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P2X receptors in sensory neurons co-cultured with cancer cells exhibit a decrease in opioid sensitivity

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Abstract

Opioids are known to control the activity of P2X receptors in the sensory neurons of rat. These receptors are important in persistent pain signaling. However, there are extremely severe pain states, such as those associated with metastatic diseases, which are refractory to opioid treatment. We have tested the possibility that cancer cells affect the sensitivity of $P2X_{2/3}$ and $P2X_2$ receptors to opiates. Sensitivity of ATP-activated currents to the selective μ-opioid receptor agonist endomorphin-1 was evaluated in rat nodose neurons co-cultured (on separate cover slips) with fibrosarcoma cells (NCTC 2472) using whole-cell patch-clamp recordings. Both in control and co-cultured neurons, P2X-mediated responses exhibited highly variable biphasic desensitization kinetics with fast and slow components. However, ATP-activated currents in co-cultured neurons acquired a new feature: the degree of their inhibition by endomorphin-1 demonstrated strong dependence on their desensitization kinetics. The neurons with “slower” responses were subjected to smaller inhibitory effect of the opioid. The “ultra-slow” responses completely lost their sensitivity to the opioid. The occurrence of such responses, rarely observed in the control neurons, was considerably increased with the duration of co-culturing. Application of endomorphin-1 to nodose neurons, co-cultured with rapidly proliferating, but non-malignant cells (fibroblasts) resulted in the data similar to control. In summary, fibrosarcoma cells release diffusible factors altering the properties of desensitization kinetics of P2X receptors and, in particular, decrease their sensitivity to opioid inhibitory control. These phenomena may increase neuronal excitability initiated by peripheral ATP release and thereby contribute to the decreased sensitivity of cancer pain to opioids.

Keywords

P2X mediated currents; rat nodose neurons; fibrosarcoma; opioid

Introduction

ATP acts as a fast neurotransmitter by activating P2X receptors, a family of ligand-gated ion channels in the central and peripheral nervous systems (Burnstock & Wood, 1996). Subtypes

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of P2X receptors are widely distributed on peripheral and central terminals of primary sensory neurons that transmit nociceptive information (Dunn *et al.*, 2001; Jarvis, 2003; Krishtal *et al.*, 1983; McGaraughty *et al.*, 2003; Staikopoulos *et al.*, 2007). It has been shown that expression of distinct subtypes of P2X receptors increased in inflammatory (Xu & Huang, 2002) and neuropathic pain (Kage *et al.*, 2002; Zhou *et al.*, 2001). Also, the up-regulation of $P2X_3$ receptors in sensory nerve terminals in the skin has been found in a model of bone cancer pain (Gilchrist *et al.*, 2005). Thus, peripheral P2X receptors represent an attractive target for the treatment of various pain states.

There is a great deal of evidence suggesting that opioid peptides can produce analgesia not only through central, but also via peripheral mechanisms (Janson & Stein, 2003; Reichert *et al.*, 2001). Recently it has been found that P2X and μ-opioid receptors are functionally coupled in sensory neurons. Responses of individual sensory neurons to ATP consist of a fast (mediated by $P2X_3$ receptors) and a slow (mediated by heteromeric $P2X_{2/3}$ and $P2X_2$ receptors) current; both currents are inhibited by μ-opioid receptor agonists (Chizhmakov *et al.*, 2005).

Although activation of μ-opioid receptors can produce analgesia and attenuate responses of nociceptors under certain conditions such as inflammation (Janson & Stein, 2003; Reichert *et al.*, 2001; Schafer, 1999), opioids are less effective for other chronic pain states such as cancer pain, and escalating doses are often required (Lesage & Portenoy, 1999; Mercadante, 1997; Mercadante & Arcuri, 1998; Portenoy & Lesage, 1999), which limit their use. It is unclear why opioids are less effective in treating cancer pain, but one possibility is that cancer cells, or substances released from these cells, alter opioid signaling.

