropic effect 27 as well as a positive 28,29 and a negative 30-33 inotropic effect. A positive inotropic action of enkephalins has been described in cultured chick embryo heart cells. 34,35 However, it is still unclear whether opioid peptides have a direct action on mammalian myocardium rather than an effect due to modulation of other neurotransmitter release. Additionally, the specific effect of agonists to each of the three known opioid receptors, 36-38 , δ, and κ, on myocardial function is still unknown. The purpose of this study was 1) to characterize the effect of opioid agonists specific for the μ-, δ-, and κ-receptors on the contraction and cytosolic Ca2+ (Ca i ) transient of adult rat left ventricular myocytes, 2) to determine the effect of opioid receptor stimulation on Ca2+ homeostasis of myocardial cells, 3) to define a possible mechanism that couples receptor stimulation to the effects of opioids on myocardial function, and 4) to determine whether similarities exist in the mechanism of action of opioid receptor agonists between myocardial and neuronal cells, which would help establish whether there may be a general mechanism for the action of opioids.

Materials and Methods

Myocyte Isolation Procedure

Left ventricular myocytes were enzymatically dissociated as previously described. 39 Briefly, 4-6-month-old male Wistar rats from the Gerontology Research Center Colony were killed by decapitation; the heart was quickly removed and retrogradely perfused with 25 ml nominally Ca2+-free buffer of the following composition (mM): NaCl 116.4, NaHCO3 26.2, Na2HPO4 1.0, KCl 5.4, MgSO4 0.8, and D-glucose 5.5. This medium was not recirculated and was continuously gassed with 95% O2-5% CO2 to keep the pH at 7.35±0.05. Temperature was 36±1°C. The perfusate was then switched to a similar solution to which collagenase and CaCl2 had been added to achieve a final concentration of 160 units/ml and 60 μM, respectively. After 15-30 minutes of perfusion with this medium, the left ventricle was isolated, and single cardiac myocytes were mechanically disaggregated and resuspended in a bicarbonate buffer with 1.8 mM bathing Ca2+ (Ca o ).

Contractile Measurements

The contractile properties of isolated myocytes were assessed as previously described. 39,40 Briefly, a 0.2-ml cell suspension was added to a lucite chamber of 0.7-ml volume, which was placed on the stage of an inverted microscope equipped with phase-contrast optics. A glass coverslip constitutes the bottom of this chamber. The time required for complete turnover of the perfusate in this chamber is ~30-60 seconds. The cells were continuously superfused with a buffer of the following composition (mM): NaCl 137, MgSO4 1.2, CaCl2 1.5, KCl 5, HEPES 20, and d-glucose 15 (pH 7.4). Ca i was 1.5 mM unless otherwise indicated. Experiments were done at 23°C. Two platinum electrodes placed in the bathing fluid were connected to a stimulator (model SD9, Grass Instrument Co., Quincy, Mass.) and were used to field-stimulate the myocyte to twitch at 1 Hz with 2-4-msec pulses. The image of the individual cell under study was projected onto a photodiode array, and changes in length were quantified through edge-motion detection. The signal was then transmitted to a chart recorder (Brush 220, Gould, Cleveland, Ohio) and to a VAX 11/730 computer for on-line analysis of twitch parameters. The extent of shortening during the twitch was expressed as percent of the resting cell length. The time course of the contraction was measured as the time from the electrical stimulus to 90% relaxation.

Simultaneous Measurements of Cell Length and Ca i

In some experiments, cell length and Ca i were measured simultaneously as recently described. 40 Briefly, dissociated myocytes bathed in HEPES-buff ered medium were loaded at 23°C with the ester derivative (AM form) of the Ca2+ probe indo-1, 41 dissolved in dimethyl-sulfoxide, and mixed with fetal calf serum and a dispersing agent (Pluronic F-12, BASF Wyandotte, Wyandotte, Mich.). 42 Indo-1 fluorescence was excited by epi-illumination with 10-μsec flashes of 350±5 nm light. Paired photomultipliers collected indo-1 emission by simultaneously measuring spectral windows of 391-434 and 457-507 nm selected by band-pass interference filters. The ratio of indo-1 emission at the two wavelengths was calculated as an index of [Ca2+] i by using a pair of fast integrator sample-and-hold circuits under the control of a VAX 11/730 computer. Cell length was monitored from the bright field image of the cell, which was projected onto a photodiode array (1024SAQ/RC1024 LNA Starlight, Reticon, Sunnyvale, Calif.) with a 5-msec time-scan rate. By using red light (650-750 nm) for the bright field image and a dichroic mirror (600-nm short pass) to transmit the fluorescent light (395-510 nm), cell length and Ca i were measured simultaneously without cross talk. Experiments were implemented at 23°C. It has recently
been shown that, when cardiac myocytes are loaded with the ester derivative of indo-1, significant compartmentalization of the indicator occurs in the mitochondria. Because the degree of compartmentalization is not identical in all myocytes, this prevents the use of a standard calibration curve to determine the absolute value of Ca. For this reason, the indo-1 signals shown in this study are not calibrated; rather, directional changes in indo-1 fluorescence ratio transient are taken as an indicator of Ca.

**Indo-1 Fluorescence Measurement During Caffeine Exposure**

In the absence of electrical stimulation, the Ca$^{2+}$ content of the sarcoplasmic reticulum (SR) in cardiac myocytes and of an intracellular Ca$^{2+}$ pool in neuroblastoma-2a cells (American Type Culture Collection, Rockville, Md.) was assessed from the increase in indo-1 fluorescence ratio obtained on the addition of caffeine. Release of Ca$^{2+}$ was elicited by rapid extracellular application of caffeine (15 mM dissolved in the superfusion buffer) from a micropipette positioned directly above the cell. Pressure pulses of 20 psi were applied to the pipette with a Picospritzer II (General Valve Corp., Fairfield, N.J.). The duration of these pulses was 200 msec for the experiments with myocytes, and 6 minutes for the studies with neuroblastoma-2a cells. Cells were perfused at room temperature with HEPES buffer.

**Inositol 1,4,5-Trisphosphate Assay**

Left ventricular myocytes resuspended at a final protein concentration of 1.7–1.9 mg/mL were incubated for 10 minutes in the presence of 10 mM LiCl before the addition of the opioid receptor agonist. Each incubation reaction was quenched, at a specified time, with cold HClO$_4$ (0.6 M). After centrifugation at 2,500g for 10 minutes, the pellet was dissolved in 0.3 M NaOH, and the protein content was assayed using bovine serum albumin as a standard. The acid supernatant was neutralized with 1.5 M KOH, containing 60 mM HEPES, to pH 7.0, and then the inositol 1,4,5-trisphosphate (IP$_3$) content was estimated using an IP$_3$ binding protein assay (Amersham Corp., Arlington Heights, Ill.).

