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Inotropic action of σ receptor ligands in isolated cardiac myocytes from adult rats

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Abstract

High affinity binding sites for σ receptor ligands were found in membranes of cardiac myocytes from adult rats. The σ receptor ligand (+)-3-hydroxyphenyl-*N*-(1-propyl)piperidine ((+)-3-PPP) binds with a K_d of 17.9 ± 4.0 nM and a B_{max} of 275 ± 32.1 fmol/mg protein. Competition experiments of (+)-pentazocine with [^{3}H]1,3-di-*O*-tolylguanidine ([^{3}H]DTG) binding yielded a K_i of 6.1 ± 1.3 nM. The majority of the sites (> 80%) were of the σ_1 subtype. Exposure of isolated cardiomyocytes from adult rats to (+)-3-PPP (10 nM–1.0 μM) caused a marked concentration-dependent increase in the amplitude of systolic cell contraction, reaching 149% of control level, with an apparent ED_{50} value of 4.5 nM. The increase in the contraction amplitude was markedly inhibited by pretreatment with verapamil or thapsigargin. An increase in the amplitude of $[Ca^{2+}]_i$ transients, similar to that in the amplitude of cell contraction, was observed in indo-1-loaded cardiomyocytes exposed to 0.1 μM (+)-3-PPP. Exposure to 10 nM of haloperidol or (+)-pentazocine induced an increase in the amplitude of contraction, reaching 188% and 138% (respectively) of control level. A lower concentration of haloperidol or (+)-pentazocine (1 nM) did not induce an increase in the contraction amplitude but rather reduced the amplitude to 70–80% of control.

Keywords: Cardiac myocyte; σ Receptor; Contractility; $[Ca^{2+}]_i$ transient

1. Introduction

σ Receptors are currently defined as binding sites exhibiting high affinity for (+)-3-hydroxyphenyl-*N*-(1-propyl)piperidine ((+)-3-PPP), 1,3-di-*O*-tolylguanidine (DTG), (+)-pentazocine and haloperidol (Quirion et al., 1992). These sites are present in various regions of the central nervous system (CNS) and are particularly abundant in dopamine-rich, as well as cortical and limbic areas (Walker et al., 1990). In addition, σ binding sites are present in peripheral tissues such as gastrointestinal tract, vas deferens, various endocrine tissues, blood vessels and cardiomyocytes from neonatal rats (Su et al., 1988; Wolfe et al., 1988, 1989; Kennedy and Henderson, 1989; Ela et al., 1994; Paul et al., 1993).

Neither the functional roles nor the mechanism(s) of action of the σ receptors are fully understood (for recent reviews see Walker et al., 1990; Itzhak, 1994; Su and Junien, 1994; Quirion et al., 1992). Results of several studies have indicated that σ receptor ligands affect regulation of movement and posture (Goldstein et al., 1989; Matsumoto et al., 1990; Walker et al., 1988, 1993; Clissold et al., 1993), cause inhibition of agonist-stimulated phosphoinositide turnover in brain synaptoneurosomes (Bowen et al., 1988), modulate the activity of the hippocampal and dopaminergic system (Poncelet et al., 1993; Booth and Baldessarini, 1991; Patrick et al., 1993), potentiate neurogenic twitch contraction of isolated mouse vas deferens (Campbell et al., 1987; Matsuno et al., 1993), inhibit contraction of the longitudinal muscle/myenteric plexus of guinea pig

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ileum (Campbell et al., 1989), induce stimulation of duodenal alkaline secretion (Pascaud et al., 1993), modulate activity of glutaminergic neurons (Karbon et al., 1990) and NMDA-receptor mediated response (Rao et al., 1990; Monnet et al., 1990), and cause inhibition of a tonic, outward K^+ current in NCB-20 neuroblastoma cells (Wu et al., 1991; Morio et al., 1994). However, most of these effects are exerted by concentrations of ligands two or three orders of magnitude greater than the K_d values obtained in binding studies.

Recently we have reported the presence of σ binding sites on cardiomyocytes from neonatal rats (Ela et al., 1994). In these cells σ receptor ligands, at nanomolar concentrations, exert a complex effect on the amplitudes and frequencies of contraction, Ca^{2+} fluxes and $[Ca^{2+}]_i$ transients.

The results obtained in neonatal rat cardiomyocytes indicate that σ receptors may have an important role in the regulation of cardiac function. In view of age-related differences in σ binding parameters to membranes from rat brains, and the differences in behavioral responses of old and young rats to injected σ receptor ligands (Matsumoto et al., 1989), it was important to determine the pattern of response of cardiomyocytes isolated from adult rats to σ receptor ligands. In the present study we have determined the binding parameters of (+)-3-PPP to membranes of cardiomyocytes isolated from adult rats, the inhibition constant of (+)-pentazocine to [^{3}H]DTG binding and the effects of several prototypic σ receptor ligands on contractility and on $[Ca^{2+}]_i$ transients in adult rat cardiac myocytes. We found that (+)-3-PPP, (+)-pentazocine, and haloperidol, at concentrations of 10 nM and higher, markedly increased the amplitude of systolic cell contraction and the amplitude of $[Ca^{2+}]_i$ transients in these cells. At 1 nM of these ligands, a decrease in the amplitude was observed. The pattern of response of cardiac myocytes from adult rats to σ receptor ligands is different from that of neonatal cardiomyocytes; this suggests changes in response to σ receptor ligands during the development of the myocardium.

2. Materials and methods

2.1. Membrane preparation and binding of [^{3}H]3-PPP and [^{3}H]DTG σ receptors

Frozen hearts from adult rats were homogenized in 50 mM Tris-HCl buffer (pH 8) at 4°C using a polytron homogenizer. The homogenate was centrifuged at 1000 $\times g$ for 10 min and the pellet discarded. The supernatant was centrifuged at 20000 $\times g$ for 25 min and the pellet of crude membranes was resuspended in Tris buffer with a Dounce homogenizer. Binding of [^{3}H]3-PPP and [^{3}H]DTG to σ receptors in these membrane preparations was carried out as previously described (Ela et al., 1994; Barg et al., 1994). In brief, crude membranes (0.10–0.25 mg protein) were incubated at 25°C for 2 h in 0.5 ml of 50 mM Tris-HCl buffer (pH 8) with 3 nM [^{3}H](+)-3-PPP (80 Ci/mmol) or 3 nM [^{3}H]DTG (36.8 Ci/mmol) (NEN-DuPont, Boston, MA, USA) in the presence or absence of the competing ligand. The mixture was filtered through Whatman GF/B filters pre-soaked in 0.5% polyethylenimine (for 1–2 h, to reduce non-specific binding). K_d and B_{max} values were determined by the Implot4 computer program (Graph Pad Software, San Diego, CA, USA). The competition binding assay curve was generated with the Sigma Plot 4.11 computer program (Jandel Scientific, Corta Madera, CA, USA) using an equation from the ALLFIT program (DeLean et al., 1988). Protein concentration was determined by the method of Bradford (1976).

