Differential Effects of Fentanyl and Morphine on Intracellular Ca²⁺ Transients and Contraction in Rat Ventricular Myocytes

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Background: Our objective was to elucidate the direct effects of fentanyl and morphine on cardiac excitation–contraction coupling using individual, field-stimulated rat ventricular myocytes.

Methods: Freshly isolated myocytes were loaded with fura-2 and field stimulated (0.3 Hz) at 28°C. Amplitude and timing of intracellular Ca²⁺ concentration (at a 340:380 ratio) and myocyte shortening (video edge detection) were monitored simultaneously in individual cells. Real time Ca²⁺ uptake into isolated sarcoplasmic reticulum vesicles was measured using fura-2 free acid in the extravesicular compartment.

Results: The authors studied 120 cells from 30 rat hearts. Fentanyl (30–1,000 nM) caused dose-dependent decreases in peak intracellular Ca²⁺ concentration and shortening, whereas morphine (3–100 μM) decreased shortening without a concomitant decrease in the Ca²⁺ transient. Fentanyl prolonged the time to peak and to 50% recovery for shortening and the Ca²⁺ transient, whereas morphine only prolonged the timing parameters for shortening. Morphine (100 μM), but not fentanyl (1 μM), decreased the amount of Ca²⁺ released from intracellular stores in response to caffeine in intact cells, and it inhibited the rate of Ca²⁺ uptake in isolated sarcoplasmic reticulum vesicles. Fentanyl and morphine both caused a downward shift in the dose–response curve to extracellular Ca²⁺ for shortening, with no concomitant effect on the Ca²⁺ transient.

Conclusions: Fentanyl and morphine directly depress cardiac excitation–contraction coupling at the cellular level. Fentanyl depresses myocardial contractility by decreasing the availability of intracellular Ca²⁺ and myofilament Ca²⁺ sensitivity. In contrast, morphine depresses myocardial contractility primarily by decreasing myofilament Ca²⁺ sensitivity. (Key words: Cardiomyocytes; negative inotrope; opioids.)

OPIOIDS are widely used as analgesics or anesthetics in patients with intolerable pain, limited cardiovascular performance, or ischemic heart disease. Despite their prevalent use, the direct effects of opioids on cardiac contractility are poorly understood and controversial. Opioids can indirectly alter cardiac function *via* inhibitory actions on the autonomic or central nervous systems.[1–3] In addition, opioids may alter cardiac contractility directly *via* activation of opioid receptors[4,5] or by membrane interactions because of their chemical properties and structures *per se*.[6] Morphine has been reported to cause positive[7,8] inotropic effects in dogs and negative[9] inotropic effects in rats. Fentanyl has been reported to have little or no effect on myocardial contractility[10] or to exert a negative inotropic effect.[11,12] The differences among these findings may be related to the difficulty in assessing the direct effects of opioids on cardiac function *in vivo*, where concomitant changes in preload, afterload, baroreflex activity, and central nervous system activity may be confounding factors.

In vitro studies provide a more direct approach to evaluate the specific effects of opioids on myocardial contractility. Morphine induces a negative inotropic effect in human and rat atrial preparations,[13,14] rat and cat papillary muscle,[15,16] and perfused rat hearts,[17] whereas no inotropic effect was observed in cultured rat cardiac myocytes.[18] Fentanyl is reported to cause a negative inotropic effect in isolated ventricular myocardium[15] and papillary muscle.[19] Whether opioids exert their actions *via* alterations in intracellular free Ca²⁺ concentration ([Ca²⁺]ᵢ) or myofilament Ca²⁺ sensitivity is not known. In cultured neonatal cardiac myocytes, mor-

phine did not cause myocardial depression and increased intracellular free Ca^{2+} concentration in a dose-dependent manner.[18] However, contractility is regulated differently in adult cardiomyocytes than in neonatal cardiomyocytes.[20] Neonatal myocytes have an underdeveloped sarcoplasmic reticulum (SR) and express different isoforms of contractile proteins and second messengers (*e.g.*, protein kinase C).[20-23] The direct effects of opioids on cellular mechanisms that regulate contractility in adult cardiac myocytes have not been investigated.

Our goal was to determine whether fentanyl or morphine, or both, alter cardiac excitation–contraction coupling at the cellular level in freshly isolated, field-stimulated, adult rat ventricular myocytes. This experimental model allows us to simultaneously measure changes in the amplitude and timing of $[Ca^{2+}]_i$ and myocyte shortening, independent of any hemodynamic, neural, humoral, or locally derived factors. Our hypothesis was that opioids cause myocardial depression by decreasing the availability of $[Ca^{2+}]_i$ or the myofilament Ca^{2+} sensitivity, or both. We also assessed the effects of these opioids on Ca^{2+} uptake and release in isolated SR vesicles.

Materials and Methods

Ventricular Myocyte Preparation

Isolated adult ventricular myocytes from rat hearts were obtained as previously described.[24] Briefly, the hearts were excised, cannulated *via* the aorta, attached to a modified Langendorff perfusion apparatus, and perfused with oxygenated (95% oxygen and 5% carbon dioxide) Krebs-Henseleit buffer (37°C) containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 37.5 mM $NaHCO_3$, and 16.5 mM dextrose, with a pH of 7.35. After a 5-min equilibration period, the perfusion buffer was changed to a Ca^{2+}-free Krebs-Henseleit buffer containing 30 mg collagenase type II (Worthington Biochemical, Freehold, NJ; lot M6C152; 347 units/ml). After collagenase digestion (20 min), the ventricles were minced and shaken in Krebs-Henseleit buffer, and the resulting cellular digest was washed, filtered, and resuspended in phosphate-free HEPES-buffered saline containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 1.25 mM $CaCl_2$, 11 mM dextrose, 25 mM HEPES, and 5 mM pyruvate, with a pH of 7.35 and vigorously bubbled immediately before use with 100% oxygen. Typically, $6-8 \times 10^6$ cells/rat heart were obtained using this procedure. Viability, as assessed by the percentage of

cells that retained a rod-shaped structure with no blebs or granulations, was routinely between 80% and 90%. Myocytes were suspended in HEPES-buffered saline (1×10^6 cells/ml) and stored in an oxygen hood until they were used.

