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Chronic U50,488H abolishes inositol 1,4,5-trisphosphate and intracellular Ca^{2+} elevations evoked by κ-opioid receptor in rat myocytes

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Abstract

The inositol 1,4,5-trisphosphate (IP_3) content and intracellular free Ca^{2+} ($[Ca^{2+}]_i$) level in response to κ-opioid receptor stimulation with selective κ-opioid receptor agonists, dynorphin-(1-13) and *trans*-3,4-dichloro-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeacetamidel (U50,488H) were determined in ventricular myocytes. Both IP_3 and $[Ca^{2+}]_i$ were increased following κ-opioid receptor stimulation. The responses of IP_3 and $[Ca^{2+}]_i$ to κ-opioid receptor stimulation were abolished in myocytes of rats that had received chronic injection of U50,488H for 4 days. κ-Opioid receptor stimulation with U50,488H also reduced the $[Ca^{2+}]_i$ transient, induced by electrical stimulation and caffeine, both known to mobilize $[Ca^{2+}]_i$. The effect was abolished after the myocytes had been incubated with U50,488H at a subthreshold concentration for its effect on $[Ca^{2+}]_i$ for 24 h. The present study showed for the first time that, upon the development of tolerance to a κ-opioid receptor agonist, the responses of IP_3 and $[Ca^{2+}]_i$ to κ-opioid receptor stimulation were abolished. The lack of response in $[Ca^{2+}]_i$ was due to a failure of mobilization of Ca^{2+} from its intracellular pool. Further study is needed to determine the events that occur after the κ-opioid receptor stimulation to production of IP_3 upon the development of tolerance to a κ-opioid.

Keywords: κ-Opioid receptor, tolerance; Ca^{2+}, intracellular, free; Inositol 1,4,5-trisphosphate; Myocyte, ventricular; U50,488H

1. Introduction

Our previous studies showed that chronic treatment of rats with *trans*-3,4-dichloro-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeacetamidel (U50,488H), a selective κ-opioid receptor agonist, for 4 days, that induced tolerance to the agonist as indicated by an attenuated hypothermic response to U50,488H (Bhargava et al., 1989), significantly attenuated the negatively chronotropic, negatively inotropic and arrhythmogenic effects of the agonist in the isolated perfused rat heart (Xia et al., 1994). The maximum binding capacity and the dissociation constant of the cardiac κ-opioid receptor were, however, unchanged and increased slightly, respectively (Xia et al., 1994), suggesting that the altered cardiac functions upon the development of tolerance to a κ-opioid may not be mainly due to the changes in the binding properties of the κ-opioid receptor.

It has been found that in the rat heart, which has been shown to contain substantial κ-opioid receptor binding sites (Ventura et al., 1989; Tai et al., 1991), κ-opioid receptor stimulation with specific κ-opioid receptor agonists increased intracellular free Ca^{2+} ($[Ca^{2+}]_i$) concentration through mobilization of Ca^{2+} from its intracellular store (Tai et al., 1992; Ventura et al., 1992), and inositol 1,4,5-trisphosphate (IP_3) content (Ventura et al., 1992). In a subsequent study, the Ca^{2+} response was shown to be secondary to the production of IP_3 as interference with the production of IP_3 also interfered with the Ca^{2+} responses to κ-opioid receptor stimulation in the same direction (Wong et al., 1995).

The responses of IP_3/Ca^{2+} to a κ-opioid receptor agonist upon the development of tolerance has, however, not been studied. The purpose of the present study was therefore to test whether the responses in IP_3/Ca^{2+} to κ-opioid receptor stimulation with specific κ-opioid receptor agonists in the heart were altered upon the development of tolerance to the κ-opioid. We found that upon the development of tolerance to U50,488H, the elevations in IP_3 and $[Ca^{2+}]_i$ in responses to κ-opioid receptor stimulation were abolished and that the lack of elevation in $[Ca^{2+}]_i$ might be due mainly to the failure of mobilization of Ca^{2+} from its intracellular store. The preliminary results

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of part of the study have been presented in proceeding form (Sheng et al., 1994; Sheng and Wong, 1995).

2. Materials and methods

2.1. Induction of tolerance in the rat by daily injection with U50,488H

Male Sprague-Dawley rats (190–210 g) were divided into two groups. One group of rats was injected with U50,488H (25 mg/kg), while the other with vehicle, twice daily for 4 days as described previously (Bhargava et al., 1989). The treatment has been shown to produce a high degree of tolerance to the hypothermic effect (Bhargava et al., 1989; Xia et al., 1994) and the arrhythmogenic, and negative inotropic and chronotropic effects in the isolated heart (Xia et al., 1994) of U50,488H in the rat.

