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Highly selective σ receptor ligands elevate inositol 1,4,5-trisphosphate production in rat cardiac myocytes

Marie Novakova [a,1], Catherine Ela [a], Wayne D. Bowen [b], Yonathan Hasin [c], Yael Eilam [a,*]

[a] *Department of Bacteriology, Hebrew University-Hadassah Medical School, Jerusalem, Israel*
[b] *Laboratory of Medicinal Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Health, Bethesda, MD 20892, USA*
[c] *Department of Cardiology, Hadassah University Hospital, Jerusalem, Israel*

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Abstract

Exposure of cardiac myocytes from adult rat ventricles to the highly selective, high affinity σ receptor ligands 1*S*,2 *R-cis-N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(1-pyrrolidinyl)-cyclohexylamine (BD-737) (0.1–100 nM) and *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*,*N*′,*N*′-trimethylethylenediamine (BD-1047) (0.01–10 nM), caused potentiation of electrically-evoked amplitudes of contraction and Ca^{2+} transients, while exposure to 100 nM BD-1047 caused attenuation of these amplitudes. In addition, BD-737 (1–100 nM) and BD-1047 (10–100 nM) caused an increase in the incidence of spontaneous twitches. These effects were inhibited when the incubation with BD-737 was done in the presence of the phospholipase C inhibitor, neomycin, or after pre-incubation with thapsigargin or caffeine which deplete the sarcoplasmic reticulum Ca^{2+} stores. Inositol 1,4,5-trisphosphate (IP_3) production in cardiac myocytes was determined by the IP_3 binding protein assay. Both substances caused an increase in the intracellular concentration of IP_3. BD-737 caused a rapid transient increase to 3.2-fold in 1 min and stabilization at 2.1-fold of control thereafter. BD-1047 caused a gradual increase reaching 4.4-fold after 5 min. The results suggest that the effects of these σ receptor ligands on contractility and spontaneous contractions are mediated by activation of phospholipase C and elevation of intracellular IP_3 level.

Keywords: σ Receptor; Cardiac myocyte; Contractility; Ca^{2+}, cytosolic; Inositol 1,4,5-trisphosphate; Phospholipase C

1. Introduction

The σ receptors are widely distributed in mammalian brain, immune and endocrine systems and in many peripheral organs, though the exact cellular function of these receptors is still largely unknown. The σ receptors have been implicated in modulation of a number of biochemical, physiological and behavioral processes in the central nervous system as well as in the endocrine, immune and gastric systems. However, the signal transduction pathway(s) mediating the functional response to σ ligand–receptor interaction in different tissues are still unknown (for reviews see the works of Walker et al. (1990), Itzhak and Stein (1990), Su (1991), Su and Junien (1994), Bowen (1994), Walker et al. (1994) and Wolfe and De Souza (1994)).

Recently, the σ receptor has been cloned from guinea pig liver (Hanner et al., 1996) and from human placental choriocarcinoma cells (Kekuda et al., 1996). Expression of the human σ_1 receptor in several human tissues, including human heart tissue, has been reported (Kekuda et al., 1996).

Our group has shown the presence of authentic σ binding sites, mainly of the σ_1 subtype, on cardiac myocytes from neonatal and adult rats (Ela et al., 1994; Novakova et al., 1995). In these cells, nanomolar concentrations of the prototypic σ receptor ligands (+)-3-hydroxyphenyl-*N*-(1-propyl)piperidine ((+)-3-PPP), (+)-pentazocine, or haloperidol exerted effects on contractility, Ca^{2+}-transients and Ca^{2+} fluxes (Ela et al., 1994, 1996; Novakova et al., 1995). The unique features of this study are: (1) effects are induced by very low concentrations of σ receptor ligands, at the nanomolar range; (2) an effect was found on a well characterized second messenger,

* Corresponding author. Tel.: +972-2-6758240; Fax: +972-2-6784010; E-mail: eilam@md.huji.ac.il
[1] Present address: Department of Physiology, Medical Faculty of Masaryk University, Brno, Czech Republic.

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cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$); (3) our work describes significant effects of σ receptor ligands on cardiac myocytes; this work may suggest an important role of endogenous σ receptor ligands (still not disclosed, although suggested, see the work of Patterson et al. (1994)) in the regulation of cardiac functions, and might suggest that cardiac side-effects may be induced by drugs in clinical use which have a high affinity for σ receptors.

The synthesis and characterization of a novel class of σ receptor ligands based on the aryl ethylenediamine pharmacophore have been reported (Radesca et al., 1991; Bowen et al., 1992b; De Costa et al., 1992a,b, 1993). Radioligand binding studies revealed that the novel receptor ligands 1*S*,2*R*-*cis*-*N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(1-pyrrolidinyl)-cyclohexylamine (BD-737) and *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*,*N*′,*N*′-trimethylethylenediamine (BD-1047) are among the most selective σ receptor ligands existent with exceptionally high affinity (Bowen et al., 1992b; Matsumoto et al., 1995). Functional studies have indicated that BD-737 produces alterations in motor behavior, causes inhibition of muscarinic cholinergic phosphoinositide response (Bowen et al., 1992b) and potentiates *N*-methyl-D-aspartate (NMDA)-evoked noradrenaline release from rat hippocampal slices (Monnet et al., 1996), thus acting as an agonist. BD-1047 has been classified as a putative antagonist since this ligand attenuated the dystonia produced by 1,3-di-*o*-tolylguanidine in rats (Matsumoto et al., 1995).

In the present study we investigated the effects of the σ receptor agonist BD-737 and the putative antagonist BD-1047 on the modulation of the amplitudes of electrically evoked $[Ca^{2+}]_i$-transients and contractions in cardiac myocytes isolated from adult rat ventricles. It was shown that both ligands caused significant, dose dependent, transient increases in contractile amplitude. This effect appears to be mediated by elevated production of inositol 1,4,5-trisphosphate (IP_3), which in turn potentiates the systolic release of Ca^{2+} from sarcoplasmic reticulum-Ca^{2+} stores and as a result causes transient increase in contractile amplitudes. At the higher concentration range, a longer exposure to BD-1047 caused sustained elevation of IP_3, subsequent depletion of Ca^{2+} stores and as a result attenuation of $[Ca^{2+}]_i$-transients and contractility.

2. Materials and methods

2.1. Preparation of isolated ventricular myocytes

Preparation of isolated rat cardiac myocytes, using variations of the methods described by Kramer et al. (1991) and Hansford and Lakatta (1987), has been described previously (Novakova et al., 1995). Briefly, hearts were removed from adult Sabra rats (220–250 g) anesthetized with diethyl ether. The heart was immediately attached to an aortic cannula and retrogradely perfused at constant pressure with Krebs–Henseleit physiological salt solution containing (in mM): 118 NaCl, 4.5 KCl, 24 $NaHCO_3$, 5 glucose, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 20 taurine and 1.2 $CaCl_2$, which was equilibrated with an atmosphere of 95% O_2 and 5% CO_2, and maintained at 37°C throughout the experiment. After 5 min, the solution was switched to a similar Krebs–Henseleit physiological salt solution, but containing 5 μM $CaCl_2$ for 5 min, and subsequently to a similar salt solution, containing 5 μM $CaCl_2$, 0.03% collagenase and 0.01% protease (Sigma) for 12 min. The ventricle was then removed, cut into small pieces and shaken in Krebs–Henseleit physiological salt solution containing 0.8% albumin and 50 μM $CaCl_2$. The cells were then collected by centrifugation in Krebs–Henseleit salt solution containing 0.8% albumin and 1.2 mM $CaCl_2$, and allowed to attach themselves to circular glass coverslips (22 mm) coated with poly-L-lysine.