Contractility and Intracellular Ca^{2+} Measurements

Simultaneous measurement of shortening and $[Ca^{2+}]_i$ was performed as previously described.[25] Ventricular myocytes (0.5×10^6 cells/ml) were incubated in HEPES-buffered saline containing 2 μM fura-2/AM (Texas Fluorescence Labs, Austin, TX) at 37°C for 20 min. Fura-2–loaded ventricular myocytes were placed in a temperature-regulated (28°C) chamber (Bioptechs, Butler, PA) mounted on the stage of an Olympus IX-70 (Olympus America, Lake Success, NY) inverted fluorescence microscope. The volume of the chamber was 1.5 ml. The cells were superfused continuously with HEPES-buffered saline at a flow rate of 2 ml/min and were field stimulated *via* bipolar platinum electrodes at a frequency of 0.3 Hz and a duration of 5 ms using a Grass SD9 stimulator (Grass Instruments, West Warwick, RI). Myocytes were chosen for study according to the following criteria: (1) a rod-shaped appearance with clear striations and no membrane blebs, (2) a negative staircase of twitch performance (typical for rats) when stimulated from rest, and (3) the absence of spontaneous contractions.

Fluorescence measurements were performed on single ventricular myocytes using a dual-wavelength spectrofluorometer (Deltascan RFK6002; Photon Technology International, South Brunswick, NJ) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. The cells also were illuminated with red light at a wavelength of more than 600 nm for simultaneous video edge detection. An additional postspecimen dichroic mirror deflects light at wavelengths of more than 600 nm into a charge-coupled device video camera (Phillips VC 62505T; Marshall Electronics, Culver City, CA) to measure myocyte shortening and relengthening. The fluorescence sampling frequency was 100 Hz, and data were collected using a software package (Felix) from Photon Technology International. The $[Ca^{2+}]_i$ was estimated by comparing the cellular fluorescence ratio with fluorescence ratios acquired using fura-2 (free acid) in buffers containing known Ca^{2+} concentrations.

Simultaneous measurement of cell shortening was monitored using a video edge detector (Crescent Electronics, Sandy, UT) with 16-ms temporal resolution. The video edge detector was calibrated using a stage micro-

meter so cell lengths during shortening and relengthening could be monitored. Myocytes typically contracted on one end with the other end lightly attached to the chamber. Thus contraction represented unloaded isotonic shortening. Myocyte length in response to field stimulation was measured (in micrometers) and is expressed as the change from resting cell length (twitch amplitude). Lab View (National Instruments, Austin, TX) was used for data acquisition of cell shortening using a sampling rate of 100 Hz.

Analysis of Ca^{2+} Transients and Contractile Data

Fluorescence data for the Ca^{2+} transients were imported into Labview, and both the Ca^{2+} transients and the myocyte contractile responses were analyzed synchronously and simultaneously. The following parameters were calculated for each contraction: diastolic and systolic $[Ca^{2+}]_i$ and cell length; change in $[Ca^{2+}]_i$ and twitch amplitude; time to peak (Tp) for $[Ca^{2+}]_i$ and shortening; and time to 50% recovery (Tr) for $[Ca^{2+}]_i$ and shortening. Parameters from 15 contractions were averaged to obtain mean values at baseline and in response to the various interventions. Averaging the parameters progressively minimizes beat-to-beat variation.

Changes in twitch amplitude in response to the interventions are expressed as a percentage of baseline shortening. Changes in timing were measured in milliseconds and were normalized to changes in amplitude. Changes in $[Ca^{2+}]_i$ were measured as the change in the 340:380 ratio from baseline. Changes in the 340:380 ratio in response to the interventions were expressed as a percentage of the control response in the absence of any intervention.

Purification of Sarcoplasmic Reticulum Vesicles. Freshly isolated adult rat hearts were homogenized in five volumes of MOPS buffer (10 mm, pH 7.4, 4°C) containing 290 mm sucrose, 3 mm NaN$_3$, 1 mm dithiothreitol, 1 μm pepstatin A, 1 μm leupeptin, and 0.8 mm phenylmethylsulfonyl fluoride using a Brinkmann Polytron homogenizer (Westbury, NY). The homogenate was centrifuged at 7,500g for 20 min. The supernatant was saved and centrifuged again at 40,000g for 60 min. The resultant pellet was suspended in three volumes of MOPS (10 mm, pH 6.8, 4°C) containing 600 mm KCl, 3 mm NaN$_3$, 1 mm dithiothreitol, and protease inhibitors. The material was centrifuged at 140,000g for 40 min, and the final pellet was resuspended in a Ca^{2+}-free sucrose buffer and stored at −80°C until it was used.

Measurement of Ca^{2+} Uptake and Content in Sarcoplasmic Reticulum Vesicles. Double-distilled tap water was deionized using a Milli-Q reagent water system (Millipore, Bedford, MA) and further purified by dual ion-exchange chromatography and a Ca^{2+} Sponge-S (Molecular Probes, Eugene, OR) to remove residual Ca^{2+}. A buffering system representing intracellular conditions and capable of regenerating adenosine triphosphate was used to suspend the vesicles and contained 20 mm HEPES, 100 mm KCl, 5 mm NaCl, 5 mm MgCl$_2$, and 5 mm creatine phosphate (pH 7.2, 37°C) and creatine phosphokinase (0.4 units/ml). Oxalate (10 mm) was added to act as a Ca^{2+}-precipitating anion inside the vesicles to minimize leakage of Ca^{2+} and to maintain the Ca^{2+} gradient across the vesicular membrane.[26] The solutions were prepared using an iterative solution-mixing program (Solwin v2.0, Philadelphia, PA). Binding constants for the ionic compounds were corrected for temperature and ionic strength. CaCl$_2$ was added back to the buffer to yield a free Ca^{2+} concentration of 1 μm (pCa 6).

Measurements of Ca^{2+} uptake and release were evaluated in real time using suspensions of SR vesicles and 2 μm fura-2 free acid (Texas Fluorescence Labs) in the extravesicular compartment. Fluorescence experiments were performed using dual-wavelength fluorometry in a temperature-regulated (37°C) sample compartment. Microcuvettes (250 μl) were washed in EGTA (2 mm) solution to remove all Ca^{2+} and then thoroughly rinsed with Ca^{2+}-free buffer and allowed to dry. The addition of adenosine triphosphate (1 mm) to the vesicular suspension triggered the uptake of Ca^{2+} into the vesicles, which was measured as a decrease in the fluorescence signal (340:380 ratio) from the extravesicular compartment. Caffeine (20 mm) was used to release Ca^{2+} from the vesicles to evaluate vesicular Ca^{2+} content. Fluorescence data were collected using the Felix program at a sampling frequency of 20 Hz. The rate of Ca^{2+} uptake was measured as the decrease in the fluorescence signal during a 45-s period in the presence or absence of opioid. The addition of 1,000 nm fentanyl or 100 μm morphine did not alter the pH of the suspension buffer.

Experimental Protocols

Protocols were designed so each cell could be used as its own control.