2.2. Isolation and culture of ventricular myocytes with U50,488H for induction of tolerance

Rats were killed by cervical dislocation. Ventricular myocytes were isolated as previously described (Tai et al., 1992). Retrograde perfusion of the coronary arteries with a Joklik-modified Eagle's medium (MEM) supplemented with 10 mM $NaHCO_3$, 10 mM Hepes, 0.1% bovine serum albumin, 125 U/ml collagenase (type I; Sigma) and 30 μM Ca^{2+} was employed for separation and isolation of the ventricular myocytes. After the cells had been isolated, they were suspended in MEM supplemented with 10 mM $NaHCO_3$, 10 mM Hepes, 1% bovine serum albumin and 1 mM Ca^{2+} (Hepes buffer-MEM, pH 7.2).

For the study involving incubation of ventricular myocytes with U50,488H, the cells were incubated, under 5% CO_2 atmosphere at 37°C, in culture dishes at a density of 2×10^{-5} cells/dish in 3 ml of MEM containing 0.2% bovine serum albumin, 10^{-8} M insulin, 100 U/ml of penicillin G, 100 μg/ml of streptomycin. One group of myocytes was incubated in the presence of U50,488H at 10^{-6} M, a subthreshold concentration for inhibiting the electrically induced $[Ca^{2+}]_i$ transients while the control group was in the absence of the κ-opioid receptor agonist.

2.3. IP_3 assay

Ventricular myocytes were resuspended at a final protein concentration of 1.8–2.0 mg/ml before the addition of the κ-opioid receptor agonist, dynorphin-(1-13). Each incubation with a κ-opioid receptor agonist dynorphin-(1-13) was quenched, at a specified time, with cold $HClO_4$ (0.6 M). After centrifugation at $2500 \times g$ for 10 min, the pellet was dissolved in 0.3 M NaOH, and the protein content was assayed using bovine serum albumin as a standard (Lowry et al., 1951). The acid supernatant was neutralized with a solution containing 1.5 M KOH and 60 mM Hepes to pH 7.0, and then the IP_3 content was estimated using an IP_3 binding protein [^{3}H] assay system (Amersham).

2.4. Measurement of $[Ca^{2+}]_i$ in ventricular myocytes

Both cell suspension and single cell preparations were used. Ventricular myocytes were loaded with 2 μM fura-2-AM at room temperature for 20 min in Hepes buffer-MEM. The cells loaded with fura-2 were resuspended in Krebs-Henseleit buffer ($NaHCO_3$ replaced with 25 mM Hepes) containing 1 mM Ca^{2+} and stored at room temperature for at least 1 h before use.

For the study of effect of κ-opioid receptor stimulation on the $[Ca^{2+}]_i$, a cell suspension preparation was employed. The $[Ca^{2+}]_i$ of ventricular myocytes of chronically U50,488H-treated rats was measured with the method of single-excitation wavelength as previously described (Tai et al., 1992). Briefly, $1-2 \times 10^4$ cells suspended in 1.5 ml of Krebs-Henseleit buffer were transferred into a temperature-controlled quartz cuvette equipped with a magnetic stirrer. The excitation wavelength was established using a 340 nm interference filter with 3 nm bandbass and the emitted light was collected through a 500 nm filter with a 10 nm bandbass in a Hatachi F-4000 Fluorescence Spectrofluorimeter. $[Ca^{2+}]_i$ was calculated according to the equation: $[Ca^{2+}]_i$ (nM) $= 224\,(F - F_{min})/(F_{max} - F)$. F_{max} was determined as the fluorescence of fura-2 after the cells were permeabilized with 0.48 mM digitonin followed by 100 μl 6% Triton-X-100 to lyse all subcellular membranes. F_{min} was determined as the fluorescence after addition of 3.33 mM EGTA, which sequestered Ca^{2+}. Preliminary experiments in our laboratory showed that the Ca^{2+} response reached a plateau at 1–2 min after administration of a κ-opioid receptor agonist and the plateau could last for at least 20 min in the presence of the agonist. In the present study, we observed the Ca^{2+} response for at least 2 min for each dose.

In the experiments for the study of electrically and caffeine-induced $[Ca^{2+}]_i$ transients, a single cell preparation was used. The myocytes selected for the study were rod shaped with clear striations. They exhibited a synchronous contraction (twitch) in response to suprathreshold 4 ms stimuli at 0.2 Hz delivered by a stimulator (Grass S88) through two platinum field-stimulation electrodes in the bathing fluid. Both electrical stimulation (DuBell et al., 1993 Janczewski et al., 1993) and caffeine (Bassani et al., 1993; Seki and MacLeod, 1995) were shown to mobilize intracellular Ca^{2+} from its store, leading to $[Ca^{2+}]_i$ transients.