2.2. Contractility measurements

The glass coverslips with the attached isolated adult ventricular myocytes were placed in a cell chamber equipped with a glass bottom and constantly superfused with Krebs–Henseleit salt solution. The temperature of the superfusion solution was maintained at 37°C throughout the experiments. The cell chamber was mounted on the stage of an inverted microscope. Myocytes were field-stimulated about 25% above threshold with 1-ms square wave pulses (30–40 V), through two platinum electrodes placed in the superfusion solution, and connected to a stimulator (Grass Instruments, MA).

Cell motion was measured by recording cell-edge movement using a phase-contrast microscope–video motion detector system, as previously described (Ela et al., 1994). After equilibration with the superfusion solution and stabilization of the contractile amplitude of the cardiomyocytes, the drugs, dissolved in Krebs–Henseleit solution, were added by superfusion. The results are expressed as a percentage of contractile amplitude of the same cell before drug addition. The contractile amplitude of control cells did not change by more than ±3% during the time of the experiments.

2.3. Loading cells with indo-1 and measurement of $[Ca^{2+}]_i$-transients

Stock solutions of indo-1-acetoxymethylester (indo-1/AM) from Molecular Probes (Eugene, OR) were prepared as described (Du Bell et al., 1988). Indo-1/AM loading solution was composed of Krebs–Henseleit physiological salt solution equilibrated with an atmosphere of 95% O_2 and 5% CO_2 and 6 μM indo-1. Cells attached to glass coverslips were incubated in indo-1 loading solution in the dark, in 5% CO_2–95% O_2 atmosphere, at 37°C for 15 min.

Measurement of $[Ca^{2+}]_i$-transients was done as described previously (Novakova et al., 1995) by using a FM-1000 dual wavelength fluorescence microphotometer (Rincon, San Paulo, CA). The glass coverslips with the indo-1-loaded cells were placed in a cell chamber with a glass bottom which was mounted on the stage of the microphotometer. The cells were superfused constantly with Krebs–Henseleit physiological salt solution equilibrated with an atmosphere of 95% O_2 and 5% CO_2, at 37°C. After a 10-min wash, the cells were field stimulated as described above. Indo-1 fluorescence ratio at 410/480 nm was recorded from single cells before and at different time intervals after drug addition. Each measurement continued approximately 5 s. Between measurements, the cells were kept in the dark to minimize indo-1 bleaching. The results were not calibrated to $[Ca^{2+}]_i$ values but are presented as indo-1 fluorescence ratios. In rat cardiomyocytes loaded with indo-1 it has been indicated that calibration is not precise due to the subcellular compartmentalization of indo-1 (Spurgeon et al., 1990). Because the fluorescence ratio is a monotonic function of $[Ca^{2+}]_i$ the difference between the peak-systolic and diastolic indo-1 fluorescence ratio was measured in each cell before and after drug addition and expressed as percent change in the value of this difference induced by the drugs, as compared to the value in the same cell before drug addition.

2.4. IP_3 assay

Ventricular myocytes were isolated, suspended in Krebs–Henseleit salt solution at protein concentration of 1.0–1.2 mg/ml and divided into 100 μl samples in centrifuge tubes which were maintained at 37°C. Each sample was pre-incubated for 10 min with 10 mM LiCl (to inhibit inositol phosphate metabolism, Lynch et al., 1997) before the addition of BD-737 or BD-1047, for the specified time. The reaction was stopped by the addition of cold $HClO_4$ (0.6 M). The tubes were maintained in ice for 20 min, then centrifuged, and the pellet was kept for protein determination by the Lowry method (Lowry et al., 1951) after dissolving the pellets in 0.3 M NaOH. The supernatant was neutralized to pH 7.0 with 1.5 M KOH containing 60 mM HEPES. IP_3 content was determined by an IP_3 binding protein assay according to the manufacturer's instructions (Amersham, Arlington Heights, IL)

3. Results

3.1. Effects of BD-737 and BD-1047 on contractility

Fig. 1a shows a representative tracing of ventricular myocyte twitch amplitudes before and after exposure to 10 nM BD-737. The response is characterized by potentiation of the twitch amplitudes elicited by electrical stimulation. The potentiation of the twitch amplitude was often accompanied by one or two bursts of a few spontaneous twitches

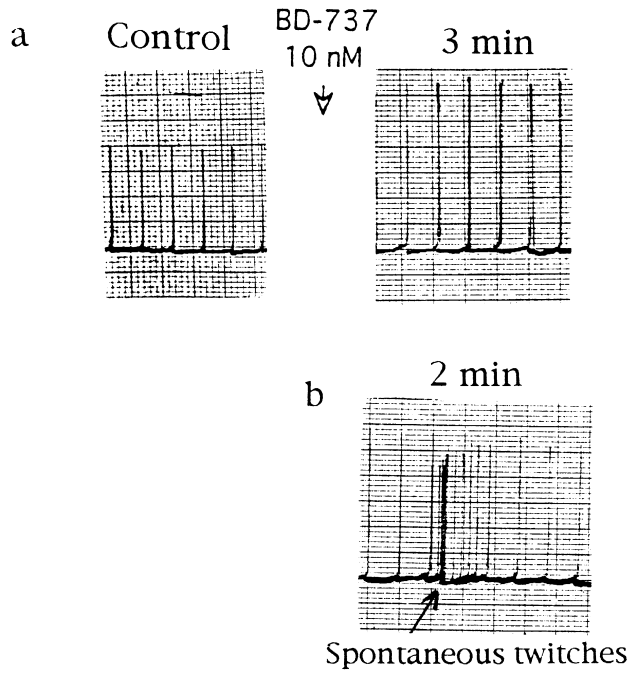

Fig. 1. Original recording showing the effects of BD-737 on contractility of a single cardiac myocyte isolated from adult rat. The recording, done before and after the addition of 10 nM BD-737 to the superfusion solution, shows (a) an increase in contractile amplitude after the addition of BD-737, and (b) a burst of spontaneous contractions which occurred 2 min after drug addition.

as shown in Fig. 1b. The incidence of spontaneous twitches was significant at 1 nM BD-737 and 10 nM BD-1047, it increased with the elevation of drug concentration, and was higher in cells exposed to BD-737 than in cells exposed to BD-1047 (Fig. 2).