2.2. Preparation of isolated cardiac myocytes

Isolated rat cardiac myocytes were prepared using variations of the methods described by Kramer et al. (1991) and Hansford and Lakatta (1987). 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 flow 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 $CaCl_2$. The solution was equilibrated with an atmosphere of 95% air and 5% CO_2, and was maintained at 37°C throughout the experiment. After 10 min, the solution was switched to a similar physiological salt solution, but containing 5 μM $CaCl_2$ for 5 min and subsequently to a 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 physiological salt solution containing 0.8% albumin and 50 μM $CaCl_2$. The cells were collected by centrifugation in salt solution containing 0.8% albumin and 1.2 mM $CaCl_2$. The cells were allowed to attach to circular glass coverslips (12 mm) coated with L-polylysine.

2.3. Contractility measurements

The glass coverslip with the attached isolated adult ventricular myocytes was 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 2.0-ms square wave pulses (30–40 V),

through two platinum electrodes based in the superfusion solution, and connected to a stimulator (Grass Instruments, MA, USA).

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 $\pm 3\%$ during the time of the experiments.

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

Stock solutions of indo-1 from Molecular Probes (Eugene, OR, USA) were prepared as described (Du Bell et al., 1988). Indo-1 loading solution was composed of Krebs-Henseleit physiological salt solution equilibrated with an atmosphere of 95% air 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% air atmosphere, at 37°C for 15 min.

Measurements of $[Ca^{2+}]_i$ transients were done as described previously (Ela et al., 1994) by using an FM-1000 dual wavelength fluorescence microphotometer (Ricon, San Paulo, CA, USA). 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% air 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 10 s. Between measurements, the cells were kept in the dark to minimize indo-1 bleaching. Results have not been calibrated to $[Ca^{2+}]_i$ values but presented as indo-1 fluorescences ratio. It has been indicated in rat cardiomyocytes loaded with indo-1 that calibration is not precise due to the subcellular compartmentalization of indo-1 (Spurgeon et al., 1990). Because 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.

3. Results

3.1. Binding of [^{3}H](+)-3-PPP and [^{3}H]DTG to membranes of rat hearts

As shown in Fig. 1, unlabeled (+)-3-PPP reduced the binding of [^{3}H](+)-3-PPP in a concentration-dependent manner. The B_{max} and K_d values for (+)-3-PPP binding are 275.6 ± 32.1 fmol/mg protein and 17.9 ± 4.0 nM respectively. These results strongly suggest the presence of an authentic σ binding sites in rat heart membranes with an affinity similar to that of neural tissue (Barg et al., 1994). In order to characterize these binding sites and to distinguish between σ_1 and σ_2, [^{3}H]DTG binding was examined in the presence of increasing concentrations of (+)-pentazocine. (+)-Pentazocine has about 200-fold higher affinity for the σ_1 site than the σ_2 site, whereas DTG binds with approximately equal affinities to σ_1 and σ_2 (as determined in rat brain and other tissues) (Bowen et al., 1993). Therefore, nanomolar concentrations of (+)-pentazocine selectively block the binding of [^{3}H]DTG only to the σ_1 site. Heterologous competition experiments, in which [^{3}H]DTG binding is measured in the presence of increasing concentrations of (+)-pentazocine, enable to determine the relative amounts of the two sites and to calculate the affinity of (+)-pentazocine to the σ_1 site (Bowen et al., 1993). The results of such experiments, shown in Fig. 2, indicate that at least 80% of the σ sites are σ_1 (assuming that the affinity of (+)-pentazocine to σ_2 in rat cardiac mem-

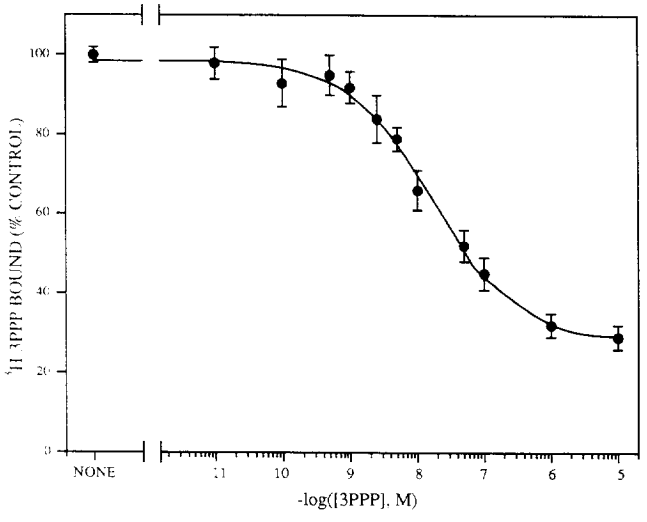


Fig. 1. Homologous competition curve of [^{3}H](+)-3-PPP binding to σ receptors in rat heart membranes. Binding was determined as described in Materials and methods and presented as percentage of control. Values of the binding parameters, B_{max} (275.6 ± 32.1 fmol/mg protein) and K_d (17.9 ± 4.0 nM), were calculated from the specific binding at each concentration of (+)-3-PPP. Data are the mean $\pm$ S.E.M. of three experiments performed in duplicate.