Fluorescence measurements were carried out on a Nikon inverted microscope equipped with a fluorimetric system (PTI). A small aliquot of fura-2-loaded cells was placed on a cover-slip mounted at the bottom of the experimental chamber and then superfused with Krebs-Henseleit buffer containing 1.0 mM Ca^{2+} at 22°C. The Ca^{2+}-dependent

Table 1
Effects of U50,488H on $[Ca^{2+}]_i$ (nM) in isolated ventricular myocytes of rats treated with U50,488H for 4 days

	Before	Dose (M)		
		10^{-5}	3×10^{-5}	10^{-4}
Control	148.4±5.7	190.4±7.4	224.7±7.6	282.6±12.4
Chronic U50,488H-treated	140.1±10.2	153.4±12.2 [a]	167.9±15.0 [a]	182.4±18.3 [b]

Values are mean±S.E.M.; $n=8$.
[a,b] Significantly different from the corresponding control at $P<0.05$ and <0.01, respectively.

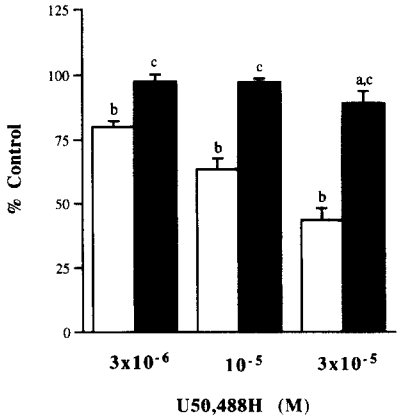


Fig. 1. Effects of U50,488H at 3×10^{-6}–3×10^{-5} M on the electrically induced $[Ca^{2+}]_i$ transient in a single isolated ventricular myocyte in the presence (■) or absence (□) of Mr2266 at 10^{-6} M. The amplitude of the $[Ca^{2+}]_i$ transient before drug treatment is 100%. Since the lowest point was reached at 20 min after administration of U50,488H, the values at this time point were used. Values are mean±S.E.M.; $n=8$ in the presence of Mr2266 and $n=7$ in the absence of Mr2266.
[a,b] Significantly different from the corresponding controls before drug treatment at $P<0.05$ and <0.01.
[c] Significantly different from the corresponding group with U50,488H only at $P<0.01$.

signal of fura-2 was obtained by illuminating at 340 and 380 nm, and recording the emitted light at 510 nm. The background fluorescence was subtracted. $[Ca^{2+}]_i$ transients were presented as fluorescence ratio with no attempt to calculate Ca^{2+} concentration, as absolute values of $[Ca^{2+}]_i$ were not essential for interpretation of the results.

In order to specifically assess the Ca^{2+} content in the sarcoplasmic reticulum in cardiac myocytes, rapid application of 10^{-2} M caffeine from a micropippette positioned directly above the cell, known to produce $[Ca^{2+}]_i$ transients by releasing Ca^{2+} from the sarcoplasmic reticulum (Bassani et al., 1993; Seki and MacLeod, 1995), was carried out following the administration of 10^{-5} M U50,488H. Caffeine was applied 10 s after cessation of electrical stimulation in cardiac myocytes. Normal amplitude of the electrically induced $[Ca^{2+}]_i$ transients could be restored in 60 s after washout of caffeine. For experiments involving the determination of the effect of U50,488H on the caffeine-induced $[Ca^{2+}]_i$ transient, caffeine was first applied to determine if the cell responded to the drug. 5 min later U50,488H was administered. The 5 min interval was to ensure that all caffeine had diffused out of the cell.

2.5. Statistical analysis

Paired Student's test was used to determine the difference between control and drug treatment and one way analysis of variance (ANOVA) was used to determine the difference among groups. Significance level was set at $P<0.05$.