**Materials**

Methionine enkephalin and leucine enkephalin are naturally occurring opioid peptides that act predominantly as δ-agonists and were purchased from Serva Fine Biochemicals, Westbury, N.Y. (trans-(dl)-3,4-Dichloro-N-methyl-N-[2-[(1-pyrrolidinyl)cy clohexyl]benzeneacetamide) methanesulfonate hydrate (U-50,488H) is a selective κ-agonist and was purchased from The Upjohn Co., Kalamazoo, Mich. Dynorphin A (fragment 1–17) is a naturally occurring peptide that binds to κ-receptors, and it was purchased from Peninsula Laboratories, Belmont, Calif. [d-Ala$^2$,N-Me-Phe$^4$,Gly$^8$-ol]-enkephalin (DAGO), a selective μ-agonist, was purchased from Sigma Chemical Co., St. Louis, Mo. Naloxone hydrochloride, a δ-receptor antagonist, was a gift of duPont de Nemours & Co., Wilmington, Del. (−)-N-(3-Furylmethyl)-α-normetazocine methanesulfonate (Mr 1452), a selective κ-receptor antagonist, was a gift of Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, Conn. Caffeine was purchased from Sigma. Ryanodine was purchased from Progressive Agri Systems, Windgap, Pa.

**Statistical Analysis**

The data were analyzed as the percentage of the value in the absence of the drug, and each cell served as its own control. The results are expressed as mean±SEM.

The concentration–response curves and the maximum effect of the various agonists were compared by a one-way analysis of variance (ANOVA), followed by a Bonferroni test. Comparison of the different agonists across time was done with a two-way repeated-measures ANOVA. A value of p<0.05 was taken to indicate significance.

**Results**

**Effect of μ, δ, and κ Opioid Agonists on the Twitch**

The role of each of the three opioid receptors, μ, κ, and δ, to modulate the contraction of the myocardial cell was initially assessed in experiments with specific agonists and antagonists in myocytes not loaded with indo-1.

The selective μ-agonist DAGO had no effect on twitch amplitude at concentrations up to 10$^{-5}$ M (Figure 1A). In contrast, both methionine enkephalin (Figure 1B) and leucine enkephalin (Figure 1C), which bind predominantly to the δ-receptor and to a much smaller extent to the μ-receptor, had a significant negative inotropic action. The selective synthetic κ-agonist U-50,488H had a biphasic effect on contraction that increased transiently before a progressive diminution and also decreased diastolic cell length (Figure 1D).

It is noteworthy that the response to the δ- and κ-agonists differed in several ways. Methionine enkephalin and leucine enkephalin caused a monotonic decrease in the amplitude of the twitch (Figures 1B and 1C), whereas U-50,488H had a biphasic effect; that is, a transient increase in twitch amplitude preceded the negative inotropic action (Figure 1D). The κ-agonist, unlike the δ-agonists, caused a reduction of diastolic cell length (dotted line in Figure 1D). Finally, washout of methionine enkephalin (Figure 1B) and leucine enkephalin (Figure 1C, lower tracing) did not reverse their negative inotropic effect unless concentrations as low as 10$^{-10}$ M were used (Figure 1C, upper tracing for leucine enkephalin). In contrast, the negative inotropic effect of U-50,488H was reversible even at concentrations as high as 10$^{-3}$ M (Figure 1D).

Opioid receptor blockers were used to assess the specificity of the response to the δ- and κ-agonists. Figure 2 shows that naloxone and Mr 1452, which
FiguRe 1. Effect of opioid receptor agonists on contraction during electrical stimulation at 1 Hz in 1.5 mM Ca+. Panel A: Representative tracing showing the effect of μ opioid receptor stimulation on twitch amplitude. The μ-agonist DAGO (10^-5 M) had no effect on contraction. Panel B: Representative tracing showing the effect of δ opioid receptor stimulation with methionine enkephalin (10^-8 M) on twitch amplitude. This δ-agonist caused a monotonic decrease in contraction that was not reversible on washout. Panel C: Representative trac-ings showing the effect of δ opioid receptor stimulation with leucine enkephalin on twitch amplitude. Upper tracing shows the effect of 10^-10 M leucine enkephalin. At this concentration, the peptide decreased the amplitude of the contraction, which was reversible with washout. Lower tracing shows the effect of 10^-6 M leucine enkephalin. At this concentration, the peptide had a negative inotropic action qualitatively similar to that of 10^-8 M methionine enkephalin, and its effect was not reversible with washout. Panel D: Representative tracing showing the effect of the κ-agonist U-50,488H (10^-7 M) on contraction. This agonist had a marked negative inotropic action, which was preceded by a transient increase in twitch amplitude and was associated with a reduction in diastolic cell length. Both the decrease in the amplitude of the twitch and in diastolic cell length were reversible on washout of the drug.

bind respectively to the δ- and κ-receptors had no effect alone but fully antagonized the negative inotropic action of leucine enkephalin (panel A) as well as the transient increase in contractility, the decrease in diastolic cell length, and the sustained decrease in twitch amplitude due to U-50,488H (panel B). Similar results were obtained with methionine enkephalin and naloxone (not shown). It is noteworthy that the addition of naloxone during exposure to saturating concentrations of δ opioid receptor agonists reversed the negative inotropic action of these substances (not shown).

Figure 3A shows the concentration–response curve for the effect of methionine enkephalin, leucine enkephalin, and U-50,488H on the contractile properties of ventricular myocytes. Methionine enkephalin and leucine enkephalin had a concentration-dependent negative inotropic action that became evident at 10^-10 M, saturated at 10^-8 M, and was associated with a prolongation of the time course of the contraction. Methionine enkephalin at 10^-6 M decreased the amplitude of the contraction to 42.0±5.3% of control and prolonged the time to 90% relaxation to 176.0±7.5% of control. Leucine enkephalin at 10^-6 M decreased the amplitude of the contraction to 18.9±4.0% of control and prolonged the time to 90% relaxation to 248.1±16.6% of control. The negative inotropic effect of 10^-6 M leucine enkephalin was significantly more marked than that of 10^-6 M methionine enkephalin. The negative inotropic effect of U-50,488H became apparent at 10^-7 M and saturated at 10^-5 M. At 2x10^-5 M the κ-agonist decreased twitch amplitude to 8.6±3.9% of control and prolonged the time to 90% relaxation to 192.2±21.4% of control. The negative inotropic effect of 2x10^-5 M U-50,488H was more pronounced than that of 10^-6 M methionine enkephalin but not statistically different from that of 10^-6 M leucine enkephalin. Figure 3B shows the average time course of the effect of 10^-8 M methionine enkephalin, 10^-8 M leucine enkephalin, and 10^-5 M U-50,488H on twitch characteristics. The decrease in the amplitude of the contraction and the prolongation of the time to 90% relaxation produced by methionine enkephalin was already evident within the initial 5 minutes of exposure to the opioid, and no further decrease in twitch amplitude occurred after 15 minutes of superfusion.