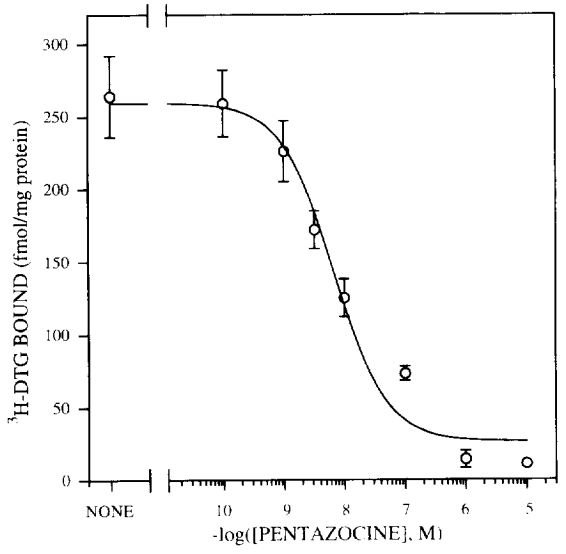


Fig. 2. Heterologous competition curve of [^{3}H]DTG binding to σ receptors in rat heart membranes in the presence of the indicated concentrations of (+)-pentazocine. Binding was determined as described in Materials and methods and presented as fmol/mg protein. Values of the binding parameters, B_{max} (259.7 ± 34.8 fmol/mg protein) and K_i (6.1 ± 1.3 nM), were calculated from the specific binding. Data are the mean ± S.E.M. of three experiments performed in duplicate.

branes is similar to that in rat brain membranes (K_d = 1.36 μM, Bowen et al., 1993). The affinity of (+)-pentazocine to the σ_1 site, K_i = 6.1 ± 1.3 nM, was determined from the results shown in Fig. 2. This value is very similar to the value determined in rat brain membranes in which the K_i value for (+)-pentazocine binding to σ_1 is 6.7 ± 1.2 nM (Bowen et al., 1993).

3.2. Effects of (+)-3-PPP on contractility

Fig. 3 shows representative tracings of cell motion of isolated ventricular myocytes from adult rats, before and at different times after the addition of 100 nM

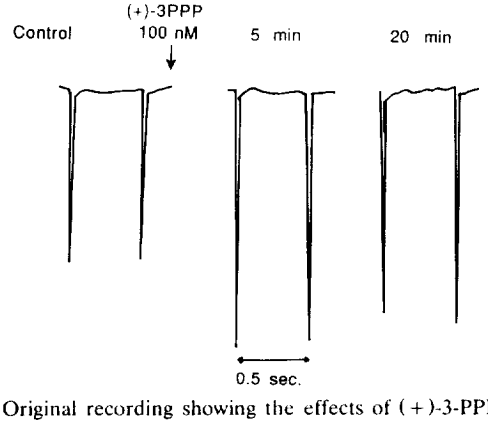
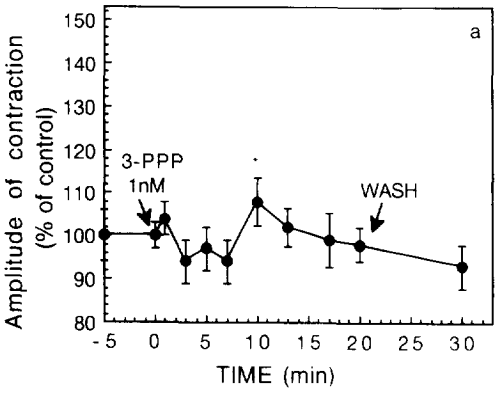


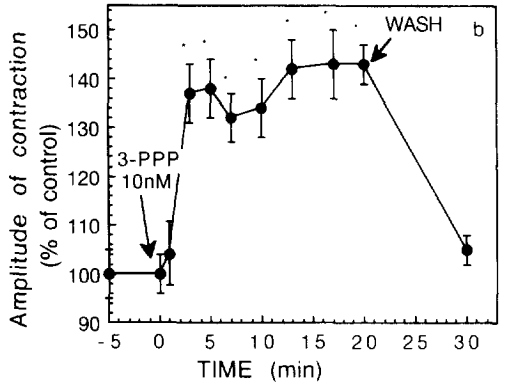
Fig. 3. Original recording showing the effects of (+)-3-PPP on cell motion of a single cardiomyocyte isolated from adult rat heart. The recording was done before and after the addition of 100 nM (+)-3-PPP to the superfusion solution.

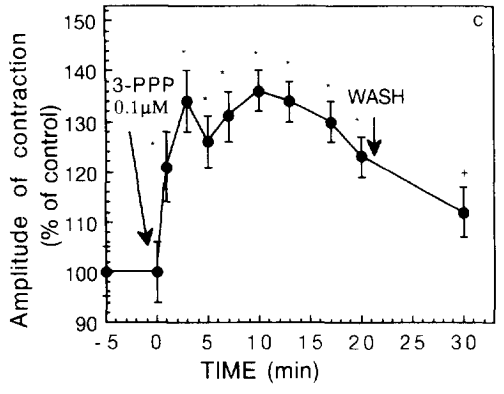
(+)-3-PPP. Effects of various concentrations of (+)-3-PPP on cell motion are shown in Fig. 4. Exposure to 1 nM (+)-3-PPP induced a very slight increase in the amplitude of contraction 10 min after exposure (Fig. 4a). Exposure to 10 nM (+)-3-PPP caused an increase in the amplitude of contraction to 137 ± 6% of control level at 3 min. The amplitude remained at this high level, reaching 143 ± 4% after 20 min of exposure. Subsequent superfusion with drug-free medium caused a complete reversal of the effect (Fig. 4b). About 25% of the cells exposed to 10 nM (+)-3-PPP did not respond with the typical increase in the amplitude of contraction. In these cells, the amplitude gradually decreased and stabilized at approximately 75% of control level (not shown). Exposure to 100 nM (+)-3-PPP caused an increase in the amplitude of contraction reaching 134 ± 6% of control at 3 min. However, the increase was transient and the amplitude started to decrease after 10 min of exposure (Fig. 4c). This pattern of response became more pronounced using 1 μM (+)-3-PPP. The amplitude of cell motion increased to the maximal value (149 ± 3%) within 1 min after exposure and started to decrease thereafter (Fig. 4d). Fig. 5 shows a dose-response plot of the maximal values of the amplitude of cell motion, obtained with various (+)-3-PPP concentrations. The apparent ED_{50} value for the maximal increase in the contractile amplitude, calculated from the data in Fig. 5, is 4.5 nM. This value is only slightly lower than the K_d value, determined in binding experiments (17.9 ± 4.0 nM, see Fig. 1).