3. Results

3.1. Effects of κ-opioid receptor stimulation on IP_3 and $[Ca^{2+}]_i$

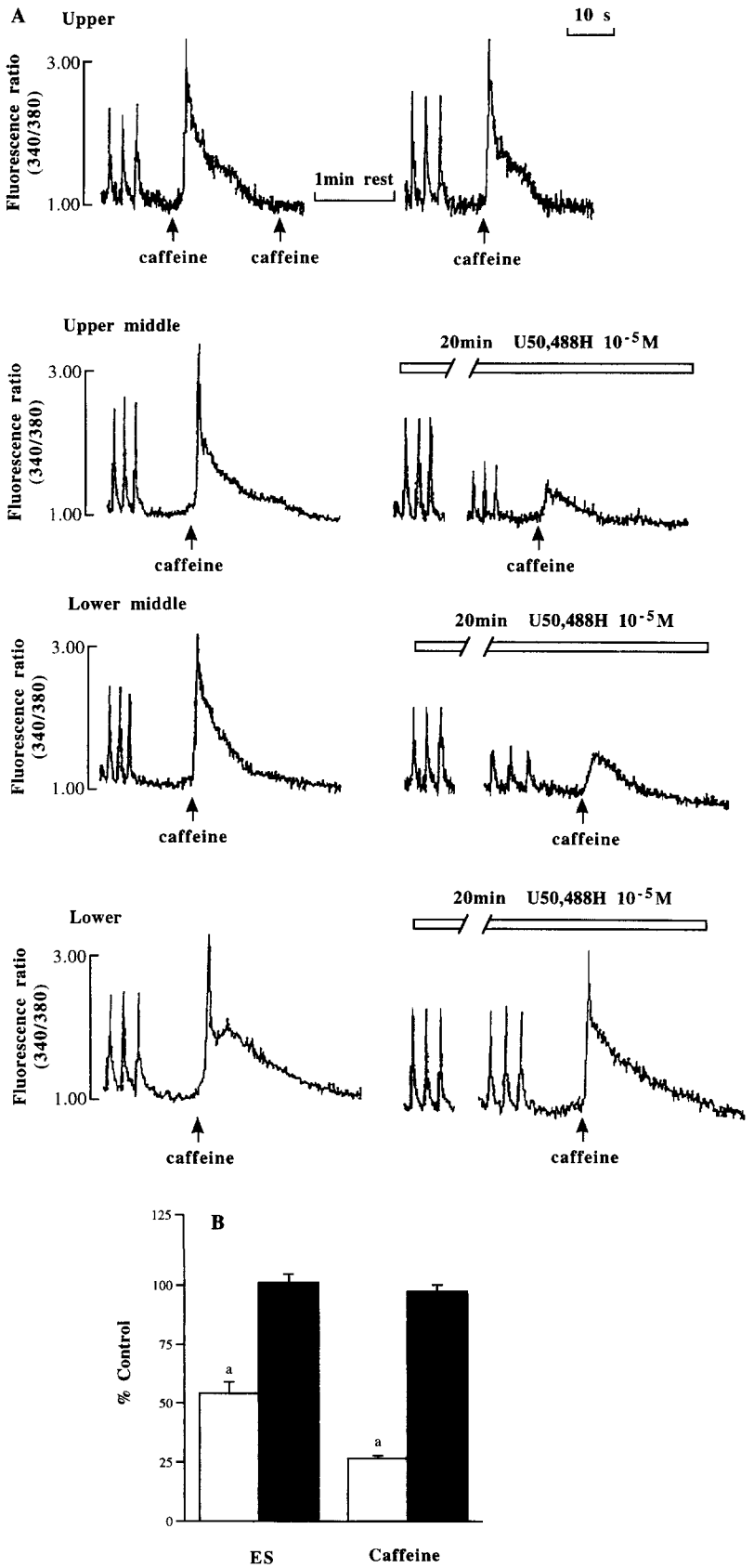
Since previous study in our laboratory showed that both U50,488H and dynorphin-(1-13) were equipotent in elevating $[Ca^{2+}]_i$ (Tai et al., 1992), both agonists were used for κ-opioid receptor stimulation in the present study. κ-Opioid receptor stimulation with U50,488H at 10^{-5}–10^{-4} M, the effects of which had been shown to be antagonized by specific κ-opioid receptor antagonists, (−)-3-furylmethyl-5,9,-diethyl-2-hydroxy-6,7-benzomorphinan (Mr2266) (Tai et al., 1992) and (−)-*N*-(3-furylmethyl)-α-normetazocine methanesulfonate (Mr1452) (Ventura et al., 1992), in the ventricular myocyte, increased $[Ca^{2+}]_i$ dose-dependently (Table 1). This was in agreement with the previous observations (Tai et al., 1992; Ventura et al., 1992). In rats injected with U50,488H for 4 days, the $[Ca^{2+}]_i$ did not show significant increases in response to κ-opioid receptor stimulation by U50,488H (Table 1).