The effects of BD-737 and BD-1047 on contractility of myocardial cells were assessed by recording the twitch amplitudes during 30 min after exposure to various con-

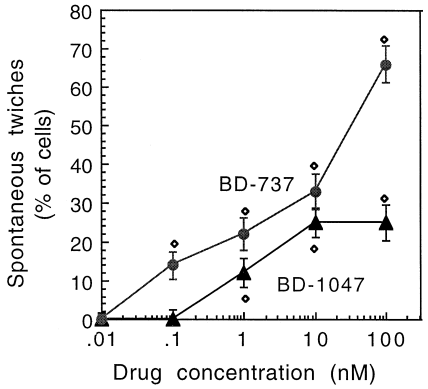

Fig. 2. Effects of BD-737 and BD-1047 on the frequency of the incidence of spontaneous contractions. The data show the percentage of cells in which at least one burst of spontaneous contractions occurred during a period of 30 min after drug addition to the superfusion physiological salt solution. Means ± S.E.M. of six different preparations are shown. * Significant difference ($P \leq 0.01$) from control, determined by *t*-test for small samples.

centrations of BD-737 and BD-1047 (0.01–100 nM). Slow oscillations in amplitude were observed in cardiac myocytes exposed to all examined concentrations of the two compounds, but the magnitude of the maximal and minimal amplitudes varied according to the concentrations and the compounds used (Fig. 3). In most of the experiments

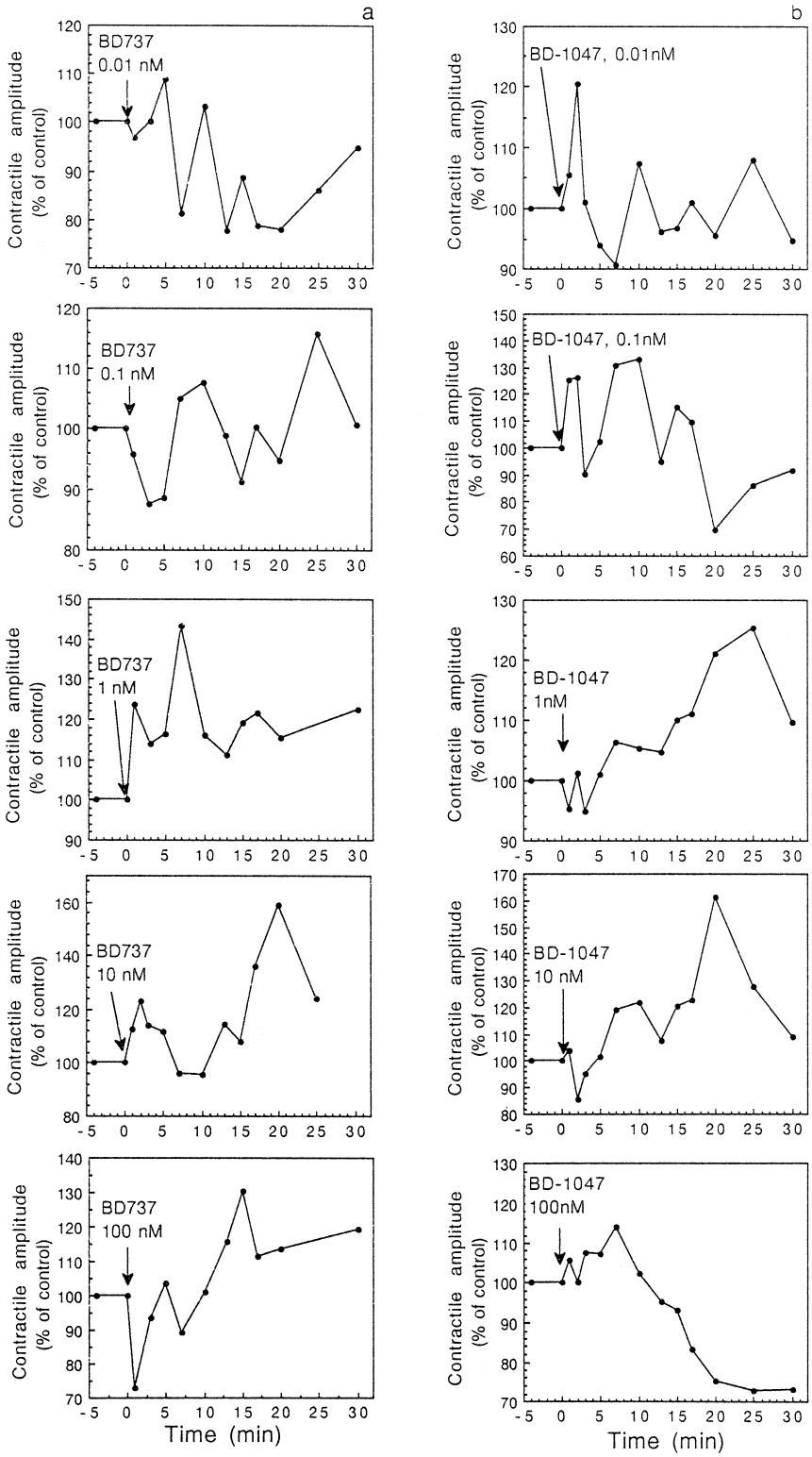

Fig. 3. Time-courses of the effects of BD-737 and BD-1047, at different concentrations, on the amplitude of contraction in cardiac myocytes from adult rats. Cell motion was continuously recorded in single cardiomyocytes isolated from adult rats and superfused with physiological salt solution. After stabilization of the contractile amplitude, (a) BD-737 or (b) BD-1047, at concentrations indicated in the figure, was added to the superfusion solution for 30 min. The data represent measurements from single cells. Similar results were obtained in six to nine cells exposed to each concentration.

two peaks in amplitude were observed during the time of measurement. Recordings from six to nine cells exposed to each of the concentrations were used to calculate the mean ± S.E.M. of the major and the second peak, the lowest and second lowest minimal amplitude, as well as the amplitude at the end of the experiments at 30 min. All data were calculated as percentages of the amplitude of contraction measured in the same cell before the exposure to the σ receptor ligand (Fig. 4). At a concentration of 0.01 nM, BD-737 caused a predominantly inhibitory response. Potentiation of contraction was observed from 0.1 to 10 nM BD-737, at which concentration the maximal contractile amplitude reached 197.8% ± 16.4% of control, and amplitudes at the end of the experiment reached 176.7% ± 27% of control. Cardiomyocytes exposed to 100 nM BD-737 responded with a smaller potentiation of contraction as compared with the myocytes exposed to 10 nM. The maximal peak amplitude and the amplitude at the end of the experiment reached 143.1% ± 7.3% and 139.4% ± 8.7% of control, respectively. Thus, BD-737 exerted a biphasic effect on contraction triggered by electrical stimulation (Fig. 4a). The response of the cardiomyocytes to BD-1047 was somewhat different. At 0.01 nM the peak amplitude reached 125.3% ± 4.2% of control, the smallest amplitude was 89.9% ± 4.9% and the end point was at the control level. The values of the peak amplitude increased dose dependently up to 10 nM BD-1047 whereas the smallest amplitudes remained between 83%–91% of control. The increase in the peak amplitude was less pronounced than in myocytes exposed to BD-737; at 10 nM BD-1047 the major peak reached 141.8% ± 13.9% of control. At 100 nM the response of the myocytes to BD-1047 was predominantly inhibitory. After an initial small increase (116.9% ± 9.6%) the amplitude decreased below control level and the end point was at 80.8% ± 4.9% of control (Fig. 4b). Thus, a biphasic effect was exerted by BD-1047 on cardiomyocyte contractility; at 100 nM, BD-1047 acted as an inverse agonist.