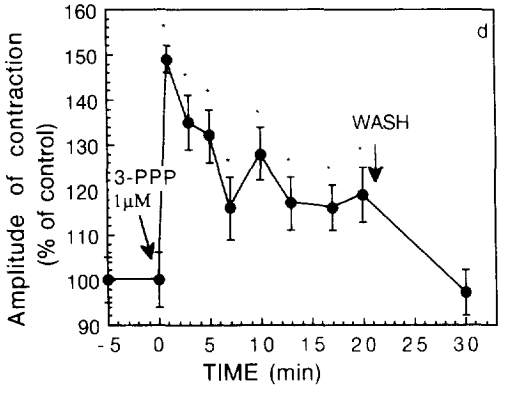
3.3. Effects of haloperidol, (+)-pentazocine and DTG on contractility

Effects of haloperidol and (+)-pentazocine were examined at the lower concentration range since these ligands bind to the σ_1 receptor subtype with a very high affinity (K_i = 5 nM) in guinea pig brain homogenates (De Costa et al., 1989). Exposure to 10 nM haloperidol induced a gradual increase in the amplitude of cell motion, starting after 13 min of exposure and reaching 188 ± 7% of control level at the end of the measurement at 20 min (Fig. 6a). Exposure to 10 nM (+)-pentazocine also induced a gradual increase in the amplitude of systolic motion, starting already after 3 min of exposure and reaching 138 ± 5% of control level at the end of the measurement, at 25 min (Fig. 6b). In similarity to the results observed with (+)-3-PPP, about 25% of the examined cells responded differently to 10 nM (+)-pentazocine or haloperidol; there was no increase in the amplitude of contraction but rather a gradual decrease which stabilized after 3 min at approximately 70–80% of control level (not shown). A lower concentration of haloperidol (1 nM) did not cause an increase in the contractile amplitude but a









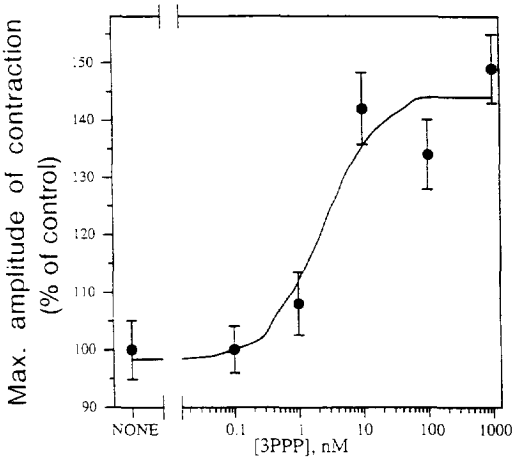


Fig. 5. The maximal increase in the amplitude of contraction as a function of the applied concentration of (+)-3-PPP. Values of the maximal increases in the amplitude of cell contraction, obtained with the different concentrations of (+)-3-PPP, were plotted as a function of log (+)-3-PPP concentration. Data are mean ± S.E.M., ($n = 6$). ED_{50} value (4.5 nM) was determined by Inplot4 computer program (Graph Pad Software, San Diego, CA, USA). The curve was generated with the Sigma Plot 4.11 computer program (Jandel Scientific, Corta Madera, CA, USA).

gradual decrease which stabilized at approximately 70% of control level (Fig. 6a). Similarly, exposure to 1 nM (+)-pentazocine caused a gradual decrease in the amplitude of cell contraction, which stabilized at approximately 75% of control level (Fig. 6b). Exposure to 10 nM DTG did not yield any effect; higher concentrations of DTG, 0.1 μM and 1.0 μM, caused a transient decrease in the amplitude of contraction, which returned to the control level after 20 min (Fig. 6c).

3.4. Oscillations in the response of individual cells to σ receptor ligands

The results depicted in Figs. 4 and 6 represent average values calculated from data obtained from 8–10 cells. When we examined results obtained from single cells we occasionally observed oscillations in the response to σ receptor ligands such as those shown in

Fig. 4. Time courses of the effects of (+)-3-PPP, at different concentrations, on the amplitude of contraction in cardiomyocytes from adult rats. Cell motion was continuously recorded in single cardiomyo-cytes isolated from adult rats and superfused with physiological salt solution (as described in Materials and methods). After stabilization of the contractile amplitude, (+)-3-PPP at the concentrations indicated in Fig. 4a–d was added to the superfusion solution for 20 min. The solution was then changed to physiological salt solution without (+)-3-PPP for a 15 min wash. In each graph the data represent means ± S.E.M. of measurements done on eight cells. Significance was determined by t-test for small independent samples. $^{*}P < 0.01$.

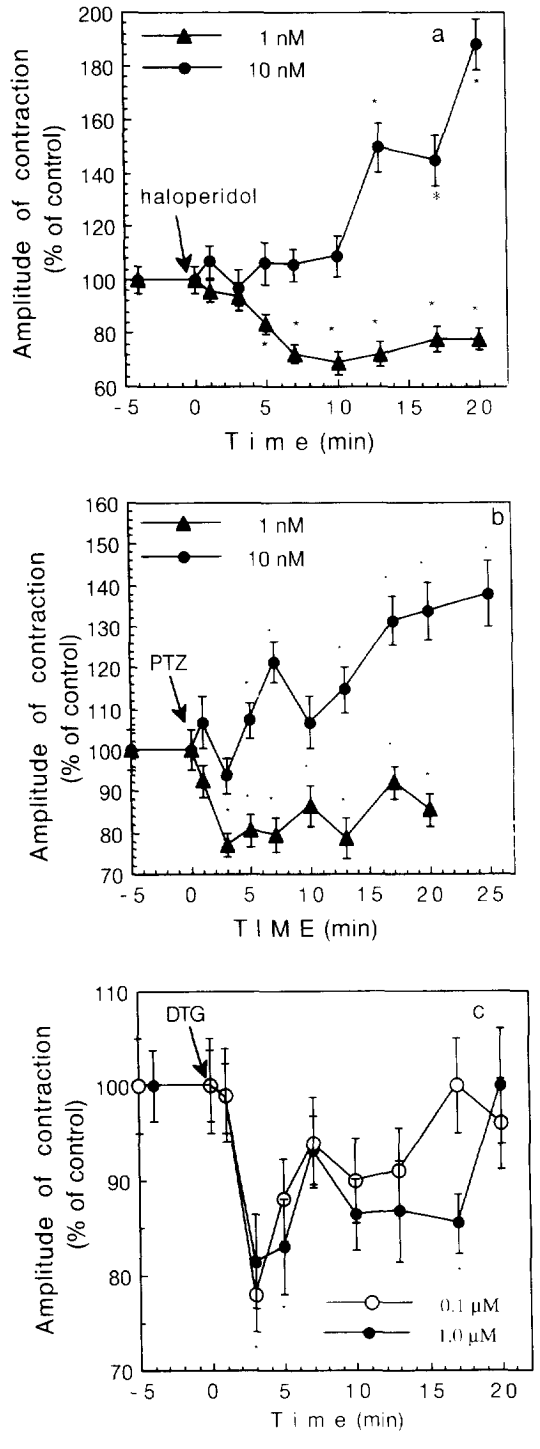


Fig. 6. Effects of haloperidol, (+)-pentazocine and DTG on the amplitude of contraction of cardiac myocytes from adult rat. Cell motion was continuously recorded in single cardiomyocytes isolated from adult rats and superfused with physiological salt solution (as described in Materials and methods). After stabilization of the contractile amplitude, haloperidol (a), (+)-pentazocine (PTZ) (b), or DTG (c), at the indicated concentration, was added to the superfusion solution for 20 min. In each graph the data represent means ± S.E.M. of measurements done on 6–8 cells. Significance was determined by *t*-test for small independent samples. $^{*}P < 0.01$.