Table 2
Effects of dynorphin-(1-13) at 50 μM on inositol 1,4,5-trisphosphate (pmol/mg protein) in isolated ventricular myocytes of rats treated with U50,488H for 4 days

	Before	Time (min)			
		0.25	0.5	1	2
Control	0.89±0.19	2.28±0.36	3.00±0.64	1.40±0.29	1.70±0.33
Chronic U50,488H-treated	0.97±0.33	0.74±0.15 [b]	1.06±0.33 [a]	1.21±0.28	0.53±0.10 [b]

Values are mean±S.E.M.; $n=8$.
[a,b] Significantly different from the corresponding control at $P<0.05$ and <0.01, respectively.

Similarly, κ-opioid receptor stimulation with another κ-opioid receptor agonist, dynorphin-(1-13) at 50 μM a concentration shown previously to produce Mr2266-reversible effects on $[Ca^{2+}]_i$ (Tai et al., 1992), also increased the IP_3 content in agreement with our previous observation (Wong et al., 1995). Time course changes showed that the


A
Upper
10 s
Fluorescence ratio (340/380)
3.00
1.00
1min rest
caffeine
caffeine
caffeine
Upper middle
20min U50,488H 10⁻⁵M
caffeine
caffeine
Lower middle
20min U50,488H 10⁻⁵M
caffeine
caffeine
Lower
20min U50,488H 10⁻⁵M
caffeine
caffeine
B
% Control
125
100
75
50
25
0
a
a
ES
Caffeine

IP_3 level increased rapidly, reaching the peak at 15–30 s followed by a sustained elevation lasting for over 2 min (Table 2). The same responses were previously observed following the administration of U50,488H (Ventura et al., 1992). The effects of κ-opioid receptor stimulation on IP_3 was also abolished following chronic injection with U50,488H for 4 days – both the peak response at 30 s and the secondary sustained elevation were absent (Table 2).

3.2. Effects of κ-opioid receptor stimulation on electrically and caffeine-induced $[Ca^{2+}]_i$ transients

In agreement with the previous observation (Ventura et al., 1992), U50,488H at 3×10^{-6}–3×10^{-5} M dose dependently reduced the electrically induced $[Ca^{2+}]_i$ transient in single ventricular myocytes (Fig. 1). At 10^{-6} M U50,488H did not affect the $[Ca^{2+}]_i$ transient (data not shown). Mr2266 at 10^{-6} M, which itself did not produce any effect, completely abolished and attenuated significantly the inhibitory effects of U50,488H on the electrically induced $[Ca^{2+}]_i$ transient at 3×10^{-6}, and 10^{-5} and 3×10^{-5} M, respectively (Fig. 1). The effect of U50,488H at 10^{-5} M on the $[Ca^{2+}]_i$ transient was absent in myocytes incubated with U50,488H at 10^{-6} M, a dose that had no inhibitory effect on the $[Ca^{2+}]_i$ transient, for 24 h (Fig. 2). However, in myocytes incubated with U50,488H at 10^{-6} M for 16 h the inhibitory effect of κ-opioid receptor stimulation was still present (data not shown).

Caffeine at 10^{-2} M induced a $[Ca^{2+}]_i$ transient, which was restored at 1 min after removal of the drug by washing (Fig. 2A), a phenomenon observed previously (Bassani et al., 1993; Seki and MacLeod, 1995). In ventricular myocytes perfused with U50,488H at 10^{-5} M for 20 min, that reduced the electrically induced $[Ca^{2+}]_i$ transient, also reduced the caffeine-induced $[Ca^{2+}]_i$ transient to as low as 27% of the control (Fig. 2B). In ventricular myocytes incubated with U50,488H for 24 h, U50,488H had no effect on the caffeine-induced $[Ca^{2+}]_i$ transient as in the case of the electrically induced $[Ca^{2+}]_i$ transient (Fig. 2).