Figure 2. Effect of opioid receptor antagonists on twitch stimulation by δ- and κ-receptors. Panel A: Tracing showing that pretreatment with 10^-8 M naloxone, a δ-receptor antagonist, prevented the negative inotropic action of 10^-8 M leucine enkephalin. Panel B: Tracing showing that pretreatment with 10^-6 M Mr 1452, a κ-receptor antagonist, prevented the decrease in the amplitude of the twitch and in diastolic cell length as well as the transient positive inotropic action produced by 10^-5 M U-50,488H, a κ-agonist.
Figure 3. Average effect of δ-agonists methionine enkephalin and leucine enkephalin and of κ-agonist U50,488H on twitch characteristics. Results are expressed as the percentage of the values before the exposure to the agonist. Each cell was taken as its own control. Absolute values for the control condition for methionine enkephalin (n=20) were as follows: twitch amplitude (●), 6.6±0.4% of the resting cell length; time from the stimulus to 90% relaxation (○), 369.5±26.6 msec. Absolute values for the control condition for leucine enkephalin (n=20) were as follows: twitch amplitude (▲), 7.5±0.5% of the resting cell length; time from the stimulus to 90% relaxation (△), 359.7±18.5 msec. Absolute values for the control condition for U-50,488H (n=22) were as follows: twitch amplitude (■), 9.9±0.7% of the resting cell length; time from the stimulus to 90% relaxation (□), 459.7±23.3 msec. Panel A: Concentration–response curve. Myocytes were exposed to only one concentration of the agonist, and each point represents the mean±SEM of four or five cells. All measurements were obtained under steady-state conditions after 20 minutes of superfusion with either methionine enkephalin or leucine enkephalin or after 15 minutes of superfusion with U-50,488H. Note the decrease in the amplitude of the contraction and prolongation of the time to 90% relaxation. Panel B: Time course showing the effect of 10⁻⁸ M methionine enkephalin, 10⁻⁹ M leucine enkephalin, and 10⁻⁵ M U-50,488H. Note that the κ-agonist develops with a faster time course than either methionine enkephalin or leucine enkephalin (see text). Myocytes are the same as depicted in panel A for the same agonist concentrations.

Effect of δ and κ Opioid Agonists on the Simultaneously Recorded Twitch and CaTransient

Experimental protocols similar to those previously described were used for the experiments to be reported in this section. Myocytes were loaded with the Ca²⁺ probe indo-1, and changes in cell length and Caᵢ were monitored simultaneously during electrical stimulation at 1 Hz in 1.5 mM Caᵢ. Figure 4 depicts the effect of 10⁻⁸ M leucine enkephalin on the twitch and Ca transient. The continuous tracing in panel A and the average data in panel B of this figure show that the effect of leucine enkephalin in these indo-1–loaded cells is similar to that in unloaded myocytes depicted in Figures 1C and 3. Additional insights on the action of leucine enkephalin can be gained from simultaneously recording indo-1 fluorescence and cell length. In Figure 4A, below the continuous upper recording of cell length, are shown the twitch and Ca transient under control conditions (a) and when the effect of the opioid was maximal (b). When these signals are superimposed (c), it is apparent that the

The effect of 10⁻⁸ M leucine enkephalin on the twitch was noticeable after 1 minute of exposure. The time course of the decrease in the amplitude of the contraction caused by leucine enkephalin was faster than that caused by a similar concentration of methionine enkephalin.

The response to U-50,488H (10⁻⁵ M) occurred more rapidly than the response to either δ-agonist. It is noteworthy that the transient increase in twitch amplitude depicted in Figure 1 represents a consistent finding of the response to U-50,488H. However, this increase occurred shortly after exposure to the κ-agonist, and it usually resolved within 1 minute. For this reason, it is not reflected in the average data in Figure 3B.

To determine whether the changes in twitch contraction caused by δ and κ opioid receptor agonists were paralleled by similar changes in Caᵢ in other experiments we monitored the effect of opioid peptides on the simultaneously recorded changes in cell length and indo-1 fluorescence ratio.
negative inotropic action of the peptide was associated with a decrease in the peak systolic indo-1 ratio. Additionally, a decrease in diastolic indo-1 fluorescence also occurred, and it was paralleled by an increase in diastolic cell length. When these tracings are superimposed and also normalized to the same peak amplitude (d), the effect of leucine enkephalin to prolong the time to 90% relaxation of the contraction and of the Ca transient becomes evident. Average data on the effect of leucine enkephalin (10^-8 M) on twitch parameters and the simultaneously recorded indo-1 fluorescence ratio are shown respectively in Figures 4B and 4C. Thus, leucine enkephalin

FIGURE 4. Effect of 10^-8 M leucine enkephalin on the simultaneously recorded indo-1 fluorescence ratio and cell length. Panel A: Continuous recording showing the negative effect of leucine enkephalin on the twitch (upper tracing). The lower tracings were obtained at the times indicated by the letters in the continuous tracing and show the simultaneously recorded Ca transient and twitch under control conditions (a) and when the effect of the peptide was maximal (b). The tracings from a and b have been superimposed at c, and the tracings from a and b have been superimposed and normalized to the same peak amplitude at d (see text). Arrows indicate control tracings. Panel B: Average time course showing the effect of leucine enkephalin on twitch characteristics (n=4). Results are expressed as percentage of control. Absolute values for the control condition were as follows: twitch amplitude (m), 8.6±0.9% of the resting cell length; time from the stimulus to 90% relaxation (b), 423.0±15.3 msec. Panel C: Average time course showing the effect of leucine enkephalin on indo-1 fluorescence ratio for the same myocytes as in panels A and B. The results are expressed as percentage of control. Absolute values for the control condition were as follows: the amplitude of the Ca transient (m, systolic−diastolic indo-1 ratio), 0.16±0.05; time from the stimulus to 90% relaxation of the indo-1 transient (b), 451.2±38.8 msec.