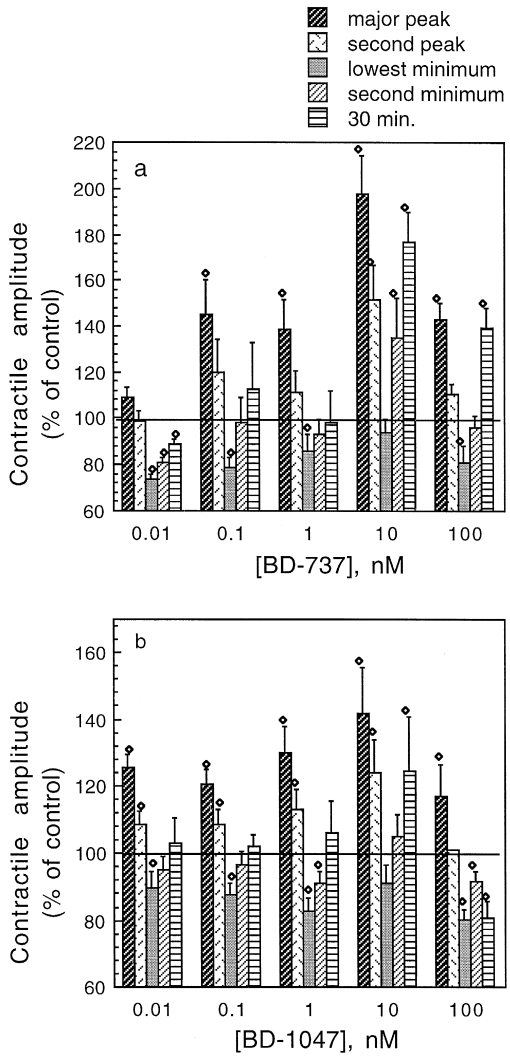

Fig. 4. The maximal and minimal amplitudes of contraction as a function of the applied concentration of BD-737 and BD-1047. Cell motion was continuously recorded in single cardiomyocytes isolated from adult rats and superfused with physiological salt solution, containing the indicated concentration of BD-737 or BD-1047. Slow oscillations in amplitudes were observed in cardiac myocytes exposed to all examined concentrations of the two compounds. Recordings from six to nine cells exposed to each concentration of (a) BD-737 and (b) BD-1047 were used to calculate the mean ± S.E.M. of the major and second maximal contractile amplitudes, the lowest and the second minimal contractile amplitudes, as well as the amplitudes at the end of the experiments at 30 min. All data were calculated as percentages of the amplitude of contraction measured in the same cell before the exposure to the σ receptor ligand. * Significant difference ($P \leq 0.01$) from control, determined by *t*-test for small samples.

3.2. Effects of BD-737 and BD-1047 on cytosolic Ca^{2+} transients

Cytosolic Ca^{2+} ($[Ca^{2+}]_i$) transients were recorded in indo-1 loaded cardiomyocytes during electrical stimulation, before and at various time intervals after the addition of BD-737 (10 nM) and BD-1047 (100 nM). The effects of both σ receptor ligands on $[Ca^{2+}]_i$ transients were similar to the corresponding effects on contractility. Both ligands induced slow oscillations in amplitude of $[Ca^{2+}]_i$ transients. Whereas 10 nM BD-737 potentiated $[Ca^{2+}]_i$ transient amplitudes, which reached 192% of control, the effect of 100 nM BD-1047 was predominantly inhibitory; $[Ca^{2+}]_i$ transient amplitudes oscillated between 80% and 93% of control. Representative recordings of $[Ca^{2+}]_i$ transients are shown in Fig. 5. These results indicate that the changes in twitch contraction induced by both σ receptor ligands are at least in part due to the changes in $[Ca^{2+}]_i$ transients which trigger the contractions.

3.3. The mechanism of action of σ receptor ligands

To assess the contribution of sarcoplasmic reticulum Ca^{2+} stores to the increases in the amplitude of contraction in cardiac myocytes exposed to BD-737 the cells were

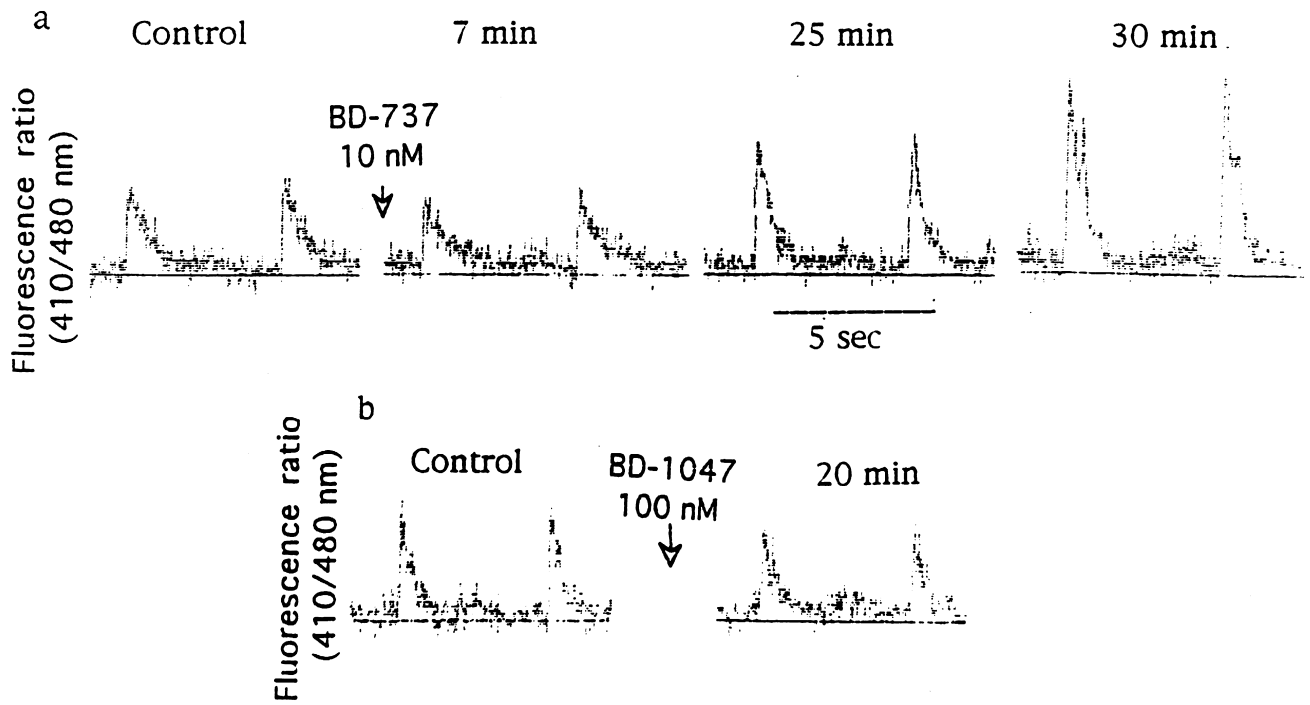

Fig. 5. Effects of BD-737 and BD-1047 on the amplitude of $[Ca^{2+}]_i$-transients in single cardiac myocytes from adult rats. Isolated cardiac myocytes were loaded with indo-1 followed by measurements of the indo-1 fluorescence ratio as described in Section 2. Original recording of $[Ca^{2+}]_i$-transients in indo-1-loaded cardiac myocytes before and after the addition of (a) BD-737 (10 nM) and (b) BD-1047 (100 nM) to the superfusion medium are shown. The recording shows two different responses: an increase, and a decrease in the amplitude of $[Ca^{2+}]_i$-transients.

pre-treated with thapsigargin which blocks the sarcoplasmic reticulum Ca^{2+} pump and therefore depletes the sarcoplasmic reticulum Ca^{2+} stores (Janczewski and Lakatta, 1993). After 15-min exposure to 50 nM thapsigargin, 10 nM BD-737 was added. There was no increase in the amplitude of contraction but a continuous decrease (Fig. 6). This result may indicate that the increase in twitch amplitude in the absence of thapsigargin was mediated by the release of Ca^{2+} from sarcoplasmic reticulum stores.