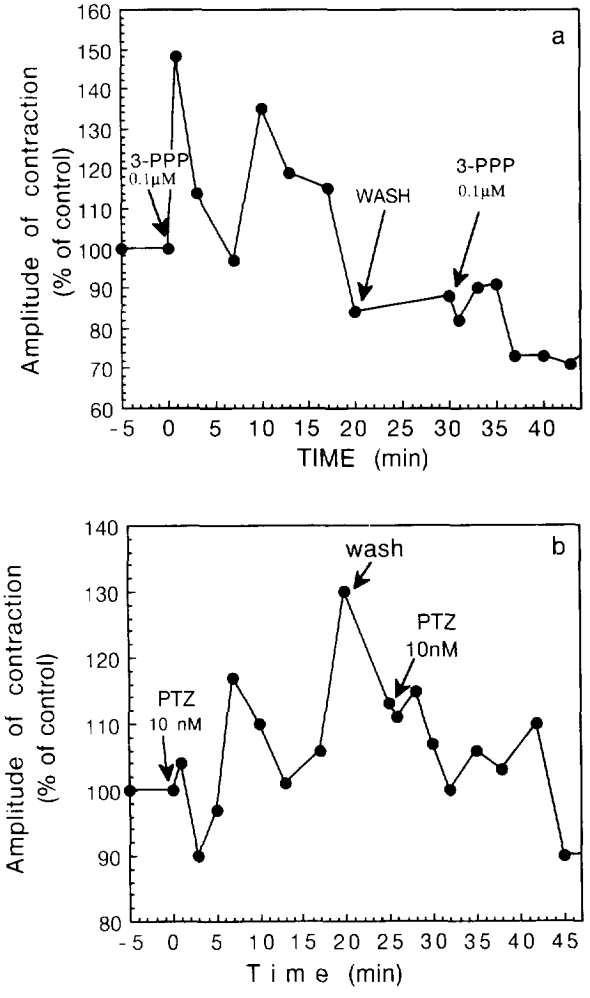


Fig. 7. Effects of repeated application of (+)-3-PPP and (+)-pentazocine on the amplitude of contraction of cardiac myocytes from adult rats. The graphs show results of experiments in which oscillations in the amplitude of contraction were induced following exposure to (+)-3-PPP (a), or to (+)-pentazocine (PTZ) (b). The experiments were done as described in the legend to Fig. 4, but after the cells have been washed for 15 min, (+)-3-PPP (100 nM) (a) or (+)-pentazocine (10 nM) (b) was added again. Cell motion was continuously recorded. Similar results regarding the second application of (+)-3-PPP or (+)-pentazocine were obtained in five and four experiments respectively.

Fig. 7. The oscillations are masked when the average changes at each time-point are calculated.

3.5. Desensitization of the positive inotropic effect of σ receptor ligands

The increase in the amplitude of cell motion which was induced by 0.1 and 1 μM (+)-3-PPP was followed by a rapid decrease (Fig. 4c and d). This decrease may be mediated by desensitization of the receptor. In order to examine this hypothesis, the cells which had been incubated with 0.1 μM (+)-3-PPP for 20 min were washed by superfusion with drug-free medium

during 10 min. (+)-3-PPP (0.1 μM) was then added again to the superfusion medium and the amplitude of cell motion was measured. As shown in Fig. 7a, there was no second increase in the amplitude of cell motion, but it continued to decrease and stabilized at about 75% of control level. Similar experiments were done with cells exposed to 10 nM (+)-pentazocine (Fig. 7b). After a 10 min exposure, when the amplitude of celll motion was still increasing, the cells were washed and (+)-pentazocine (10 nM) was re-applied. The wash abolished the drug-induced increase in the amplitude of cell contraction. The second application of (+)-pentazocine did not cause a second increase in the amplitude, but it continued to decrease. These results strongly suggest that the decrease in the amplitude of cell contraction, which follows the increase, is mediated by desensitization of a receptor subtype. Alternative interpretations may assume conformation changes in the receptor or in the channel which mediates the increase in $[Ca^{2+}]_i$.

3.6. Effects of verapamil and thapsigargin on the response to (+)-3-PPP

To obtain further information on the mechanism of action of σ receptor ligands, the cells were exposed to verapamil (5×10^{-7} M), 15 min before exposure to 100 nM (+)-3-PPP (in the presence of verapamil). Verapamil alone decreased the amplitude of contraction to

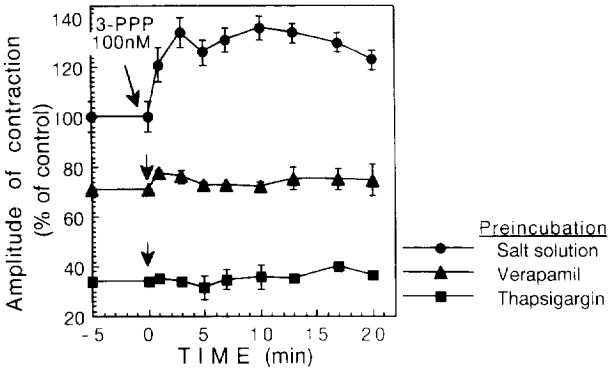


Fig. 8. Effects of pre-treatment with verapamil and thapsigargin on the (+)-3-PPP induced increase in contractile amplitude in adult rat cardiac myocytes. Cell motion was continuously recorded in isolated cardiomyocytes from adult rats. The cardiomyocytes were first preincubated during 15 min with verapamil (0.5 μM) or thapsigargin (50 nM) (each added to the superfusion physiological salt solution) or with the salt solution alone. Preincubation with thapsigargin decreased the amplitude of contraction to 33.7%, and preincubation with verapamil to 71.2% of the contractile amplitude in the same cells before preincubation. Then, the superfusion solution was replaced with salt solution containing (+)-3-PPP (100 nM), together with the compound used for preincubation. Cell motion was recorded for an additional 20 min. Results are expressed as a percentage of the amplitude of contraction measured in the same cells before the preincubation. The arrow indicates the addition of 100 nM (+)-3-PPP. Results are mean ± S.E.M. ($n = 6$).