FIGURE 5. Effect of k-agonist U-50,488H (10^-5 M) on the simultaneously recorded indo-1 fluorescence ratio and cell length. Panel A: Continuous recording showing the effect of U-50,488H on the twitch (upper tracing). The lower tracings were obtained at the times indicated by the letters in the continuous tracing and show the simultaneously recorded Ca transient and twitch under control conditions (a), during the transient increase in contractility (b), and when the effect of the agonist was maximal (c). The tracings from a, b, and c have been superimposed and normalized to the same peak amplitude at d (see text). Arrows indicate control tracings. Panel B: Average time course showing the effect of U-50,488H on twitch characteristics (n=7). Results are expressed as percentage of control. Absolute values for the control condition were as follows: twitch amplitude (m), 10.2±0.8% of the resting cell length; time from the stimulus to 90% relaxation (b), 507.6±29.2 msec. Panel C: Time course showing the effect of U-50,488H on indo-1 fluorescence ratio for the same myocytes as in panel A. The effect of the agonist on indo-1 ratio is expressed as percentage of control. Absolute values for the control condition were as follows: amplitude of the Ca transient (m, systolic−diastolic indo-1 ratio), 0.29±0.01; time from the stimulus to 90% relaxation of the indo-1 transient (b), 650.6±35.7 msec.
decreased the amplitude of the twitch and prolonged the time to 90% relaxation. These changes in contraction were associated with a decrease in the amplitude of the indo-1 ratio as well as a prolongation of the time from the electrical stimulus to 90% relaxation of the Ca transient.

Figure 5 depicts the effect of 10−3 M U-50,488H on the simultaneously recorded twitch and Ca transient. Figure 5A shows a recording from a representative myocyte. The upper continuous tracing confirms that shortly after the addition of the κ-agonist there are a transient increase in contractility and a decrease in diastolic cell length that precede the negative inotropic action of U-50,488H (also see Figure 1D). The lower tracings were obtained at the times indicated in the upper continuous recording during control conditions (a), during the transient increase in twitch amplitude (b), and when the negative inotropic action of U-50,488H was maximal (c). When the tracings from a, b, and c are superimposed (d), it is apparent that the transient increase in twitch amplitude and decrease in diastolic cell length were paralleled by an increase in both peak systolic and diastolic Ca, whereas the negative inotropic action of the κ-agonist was associated with an increase in diastolic cell length and a decrease in resting indo-1 fluorescence. During the transient positive inotropic action of U-50,488H, a small increase in Ca transient amplitude was associated with a relatively more marked increase in the amplitude of the contraction, a result that may be accounted for by an enhanced myofilament responsiveness to Ca2+. In this example, the transient diastolic cell shortening and increase in resting indo-1 ratio that occur at the onset of the superfusion with U-50,488H have resolved at the time that the negative inotropic action of the κ-agonist is maximal. It is noteworthy that in some cells diastolic cell length was still decreased despite a resting indo-1 ratio lower than in control after 15-minute exposure to the κ-agonist. When the tracings from a and c are normalized to the same peak amplitude (e), it is apparent that the negative effect of U-50,488H is associated with a prolongation of the time course of the contraction and of the Ca transient. These findings are confirmed by the average values for contraction and Ca transient shown in Figures 5B and 5C, respectively. Because the first time point was obtained 1 minute after beginning the superfusion with U-50,488H, the transient increase in the amplitude of the Ca transient and of the contraction are not reflected in these plots. Comparison of the average data in Figures 4 and 5 confirms that the effects of U-50,488H developed with a time course faster than that of leucine enkephalin.

In other experiments we used the endogenous κ-selective agonist dynorphin A to assess whether its actions were qualitatively similar to those of U-50,488H. Because dynorphin A (fragment 1-17) is unstable and may stick to the plastic tubing used for superfusion, it was directly injected into the bathing medium from a glass micropipette placed above the myocyte. This technique is described in detail in a subsequent section of the present article; its limitation is that it does not allow a close control of the concentration of the agonist to which the cell is exposed, because significant dilution occurs after its injection into the bathing medium. Figure 6 shows a representative example of the effect of dynorphin A (fragment 1-17) on a rat myocyte loaded with the Ca2+ probe indo-1. The upper continuous recording shows that dynorphin A (fragment 1-17), similar to U-50,488H, had a marked negative inotropic action that was preceded by a transient increase in twitch amplitude and was associated with diastolic cell shortening. The lower tracings of Ca and contraction in Figure 6 were obtained during the control period (a), shortly after beginning the superfusion with the agonist (b), and when the negative inotropic action of dynorphin A (fragment 1-17) was maximal (c). The tracings from a and c were superimposed (d), and the same tracings were also normalized to the same peak amplitude (e). The negative inotropic action of dynorphin A (fragment 1-17) was associated with a decrease in peak systolic Ca (d) and with prolongation in the time course of relaxation both of the twitch and of the Ca transient (e). It is noteworthy that at the time the tracing from b was obtained, peak systolic Ca was lower, and twitch shortening was enhanced in comparison with the recording at a. It cannot be excluded that data collection for the tracing from b was initiated after a transient increase in peak systolic Ca, similar to that caused by U-50,488H (Figure 5A, tracing from b); however, it still remains to be explained how the κ-agonist could enhance the amplitude of the contraction while decreasing systolic Ca. Additionally, after reaching steady state, there was persistent diastolic cell shortening in spite of a resting Ca lower than control (d). A similar relation between resting indo-1 ratio and cell length was also observed in some cells exposed to U-50,488H. These findings are consistent with the enhancement of myofilament responsiveness to Ca2+ produced by κ opioid receptor stimulation.

Effect of δ and κ Opioid Agonists on Myofilament Responsiveness to Ca2+

In myocardial tissue, myofilament responsiveness to Ca2+ can be assessed as the relation between the twitch and the simultaneously recorded systolic Ca. To obtain a spectrum of contractions and Ca transients of different amplitudes in single myocytes, we varied both the pattern of stimulation and/or Ca. Electrical stimulation was resumed after a period of rest, and in rat myocardium studied under our experimental conditions, this intervention determines a negative staircase in the amplitude of the contraction and of the associated Ca transient. This experimental protocol can be repeated after varying Ca. To use this technique to assess the effect of a substance on myofilament responsiveness to Ca2+, it is necessary to allow enough time for the substance to complete its action, that is, after its effect on twitch...
amplitude has achieved steady state. Subsequently, the effect on myofilament responsiveness to Ca\(^{2+}\) is assessed from the shift in the twitch amplitude–systolic Ca\(^{2+}\) relation from control. Thus, the effects of leucine enkephalin and U-50,488H were assessed after 20 and 15 minutes of exposure, respectively. Figure 7A shows a representative example of the effect of 10\(^{-8}\) M leucine enkephalin. The \(\delta\) opioid agonist did not affect the twitch–systolic Ca\(^{2+}\) relation. In contrast, the \(\kappa\)-agonist U-50,488H (Figure 7B) shifted this relation to the left; that is, a Ca\(^{2+}\) transient that achieved a given value of systolic indo-1 ratio was associated with an amplitude of the twitch that was more marked in the presence of U-50,488H than in control. This action was reversible with washout of the agonist. These results indicate that leucine enkephalin had no effect, whereas the \(\kappa\)-agonist reversibly increased myofilament responsiveness to Ca\(^{2+}\).