Protocol 1: Dose-dependent Effects of Opioids on $[Ca^{2+}]_i$ and Myocyte Shortening. Changes in myocyte shortening and $[Ca^{2+}]_i$ during exposure to fentanyl or morphine were determined. Baseline measurements were collected from individual myocytes for 1.5 min in

the absence of any intervention. Myocytes were exposed to sequential doses of the same opioid at four different concentrations (30, 100, 300, and 1,000 nM fentanyl; 3, 10, 30, and 100 μM morphine). This was achieved by rapidly exchanging the buffer in the dish with new buffer containing the opioid at the desired concentration. Individual myocytes were exposed to only one opioid. Data were acquired for 1.5 min after a 5-min equilibration period in the presence of the opioid.

Protocol 2: Effects of Opioids on Sarcoplasmic Reticulum Ca^{2+} Stores. To determine whether fentanyl or morphine alters Ca^{2+} release from SR Ca^{2+} stores, we measured caffeine-induced Ca^{2+} release in the presence or absence of the opioid. Baseline $[Ca^{2+}]_i$ transients were collected from individual myocytes for 1.5 min. Fentanyl (100, 1,000 nM) or morphine (10, 100 μM) was then added to the superfusion buffer and allowed to equilibrate for 5 min. Field stimulation of the myocyte was discontinued and caffeine (20 mM) was applied to the cell 15 s later. The amplitude of the $[Ca^{2+}]_i$ transient induced by caffeine was compared with the amplitude of the field-stimulated $[Ca^{2+}]_i$ transient before the respective drugs were added and is reported as a percentage of the control amplitude.

Protocol 3: Effects of Opioids on Myofilament Ca^{2+} Sensitivity. To determine whether fentanyl or morphine alters myofilament Ca^{2+} sensitivity, we evaluated the dose–response curve to extracellular Ca^{2+} in the presence or absence of the opioids. Baseline parameters were collected from individual myocytes for 1.5 min. Dose–response curves to extracellular Ca^{2+} were performed by exchanging the buffer in the dish with a new buffer containing Ca^{2+} at the desired concentration. Data were acquired for 1.5 min after a new steady state was established. Dose–response curves to extracellular Ca^{2+} were then performed in the presence of either 100 nM fentanyl or 10 μM morphine. Cells were allowed to stabilize for 5 min after each intervention. Changes in myocyte shortening and the $[Ca^{2+}]_i$ transient were expressed as a percentage change from baseline in the control group. Similarly, changes in myocyte shortening and the $[Ca^{2+}]_i$ transient in the presence of fentanyl or morphine were expressed as percentage changes from baseline 5 min after exposure to the opioids.

Materials

Fentanyl and morphine were obtained from the Cleveland Clinic Pharmacy. Caffeine was purchased from Sigma Chemical Company (St. Louis, MO).

Statistical Analysis

Each experimental protocol was performed on multiple myocytes from the same heart and repeated in at least four hearts. Results obtained from myocytes in each heart were averaged so all hearts were weighted equally. The dose-dependent effects of fentanyl or morphine on myocyte shortening and $[Ca^{2+}]_i$ were assessed using one-way analysis of variance with repeated measures and the Bonferonni/Dunn *post hoc* test. Comparisons between groups were made by two-way analysis of variance. Results are expressed as the mean ± SEM. Differences were considered significant at $P < 0.05$.

Results

Baseline Parameters for Myocyte Shortening and $[Ca^{2+}]_i$

Baseline $[Ca^{2+}]_i$ and the diastolic cell length were 80 ± 10 nM and 124 ± 2 μm, respectively. Peak $[Ca^{2+}]_i$ was 360 ± 30 nM. Twitch amplitude was 11% (14.0 ± 0.7 μm) of the baseline resting diastolic cell length. Time to peak $[Ca^{2+}]_i$ and shortening were 166 ± 3 and 182 ± 3 ms, respectively. The Tr for $[Ca^{2+}]_i$ and shortening was 307 ± 4 and 326 ± 5 ms, respectively. Baseline measurements were stable during the course of these experiments.

Effects of Fentanyl on Myocyte Shortening and $[Ca^{2+}]_i$

Figure 1A shows that the addition of fentanyl to an individual, field-stimulated ventricular myocyte results in dose-dependent inhibition of myocyte shortening and a concomitant decrease in peak $[Ca^{2+}]_i$. The myocardial depressant effects of fentanyl were completely restored after washout of fentanyl. Figure 1B shows individual contractions and $[Ca^{2+}]_i$ transients. Figure 2 displays the summarized data. Fentanyl caused dose-dependent decreases in myocyte shortening and peak $[Ca^{2+}]_i$. Fentanyl prolonged Tp and Tr for shortening and $[Ca^{2+}]_i$ at concentrations more than 30 nM (fig. 3).

Effects of Morphine on Myocyte Shortening and $[Ca^{2+}]_i$

Figure 4A shows that the addition of morphine to an individual, field-stimulated ventricular myocyte results in a decrease in myocyte shortening with no concomitant change in the amplitude of the $[Ca^{2+}]_i$ transient. The inhibitory effect reached a plateau at approximately 10 μM morphine, higher concentrations had no additional

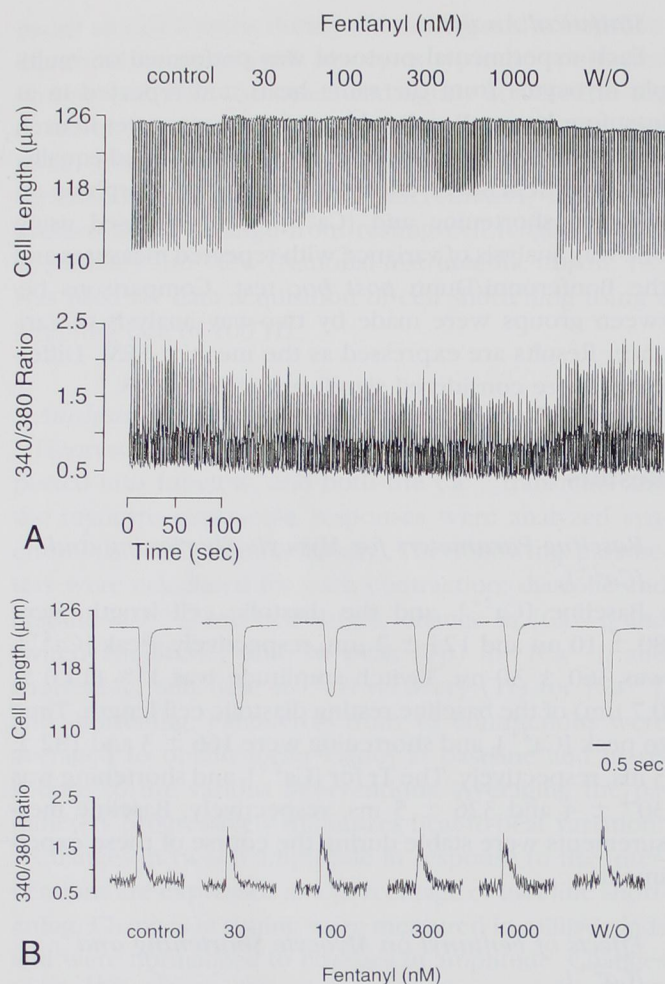Fig. 1. (*A*) Representative trace showing the dose-dependent effects of fentanyl on myocyte shortening (*top*) and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (*bottom*). Fentanyl was added to individual field-stimulated myocytes at the concentrations depicted, followed by washout (w/o). Changes in cell length were measured in micrometers. $[Ca^{2+}]_i$ was measured as the 340:380 ratio. (*B*) Expanded view of individual contractions and $[Ca^{2+}]_i$ transients taken from part A.