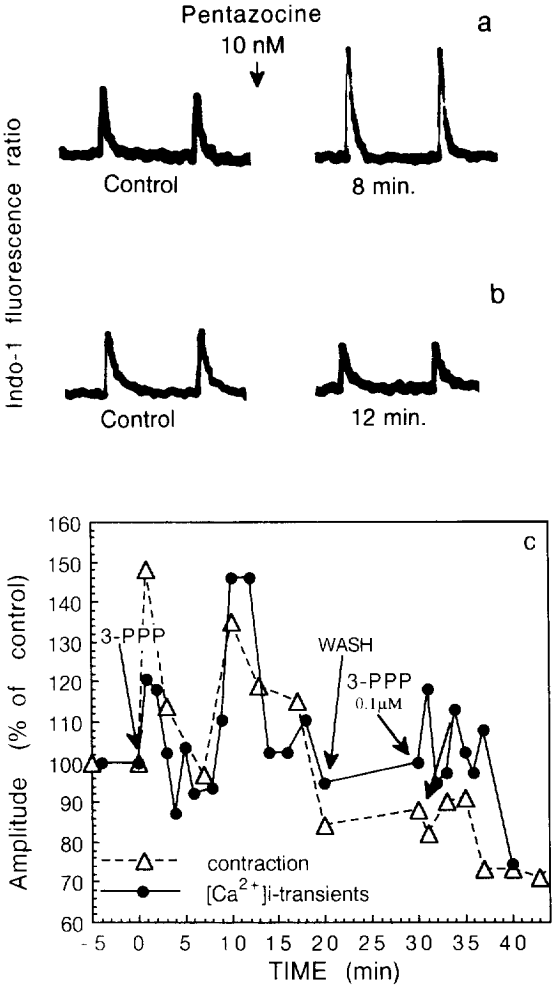


Fig. 9. Effects of (+)-3-PPP and (+)-pentazocine on the amplitude of $[Ca^{2+}]_i$ transients in single cardiac myocytes from adult rats. Isolated cardiac myocytes were loaded with indo-1 following by measurements of indo-1 fluorescence ratio as descried in Materials and methods. Original recordings of $[Ca^{2+}]_i$ transients in indo-1-loaded cardiac myocytes before and after the addition of (+)-pentazocine (10 nM) to the superfusion medium are shown in (a) and (b). The recording shows two different responses: an increase (a), and a decrease (b) in the amplitude of $[Ca^{2+}]_i$ transients. In (c), the amplitudes of $[Ca^{2+}]_i$ transients were measured in experiments done as in Fig. 7 but fluorescence ratio (410/480 nm) was recorded instead of cell motion. The graph shows results obtained in a single cardiac myocyte. For comparison, the broken line shows the results of a similar experiment in which the amplitudes of contraction were measured.

71.2 ± 5.1% of control level (mean ± S.E., $n = 5$). Exposure to verapamil almost completely abolished the effect of (+)-3-PPP on the amplitude of contraction (Fig. 8).

To assess the contribution of the Ca^{2+} stores in the sarcoplasmic reticulum to the observed changes in contractility, the cells were preincubated with thapsigargin (50 nM) 15 min before the addition of (+)-3-PPP. Thapsigargin blocks the sarcoplasmic reticulum Ca^{2+}-ATPase and depletes the sarcoplasmic reticulum from Ca^{2-} (Wrzosek et al., 1992). Thapsigargin alone de-

creased the amplitude of contraction to 33.7 ± 6.2% of control (mean ± S.E.M., $n = 6$). After the stabilization of the contractile amplitude, (+)-3-PPP (100 nM) was added (together with thapsigargin) and the changes in the amplitude were monitored. Thapsigargin substantially decreased the (+)-3-PPP-induced effects on the amplitude of contraction (Fig. 8).

3.7. Effects of σ receptor ligands on $[Ca^{2+}]_i$ transients

Changes in the amplitude of contraction in cardiac myocytes may be mediated by changes in the availability of Ca^{2+} to the myofilaments (measured as amplitude of $[Ca^{2+}]_i$ transients) or by changes in the responsiveness of the myofilaments to activation by $[Ca^{2+}]_i$. To distinguish between these mechanisms we examined the effects of 0.1 μM (+)-3-PPP on $[Ca^{2+}]_i$ transients in isolated cardiac myocytes loaded with indo-1. Fig. 9c shows the changes in the amplitude of $[Ca^{2+}]_i$ transients induced by 0.1 μM (+)-3-PPP in a single cardiac myocyte. The graph is superimposed on the graph showing corresponding changes in the amplitude of contraction (Fig. 7a). As in Fig. 7a, the amplitude of the $[Ca^{2+}]_i$ transients shows oscillations. Second application of (+)-3-PPP after washing the cell did not cause a second increase in the amplitude of $[Ca^{2+}]_i$ transients. Although the results were obtained in different experiments, the patterns of changes in the amplitudes of contraction and $[Ca^{2+}]_i$ transients are very similar (Fig. 9c). Changes induced in the amplitude of $[Ca^{2+}]_i$ transients by 10 nM pentazocine were also similar to the corresponding changes in the contractile amplitude (Fig. 9a and b).

4. Discussion

4.1. Dual effect of σ receptor ligands on contractility suggests involvement of two receptor subtypes or conformations

The present study shows that the σ receptor ligands, (+)-3-PPP, (+)-pentazocine, and haloperidol, at concentrations of 10 nM and higher, cause substantial increases in the amplitude of cell contraction in cardiac myocytes from adult rat. Plotting the maximal increase in the amplitude of cell motion versus the concentration of (+)-3-PPP yielded an apparent ED_{50} value of 4.5 nM. This value is slightly lower than the K_d determined for (+)-3-PPP binding to membranes of adult rat hearts (17.9 ± 4.0 nM, Fig. 1). At the higher concentration range (0.1 and 1.0 μM) the increase in the amplitude of contraction was followed by a dose-dependent gradual decrease. A different response, consisting of a decrease in the amplitude of systolic motion which stabilized at 70%–80% of control level, was observed under the following conditions: (a) in cells exposed to 1 nM haloperidol or 1 nM (+)-pentazocine; (b) in about 25% of cells exposed to 10 nM haloperidol, 10 nM (+)-pentazocine and 10 nM (+)-3-PPP; (c) in cells exposed to 0.1 and 1.0 μM DTG.