4. Discussion

The most important finding in the present study is that after tolerance to U50,488H had been induced by chronic injection of the κ-opioid for 4 days, the responses in IP_3 production and Ca^{2+} to κ-opioid receptor stimulation were abolished. In addition, incubation of ventricular myocytes for 24 h with U50,488H at 10^{-6} M, a subthreshold concentration for its effect on $[Ca^{2+}]_i$, also abolished the inhibitory effects of the agonist on the $[Ca^{2+}]_i$ transient induced by electrical field stimulation of the ventricular myocyte or caffeine The observation indicates that tolerance was also induced by incubation with the κ-opioid receptor agonist for 24 h. It also confirms that Ca^{2+} response to κ-opioid receptor stimulation was abolished upon the development of tolerance. In addition, since both electrical stimulation and caffeine are known to mobilize $[Ca^{2+}]_i$, the abolition of the inhibitory effects of κ-opioid receptor stimulation on electrically and caffeine-induced $[Ca^{2+}]_i$ transients upon the development of tolerance indicates failure of mobilization of intracellular Ca^{2+} from its intracellular pool. Thus, after the development of tolerance, the κ-opioid receptor stimulation failed to trigger the cascade of events – increased production of IP_3, mobilization of Ca^{2+} from its intracellular store and as a consequence increased $[Ca^{2+}]_i$. Since the development of tolerance was accompanied by a slight reduction in the affinity of the binding sites to κ-opioid receptor agonist, but not by any change in the number of κ-opioid receptor binding sites (Xia et al., 1994), it appears that the failure to elicit responses in IP_3/Ca^{2+} following the development of tolerance of κ-opioid receptor is due mainly to abnormalities distal to receptor stimulation. Further studies are needed to address the underlying cellular mechanisms upon the development of tolerance in κ-opioid receptor. Of particular interest will be the coupling between receptor and G-proteins, which has been shown to mediate the physiological effects of κ-opioid receptor stimulation (Baraban et al., 1995; Ma et al., 1995). Tao et al., 1993 has shown that tolerance to μ- and δ-opioids may result from uncoupling between opioid receptors and G-proteins whereas Polastrom et al., 1993 were unable to find a modification of the physical coupling between the μ receptor and its G-protein following chronic morphine treatment. Abundance of G-protein is also important as development of tolerance to morphine has been shown to increase G-proteins in human neuroblastoma cells (Ammer and Schulz, 1993). The biochemical events from G-proteins to IP_3 formation, which are not well understood in signal transduction in opioid

Fig. 2. Effects of U50,488H (10^{-5} M) on the electrically and caffeine-induced $[Ca^{2+}]_i$ transient in a single isolated ventricular myocyte incubated with U50,488H (10^{-6} M).

(A) Representative tracings. Upper: $[Ca^{2+}]_i$ transients induced by both electrical stimulation and caffeine; note it took 1 whole min before caffeine was able to induce another $[Ca^{2+}]_i$ transient. Upper middle: $[Ca^{2+}]_i$ transients induced by both electrical stimulation and caffeine in the presence of U50,488H (10^{-5} M). Lower middle: $[Ca^{2+}]_i$ transients induced by both electrical stimulation and caffeine in the presence of U50,488H (10^{-5} M) following incubation with vehicle for 24 h. Lower: $[Ca^{2+}]_i$ transients induced by both electrical stimulation and caffeine in the presence of U50,488H (10^{-5} M) following incubation with U50,488H (10^{-6} M) for 24 h.

(B) Effects of U50,488H (10^{-5} M) on both electrically and caffeine-induced $[Ca^{2+}]_i$ transient following incubation of the myocytes with U50,488H (10^{-6} M) for 24 h. □, vehicle; ■, incubated with U50,488H. The amplitude of the $[Ca^{2+}]_i$ transient before drug treatment is 100%; ES, electrical field stimulation. Values are mean ± S.E.M.; $n = 5$. [a] Significantly different from the corresponding group without incubation with U50,488H at $P < 0.01$.

receptor upon the development of tolerance, also warrant further studies.

A major concern with the present study is the high concentrations of κ-opioid receptor agonists (10^{-5}–10^{-4} M) used. In previous studies in the same ventricular myocyte preparation, the effects of the same κ-opioid receptor agonist, U50,488H, in the same concentration range on $[Ca^{2+}]_i$ were shown to be antagonized by κ-opioid receptor antagonist, Mr1452 at 10^{-6} M (Ventura et al., 1992). Similarly, in the present study the effects of U50,488H on the electrically induced $[Ca^{2+}]_i$ transient were also antagonized by Mr2266 at 10^{-6} M. That the effects of high concentration of the κ-opioid receptor agonist were blocked by κ-opioid receptor antagonists indicates that the effects were κ-opioid receptor mediated. High concentrations of κ-opioid receptor agonists may be required to act on the low affinity κ-opioid receptor site, which has been demonstrated to be present in the heart (Jin et al., 1995). High concentrations of the κ-opioid receptor agonist have also been reported to produce κ-opioid receptor-mediated effects in other tissues. In a catecholamine neuronal cell line, U50,488H at 10^{-5} M inhibited the voltage-activated K^+ current and the effects of U50,488H on the K^+ current were blocked by a specific κ-opioid receptor antagonist, nor-binaltorphimine (Baraban et al., 1995). Similarly, in three related thymoma cell lines, which expressed a single high-affinity κ_1-opioid receptor, U50,488H at 10^{-5} M suppressed forskolin-stimulated adenylyl cyclase activity (Lawrence et al., 1995). It is also not uncommon to find that very high concentrations of non-opioid substances are required to elicit a receptor-mediated effect. For example, carbachol, a muscarinic receptor agonist, at 10^{-4} M, increased the force of contraction of the chick atrium, which was blocked by atropine (Tajima et al., 1987). Carbachol also increased a calcium current in the guinea-pig ventricular myocyte, which was blocked by a M_1 muscarinic receptor antagonist, pirenzepine (Gallo et al., 1993). An obvious question is the physiological implication of the effects of such high concentrations. Further studies are needed to address the question.