In a murine model of cancer pain, osteolytic fibrosarcoma cells were implanted into and around the calcaneous bone of the hind paw. This produced ongoing nocifensive behavior, hyperalgesia (Wacnik *et al.*, 2001) and sensitization of C-fiber nociceptors in skin overlying the tumor (Cain *et al.*, 2001). In order to address the mechanisms by which cancer cells interact with and alter excitability of nociceptors, we used a recently developed *in vitro* model, in which fibrosarcoma cells were co-cultured with dorsal root ganglion neurons. Using this model, it was found that excitability of small co-cultured neurons increased as evidenced by enhanced responses to potassium and to capsaicin evaluated by calcium imaging (Khasabova *et al.*, 2007). In the present study, fibrosarcoma cells were co-cultured with nodose neurons carrying out visceral sensory function. The use of these neurons, extensively expressing slow $P2X_2$ and $P2X_{2/3}$ receptors (Gever *et al.*, 2006), was robust for determining the effect of cancer cells on ATP-evoked currents and on their modulation by opioids. Our data indicate that tumor-derived factors diminish opioid modulation of ATP responses in nodose neurons when co-cultured with fibrosarcoma cells.

Materials and Methods

Co-culture of rat nodose ganglia neurons with NCTC fibrosarcoma cells and fibroblasts

Wistar rats (8–10 days old and weighing 15–20 g) were euthanized with CO_2 and decapitated. All procedures were carried out in accordance with the European Communities Council Directive and National Institutes of Health guidelines for care and use of laboratory

animals. After decapitation nodose ganglia were removed as described elsewhere (Chizhmakov *et al.*, 2005) and were incubated for 30–50 min in PBS solution containing 0.3% trypsin and 0.1% collagenase at 32 °C. Throughout the entire procedure the media was continuously saturated with 95% O_2 + 5% CO_2 gas mixture (pH 7.4). Neurons were isolated by trituration and plated on 13 mm circular uncoated glass cover-slips (Thomas Scientific, Swedesboro, NJ, USA). Similar cover-slips were used for all the cultures described below.

Fibrosarcoma cells (NCTC clone 2472) obtained from American Type Cell Culture (Manassas, VA, USA) were prepared as described previously (Khasabova *et al.*, 2007) with slight modification. Cancer cells were cultured in NCTC135 medium (Sigma, MO, USA), supplemented with 10% horse serum (Gibco, Carlsbad, CA, USA) and 4 mM glutamine and incubated in 95% O_2 and 5% CO_2. Approximately $1.5{\cdot}10^5$ cells/cm^2 were plated on glass cover-slips in 3.5 cm Petri dishes (Corning, USA). After 24 hours of incubation, the medium was replaced with DMEM (Sigma, USA), containing 10% heat inactivated fetal calf serum (Gibco, Carlsbad, CA, USA) and 5 mg/ml insulin. Isolated nodose neurons, seeded on separate glass cover-slips, were placed into the Petri dishes next to the cover-slips with the NCTC2472 cells and incubated for 2–5 days. This approach prevented physical contact between co-cultured cells.

To address the possibility of non-specific metabolic effects of the co-culture condition, nodose neurons were co-cultured with fibroblasts. To isolate and culture the fibroblasts we used a modified variant of the procedure, described previously (Brockes *et al.*, 1979). Namely, fibroblasts were isolated from peripheral nerves of 5 days old rats. After fermentation, the cells were seeded on glass cover-slips at an initial density of $5–10{\cdot}10^3$/cm^2 cells. The media contained 90% DMEM and 10% FBS. On the 5th day after cultivation the media was replaced with an equal volume of fresh media, and the cells (at an increased density of up to 10^5/cm^2 which was comparable to the initial density of cancer cells) were used for co-culturing with nodose ganglia neurons.

Drugs

The P2X receptor agonists Na-ATP (20 μM), α,β-methylene ATP (60 μM), and the μ-opioid receptor agonist, endomorphin-1 (1 μM), were dissolved in the extracellular solution just before use. Na-ATP and α,β-methylene ATP were obtained from Sigma (USA), endomorphin-1 was from TOCRIS (Ellisville, MO, USA).