effect on shortening. At the highest concentration tested (100 μM), morphine increased the amplitude of the $[Ca^{2+}]_i$ transient. A decrease in diastolic $[Ca^{2+}]_i$ (change in 340:380 ratio = -0.5 ± 0.1) paralleled by an increase in diastolic cell length (3.0 ± 0.2 μm) was also observed ($P < 0.05$). The changes in myocyte shortening and $[Ca^{2+}]_i$ were not immediately reversible after washout of morphine. Figure 4B shows individual myocyte twitches and $[Ca^{2+}]_i$ transients. Figure 5 displays summarized data. Morphine caused a decrease in myocyte shortening without a concomitant decrease in the amplitude of the $[Ca^{2+}]_i$ transient, which was actually increased by 100 μM morphine. Morphine prolonged Tp and Tr for myo-

cyte shortening with no concomitant effect on Tp or Tr for $[Ca^{2+}]_i$ (fig. 6).

Effects of Fentanyl and Morphine on Ca^{2+} Uptake and Content in Isolated Sarcoplasmic Reticulum Vesicles

Ca^{2+} uptake by the vesicles was measured in real time as a decrease in the 340:380 ratio from the extravesicular compartment. Caffeine (20 mM) was used to release Ca^{2+} from the vesicles. Figure 7A shows that fentanyl had no effect on the rate of Ca^{2+} uptake at any concentration tested. Summarized data for fentanyl are shown in figure 7B. Morphine only had an effect on the rate of Ca^{2+} uptake into the SR vesicles at the highest concentration tested (fig. 8A). Summarized data for morphine are shown in figure 8B. The total amount of Ca^{2+} released from the vesicles in response to caffeine was unaltered by fentanyl ($96 \pm 2\%$ of control) or morphine ($97 \pm 4\%$ of control) compared with the vehicle control (figs. 7A and 8A).

Effects of Fentanyl and Morphine on Sarcoplasmic Reticulum Ca^{2+} Stores in Intact Cardiomyocytes

We also assessed the extent to which fentanyl or morphine altered the amount of Ca^{2+} released from the SR in response to caffeine (20 mM) in intact cardiac myocytes. Fentanyl (100, 1,000 nM) did not alter the amplitude of the
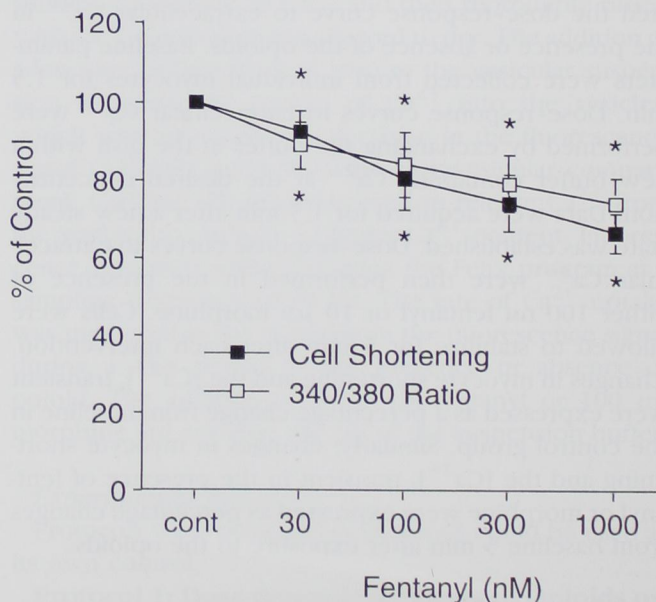

Fig. 2. Summarized data for the effects of fentanyl on the amplitude of myocyte shortening and $[Ca^{2+}]_i$ transient. Results are expressed as a percentage of control (cont) in the absence of any intervention. *Significant change from control ($P < 0.05$). n = 20 cells per five hearts.

Fig. 3. Summarized data for the effects of fentanyl on time to peak (Tp) and time to 50% recovery (Tr) for myocyte shortening and $[Ca^{2+}]_i$ transient. Changes in timing were measured in milliseconds and were normalized to changes in peak amplitude. *Significant change from control ($P < 0.05$). n = 20 cells per five hearts.
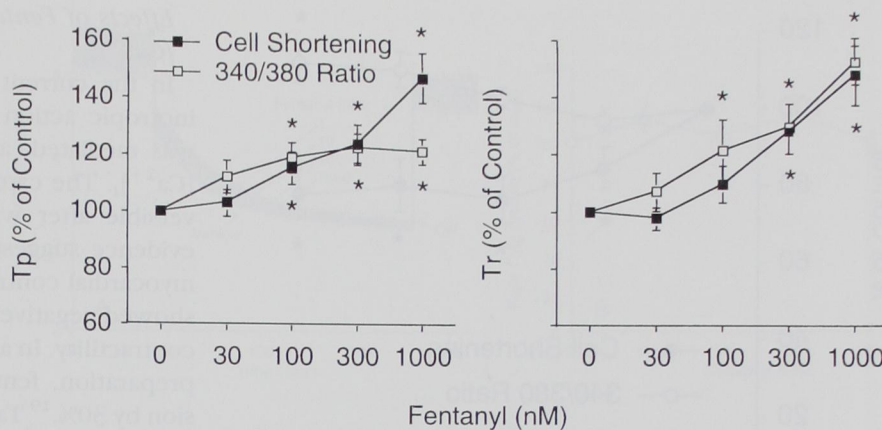
caffeine-induced $[Ca^{2+}]_i$ transient compared with control (fig. 9). Only the highest dose of morphine (100 μM) significantly decreased the caffeine-induced $[Ca^{2+}]_i$ transient.