Our results, showing effects of σ receptor ligands at nanomolar concentrations, are unique, as most of the previous studies have shown effects of σ receptor ligands only at concentrations which are two or three orders of magnitude above the binding affinity (reviewed by Su and Junien, 1994). The low concentration range of the prototypical σ receptor ligands which induce physiological effects on cardiac myocytes indicates that the effects are indeed mediated by the σ receptors and not by cross-reactivity of the σ receptor ligands with other receptors. In addition, the low concentrations of (+)-pentazocine which induce the effects indicate the involvement of the σ_1 receptor subtype. (+)-Pentazocine and (+)-SKF-10,047 bind with high affinity to the σ_1 subtype and with low affinity to σ_2 subtype (Bowen et al., 1989, 1993; Rothman et al., 1991; McCann et al., 1994). These ligands are therefore classified as a discriminators for σ receptor subtypes 1 and 2 (Quirion et al., 1992; McCann et al., 1994; reviewed by Itzhak, 1994). Consistent with this suggestion are the results of binding of [^{3}H]DTG in the presence of increasing concentrations of (+)-pentazocine, to cardiac membranes. Analysis of the results indicates that at least 80% of the sites are σ_1. The value of the K_i for (+)-pentazocine binding to σ_1 in cardiac membranes, $K_i = 6.1$ nM, is similar to values obtained in rat brain membranes and in other tissues (Bowen et al., 1993).

However, two opposite effects are exerted by the σ ligands, depending on the particular ligand and its concentration: one effect is an increase and the other is a decrease in the amplitude of contraction. Both effects seem to be mediated by high affinity receptors, but the decrease in the contractile amplitude is observed at the lower concentration range. The results may be interpreted by assuming that the two effects are mediated by two subtypes of the σ_1 receptor, tentatively designated as σ_1^{dec} (mediating the decrease) and σ_1^{inc} (mediating the increase in the contractile amplitude). An alternative interpretation may assume two conformations of the same receptor subtype, designated similarly. It is impossible, at this stage, to decide between these two models. When cells are exposed to 10 nM haloperidol, (+)-pentazocine, or (+)-3-PPP, about 25% of the cells reveal only the function of σ_1^{dec} whereas 75% of the cells reveal only the function of the σ_1^{inc}. Exposure to (+)-3-PPP at concentrations of 0.1 and 1.0 μM induced in all examined cells a transient increase in the amplitude of systolic motion which was followed by a decrease. This decrease appears to be caused by desensitization or conformational change

of σ_1^{inc} since when the cells are washed after exposure to the σ receptor ligand and the ligand is re-applied, there is no second increase in the contractile amplitude but rather a continued decrease to a level of 75–80% of control. This decrease may suggest that effect of σ_1^{dec} is revealed after desensitization of σ_1^{inc}. Exposure of cardiac myocytes to 1 nM haloperidol or (+)-pentazocine or to 100 nM DTG induces only the effect of σ_1^{dec}.

Several recent studies in different tissues suggest the presence of σ receptor subtypes in addition to the subtypes classified as 1 and 2. For example, it was shown that (+)-3-PPP and DTG bind to pharmacologically distinct σ sites in guinea pig brain membranes (Karbon et al., 1991) and in rat brain membranes (Itzhak et al., 1991; Chaki et al., 1993; Matsumoto and Walker, 1992). In guinea pig brains, DTG binds to at least three σ binding sites (Connick et al., 1992). A new subtype of σ receptor in rat brain has been recently suggested on the basis of binding and functional studies (Booth et al., 1993).

Conformational changes in σ receptors have also been suggested, mainly on basis of radioligand binding: (+)-pentazocine binding to guinea pig cerebellum is modulated by divalent cations (Basile et al., 1992). DTG binding to guinea pig brain is allosterically regulated by Ca^{2+} channel antagonists (Rothman et al., 1991). In adrenal chromaffin cells σ_1 receptor ligands inhibit nicotine-stimulated catecholamine release and $[Ca^{2+}]_i$ increase. In these cells, nicotine allosterically modulates the binding of (+)-pentazocine to σ receptors (Paul et al., 1993). Allosteric inhibition of (+)-3-PPP binding to σ receptors by polyamines has also been reported (Paul et al., 1990).

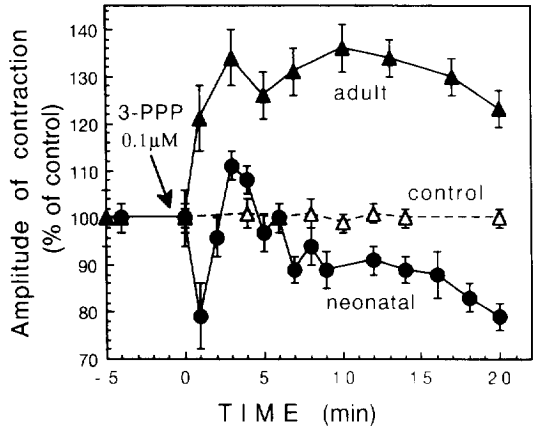


Fig. 10. A comparison between the responses of cardiomyocytes from neonatal and adult rats to exposure to (+)-3-PPP. Cell motion was continuously recorded, before and after the addition of 100 nM (+)-3-PPP to cultured cardiac myocytes from neonatal rats or to isolated cardiac myocytes from adult rats. Control was measured in neonatal rat cardiac myocytes. Similar control results were obtained in adult rat cardiomyocytes. Results are expressed as percentage of the amplitude of contraction measured in the same cells before exposure to (+)-3-PPP. Data are mean ± S.E.M. ($n = 8$).

4.2. The changes in contractility are mediated by changes in intracellular Ca^{2+}

Measurements of the effects of (+)-3-PPP (100 nM) and pentazocine (10 nM) on $[Ca^{2+}]_i$ transients revealed patterns of changes very similar to the patterns observed in the corresponding changes in the amplitude of contraction. These experiments indicate that the effects on contractility are mediated by changes in the availability of Ca^{2+} to the myofilaments. Both types of responses, the increase and the decrease in the amplitudes of $[Ca^{2+}]_i$ transients, were observed. The changes in the contractile aplitude were markedly diminished by preincubating the cells with verapamil or thapsigargin. This result indicates that in cardiac myocytes from adult rats, σ receptor ligands affect both Ca^{2+} transport across the sarcolemma and the sarcoplasmic reticulum Ca^{2+} uptake/release systems.