Electrophysiological recording

Conventional whole-cell patch clamp was used in combination with concentration clamp technique. We used a “square-pulse” application technique combining two separate U-tubes (Krishtal & Pidoplichko, 1980) targeted to the surface-attached cell. The rate of solution application and washout in this modification was 30–50 ms and 50–100 ms correspondingly. P2X receptor agonists (ATP or α,β-methylene ATP) were applied for 2.5 s every 0.5 min or for 10 s every 2.5 min. This protocol ensured complete recovery from desensitization. ATP was applied 2–3 times before the application of endomorphin-1. Neurons demonstrating run-down in the course of control applications were not tested further. Therefore, sufficiently long recordings of the ATP-elicited responses were not accompanied by any significant run-

down. Short (2.5 s) pulses allowed tracking the time course of inhibition of P2X-mediated currents by opioid peptide. Long (10 s) pulses were used to study desensitization kinetics of P2X-mediated currents in detail. Endomorphin-1 was added to extracellular and P2X-agonist containing solutions, so that it was constantly applied to the cell during the experiment. The opioid agonist was superfused on the tested neuron until the steady-state level of P2X-current inhibition was reached (usually within 3–4 min).

Whole-cell patch-clamp recordings were performed at room temperature (22±1°C) using a patch-clamp amplifier Axopatch 200B (Axon Instruments, Union City, CA, USA). The data were filtered at 2 kHz, digitized at 5 kHz, and stored on a laboratory computer using a Digidata 1200B interface and pClamp 8.0 software (Axon Instruments, Union City, CA, USA). All recordings were made at holding and test potentials of −70 mV. The extracellular solution contained (in mM) 150 NaCl, 2 $CaCl_2$, 5 KCl, 10 glucose, 10 HEPES (pH was adjusted to 7.2–7.4). The patch pipette solution (artificial intracellular medium) contained (in mM) 140 KCl, 10 HEPES, 10 EGTA, 0.3 GTP, 5 Mg-ATP (pH was adjusted to 7.2–7.4). Intervals of 2–3 minutes were allowed between patch rupture and application of stimuli to allow for equilibration with the patch pipette solution.

Data analysis

To determine changes produced by the fibrosarcoma cells in the kinetics of the total ATP-activated current in neurons with different desensitization rates, ATP was applied for 10 s. The total transmembrane P2X current (I) was described as a sum of “fast” and “slow” components of integrative currents, according to the equation $I=I_f \cdot \exp(-t/\tau_f)+I_s \cdot \exp(-t/\tau_s)$, where I_f, I_s and τ_f, τ_s are the amplitudes and time constants of fast and slow desensitized currents, respectively. The contribution of the slow desensitization component to the decay of ATP-induced current was calculated as $I_s/(I_f+I_s)$.

In studies of the effects of fibrosarcoma cells on opioid modulation of ATP currents evoked by brief (2.5 s) application of ATP, the magnitude of desensitization was estimated by the ratio $I_{2.5}/I_{max}$, where $I_{2.5}$ is the current 2.5 s after application of a P2X agonist and I_{max} is the maximal peak amplitude of current. Thus, neurons with fast developing desensitization were characterized by a lower index whereas neurons with slow desensitization had a higher index. The inhibitory effect of endomorphin-1 on P2X current was evaluated as a magnitude of inhibition $(I_{max} - I_{ss})/I_{max}$, where I_{max} is the amplitude of the current induced by the P2X agonist prior to application of endomorphin-1 and I_{ss} is the peak current measured when the inhibitory effect of opioid reached maximum.

The relationship between the rate of desensitization of ATP-activated currents and endomorphin-evoked inhibition was fitted by the least-square linear regression $y=a+b \cdot x$ and the squared value of correlation coefficient (R^2) was calculated. All data were analyzed with Origin 7.5 (OriginLab Corp., Northampton, MA, USA) and Microsoft Excel software. Values are given as mean ± SD. Statistical analysis was performed using single factor ANOVA. The differences were considered to be significant if $p<0.05$.