Effects of Fentanyl and Morphine on Myofilament Responsiveness to Ca^{2+}

Myofilament responsiveness to Ca^{2+} can be assessed by evaluating the relation between shortening and $[Ca^{2+}]_i$. To obtain a range of values for myocyte shortening and $[Ca^{2+}]_i$, we performed a dose–response curve to extracellular Ca^{2+}($[Ca^{2+}]_o$). Increasing $[Ca^{2+}]_o$ from 1 to 4 mM without opioids resulted in a dose-dependent increase in myocyte shortening and a concomitant increase in peak $[Ca^{2+}]_i$ (fig. 10). Five minutes after 100 nM fentanyl, myocyte shortening and the $[Ca^{2+}]_i$ transient decreased ($P < 0.05$) to 85 ± 3% and 82 ± 5% of preadministration values, respectively. Five minutes after 10 μM morphine, myocyte shortening decreased ($P < 0.05$) to 93 ± 2% of the preadministration value, whereas no change (100 ± 3%) in the $[Ca^{2+}]_i$ transient was observed. Fentanyl (100 nM) and morphine (10 μM) caused a downward shift in the dose–response curve to $[Ca^{2+}]_o$ for shortening with no concomitant effect on $[Ca^{2+}]_i$ (fig. 10). Fentanyl and morphine caused a rightward shift in the relation between cell shortening and peak $[Ca^{2+}]_i$ (fig. 11).

Discussion

Previous studies that evaluated the effects of opioids on mammalian myocardial function yielded varying results, including evidence for positive[7,8] and negative[13,14,17] inotropic actions and no inotropic effect.[27] Those studies used intact animals, isolated perfused hearts, isometrically contracting papillary muscles, or
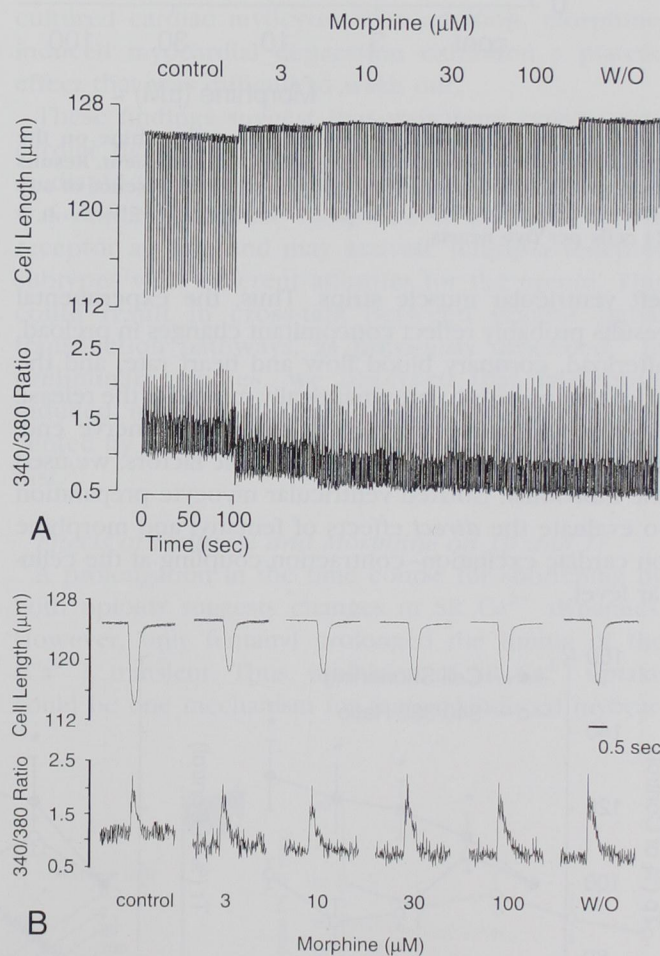

Fig. 4. (*A*) Representative trace showing the dose-dependent effects of morphine on myocyte shortening (*top*) and $[Ca^{2+}]_i$ (*bottom*). Morphine was added to individual field-stimulated myocytes at the concentrations depicted. Changes in cell length were measured in micrometers. $[Ca^{2+}]_i$ was measured as the 340:380 ratio. (*B*) Expanded view of individual contractions and $[Ca^{2+}]_i$ transients taken from part A.
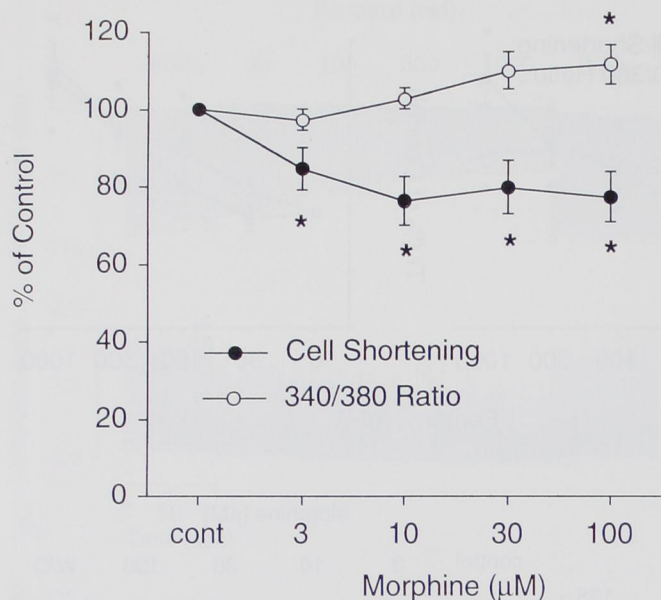
Fig. 5. Summarized data for the effects of morphine on the amplitude of myocyte shortening and $[Ca^{2+}]_i$ transient. Results are expressed as a percentage of control in the absence of any intervention. *Significant change from control ($P < 0.05$). n = 24 cells per five hearts.

left ventricular muscle strips. Thus, the experimental results probably reflect concomitant changes in preload, afterload, coronary blood flow and heart rate, and the effects of opioids to presynaptically modulate the release of norepinephrine and acetylcholine from nerve endings. To avoid these and other extrinsic factors, we used the individual, isolated ventricular myocyte preparation to evaluate the *direct* effects of fentanyl and morphine on cardiac excitation–contraction coupling at the cellular level.