**Prolongation of the Time Course of Relaxation by \(\delta\) and \(\kappa\) Opioid Agonists**

The results presented in the previous figures show that both \(\delta\) and \(\kappa\) opioid receptor agonists have a negative inotropic action and also prolong the time course of relaxation. It is unclear whether the prolongation of the time from the stimulus to 90% relaxation results from a decrease in the Ca\(^{2+}\) transient and less activation of the Ca\(^{2+}\)-dependent mechanisms responsible for Ca\(^{2+}\) extrusion from the cytosol or whether it is due to a specific effect of the opioid agonists.

It has been previously reported that in rat55 and ferret56 papillary muscles injected with aequorin the time course of tension and Ca\(^{2+}\) are not affected by a change in Ca\(_i\). Similar results were obtained in rat ventricular myocytes loaded with indo-1 when Ca\(_i\) was varied between 1.5 and 3 mM.57 However, we are not aware of studies that have addressed this question in rat myocytes for changes in Ca\(_i\) below 1.5 mM.

Lowering Ca\(_i\) from 1.5 to 0.5 mM caused a decrease in Ca\(^{2+}\) and twitch amplitudes and a delay in the time course of both signals, comparable to the decrease and delay due to 10\(^{-8}\) M leucine enkephalin (Table 1) and 10\(^{-7}\) M U-50,488H (Table 2) in 1.5 mM Ca\(_i\). This suggests that the effect of opioid peptides to prolong the time course of relaxation is not specific. However, it cannot be excluded that delayed relaxation may also be due to a direct effect of opioids on the SR and to the enhanced myofilament responsiveness to Ca\(^{2+}\) caused by U-50,488H. The effect of the latter mechanisms on relaxation may be masked by the prolongation of the Ca\(^{2+}\) transient caused by the marked diminution in its amplitude.
Effect of $\delta$ and $\kappa$ Opioid Agonists on a Caffeine-Sensitive Intracellular Ca$^{2+}$ Store in Myocytes and Neuroblastoma-2a Cells

The experiments reported in this section were aimed at testing the hypothesis that release of Ca$^{2+}$ from the SR, resulting in a depletion of this pool, could be a mechanism by which U-50,488H and leucine enkephalin decrease the Ca$_t$ transient (Figures 4 and 5). To test this hypothesis, the releasable SR Ca$^{2+}$ pool was measured under control conditions and after exposure to either the $\kappa$ or $\delta$ opioid receptor agonist. The former was obtained after the rapid addition of a high concentration of caffeine from a pipette above the cell. Caffeine released Ca$^{2+}$ from the SR and caused a Ca$_t$ transient that was abolished after perfusion with ryanodine$^{58}$ (Figure 8A), a substance that binds to and opens the SR Ca$^{2+}$ channel,$^{59}$ thus leading to a release and depletion of Ca$^{2+}$ from this organelle.$^{60}$ Previous studies have shown that the effect of caffeine on the Ca$_t$ transient is preserved immediately after exchanging the perfusate with a Ca$^{2+}$-free buffer.$^{58}$ This indicates that the increase in Ca$_i$ caused by caffeine is due to release of Ca$^{2+}$ from the SR rather than to influx from the extracellular space. Figure 8B shows that perfusion of a quiescent cell with U-50,488H slowly increased resting indo-1 fluorescence. The subsequent rapid addition of caffeine failed to trigger a Ca$_i$ transient, which indicates that SR Ca$^{2+}$ depletion had occurred. When U-50,488H was perfused with its antagonist, Mr 1452, no increase in resting indo-1 fluorescence occurred, and the caffeine-induced indo-1 transient was unchanged from control (Figure 8C). In Figure 9 is shown the result of a similar experiment with the $\delta$ opioid receptor agonist leucine enkephalin. Perfusion of a representative myocyte with leucine enkephalin triggered Ca$_i$ oscillations,$^{59,61,62}$ which were manifested as spikes in the fluorescence signal, and abolished the caffeine-induced indo-1 transient (Figure 9A). These effects of leucine enkephalin were prevented by naloxone (Figure 9B).

We next inquired whether the $\kappa$- and $\delta$-agonist-induced release of Ca$^{2+}$ from an intracellular pool represents a general mechanism for the action of opioids and whether this release also occurred in neuronal cells. Figure 10A shows that neuroblastoma-2a cells responded to caffeine with a rapid increase in Ca$_i$. The Ca$_t$ transient observed under these conditions was due to endogenous release of Ca$^{2+}$

![Figure 7](http://circres.ahajournals.org/)

**Figure 7.** Representative examples of the effect of $\delta$-agonist leucine enkephalin and $\kappa$-agonist U-50,488H on myofilament responsiveness to Ca$^{2+}$. This was assessed by the relation between twitch shortening (as percentage of the resting cell length) and systolic Ca$_i$ for twitches in the absence and presence of the opioid receptor agonist. To obtain contractions and Ca$_i$ transients of different amplitudes, either stimulation frequency or Ca$_o$ was varied (see text). Panel A: Plot showing that the twitch–Ca$_i$ relation obtained under control conditions (■) was not affected by $10^{-8}$ M leucine enkephalin (○), indicating that the $\delta$-agonist does not affect myofilament responsiveness to Ca$^{2+}$. Panel B: Plot showing that the twitch–Ca$_i$ relation obtained under control conditions (■) was shifted to the left by $10^{-7}$ M U-50,488H (○) and that the response was fully reversible with washout (△). This result indicates that the $\kappa$-agonist enhances myofilament responsiveness to Ca$^{2+}$.