Results

Occurrence of ATP-activated currents with slow desensitization kinetics increases with the time in co-culture with cancer cells

ATP responses in nodose neurons are mediated by non-desensitizing $P2X_2$, slowly desensitizing $P2X_{2/3}$ and fast desensitizing $P2X_3$ receptors (Zhong *et al.*, 2001). The fast $P2X_3$ receptors were not a subject of consideration in the present paper because they are rarely expressed in nodose neurons and are completely desensitized after 2–3 applications of ATP (Pankratov *et al.*, 2001). When compared in two samples of control and co-cultured neurons (2–5 days in co-culture), peak current amplitudes in control (2099±840 pA, n=15) and co-cultured cells (1659±523 pA, n=15) did not differ significantly (p=0.33). We have found that desensitization kinetics of ATP-activated currents significantly varied in control as well as in co-cultured neurons. Within 2.5 s of agonist application, the currents desensitized down to levels ranging (cell to cell) from 10 to 90% of their maximal amplitude (I_{max}) providing the estimates of $I_{2.5}$ (Fig. 1, upper panel). The slower desensitization corresponded to a higher value of $I_{2.5}/I_{max}$ and vice versa. Neurons cultured alone (control) and co-cultured with fibrosarcoma cells were separated into 2 groups according to the time in culture/co-culture: 2–3 days or 4–5 days. In control neurons cultured alone for 2–3 days or 4–5 days mean values of $I_{2.5}/I_{max}$ were not different (0.52±0.14, n=23 and 0.53±0.14, n=21, respectively, p=0.77; Fig. 1, lower panel, open bars). In the mean terms, ATP-activated currents in control nodose neurons cultured for 2–5 days were desensitized approximately to 50% within 2.5 s.

There was no significant difference (p=0.18) between the mean values of $I_{2.5}/I_{max}$, when nodose neurons were cultured alone (see above) or co-cultured with fibrosarcoma cells for 2–3 days (0.59±0.21, n=27). In contrast, the mean values of $I_{2.5}/I_{max}$ in neurons co-cultured for 4–5 days (0.66±0.19, n=22) were significantly higher when compared with corresponding control neurons ($p<0.05$). Thus, the number of neurons with slow desensitization kinetics of ATP-activated currents ($I_{2.5}/I_{max}>0.5$, see Fig. 1, upper panel: trace d) increased as a function of time in co-culture with fibrosarcoma cells.

Effects of endomorphin-1 on ATP-activated currents in co-cultured neurons depend on kinetics

We have shown previously that the selective μ-opioid receptor agonist, endomorphin-1 (0.01–1 μM), attenuates the amplitude of ATP-activated currents in nodose neurons (Chizhmakov *et al.*, 2005). The inhibitory effect of endomorphin-1 in these studies was not dependent on the kinetics of desensitization of the ATP current (this will be illustrated below, see Fig. 3 for the effect of opioid on the extremely slow response).

We used the same application protocol (2.5 s pulses of 20 μM ATP every 30 s) in order to observe the time course of blocking action or washout of endomorphin-1 on ATP-activated currents in co-cultured neurons. We have found that, in contrast to control neurons, ATP-activated currents in the neurons co-cultured with fibrosarcoma cells are inhibited by endomorphin-1 (1 μM) depending on the kinetics of the response (that is, depending on the value $I_{2.5}/I_{max}$). Fig. 2 demonstrates the effect of endomorphin-1 on the ATP-activated