Ca^{2+} may be released from the sarcoplasmic reticulum via two types of Ca^{2+} channels: ryanodine-sensitive channels and IP_3-sensitive channels. Ryanodine-sensitive channels are opened by nanomolar concentrations of ryanodine and by caffeine (Berridge, 1993). To determine whether the σ receptor ligand-induced potentiation of contraction is mediated by the release of Ca^{2+} from the ryanodine-sensitive Ca^{2+} stores, we first depleted these Ca^{2+} stores by pre-incubation with 10 mM caffeine. Subsequent addition of 10 nM BD-737 caused only a small increase in the amplitude of contraction; the amplitude increased by $12.2\% \pm 6.6\%$ above the amplitude determined immediately preceding BD-737 addition. This value should be compared with the increase of $97.8\% \pm 16.4\%$ above control in response to 10 nM BD-737 in cardiac myocytes which were not pre-incubated with caffeine (Fig. 7a).

The results described above suggest that the potentiation of twitch amplitudes by BD-737 is mediated in part by the release of Ca^{2+} from the sarcoplasmic reticulum via ryanodine-sensitive channels and perhaps also through the IP_3-sensitive channels. Formation of IP_3 is initiated by activation of phospholipase C which hydrolyzes an inositol lipid precursor stored in the plasma membrane to form two

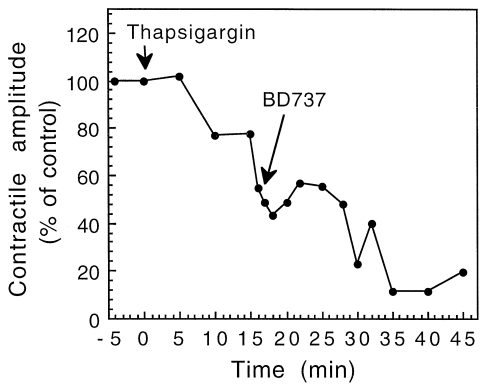

Fig. 6. Effects of pre-incubation with thapsigargin on the BD-737-induced increase in contractile amplitude in adult rat cardiac myocytes. Cell motion was continuously recorded in isolated cardiomyocytes from adult rats. The cardiomyocytes were first pre-incubated during 15 min with thapsigargin (50 nM; added to the superfusion physiological salt solution). Pre-incubation with thapsigargin decreased the amplitude of contraction to 30–40% of the contractile amplitude in the same cells before pre-incubation. The superfusion solution was then replaced with physiological salt solution containing BD-737 (10 nM) together with thapsigargin. Cell motion was recorded for an additional 30 min. Results are expressed as percentage of the amplitude of contraction measured in the same cells before the pre-incubation. Data represent measurements from a single cell. Similar results were obtained in seven different cells.

second messengers: IP_3 and diacylglycerol. Neomycin is an inhibitor of phospholipase C (Gabev et al., 1989). We examined whether neomycin would inhibit the potentiation of twitch contraction in response to BD-737. Cardiac myocytes were pre-incubated with 100 μM neomycin during 10 min. This pre-incubation did not affect the contractile amplitude, which was 100.7% ± 3.4% of control after 10 min pre-incubation. Then 10 nM BD-737 was added together with neomycin for an additional 30 min. During the exposure to BD-737 and neomycin the amplitude increased by 63.1% ± 8.5% above the level reached just before BD-737 addition. This value should be compared with the increase of 97.8% ± 16.4% above control in response to 10 nM BD-737 in cardiac myocytes which were not pre-incubated with neomycin (Fig. 7b). Thus, neomycin partially inhibits the BD-737-induced increase in contractility. Neomycin also prevented the spontaneous twitches caused by incubation with BD-737 (not shown).

The results described above suggest that the response to σ receptor activation by the σ receptor ligand BD-737 is mediated in part by an increase in IP_3 production. In the next experiments we compared the effect of 10 nM BD-737 on contractility to the effects of the α-adrenoceptor ligand phenylephrine. It has been shown that α-adrenergic stimulation by phenylephrine increases IP_3 production and twitch amplitudes (Ventura et al., 1991). Addition of 50-μM phenylephrine to cardiac myocytes induced an increase in the amplitude of contraction of 180.8% ± 25.4% above control (Fig. 7c). Pre-incubation with 100 μM neomycin followed by addition of 50 μM phenylephrine together with neomycin reduced the increase in amplitude to 73.1% ± 13.8% above the level determined immediately preceding phenylephrine addition (Fig. 7c). Thus, the response of the cardiac myocytes to σ receptor stimulation by BD-737 and its inhibition by neomycin are similar to the response to the stimulation of α-adrenoceptor by phenylephrine which is mediated by IP_3.

The response of cardiac myocytes to the σ receptor ligand BD-1047 was qualitatively similar to the response to BD-737, except at the lowest (0.01 nM) and highest (100 nM) concentrations tested. At 100 nM, BD-1047 induced a very slight transient increase in the amplitude of contraction (16.9% above control) which was followed by a decrease to 80.8% ± 4.9% of control level (Fig. 4b). Thus, it appears that BD-1047 at 100 nM acts as an inverse agonist, but the mechanism of the inhibitory effect is not