**Table 1.** Effect of Lowering Ca$_o$ From 1.5 to 0.5 mM and Effect of $10^{-8}$ M Leucine Enkephalin in 1.5 mM Ca$_o$ on the Amplitude and Time Course of the Twitch and Ca$_i$ Transient

<table>
<thead>
<tr>
<th>Effect of lowering Ca$_o$ (n=4)</th>
<th>Effect of leucine enkephalin in 1.5 mM Ca$_o$ (n=4)</th>
<th>Addition of $10^{-8}$ M leucine enkephalin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 mM Ca$_o$</td>
<td>0.5 mM Ca$_o$</td>
<td>Control</td>
</tr>
<tr>
<td>ES% 8.2±0.7</td>
<td>1.5±0.5</td>
<td>8.6±0.9</td>
</tr>
<tr>
<td>R90% (msec) 410±16.2</td>
<td>645.4±16.0</td>
<td>423.0±15.3</td>
</tr>
<tr>
<td>$\Delta$Ca$_i$ ratio 0.16±0.04</td>
<td>0.08±0.02</td>
<td>0.16±0.05</td>
</tr>
<tr>
<td>Ca$_i$, R90% (msec) 448.3±42.3</td>
<td>587.6±33.7</td>
<td>451.2±38.8</td>
</tr>
</tbody>
</table>

Values are mean±SEM. ES%, twitch amplitude as percentage of resting cell length; R90%, time to 90% relaxation of the contraction; $\Delta$Ca$_i$ ratio, Ca$_i$ transient amplitude as change in ratio (systolic–diastolic); Ca$_i$, R90%, time to 90% recovery of the Ca$_i$ transient. Leucine enkephalin in 1.5 mM Ca$_o$ had an effect on the amplitude and time course of the twitch and Ca$_i$ transient that was similar to that of lowering Ca$_o$ from 1.5 to 0.5 mM.
rather than to influx from the extracellular environment because, as in cardiac cells, it is not affected by perfusion with a Ca²⁺-free buffer (not shown). Figure 10B shows that U-50,488H slowly increased Ca, to a slightly higher level and caused frequent Ca, oscillations. The subsequent rapid addition of caffeine failed to trigger a Ca, transient similar to the control and was associated with the occurrence of two Ca, oscillations. This suggests that U-50,488H may have caused incomplete release of Ca²⁺ or that continuing reuptake and release of Ca²⁺ from an endogenous store occurred in the presence of the κ-agonist, leaving a smaller concentration of Ca²⁺ than that in control available for release by caffeine. In contrast, when the κ-agonist was perfused with its antagonist, Mr 1452, there was no effect on indo-1 fluorescence, and the subsequent caffeine-induced Ca, transient was like that in control (Figure 10C). Similar results were obtained with leucine enkephalin (Figure 11A), whose effects were blocked by naloxone (Figure 11B). Rapid release of Ca²⁺ from an endogenous pool was also observed with the κ-agonist when it was applied directly above the myocytes or neuroblastoma-2a cells and with the δ-agonist when it was added above the cardiac cells with the Picospritzer II (not shown).

**Effect of δ and κ Opioid Agonists on IP₃ Production in Myocytes**

There are conflicting reports on the mechanisms that mediate intracellular signal transduction of opioids. Previous studies have suggested that these peptides either stimulate or inhibit adenylate cyclase and cAMP production. In the myocardium, cAMP enhances SR Ca²⁺ uptake and for this reason, an opioid-mediated increase in cAMP, as seen in cultured chick embryo cardiac cells, could not account for the SR Ca²⁺ depletion observed in myocardial cells in the absence of electrical stimulation (Figures 8 and 9). A second messenger that may explain the effects of δ- and κ-receptor stimulation depicted in Figures 8 and 9 is IP₃, which in various cell types releases Ca²⁺ from an intracellular pool and increases Ca, Figure 12 shows that both U-50,488H and leucine enkephalin caused a sustained increase in the intracellular concentration of IP₃. The κ-agonist rapidly increased IP₃ sevenfold above control within 15 seconds; subsequently, IP₃ declined to a steady level, but a level that was still elevated over control at 15 minutes. In contrast, in response to δ-receptor stimulation with leucine enkephalin, the highest concentration of IP₃ was achieved after 30 seconds of exposure. The peak concentration of IP₃ was of lesser magnitude with the δ- than with the κ-agonist. The effects of δ and κ opioid receptor stimulation on IP₃ were either mark-

| Table 2. Effect of Lowering Ca, From 1.5 to 0.5 mM and Effect of κ-Agonist U-50,488H (10⁻⁵ M) in 1.5 mM Ca₅₀ on the Amplitude and Time Course of the Twitch and Ca, Transient |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| **Effect of lower Ca, (n=5)**   | **Effect of U-50,488H in 1.5 mM Ca₅₀** |
| 1.5 mM Ca₅₀ & 0.5 mM Ca₅₀       | Control                          | Addition of 10⁻⁵ M Ca₅₀          |
| ES%                            | 9.9±0.8                          | 10.2±0.8                        |
| R90% (msec)                    | 497.1±35.6                       | 507.6±29.2                      |
| ΔCa ratio                      | 0.27±0.02                        | 0.29±0.01                       |
| Ca, R90% (msec)                | 600.0±39.6                       | 650.6±35.7                      |

Values are mean±SEM. ES%, twitch amplitude as percent of the resting cell length; R90%, time to 90% relaxation of the contraction; ΔCa, ratio, Ca, transient amplitude as change in ratio (systolic-diastolic); Ca, R90%, time to 90% recovery of the Ca, transient. The κ-agonist U-50,488H, in 1.5 mM Ca,₅₀, had an effect on the amplitude and time course of the twitch and Ca, transient that was similar to that of lowering Ca, from 1.5 to 0.5 mM.
edly decreased or abolished by their specific antagonists (not shown).

Discussion

Previous studies of the effect of opioid receptor agonists on mammalian myocardial function have provided variable results and evidence for both a positive\textsuperscript{28,29} and negative\textsuperscript{30–33} inotropic action, whereas in at least another instance, no effect was found.\textsuperscript{27} However, those experimental designs used either intact animals, isolated and perfused hearts, papillary muscles, or muscle strips. Under those circumstances, the experimental results are likely to be affected by changes in preload, afterload, coronary flow, and heart rate and by the effect of opioid peptides to presynaptically modulate the release of norepinephrine and acetylcholine from the nerve endings.\textsuperscript{1–8}