In agreement with our previous observation that tolerance of ventricular myocytes to a μ-opioid receptor agonist was induced after incubation of the cells with the agonist for 24 h (Huang and Wong, 1991), the present study showed that tolerance to a κ-opioid could be induced by incubating the myocytes with the opioid at a subthreshold concentration for 24 h, a much shorter time than that required for inducing tolerance in the whole animal. This is obviously a good model for the study of cellular mechanism in tolerance as it is simple, and requires less drug and shorter time for induction of tolerance. In addition, it is devoid of the systemic and nervous influence, an obvious advantage over the whole animal preparation. The cardiac myocytes are also a suitable model for the study of cellular mechanism in the κ-opioid receptor in which IP_3/Ca^{2+} has been suggested to be involved in the signal transduction upon κ-opioid receptor stimulation (Ventura et al., 1992; Wong et al., 1995).

In a previous study, pretreatment of myocytes with ryanodine has been shown to abolish the effect of κ-opioid receptor agonists to elevate $[Ca^{2+}]_i$ (Tai et al., 1992), indicating that the ryanodine-sensitive pool overlaps with the κ-opioid receptor agonist sensitive pool. In the present study, it was found that pretreatment of the ventricular myocytes with U50,488H reduced the caffeine-induced $[Ca^{2+}]_i$ transient and that, upon the development of tolerance when U50,488H failed to mobilize Ca^{2+}, the κ-opioid receptor agonist also failed to reduce the caffeine-induced $[Ca^{2+}]_i$ transient. The observations support the suggestion that the κ-opioid receptor agonist-sensitive pool overlaps with the ryanodine-sensitive and caffeine-sensitive pools (Ventura et al., 1992).

In conclusion, the present study demonstrated for the first time that, upon the development of tolerance to a κ-opioid receptor agonist, the responses of the IP_3/Ca^{2+} system to κ-opioid receptor stimulation were abolished. The altered cardiac functions upon the development of tolerance to a κ-opioid receptor agonist may be due to the abolition of the responses of the IP_3/Ca^{2+} system to the agonist. Since there were little changes in the binding properties of κ-opioid receptor binding sites, suggesting alterations in binding may not be the main factor responsible for the altered cardiac functions upon the development of tolerance (Xia et al., 1994), further studies are needed to investigate the events that occur after the κ-opioid receptor stimulation to production of IP_3.

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References

Ammer, H. and R. Schulz, 1993, Alterations in the expression of G-proteins and regulation of adenylate cyclase in human neuroblastoma SH-SY5Y cells chronically exposed to low-efficacy μ-opioids, Biochem. J. 295, 263.

Baraban, S.C., E.W. Lothman, A. Lee and P.G. Geyenet, 1995, Kappa opioid receptor-mediated suppression of voltage-activated potassium current in a catecholaminergic neuronal cell line, J. Pharmacol. Exp. Ther. 273, 927.

Bassani, J.W.M., R.A. Bassani and D.M. Bers, 1993, Twitch-dependent SR Ca accumulation and release in rabbit ventricular myocytes, Am. J. Physiol. 265, C533.

Bhargava, H.W., A. Gulati and P. Ramarao, 1989, Effect of chronic administration of U50,488H on tolerance to its pharmacological ac-

tions and on multiple opioid receptors in rat brain regions and spinal cord, J. Pharmacol. Exp. Ther. 251, 21.

DuBell, W.H., B. Lewartowski, H.A. Spurgeon, H.S. Silverman and E.G. Lakatta, 1993, Repletion of sarcoplasmic reticulum Ca after ryanodine in rat ventricular myocytes, Am. J. Physiol. 265, H604.