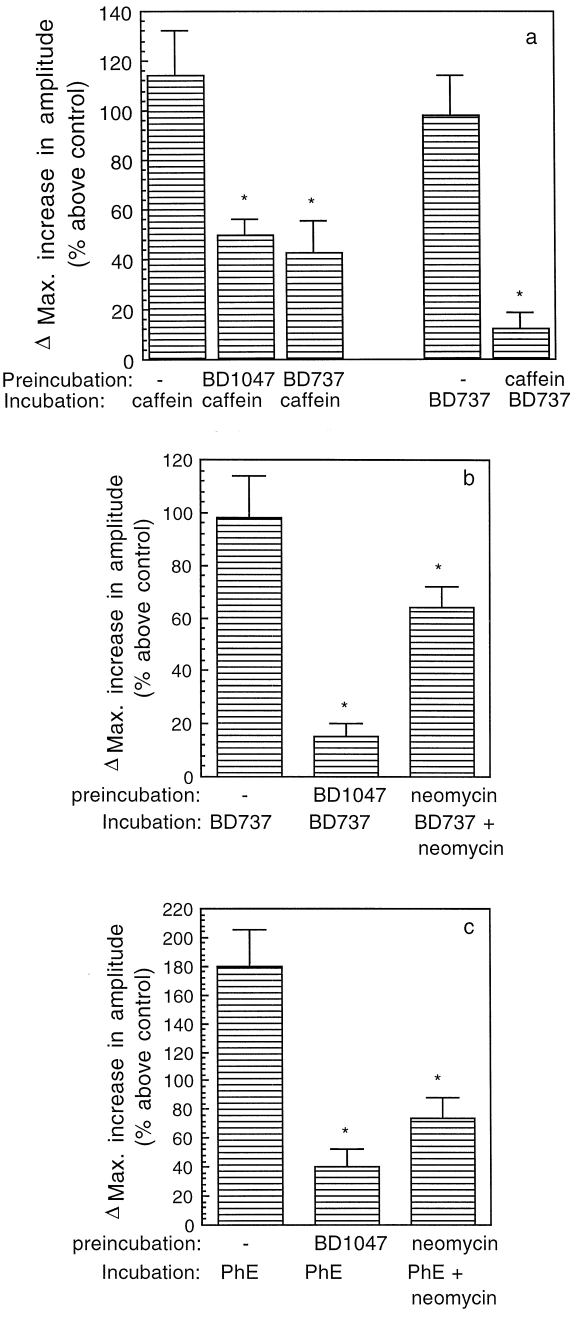

Fig. 7. Effects of pre-incubation with caffeine, neomycin or BD-1047 on the increase in contractile amplitude induced by BD-737 or phenylephrine, and effects of pre-incubation with BD-1047 or BD-737 on caffeine-induced increase in contractile amplitude in adult rat cardiac myocytes. Cell motion was continuously recorded in isolated cardiomyocytes from adult rats. (a) The cardiomyocytes were first pre-incubated during 15 min with caffeine (10 mM; added to the superfusion physiological salt solution). The superfusion solution was then replaced with salt solution containing BD-737 (10 nM). Similarly, the cells were pre-incubated with BD-737 (10 nM) or BD-1047 (100 nM; as indicated) during 30 min, then caffeine (10 mM) was added for 15 min. (b and c) The cardiomyocytes were first pre-incubated during 30 min with BD-1047 (100 nM) or 10 min with neomycin (100 μM; added to the superfusion physiological salt solution). The superfusion solution was then replaced with a similar solution containing (b) BD-737 (10 nM) or (c) phenylephrine (PhE) (50 μM) with or without the compound used for pre-incubation, as indicated. Results represent means ± S.E.M. ($n = 6$) of the maximal amplitudes obtained in cells during incubation, minus the amplitude determined immediately preceding the addition of the incubating compound. All values are expressed as percentages of the amplitude of contraction measured in the same cells before the pre-incubation. * Significant difference ($P \leq 0.01$, $n = 6$) from corresponding controls which were not pre-incubated, determined by *t*-test for small samples.

clear. We examined the hypothesis that exposure of the cardiomyocytes to 100 nM BD-1047 leads to depletion of the sarcoplasmic reticulum Ca^{2+} stores, which, in turn, causes reduced twitch amplitude. To test the suggested mechanisms, cardiac myocytes were pre-incubated with 100 nM BD-1047 during 15 min, then the superfusion solution was replaced with a solution containing BD-737 (10 nM) for an additional 30 min. Pre-incubation with BD-1047 inhibited the BD-737-induced potentiation of the twitch amplitude; the amplitude increased only by 14.1% $\pm$ 5.7% above the level determined immediately preceding BD-737 addition (Fig. 7b). This result may indicate that BD-1047 acts as an antagonist to BD-737, or it may indicate the depletion of sarcoplasmic reticulum Ca^{2+} stores by pre-incubation with BD-1047. To distinguish between these two mechanisms, cardiac myocytes were pre-incubated with BD-1047 (100 nM) during 15 min, then the superfusion solution was replaced with a solution containing phenylephrine (50 μM) for an additional 30 min. Pre-incubation with BD-1047 inhibited the subsequent increase in twitch amplitude by phenylephrine. After pre-incubation with BD-1047, the phenylephrine-induced increase in contractile amplitude reached only 41.2% $\pm$ 9.9% above the level determined immediately preceding phenylephrine addition, as compared with an increase of 180.8% $\pm$ 25.4% above control by phenylephrine alone (Fig. 7c). This result supports the assumption that exposure to 100 nM BD-1047 causes depletion of sarcoplasmic reticulum Ca^{2+} stores. This suggested mechanism was further tested by determining the effects of pre-incubation with BD-1047 (100 nM) or BD-737 (10 nM) on the caffeine-sensitive intracellular Ca^{2+} stores. Pre-incubation with each of these ligands partially inhibited the subsequent increase in twitch amplitude by caffeine (Fig. 7a). Pre-incubation with BD-1047 (100 nM) during 30 min caused a decrease in twitch amplitude to 84.6% of control. Addition of 10 mM caffeine after this pre-incubation induced a very short transient increase in contractile amplitude which started 2 min after caffeine addition and returned to the previous level after 5 min. The maximal increase reached 49.9% $\pm$ 6.1% above the level determined immediately preceding caffeine addition. This increase is much smaller and shorter than the increase induced by 10 mM caffeine alone (114.6% $\pm$ 22.2% above control during at least 15 min). These results support the assumption that BD-1047 (100 nM) causes depletion of sarcoplasmic reticulum Ca^{2+} stores. Thus, in rat ventricular myocytes BD-1047 does not act as a σ receptor antagonist.

A total of 30 min after the addition of BD-737 the mean size of contractile amplitude was 76.7% $\pm$ 27% above control. Subsequent addition of 10 mM caffeine induced a smaller and shorter increase above the level determined immediately preceding caffeine addition (42.3% $\pm$ 12.3%), compared to the increase induced by 10 mM caffeine alone (114.6% $\pm$ 22.2% above control). These results indicate that the increase in contractile amplitude induced by caffeine is not additive to that induced by BD-737.

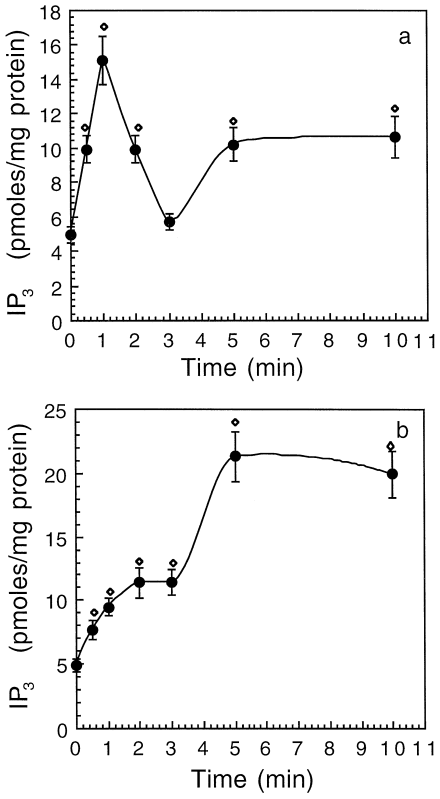

Fig. 8. Effects of BD-737 and BD-1047 on the cellular level of IP_3 in cardiac myocytes from adult rats. Suspensions of isolated cardiac myocytes in physiological salt solution were incubated for 10 min with LiCl (10 mM), then with (a) 10 nM BD-737, or (b) 100 nM BD-1047 during the indicated time intervals. The reactions were stopped and the amounts of IP_3 were determined as described in Section 2. Data represent means $\pm$ S.E.M. of four determinations. $*$ Significant difference ($P \leq 0.01$, $n = 6$) from controls determined by *t*-test for small samples.