4.3. A comparison between effects of σ receptor ligands on neonatal and adult cardiac myocytes

We have recently reported the presence of σ binding sites, predominantly of the σ_1 subtype, on membranes of cultured cardiac myocytes from neonatal rats (Ela et al., 1994). Exposure to nanomolar concentrations of the σ receptor ligands (+)-3-PPP, (+)-pentazocine and haloperidol, induced specific pattern of changes in motion amplitude of the cardiomyocytes, consisting of an initial decrease, followed by a transient increase and a final decrease in the amplitude of motion. These effects appeared to be mediated by corresponding changes in $[Ca^{2+}]_i$ transients, which were caused by changes in Ca^{2+} influx rates. The effects were pertussis toxin-insensitive and were not altered by depletion of the sarcoplasmic reticulum Ca^{2+} stores by preincubation with thapsigargin (Ela et al., 1994).

A comparison between the responses of neonatal and adult rat ventricular myocytes to σ receptor ligands (Fig. 10) reveals several differences:

(1) In adult myocytes, the initial decrease in the amplitude of systolic motion is absent, the increase in the amplitude is much higher in most of the cells. In part of the cells exposed to low concentrations of σ receptor ligands, only a decrease in the contractile amplitude is observed.

(2) The effects on the amplitude of cell contraction are inhibited by pretreatment with thapsigargin in adult rat cardiac myocytes but not in neonatal rat myocytes.

The differences between neonatal and adult cardiomyocytes in the sensitivity of σ receptor ligand effects to thapsigargin may result from the differences

in the developmental stage of the cardiomyocytes. Neonatal ventricular myocytes are immature cells with less developed sarcoplasmic reticulum than adult cells. Studies on the effects of ryanodine and nicardipine on the inotropic response of cardiac muscles of neonatal and adult rat indicated that contraction of adult rat myocardium is highly dependent on Ca^{2+} release from the sarcoplasmic reticulum, while that of neonatal rat is more dependent on trans-sarcolemma Ca^{2+} influx (Tanaka and Shigenobu, 1989). Consistent with these results are our findings, deduced from a comparison between the effects of thapsigargin on the amplitude of systolic motion in neonatal and adult cardiomyocytes. Inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase by thapsigargin in adult cells markedly decreased the amplitude of contraction (to 33.7% of control), whereas complete inhibition of the function of the sarcoplasmic reticulum in neonatal myocyte reduced the amplitude only to 66% of control (Ela et al., 1994).

These differences may help explain the inhibition of (+)-3-PPP effects by thapsigargin in adult cells but not in neonatal cells. In adult cardiac myocytes (+)-3-PPP effects are inhibited both by verapamil and by thapsigargin. This result suggests that (+)-3-PPP modulates both Ca^{2+} influx across the sarcolemma and Ca^{2+} uptake or release systems of the sarcoplasmic reticulum. In neonatal cardiac myocytes, verapamil but not thapsigargin inhibits (+)-3-PPP effects (Ela et al., 1994). Since in these cells contraction depends mainly on the trans-sarcolemma Ca^{2+} influx, effects of σ receptor ligands on the sarcoplasmic reticulum component may not be observed, or alternatively, do not exist in neonatal cardiac myocytes.

Neonatal and adult cardiomyocytes differ also in their patterns of response to σ receptor ligands. In neonatal cells (+)-3-PPP, (+)-pentazocine and haloperidol induce a decrease followed by a transient increase and a final decrease in the amplitude of contraction, whereas in adult cells, a marked increase in the amplitude with no initial decrease is observed; as discussed previously, in part of the cells, under certain conditions, only a decrease in the contractile amplitude is observed. A comparison between binding parameters of [^{3}H](+)-3-PPP to membranes of neonatal and adult cardiac myocytes reveals similar K_d values (14.2 ± 3.3 and 17.9 ± 4 nM respectively) and a somewhat higher B_{max} value in neonatal cardiomyocytes (372 ± 9 and 276 ± 32 fmol/mg protein in neonatal and adult cardiomyocytes respectively). Thus, our results suggest that the response of cardiac myocytes to σ receptor activation is altered during the development of the myocardium.

A similar pattern has been observed in rat brain. In rat brain σ binding sites are present at their highest density during the prenatal period and decline during the postnatal period of neocortical maturation (Paleos et al., 1990). Middle-aged rats had fewer σ binding sites and sites with lower affinity for the σ receptor ligand than young rats. These differences were manifested in behavioral responses to injections of σ receptor ligands (Matsumoto et al., 1989).

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

4.4. The mechanism of the effects of σ receptor ligands on contractility

The mechanism of the effects of σ receptor ligands on contraction amplitude in isolated cardiac myocytes has not yet been completely elucidated. The dose-response profile of the induced increase in the amplitude, as well as the binding parameters, indicate action through high affinity sites. The similarity in the patterns of σ receptor ligand-induced changes in the amplitude of contraction and in the amplitudes of $[Ca^{2+}]_i$ transients indicates that the effects on the contraction amplitude are mediated by changes in the availability of Ca^{2+} to the myofilaments. A similar conclusion was reached in cardiac myocytes from neonatal rats (Ela et al., 1994). The inhibition of the increase in the amplitude of contraction by verapamil indicates that the effect of σ receptor ligands is probably mediated by increased Ca^{2+} influx across the sarcolemma, similar to our previous observation in neonatal rat cardiac myocytes (Ela et al., 1994). In addition, in adult but not in neonatal cardiac myocytes, Ca^{2+} uptake or release systems of the sarcoplasmic reticulum appear to be modulated by σ receptor ligands.

Recently, (+)-3-PPP and other σ receptor ligands were found to inhibit tonic outward potassium currents in sympathetic neurons of mouse isolated hypogastric ganglion, and in NCB20 neuroblastoma cells (Wu et al., 1991; Kennedy and Henderson, 1990; Morio et al., 1994). Such a mechanism in cardiac myocytes may lead to prolonged membrane depolarization and consequently to increased calcium influx through voltage-dependent Ca^{2+} channels. Alternatively, σ receptor activation may be coupled directly or indirectly (through second messengers), to Ca^{2+} channels. Modulation of

nicotine-dependent Ca^{2+} influx in bovine cromaffin cells by σ receptor ligands has recently been reported (Paul et al., 1993).

In conclusion, both our previous (Ela et al., 1994) and present results indicate the presence and function of σ receptors in cardiac myocytes. These receptors are activated by nanomolar concentrations of σ receptor ligands, and mediate changes in contractility and Ca^{2+} fluxes. The response to σ receptor activation in adult cardiac myocytes is different from that of neonatal cardiac myocytes. Maturation of cardiac myocytes appears to involve changes in response to σ receptor ligands.

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