currents with different desensitization kinetics. Inhibitory action of endomorphin-1 reached its steady-state level within 3–3.5 min. The degree of inhibition of ATP-activated currents by endomorphin-1 was defined as $(I_{max}-I_{ss})/I_{max}$, where I_{ss} is the amplitude of the current recorded at the steady-state level of opioid-induced inhibition. The rate of desensitization was estimated by the ratio $I_{2.5}/I_{max}$ as described above. Fig. 2A shows that in the case of $I_{2.5}/I_{max}$=0.36, 61% of the initial current amplitude was inhibited by the drug. At slower desensitization of ATP-activated currents, the inhibitory action of endomorphin-1 was markedly reduced (Fig. 2B, C): to 39% ($I_{2.5}/I_{max}$=0.64) and to 24% ($I_{2.5}/I_{max}$=0.87). About 30% of the cells tested demonstrated extremely slow desensitization of ATP-activated currents, so that $I_{2.5}/I_{max}$ was close to 1. ATP-activated currents in these cells were not affected by endomorphin-1 (the degree of inhibition was 0.05±0.21, p=0.63, n=7, Fig. 3B) in contrast to control nodose neurons (the degree of inhibition was 0.48±0.16, p<0.01, n=5, Fig. 3A). This difference in sensitivity of “ultra-slow” ATP-activated currents to endomorphin-1 in control and co-cultured neurons was significant (p<0.01). The effect of the opioid on P2X-mediated currents with similar “ultra-slow” desensitization kinetics in normal and co-cultured neurons is compared in Fig. 3C. ATP-activated currents in sensory neurons are prone to gradual irreversible rundown. To minimize its effect on our measurements, we selected only cells exhibiting minimal rundown (see Methods). Still, it could not be excluded *a priori* that the rundown was smaller in the cells with slower current kinetics. This could result in their apparently lower sensitivity to the opioid. However, we found that the rundown was in fact independent on the current kinetics. Rapidly desensitizing ATP-activated currents ($I_{2.5}/I_{max}$<0.6) recovered to 0.86±0.2 (n=5) of their initial amplitude after washout of endomorphin-1. Similarly, the “ultra-slow” currents ($I_{2.5}/I_{max}\approx1$) recovered to 0.82±0.14 (n=4). Thus, the amplitudes of rapidly desensitizing and ultra-slow currents after removal of opioid declined to a similar extent (p=0.69). The unrecoverable part of the currents (possibly determined by rundown) did not depend on the degree of inhibition by endomorphin-1 (compare Fig. 2A through C, see also Fig. 3B).

In co-cultured neurons inhibition of ATP-activated currents by endomorphin-1 strongly depends on desensitization kinetics. This dependence is presented in Fig. 4 in comparison with measurements on control neurons. Fig. 4A indicates that in control neurons the degree of inhibition does not depend on desensitization kinetics (see also Fig. 3A). Fig. 4B demonstrates that slowly desensitizing ATP-activated currents in co-cultured neurons ($I_{2.5}/I_{max}\sim1$) lose sensitivity to endomorphin-1, while the fast ATP-activated currents ($I_{2.5}/I_{max}$<0.5) are inhibited by 50–60% like in control nodose neurons. The degree of inhibition of ATP-activated currents by endomorphin-1 was defined as $(I_{max}-I_{ss})/I_{max}$, where I_{ss} is the amplitude of the current recorded at the steady-state level of opioid-induced inhibition. The data points presented in Fig. 4 for control and co-cultured nodose neurons were fitted by linear equations (See Methods). Our results demonstrate that the inhibitory effect of endomorphin-1 did not correlate with kinetics of desensitization of ATP-activated currents ($R^2=10^{-5}$) in control nodose neurons (Fig. 4A), whereas inhibition of ATP currents by endomorphin-1 in the co-culture condition is strongly dependent (R^2=0.93) on desensitization kinetics (Fig. 4B).

To test whether changes in the correlation between kinetics of desensitization of ATP-activated currents and the magnitude of inhibition by endomorphin-1 reflected specific

effects of the fibrosarcoma cells, nodose neurons were co-cultured with another type of rapidly proliferating cells, fibroblasts. The effect of opioid on ATP-induced currents in the neurons co-cultured with fibroblasts was similar to that in control neurons. There was no correlation between inhibition by endomorphin-1 and desensitization kinetics of the currents ($R^2=3\cdot10^{-5}$). The effect of opioid on P2X-mediated currents was biphasic as in control, but not in the neurons co-cultured with cancer cells: opioid-induced inhibition was preceded with transient potentiation of the currents (Fig. 5) (Chizhmakov *et al.*, 2005). The current demonstrated in this Fig. had $I_{2.5}/I_{max}=0.76$, but its inhibition, $(I_{max}-I_{ss})/I_{max}=0.56$, was much stronger as compared with similar cases in fibrosarcoma co-culture conditions, cf. Fig. 2B, C. The degree of inhibition of ATP-induced currents by endomorphin-1 in the neurons co-cultured with fibroblasts (0.35±0.16, n=10) was close to that observed in control nodose neurons (0.39±0.13, n=5). These data suggest that the changes in sensitivity of nodose neurons to endomorphin-1 are not due to non-specific metabolic effects of the co-culture condition but rather indicate specific effects of fibrosarcoma cells.