3.4. Effects of BD-737 and BD-1047 on IP_3 production in cardiac myocytes

The second messenger that may explain the results described above is IP_3. Fig. 8 shows that both 10 nM BD-737 and 100 nM BD-1047 caused an increase in the intracellular concentration of IP_3. BD-737 caused a rapid increase in IP_3, by 3.2-fold above control, in 1 min; thereafter, the IP_3 level decreased and stabilized at 2.1-fold above control until the end of the experiment at 10 min. BD-1047 caused a gradual increase in IP_3 level which reached 4.4-fold above control after 5 min and remained at this high level until the end of the experiment at 10 min.

4. Discussion

It was found in the present study that exposure of isolated cardiac myocytes from adult rat ventricles to

nanomolar concentrations of the novel aryl ethylenediamine-related σ receptor ligands BD-737 and BD-1047 caused elevation in IP_3 production. Depending on the compound and its concentration, this elevation induces an increase in contractile amplitudes with oscillations (BD-737, 0.1–100 nM; BD-1047, 0.01–10 nM), or attenuation of contractile amplitudes, presumably resulting from sarcoplasmic reticulum Ca^{2+} depletion (BD-1047, 100 nM). The dose–response curves of the maximal contractile amplitudes for both ligands were found to be bell-shaped. The changes in contractility appear to be triggered by corresponding changes in amplitudes of $[Ca^{2+}]_i$ transients. Exposure to each of these σ receptor ligands causes a dose-dependent increase in incidence of spontaneous contractions.

Similar biphasic effects of σ agonists have also been observed in other systems. The σ receptor ligands were found to modulate neuronal responses to NMDA with bell-shaped dose–response curves (Rao et al., 1990; Roman et al., 1988; Monnet et al., 1992, 1996; Bergeron et al., 1995). Similarly, bell-shaped dose–response curves were observed in several behavioral models (McMillan et al., 1991; Maurice et al., 1994a,b), and in measurements of colonic motility in dogs treated with σ receptor ligands (Junien et al., 1990).

Bergeron et al. (1995) have suggested that the biphasic effect of σ receptor ligands on NMDA response might be caused by effects of these compounds on different σ receptor subtypes. In our previous investigations on effects of the σ receptor ligands (+)-3-PPP, (+)-pentazocine, haloperidol and 1,3-di-*O*-tolylguanidine (DTG) on cardiac myocytes, the presence of different receptor subtypes was suggested on the basis of desensitization experiments (Novakova et al., 1995; Ela et al., 1996). The existence of subtypes of σ_1 receptors has been previously suggested, based on different affinity values and binding profiles for the selective σ_1 probe [^{3}H](+)-pentazocine in various neuronal and non-neuronal cell lines (Vilner et al., 1992, 1995b).

The results obtained with BD-737 are essentially similar to results obtained previously by our group, using cardiac myocytes from adult rats exposed to (+)-3-PPP, (+)-pentazocine and haloperidol (Novakova et al., 1995). As with BD-737, these ligands induced attenuation of the amplitude of contraction at very low concentrations, and potentiation with slow oscillations at higher concentrations. Exposure to (+)-3-PPP yielded a dose-dependent increase in the values of the maximal amplitude, reaching a plateau at 0.1–1.0 μM (Novakova et al., 1995). Since the affinity of (+)-3-PPP to σ binding sites is about two orders of magnitude less than that of BD-737, it is plausible that a putative decreasing phase of the dose–response curve might occur at higher concentrations. Thus the results obtained with (+)-3-PPP do not contradict the pattern of bell-shaped dose–response curves obtained with the novel σ receptor ligands.

The results of the present study regarding the mechanism of action of BD-737 and BD-1047 on cardiac myocytes may suggest that stimulation of σ receptors leads to activation of phospholipase C which hydrolyzes membrane-bound phosphoinositides, resulting in an increase of cellular IP_3. This potentiates sarcoplasmic reticulum Ca^{2+} release with each electrical stimulus and subsequently modulates twitch amplitudes. The suggested mechanism is based on the following results.

(1) Our measurements revealed elevation in cellular IP_3 production triggered by exposure to BD-737 and BD-1047.

(2) Pre-incubation of cardiac myocytes with neomycin (phospholipase C inhibitor), followed by exposure to a σ receptor ligand in the presence of neomycin inhibited the effect of the σ receptor ligand on contractility. Although neomycin is not a very specific inhibitor, the finding that a similar inhibition was observed when pre-incubation with neomycin was followed by exposure to the α-adrenoceptor agonist, phenylephrine, the inotropic action of which is known to be mediated by elevation of IP_3 production (Exton, 1985; Otani et al., 1988), strengthens the suggested mechanism.

(3) The role of the sarcoplasmic reticulum Ca^{2+} pool in mediating σ receptor ligand contractile effects was confirmed by the results showing that pre-incubation with compounds which deplete the sarcoplasmic reticulum Ca^{2+} store, such as thapsigargin (an inhibitor of sarcoplasmic reticulum Ca^{2+}-ATPase; Janczewski and Lakatta, 1993) or caffeine (which opens the ryanodine-sensitive Ca^{2+}-release channels; Tsien and Tsien, 1990), inhibited the σ receptor ligand contractile response.

It has been shown in various types of cells that elevation of IP_3 production, which release Ca^{2+} from intracellular pools (Nosek et al., 1986; Fabiato, 1990; Kentish et al., 1990), may lead to both transient increase with oscillations in cytosolic Ca^{2+}, as well as to sarcoplasmic reticulum Ca^{2+} depletion (Berridge, 1993; Li et al., 1995; Tsien and Tsien, 1990; Tang et al., 1996). The oscillations appear to be related to the biphasic effect of Ca^{2+} concentration on the opening of IP_3-sensitive Ca^{2+} channels: activation of channels at low Ca^{2+} concentration and inhibition at high concentrations (Iino and Endo, 1992). Both ryanodine- and IP_3-sensitive Ca^{2+} channels are subjected to such regulation in cardiac cells (Bezprozvanny et al., 1991; Gyorke and Fill, 1993; Tang and Othmer, 1994). We suggest that the slow oscillations in amplitudes of contraction, which followed the exposure of cardiac myocytes to σ receptor ligands, are induced by oscillations in the amounts of Ca^{2+} released from the sarcoplasmic reticulum with each electrical stimulus, due to the biphasic effect of $[Ca^{2+}]_i$ on IP_3-sensitive and ryanodine-sensitive channels and to the stimulatory effect of the elevated level of IP_3 on sarcoplasmic reticulum Ca^{2+} release.

Elevation of the cellular IP_3 level may also cause a sustained increase in $[Ca^{2+}]_i$, as well as sarcoplasmic reticulum Ca^{2+} depletion (Berridge, 1993). In isolated

cardiac myocytes from adult rats, exposure to κ and δ opioid receptor agonists increased production of IP_3 and attenuated twitch amplitude due to sarcoplasmic reticulum Ca^{2+} depletion (Ventura et al., 1992). On the other hand, increased production of IP_3, by exposure of cardiac myocytes to the α-adrenoceptor agonist phenylephrine, is associated with potentiation of contractile amplitudes (Exton, 1985; Endoh and Blinks, 1988; Ventura et al., 1991 and this study). Thus, the same signal transduction element (IP_3) may mediate opposite effects: a decrease or increase in $[Ca^{2+}]_i$ leading to attenuation or potentiation of contractile amplitudes. In the present study, exposure of cardiac myocytes to 10 nM BD-737 caused potentiation of contractility, while exposure to 100 nM BD-1047 caused attenuation of contractility. Yet an increased level of IP_3 was observed in both cases, but with different kinetics. A total of 100 nM BD-1047 induced a gradual increase in IP_3, whereas 10 nM BD-737 caused a fast transient increase in IP_3. It may be suggested that these differences in the kinetics of IP_3 production lead to the opposite effects on contractility. A similar explanation of the differences between the effects of α-adrenoceptor and κ-opioidergic receptor ligands on the contractility of cardiac myocytes has been suggested (Ventura et al., 1991, 1992).