In the present study, we show that \( \delta \) and \( \kappa \) opioid receptor agonists have a negative inotropic action on isolated left ventricular myocytes, whereas \( \mu \) opioid receptor stimulation has no significant effect on the contractile properties of these myocardial cells. This finding is in agreement with a recent report that has demonstrated, in sarcolemmal rat preparations, the existence of \( \delta \)- and \( \kappa \)- but not \( \mu \)- receptors.\textsuperscript{25} We also show that leucine enkephalin and U-50,488H cause release and depletion of \( \text{Ca}^{2+} \) from the SR and trigger a rapid and sustained rise in \( I_P \), production. Release of \( \text{Ca}^{2+} \) from an endogenous pool, leading to an increase in \( C_a \), \( C_a \) oscillations, and depletion of the storage site, was also observed in neuroblastoma-2a cells. In addition, significant differences exist between \( \delta \) and \( \kappa \) opioid receptor stimulation: 1) The \( \delta \)- agonist leads to a monotonic decrease in contractility and \( C_a \), whereas the \( \kappa \)- agonist has a transient positive inotropic action, associated with an increase in the amplitude of the \( C_a \) transient, that precedes the negative effect. 2) Stimulation of the \( \kappa \)- receptor increases myofilament responsiveness to \( \text{Ca}^{2+} \), whereas leucine enkephalin appears to have no effect on myofilament responsiveness to \( \text{Ca}^{2+} \). 3) The time course of the negative inotropic action of \( \kappa \)- receptor stimulation is significantly faster than that of either methionine enkephalin or leucine enkephalin. 4) The increase in \( I_P \) is more rapid and of greater magnitude in response to the \( \kappa \)- than to the \( \delta \)-agonist.

It is of interest to discuss our results in the context of various mechanisms that may account for the inotropic action of opioid peptides. These mechanisms are discussed below.

\( \text{Ca}^{2+} \) Current

With each action potential, transsarcolemmal \( \text{Ca}^{2+} \) influx via the \( \text{Ca}^{2+} \) current \( (I_{Ca}) \) triggers \( \text{Ca}^{2+} \) release from the SR\textsuperscript{70–72} and contributes to \( \text{Ca}^{2+} \) loading of the SR\textsuperscript{70} and to direct myofilament activation.\textsuperscript{73} If \( \delta \) - and \( \kappa \)- agonists decreased \( I_{Ca} \), this would result in a smaller \( C_a \) transient and twitch. Preliminary results suggest that U-50,488H decreases \( I_{Ca} \) in guinea pig myocytes,\textsuperscript{74} and a prior report has shown that in neuroblastoma\texttimes glioma hybrid cells, the \( \delta \)- agonist D-Ala\textsuperscript{2},D-Leu\textsuperscript{–} enkephalin (DADLE) reduces \( I_{Ca} \) via the GTP binding protein \( G_{\gamma} \).\textsuperscript{75} Additionally, it has been shown that in some neuronal cell types \( \kappa \)- receptor stimulation reduces \( \text{Ca}^{2+} \) action potentials without affecting \( K^+ \) conductance\textsuperscript{76–78} and that this effect may be secondary to a decrease in \( I_{Ca} \).\textsuperscript{79}

\( K^+ \) Currents

In neurons it has been shown that \( \delta \) opioid receptor stimulation activates outward \( K^+ \) currents.\textsuperscript{78,80–82} A
A similar effect has also been reported for U-50,488H in myocardial cells and is associated with a shortening of the action potential. A decrease in action potential duration would be expected to allow less time for Ca\(^{2+}\) to flow into the cell via the L-type Ca\(^{2+}\) channels and would result in decreased SR Ca\(^{2+}\) loading.

**Ca\(^{2+}\) Efflux Into the Extracellular Space**

A decrease in SR Ca\(^{2+}\) load could also result from activation of the Ca\(^{2+}\), Mg\(^{2+}\)-ATPase in the sarclemma. Alternatively, stimulation of the Na\(^{+}\)-K\(^{+}\) pump would decrease [Na\(_i\)], increase the driving force for Na\(^{+}\)-Ca\(^{2+}\) exchange in the forward mode, and enhance the efflux of Ca\(^{2+}\) via this pathway. The effect of opioids to transiently trigger Ca\(_i\) oscillations or increase Ca\(_i\) in unstimulated myocytes (Figures 8 and 9) provides indirect evidence against these mechanisms. In addition, experiments with bovine cardiac sarcolemma have shown that morphine, methionine enkephalin, and leucine enkephalin inhibit the ouabain-sensitive Na\(^{+}\),K\(^{+}\)-ATPase and that in synapsonal plasma membranes morphine inhibited Ca\(^{2+}\),Mg\(^{2+}\)-ATPase. In the intact myocardium an inhibition of either of these transport mechanisms would be expected to raise Ca\(_i\) and SR Ca\(^{2+}\) loading and could not account for the results of this study.

**Myofilament Responsiveness to Ca\(^{2+}\)**

In addition to a diminution in the amplitude of the Ca\(_i\) transient, the other general mechanism that can decrease the inotropic state of the myocardium is a decreased myofilament responsiveness to Ca\(^{2+}\). Leucine enkephalin had no effect on myofilament response to Ca\(^{2+}\); in contrast, U-50,488H enhanced myofilament Ca\(^{2+}\) interaction (Figure 7). This may be a mechanism for the initial transient positive inotropic action and the decrease in diastolic cell length in response to \(\kappa\) opioid receptor stimulation. However, myofilament response to Ca\(^{2+}\) was assessed after 15 minutes of exposure to U-50,488H, when the negative inotropic action of \(\kappa\) opioid receptor stimulation was maximal. This indicates that the decrease in the amplitude of the Ca\(_i\) transient caused by the \(\kappa\)-agonist was of such magnitude to overcome the positive inotropic action of an enhanced myofilament responsiveness to Ca\(^{2+}\). The mechanism for this effect of \(\kappa\)-receptor stimulation is still incompletely defined. However, recent experiments from our laboratory suggest that the enhanced myofilament responsiveness to Ca\(^{2+}\) may be due to cytosolic alkalinization, secondary to protein kinase C activation of Na\(^{+}\)-H\(^{+}\) exchange. Thus, a decreased myofilament Ca\(^{2+}\) sensitivity appears to have no role in the negative inotropic action of \(\delta\) and \(\kappa\)-receptor agonists.