Effects of Fentanyl on Myocyte Shortening and $[Ca^{2+}]_i$

In the current study, fentanyl had a direct negative inotropic action on isolated ventricular myocytes that was mediated, at least in part, by a decrease in peak $[Ca^{2+}]_i$. The cardiodepressant effect of fentanyl was reversible after washout of the opioid. Although most evidence suggests that fentanyl causes little change in myocardial contractility *in vivo*, several *in vitro* studies showed negative inotropic actions of fentanyl on cardiac contractility. In a canine blood-perfused papillary muscle preparation, fentanyl (95 μM) reduced developed tension by 30%.[19] Taking into account binding of fentanyl to serum proteins,[28] the actual free plasma concentration in that study was approximately 20 μM. In rat trabeculae carneae muscle, fentanyl (150 μM) reduced peak developed tension by 30%.[15] Fentanyl (13 μM) also depressed the velocity of isometric shortening of isolated cat papillary muscle by 30%.[16] These differences in concentrations for fentanyl-induced myocardial depression are probably because of species differences or the experimental preparation. Furthermore, it is difficult in multicellular preparations to exclude the possibility that fentanyl may alter the production of locally derived factors, which can regulate myocardial contractility.

In the current study, fentanyl (1 μM) caused a 34% decrease in myocyte shortening and a concomitant 21% decrease in peak $[Ca^{2+}]_i$. Fentanyl (1 μM) was recently shown to reduce the Ca^{2+} current (I_{Ca}) in rabbit sinoatrial node by 20%.[29] Leucine enkephalin, an opioid receptor agonist, also has been shown to reduce the L-type Ca^{2+} channel current in rat ventricular myocytes by 40%.[4] Therefore, the fentanyl-induced decrease in
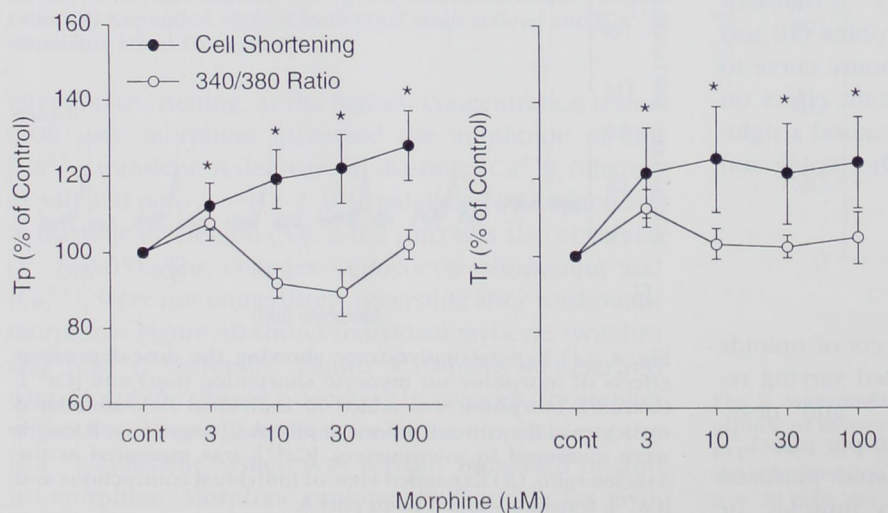

Fig. 6. Summarized data for the effects of morphine on time to peak and time to 50% recovery for myocyte shortening and $[Ca^{2+}]_i$ transient. Changes in timing were measured in milliseconds and were normalized to changes in peak amplitude. *Significant change from control ($P < 0.05$). n = 24 cells per five hearts.

Fig. 7. Original trace depicting the effects of fentanyl on the rate of Ca^{2+} uptake and content in isolated sarcoplasmic reticulum vesicles (*A*). Adenosine triphosphate was used to trigger Ca^{2+} uptake into the vesicles. Caffeine (Caf) was used to trigger Ca^{2+} release. The concentrations of fentanyl are shown. (*B*) Summarized data for the effects of fentanyl on the rate of Ca^{2+} uptake. n = 4 individual experiments.
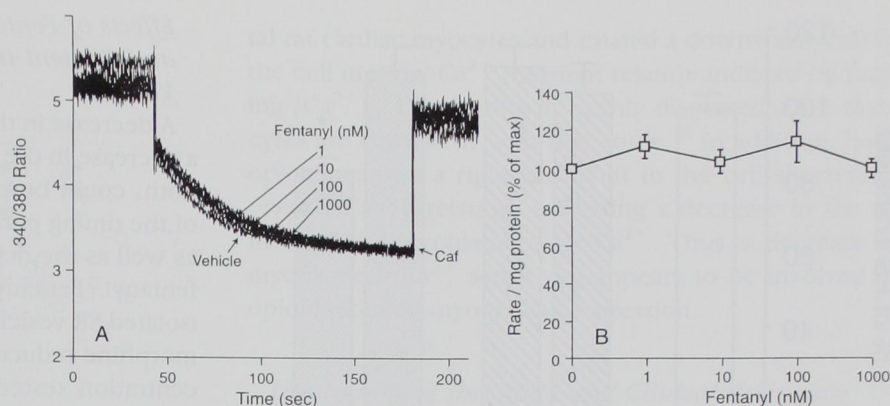
peak $[Ca^{2+}]_i$ in the current study may be a result of reduced Ca^{2+} entry *via* L-type Ca^{2+} channels.

Effects of Morphine on Myocyte Shortening and $[Ca_{2+}]_i$

Morphine causes bradycardia and hypotension *via* a direct effect on the central nervous system, which enhances parasympathetic nervous system outflow and inhibits sympathetic nervous system outflow.[30] However, the direct effects of morphine on intrinsic myocardial contractility have not been elucidated fully. Earlier *in vivo* studies reported that morphine induced a positive inotropic effect mediated by sympathoadrenal stimulation.[7,8] In contrast, other studies showed that morphine has a negative inotropic effect in isolated perfused heart and in atrial and ventricular muscle preparations.[13-17] In the current study, morphine decreased myocyte shortening, whereas $[Ca^{2+}]_i$ was either unchanged or increased at the highest concentration of morphine. These results indicate that the cardiodepressant effect of morphine is not mediated by a reduction in Ca^{2+} availability, but rather by a decrease in myofilament Ca^{2+} sensitivity. The increase in $[Ca^{2+}]_i$ with higher concentrations of mor-

phine may act to counteract the reduction in shortening and is consistent with a previous observation in cultured cardiac myocytes.[18] In addition, morphine-induced myocardial depression exhibited a plateau effect that was difficult to wash out.