Additional experiments examined whether exposing cardiac myocytes to 100 nM BD-1047 indeed caused Ca^{2+} depletion of the sarcoplasmic reticulum. Pre-incubation with 100 nM BD-1047 prevented the subsequent potentiation of twitch amplitude by BD-737. This inhibition could have resulted from BD-1047 being an antagonist at the σ site, but a similar inhibition of the potentiation of contractile amplitude was observed when the pre-incubation with 100 nM BD-1047 was followed by incubation with phenylephrine or caffeine. These results suggest sarcoplasmic reticulum Ca^{2+} depletion during pre-incubation with BD-1047. Thus, in cardiac myocytes, BD-1047 does not act as an antagonist. The 'inverse agonist' effect of this ligand at 100 nM depends on its concentration and appears to be mediated by IP_3. It has been reported in various studies that the classification of σ receptor ligands as agonists and antagonists is not definite, but may depend on the tissue studied.

It is not known whether the effects of σ receptor ligands on nerve cells are mediated by a mechanism similar to that in cardiac myocytes. It has recently been reported in rat brain microsomal fraction and in liver membrane extract, that heparin, the IP_3 receptor antagonist, inhibited binding of [^{3}H](+)-SKF-10047 to σ receptors. These results led to the suggestion that σ receptors may be situated close to IP_3 receptors, thereby affecting $[Ca^{2+}]_i$ via modulation of IP_3 channels (Tsao and Su, 1996).

It is tempting to speculate that a similar signalling mechanism mediates the effects of σ receptor activation in cardiac and nerve cells. Consistent with this suggestion are the results showing potentiation of NMDA-evoked noradrenaline release from rat hippocampal slices and potentiation of the excitatory response of pyramidal neurons to NMDA in the CA_3 region of the dorsal hippocampus in the rat (Monnet et al., 1990, 1992, 1996; Bergeron et al., 1993, 1995, 1996; Debonnel et al., 1996). These processes are normally mediated by $[Ca^{2+}]_i$.

On the other hand, nerve cells and cardiac cells differ in their cellular responses to σ_1 receptor ligands. In preparations of rat brain synaptoneurosomes and in cultures of neuron-like cell lines (including differentiated PC12 cells, N1E-115 neuroblastoma, and SK-N-SH neuroblastoma) σ_1 receptor ligands, such as haloperidol, DTG, (+)-3-PPP, BD-737, and (+)-pentazocine, failed to produce an increase in IP_3 production by themselves (Bowen et al., 1988, 1992a,b, 1993; Cutts and Bowen, 1992, Cutts et al., 1993). However, the aforementioned studies were carried out at ligand concentrations above 1 μM, and in view of the possibility of bell-shaped dose–response curves, this could have affected the results. In a recent preliminary study, carried out with (+)-pentazocine and DTG in the concentration range of 3–100 nM, using indo-1-loaded SK-N-SH neuroblastoma cells, no detectable rise in $[Ca^{2+}]_i$ was observed after addition of ligands (unpublished observation). These results, which are different from the results showing a transient $[Ca^{2+}]_i$ increase in cardiac myocytes exposed to 10 nM pentazocine (but not to DTG) (Novakova et al., 1995), indicate that in neuronal cells σ_1 receptor activation leads to a different cellular response from that in cardiac myocytes. However, the absence of an increase in $[Ca^{2+}]_i$ in neuronal cells does not exclude activation of phospholipase C. Firstly, the effect of σ receptor ligand concentrations below 100 nM on IP_3 production in neuronal cells has not yet been determined, thus it is possible that concentrations in this range could in fact lead to activation of phospholipase C and increased IP_3 production. Secondly, elevation of the cellular IP_3 level may cause an increase as well as a decrease (or no change) in $[Ca^{2+}]_i$ due to sarcoplasmic reticulum Ca^{2+} release which may lead to depletion (Berridge, 1993; Ventura et al., 1992; the present study and unpublished results). The different effects of the σ_1 receptor ligand on $[Ca^{2+}]_i$ in neuronal cells and cardiac cells may be due to the presence of different putative classes of σ_1 receptors (Vilner et al., 1992; Vilner et al., 1995a) or to a difference in responsiveness of endoplasmic reticulum vs. sarcoplasmic reticulum. It should be noted here that micromolar concentrations of σ_2 receptor ligands, but not σ_1 selective ligands, did produce a transient and thapsigargin-sensitive rise in $[Ca^{2+}]_i$ in SK-N-SH neuroblastoma cells (Vilner and Bowen, 1995; Bowen et al., 1996; submitted). Further work will be needed in order to investigate possible differences between neuronal and cardiac cells with respect to responses to σ receptor ligands.

In addition to modulating twitch amplitudes the σ receptor ligands BD-737 and BD-1047 increased, dose dependently, the occurrence of spontaneous contraction in

cardiac myocytes from adult rats. A similar increase in spontaneous contractions was observed previously in response to (+)-3-PPP, (+)-pentazocine and haloperidol (Novakova et al., 1995 and unpublished results). In cultured neonatal rat cardiac myocytes which contract spontaneously, exposure to the same σ receptor ligands caused changes in beating rates, premature beats and irregular contractions (Ela et al., 1994).

Spontaneous contractions are triggered by spontaneous sarcoplasmic reticulum Ca^{2+} release. Results from a variety of mammalian cardiac preparations indicate that the probability of spontaneous sarcoplasmic reticulum Ca^{2+} release increases with the increase in the extent to which the cytosol and sarcoplasmic reticulum become overloaded with Ca^{2+}. Spontaneous sarcoplasmic reticulum Ca^{2+} release, unlike that triggered by action potentials, occurs sporadically within cardiac cells and asynchronously among cells comprising cardiac tissue (Lakatta, 1993). Experimental results indicate that an increased incidence of spontaneous sarcoplasmic reticulum Ca^{2+} release in the myocardium can produce abnormal diastolic tonus, limited systolic function and a high probability of arrhythmias (Lakatta, 1989).

Factors which increase the IP_3 level in the myocardium are considered arrhythmogenic. Such is the release of large quantities of norepinephrine and thrombin during ischemia and reperfusion. Stimulation of α_1-adrenoceptor and cardiac thrombin receptors activates phospholipase C to release IP_3. Agents which inhibit phospholipase C activation inhibit reperfusion arrhythmia (Jacobsen et al., 1996; Steinberg and Alter, 1993). In accordance with the above results, the high frequency of spontaneous sarcoplasmic reticulum Ca^{2+} release in cardiac myocytes exposed to σ receptor ligands appears to be mediated by activation of phospholipase C which elevates the cellular IP_3 level.

Many drugs in clinical use bind to σ receptors with nanomolar affinities. Example are certain neuroleptics, monoamine oxidase inhibitors and several antihistamines (Vilner et al., 1995a). In view of the possible arrhythmogenic action of σ receptor activation, the cardiovascular side-effects of these drugs need to be examined.

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