**Release of SR Ca\(^{2+}\) by IP\(_3\)**

Our results do not establish a cause and effect relation between the increase in IP\(_3\) produced by opioids and the release and depletion of SR Ca\(^{2+}\). However, in different cell types, effects on Ca\(_i\) homeostasis similar to those described in Figures 8–11 have been observed during interventions that increase IP\(_3\) and IP\(_3\) has also been shown to release Ca\(^{2+}\) from the cardiac SR. It is also tempting to suggest a relation between the time courses of IP\(_3\) production and contraction in response to \(\kappa\) and \(\delta\)-receptor stimulation. At saturating concentrations of U-50,488H and leucine enkephalin, peak increase in IP\(_3\) was of greater magnitude and occurred with a faster time course with the \(\kappa\) than with the \(\delta\)-agonist (Figure 12), and during electrical stimulation the negative inotropic effect of U-50,488H developed with a time course

**Figure 10.** Effect of \(\kappa\)-agonist U-50,488H on the caffeine-triggered Ca\(_i\) transient in representative indo-1-loaded neuroblastoma-2a cells. Panel A: Representative tracing showing the rapid addition of caffeine (arrow) on Ca\(_i\). Caffeine triggered a Ca\(_i\) transient in all neuroblastoma-2a cells studied (n=8). In contrast to myocytes, after the rapid addition of caffeine, Ca\(_i\) returned to control only in some cells, whereas in others it showed persistent oscillations (not shown); for this reason neuroblastoma-2a cells were exposed to caffeine only once. Panel B: Representative tracing showing the effect of U-50,488H (50 \(\mu\)M). The \(\kappa\) opioid receptor agonist increased Ca\(_i\), caused Ca\(_i\) oscillations, and abolished the Ca\(_i\) transient induced by caffeine (arrow). However, the occurrence of two Ca\(_i\) oscillations during the rapid addition of caffeine suggests that the depletion of an intracellular Ca\(^{2+}\) storage site by U-50,488H might have been incomplete (see text). These effects of U-50,488H were prevented when the \(\kappa\)-antagonist Mr 1452 (5 \(\mu\)M) was perfused with U-50,488H (panel C). Under these conditions Ca\(_i\) was not increased, no Ca\(_i\) oscillations were present, and the caffeine pulse triggered a Ca\(_i\) transient similar to that in control (arrow).
faster than that of leucine enkephalin (Figure 3B). A rapid and marked increase in IP$_3$ production by the $\kappa$-agonist may facilitate the SR Ca$^{2+}$ release that occurs with each electrical stimulus and enhance Ca$_i$ transient and twitch amplitudes before the SR Ca$^{2+}$ depletion leading to the decrease in both signals (Figure 5). In contrast, a slower and less pronounced increase in IP$_3$ by the $\delta$-agonist may lead to progressive SR Ca$^{2+}$ depletion without transiently increasing contractility (Figure 4).

It has been shown that in the guinea pig hippocampus the effect of $\delta$ but not $\kappa$ opioid receptor stimulation is mediated via a pertussis toxin–sensitive G protein.$^{39}$ This suggests that a difference in the mechanism for transmembrane signal transduction may account for the different time course of the effect of $\delta$- and $\kappa$-receptor agonists.

In addition to IP$_3$, other second biochemical messengers may modulate the effect of opioids on cell function. Phosphoinositide turnover leading to IP$_3$ production is expected to be associated with enhanced protein kinase C activity.$^{69}$ In this study we did not measure protein kinase C or attempt to establish its role as a mediator of the effects that we have described. In cardiac myocytes, interventions that activate protein kinase C are known to decrease the twitch and the Ca$_i$ transient.$^{53}$ However, they also diminish the frequency of spontaneous SR Ca$^{2+}$ releases and resting indo-1 fluorescence,$^{53}$ a result opposite that reported in the present study (Figures 8 and 9). Nevertheless, the role of protein kinase C activation in the response of myocardial preparations to opioid peptides still remains to be elucidated.

**cAMP and cGMP**

Opioids may also have an effect on cAMP. It has been reported that opioids increase cAMP in the rat heart.$^{63}$ Previous work with spontaneously beating cultures of chick embryo heart cells yielded contrasting results. Some investigators have found that leucine enkephalin increases the amplitude of the contraction$^{24}$; the peak of this response was achieved within 3–5 minutes, after which it declined and approached the control value by the end of the 10th
minute, when the experiment was terminated. In a subsequent report, the same authors related this result to an increase in cell Ca\textsuperscript{2+} loading mediated by an enhancement in adenylate cyclase activity and cAMP levels.\textsuperscript{35} In contrast, in a similar preparation, others have described that opioid receptor stimulation decreases cAMP, increases cGMP, and markedly inhibits the effect of norepinephrine to stimulate cAMP and reduce cGMP.\textsuperscript{89} In rat brain homogenate, morphineline-like substances were reported to inhibit hormonally stimulated cAMP formation without affecting its basal production,\textsuperscript{64} and a similar action on adenylate cyclase activity and cAMP levels has been reported in other neuronal cell types.\textsuperscript{64,66} In unstimulated cardiac myocytes, an increase in cAMP would be expected to enhance SR Ca\textsuperscript{2+} uptake\textsuperscript{67,68} and to decrease Ca\textsuperscript{2+} as well as the frequency of oscillatory SR Ca\textsuperscript{2+} release\textsuperscript{91} whereas during electrical stimulation it would have a positive inotropic action associated with an increase in the amplitude of the Ca\textsuperscript{2+} transient that causes the contraction.\textsuperscript{40} In contrast, a decrease in the cAMP/cGMP ratio could not explain the transient increase in the amplitude of the twitch and of the associated Ca\textsuperscript{2+} transient that is determined by the \( \kappa \)-agonist. Additionally, if the negative inotropic effect of opioid peptides was due to a decrease in cAMP, the time course of relaxation should have been longer than what is achieved by lowering Ca\textsuperscript{2+} via a decrease in Ca\textsubscript{aq}, a result different from ours (Tables 1 and 2). Thus, it is unclear which role, if any, a change in cAMP and/or cGMP might have had in the results described in the present study.

In summary, we have shown that opioid peptides can have a marked effect on the function of cardiac myocytes and that this action is mediated by \( \delta \)- and \( \kappa \)-receptor, but not \( \mu \)-receptor, stimulation. Thus, neuronal release of opioids not only alters synaptic transmission but may also have a direct effect on mycardial function. Additionally, definite differences exist in the response to the two receptor agonists, and this may affect the physiological role of opioid peptides in vivo. The functional sequelae of an acute exposure to \( \kappa \)-agonists, as it may occur during the release of opioids into the synaptic cleft, may differ from those of a more chronic exposure, where opioids reach the myocardium via the systemic circulation.

It is interesting that the results on Ca\textsuperscript{2+} release from an intracellular pool obtained in unstimulated cardiac myocytes could be duplicated in neuronal cells. An increase in Ca\textsubscript{aq}, depletion of an intracellular Ca\textsuperscript{2+} pool, and Ca\textsuperscript{2+} oscillations may all reflect important mechanisms regulating the activity of the nervous system.\textsuperscript{61,62,69} Thus, coupling between opioid receptors and phosphatidylinositol turnover leading to IP\textsubscript{3} production and changes in cell Ca\textsuperscript{2+} homeostasis may reflect a general mechanism for the action of \( \kappa \) and \( \delta \) opioid peptides.

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