These findings suggest that morphine causes myocardial depression *via* a highly specific receptor-mediated pathway. A wide range of morphine doses was studied, because morphine is a mixed opioid receptor agonist and may activate multiple receptor subtypes with different affinities for the opioid. This could result in cross-talk between several signal transduction pathways, as previously described.[5] In preliminary studies, we observed that morphine-induced myocardial depression is completely prevented by naloxone, a mixed opioid receptor antagonist.[31]

Effects of Fentanyl and Morphine on Tp and Tr

A prolongation in the time course for shortening by both opioids suggests changes in SR Ca^{2+} dynamics. However, only fentanyl prolonged the timing of the $[Ca^{2+}]_i$ transient. Thus, inhibition of SR Ca^{2+} uptake could be one mechanism for fentanyl-induced myocar-

Fig. 8. Original trace depicting the effects of morphine on the rate of Ca^{2+} uptake and content in isolated sarcoplasmic reticulum vesicles (*A*). Adenosine triphosphate was used to trigger Ca^{2+} uptake into the vesicles. Caffeine was used to trigger Ca^{2+} release. The concentrations of morphine are shown. (*B*) Summarized data for the effects of morphine on the rate of Ca^{2+} uptake. *Significant change from control (*P* < 0.05). n = 4 individual experiments.
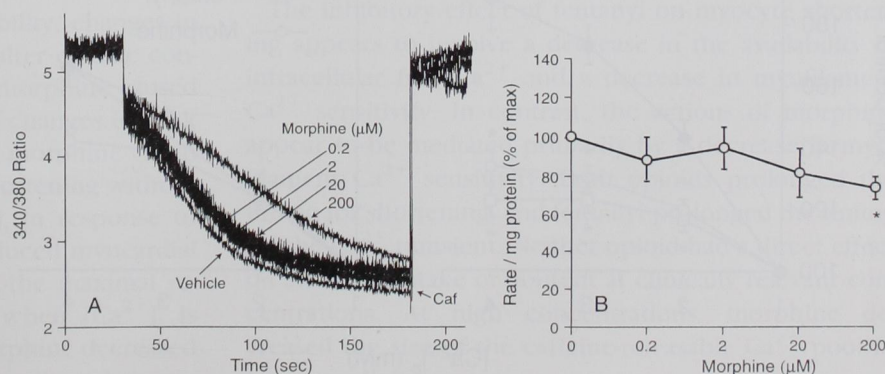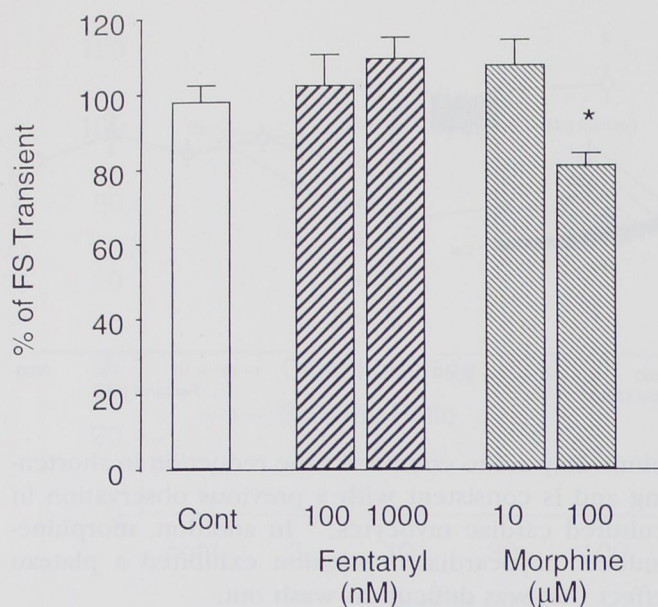
Fig. 9. Summarized data for the effects of fentanyl (100 and 1,000 nM) and morphine (10 and 100 μM) on caffeine-induced $[Ca^{2+}]_i$ release in intact cardiac myocytes. The amplitude of the caffeine-induced $[Ca^{2+}]_i$ transient was compared with the amplitude of the field-stimulated (FS) $[Ca^{2+}]_i$ transient with or without (cont) the opioids. Results are expressed as a percentage of the field-stimulated control amplitude. *Significant change from control ($P < 0.05$). n = 20 cells per six hearts for the control group; n = 16 cells per four hearts for other groups.

dial depression but would not explain the effect of morphine. The fentanyl-induced prolongation of timing parameters could be caused by the concomitant decrease in peak $[Ca^{2+}]_i$,[32] or it could be a specific effect of fentanyl on SR Ca^{2+} transport processes. To resolve this issue, we used isolated SR vesicles.

Effects of Fentanyl and Morphine on Ca^{2+} Uptake and Content in Isolated Sarcoplasmic Reticulum Vesicles

A decrease in the rate of Ca^{2+} sequestered by the SR or a decrease in the amount of Ca^{2+} available for release, or both, could be potential explanations for prolongation of the timing parameters for shortening by both opioids, as well as the depression in peak $[Ca^{2+}]_i$ observed with fentanyl. Fentanyl had no effect on Ca^{2+} uptake into isolated SR vesicles at any concentration tested, whereas morphine reduced Ca^{2+} uptake only at the highest concentration tested. This is in contrast to our previous finding that thiopental directly alters the rate of Ca^{2+} uptake into isolated SR vesicles in a dose-dependent manner.[33] Neither opioid had an effect on the amount of Ca^{2+} released from SR vesicles in response to caffeine. These results indicate that fentanyl and morphine do not directly alter SR Ca^{2+} dynamics, but this does not exclude a possible second-messenger–mediated effect of the opioids on SR Ca^{2+} function in the intact cell.[34]

Effects of Fentanyl and Morphine on Sarcoplasmic Reticulum Ca^{2+} Stores in Intact Myocytes

Because second messengers, such as diacylglycerol, and activation of protein kinase C can be involved in altering SR Ca^{2+} dynamics,[35,36] we wanted to determine whether the opioids altered the amount of Ca^{2+} released from the SR of intact myocytes in response to caffeine. Caffeine-releasable Ca^{2+} pools were unaltered by fentanyl pretreatment, indicating that the decreases in peak $[Ca^{2+}]_i$ and shortening were not caused by alterations in the amount of Ca^{2+} released from the SR. These data are
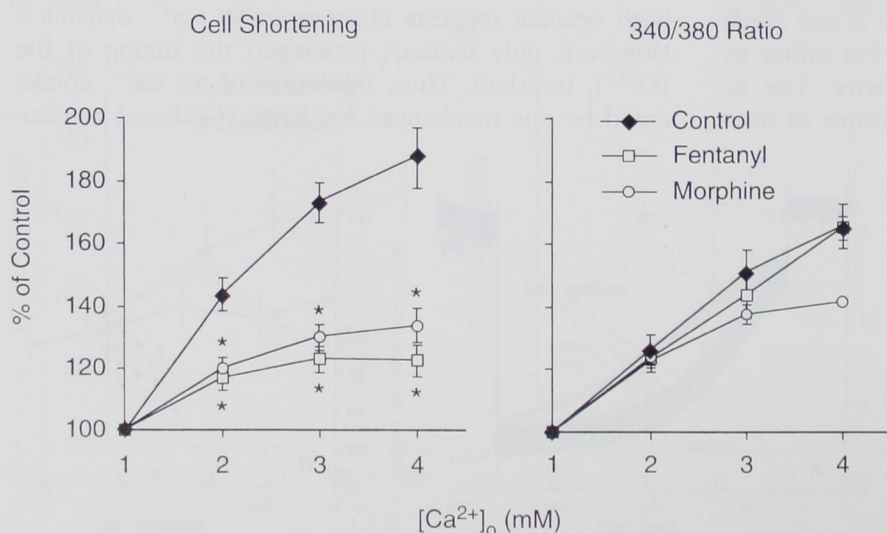