Gallo, M.P., G. Alloatti, C. Eva, A. Oberto and R.C. Levi, 1993, M1 muscarinic receptors increase calcium current and phosphoinositide turnover in guinea-pig ventricular cardiocytes, J. Physiol. 471, 41.

Huang, X.D. and T.M. Wong, 1991, Morphine and (D-Ala^2, NME-Phe^4, Gly-ol)-enkephalin increase the intracellular free calcium in isolated rat myocytes – effect of naloxone or pretreatment with morphine, Life Sci. 48, 1101.

Janczewski, A.M. and E.G. Lakatta, 1993, Thapsigargin inhibits Ca^{2+} uptake, and Ca^{2+} depletes sarcoplasmic reticulum in intact cardiac myocytes, Am. J. Physiol. 265, H517.

Jin, W.-Q., K.K. Tai, T.K.Y. Chan and T.M. Wong, 1995, Further characterization of [^{3}H]U69593 binding sites in the rat heart, J. Mol. Cell. Cardiol. 27, 1507.

Lawrence, D.M.P. D.B. Joseph and J.M. Bidlack, 1995, Kappa Opioid receptors expressed on three related thymoma cells lines, Biochem. Pharmacol. 49(1), 81.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951, Protein measurement with the folic reagent, J. Biol. Chem. 193, 265.

Ma, G.H., R.J. Miller, A. Kuznetsov and L.H. Philipson, 1995, Kappa-Opioid receptor activates an inwardly retifying K^+ channel by a G protein-linked mechanism: coexpression in *Xenopus* oocytes, Mol. Pharmacol. 47, 1035.

Polastrom, J., J.-C. Meunier and P. Jauzac, 1993, Chronic morphine induces tolerance and desensitization of μ-opioid receptor but not down-regulation in rabbit, Eur. J. Pharmacol. 266, 139.

Seki, S. and K.T. MacLeod, 1995, Effects of anoxia on intracellular Ca^{2+} and contraction in isolated guinea pig cardiac myocytes, Am. J. Physiol. 268, H1045.

Sheng, J.Z. and T.M. Wong, 1995, Effects of U50,488H on electrically-induced cytosolic Ca^{2+} transients in rat ventricular myocytes following chronic treatment with U50,488H, Analgesia 1(4–6), 723.

Sheng, J.Z., K.K. Tai, K.K. and T.M. Wong, 1994, Decreased responses in intracellular free calcium and inositol 1,4,5-trisphosphate to κ-opioid receptor stimulation in the heart of chronically U50,488H-treated rats, Regul. Pept. 54(1), 271.

Tai, K.K., W.Q. Jin, T.K.Y. Chan and T.M. Wong, 1991, Characterization of [^{3}H]-69593 binding sites in the rat heart by receptor binding studies, J. Mol. Cell. Cardiol. 23, 1297.

Tai, K.K., C.F. Bian and T.M. Wong, 1992, κ-Opioid receptor stimulation increases intracellular free calcium in isolated rat ventricular myocytes, Life Sci. 51, 909.

Tajima, T., Y. Tsuji, J.H. Brown and A.J. Pappano, 1987, Pertussis toxin-insensitive phosphoinositide hydrolysis, membrane depolarization, and positive inotropic effect of carbachol in chick atria, Circ. Res. 61, 436.

Tao, P.-L., L.-R. Chang, Y.-P. Chou, P.-Y. Law and H.H. Loh, 1993, Chronic opioid treatment may uncouple opioid receptors and G-proteins: evidence from radiation inactivation analysis, Eur. J. Pharmacol. 246, 233.

Ventura, C., L. Bastagli, P. Bernardi, C.M. Caldarera and C. Guarnieri, 1989, Opioid receptors in rat cardiac sarcolemma: effect of phenylephrine and isoproterenol, Biochim. Biophys. Acta 987, 69.

Ventura, C., H. Spurgeon, E.G. Lakatta, C. Guarnieri and M.C. Capogrossi, 1992, κ and δ Opioid receptor stimulation affects cardiac myocyte function and Ca^{2+} release from an intracellular pool in myocytes and neurons, Circ. Res. 70, 66.

Wong, N.S., J.Z. Sheng, K.K. Tai and T.M. Wong, 1995, Lithium ion alters ins(1,4,5)P_3 production and mobilization of intracellular free calcium in κ-opioid receptor agonist-stimulated rat ventricular myocytes, Analgesia 1(4–6), 842.

Xia, Q., J.Z. Sheng, K.K. Tai and T.M. Wong, 1994, Effects of chronic U50,488H treatment on binding and mechanical responses of the rat heats, J. Pharmacol. Exp. Ther. 268, 930.