Fig. 10. Summarized data for the effects of fentanyl (100 nM) and morphine (10 μM) on myocyte shortening and $[Ca^{2+}]_i$ transient in response to increasing extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). Changes in myocyte shortening and $[Ca^{2+}]_i$ transient are expressed as a percentage of the baseline values without any intervention (control group) and 5 min after fentanyl or morphine. *Significant difference from control (without opioids) values ($P < 0.05$). n = 20 cells per six hearts for control group; n = 20 cells per five hearts for other groups.
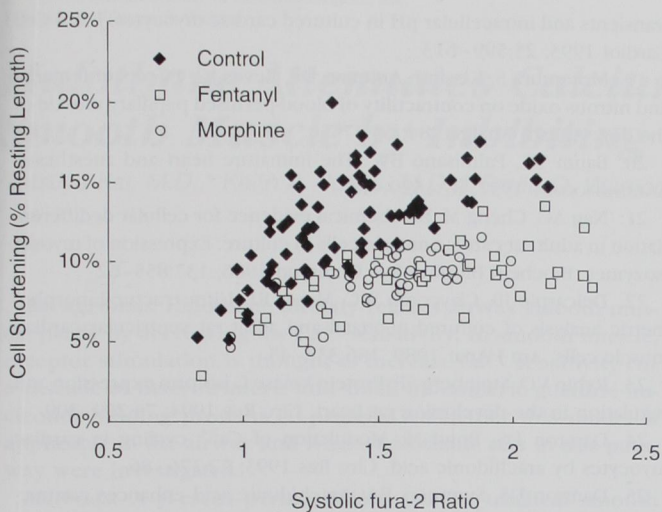
Fig. 11. The effects of fentanyl (100 nM) and morphine (10 μM) on myofilament Ca^{2+} sensitivity were assessed by plotting twitch shortening (percentage of resting cell length) as a function of peak $[Ca^{2+}]_i$ (systolic fura-2 ratio) in the absence or presence of the opioids. $[Ca^{2+}]_i$ was varied by increasing $[Ca^{2+}]_o$. The shortening-$[Ca^{2+}]_i$ relation obtained during control conditions (without opioids) was shifted to the right by fentanyl and morphine.

consistent with our findings in isolated SR vesicles. Therefore, the decrease in peak $[Ca^{2+}]_i$ induced by fentanyl probably is related to inhibition of Ca^{2+} influx across the sarcolemma.[4] Morphine decreased the amount of Ca^{2+} released by caffeine at the highest concentration tested (100 μM). Interestingly, morphine increased peak $[Ca^{2+}]_i$ in response to electric stimulation at the same concentration. Together, these results suggest that high-dose morphine may increase peak $[Ca^{2+}]_i$ by increasing Ca^{2+} influx across the sarcolemma,[18] which could counteract its negative inotropic effect and refill depleted SR Ca^{2+} stores.

Effects of Fentanyl and Morphine on Myofilament Ca^{2+} Sensitivity

In addition to decreased Ca^{2+} availability, changes in myofilament Ca^{2+} sensitivity also can alter cardiac contractile function. In the current study, morphine caused myocardial depression, independent of changes in peak $[Ca^{2+}]_i$. Furthermore, fentanyl and morphine both caused a downward shift in myocyte shortening without a concomitant change in peak $[Ca^{2+}]_i$ in response to elevated $[Ca^{2+}]_o$. Therefore, opioid-induced myocardial depression may involve a decrease in the maximal response of the myofilament to Ca^{2+} when $[Ca^{2+}]_i$ is increased. Ela *et al.*[18] observed that morphine decreased myofilament responsiveness to Ca^{2+} in cultured neona-

tal rat cardiac myocytes and caused a downward shift in the cell motion–Ca^{2+} transient relation induced by varying $[Ca^{2+}]_o$. Our results in freshly dispersed adult myocytes are consistent with that study.[18] In addition, both opioids caused a rightward shift in the cell shortening *versus* $[Ca^{2+}]_i$ relation, indicating a decrease in the affinity of the myofilament for Ca^{2+}. Thus, a decrease in myofilament Ca^{2+} sensitivity appears to be involved in opioid-induced myocardial depression.

Limitations of the Study and Clinical Relevance

Our results must be interpreted in the context of the experimental conditions (low temperature, 28°C; and low frequency of stimulation, 0.3 Hz). These conditions are necessary to maintain myocyte viability during these experiments. Peak plasma concentrations of 215 nM fentanyl have been reported after a 500-μg intravenous injection in humans.[37] Assuming 80% protein binding, the peak concentration of non-protein–bound fentanyl would be less than 50 nM. Similarly, the total serum morphine concentration after intravenous bolus injection is approximately 10 μM, resulting in a free plasma concentration of approximately 8 μM because of 20% protein binding.[38] Thus, the concentrations of opioids in this study that caused significant cardiac depression are likely to encompass the concentrations encountered in the clinical setting. Serum protein levels, their capacity to bind opioids, or both may vary in certain pathologic conditions (*e.g.*, hemodilution, liver disease, hypoproteinemia). Small changes in the amount or the binding capacity of proteins could result in an increase in the free plasma concentration of opioids. In addition, the myocardial depressant effect of fentanyl observed in this study may contribute to the profound hemodynamic depression observed during rapid infusion of high-dose fentanyl in the clinical setting.

The inhibitory effect of fentanyl on myocyte shortening appears to involve a decrease in the availability of intracellular free Ca^{2+} and a decrease in myofilament Ca^{2+} sensitivity. In contrast, the actions of morphine appear to be mediated primarily by a decrease in myofilament Ca^{2+} sensitivity. Both opioids prolonged the timing for shortening, and fentanyl prolonged the timing for the Ca^{2+} transient. Neither opioid had a direct effect on SR Ca^{2+} uptake or content at clinically relevant concentrations. At high concentrations, morphine decreased the size of the caffeine-releasable Ca^{2+} pool in intact cardiomyocytes.

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