Responses to ATP and α,β-methylene ATP demonstrate similar sensitivity to opioids in co-cultured neurons

As already mentioned, the current evoked by ATP in nodose neurons comprises cumulative activation of $P2X_{2/3}$ and $P2X_2$ receptors. The former are characterized by marked desensitization, while the latter are not measurably desensitized, at least within 10 s of ATP application, as demonstrated on recombinant receptors (Fisher *et al.*, 2004). Thus, a possible interpretation of our data summarized in Figs. 1 and 4 could be that opioid-insensitive ATP currents are determined mainly by “ultra-slow desensitizing” $P2X_2$ receptors. These receptors may not be subjected to the inhibitory action of opioids in co-cultured neurons, contrary to $P2X_{2/3}$ receptors. In order to discriminate between the currents mediated by $P2X_2$ and $P2X_{2/3}$ receptors, we used α,β-methylene ATP, which activates primarily (if not exclusively) $P2X_{2/3}$ receptors when applied at concentrations below 100 μM (Gever *et al.*, 2006). If $P2X_2$ receptors in co-cultured cells are not subjected to opioid control, ATP-induced currents should be less sensitive to endomorphin-1 than the currents elicited by α,β-methylene ATP. We have found this not to be the case. Fig. 6 demonstrates the effect of opioid on ATP and α,β-methylene ATP activated currents in the cells with fast ($I_{2.5}/I_{max}=0.45$) and slow ($I_{2.5}/I_{max}=0.97$) desensitization kinetics.

When application of 20 μM of ATP and 60 μM of α,β-meATP elicited the currents with fast desensitization kinetics, the amplitudes of ATP and α,β-meATP activated responses were identical indicating negligible contribution of $P2X_2$ receptors. Both responses were equally inhibited by endomorphin-1 (Fig. 6A). When the same agonist concentrations induced slowly desensitizing currents, the amplitude of α,β-methylene ATP activated current (reflecting the activity of $P2X_{2/3}$ receptors) was considerably smaller than the response to ATP which reflected the activity of both $P2X_2$ and $P2X_{2/3}$ receptors (Fig. 6B). It appears that the “slow” responses to both agonists in co-cultured neurons were equally resistant to endomorphin-1. In summary, these results indicate that both slowly desensitizing $P2X_{2/3}$ and $P2X_2$ receptors are insensitive to opioid in co-culture. Comparison of Figs. 6A and B indicates that $P2X_{2/3}$ mediated currents exhibit different sensitivity to opioids depending on their desensitization kinetics. Thus, these data suggest that $P2X_{2/3}$ receptors in co-cultured

neurons are under opioid control to a degree which is determined by their rate of desensitization which varies on a cell-to-cell basis. To test this assumption, we analyzed the desensitization time course of the ATP-activated currents using long (10 s) pulses of the agonist.

Opioid control of $P2X_{2/3}$ receptors in co-cultured neurons correlates with the presence of a slow component in their desensitization

Long application of ATP (10 s) elicited currents with biphasic desensitization kinetics (Eto *et al.*, 2006). ATP-activated currents can be fitted by the sum of two exponentials with different amplitudes and time constants (designated as “fast” and “slow”, see Methods). We have shown (Fig. 7) that the responses characterized in the previous sections as “fast” or “slow” (with larger or smaller $I_{2.5}/I_{max}$ value) differ by the relative contribution of the slow desensitization component, $I_s/(I_f+I_s)$. When the relative contribution of the slow component in ATP-activated currents before application of endomorphin-1 was $I_s/(I_f+I_s)=0.31$ (Fig. 7A), endomorphin-1 inhibited the current by 52%. However, the magnitude of inhibition decreased with elevated contribution of the slow component. When the slow component increased to $I_s/(I_f+I_s)=0.63$ (Fig. 7B) or $I_s/(I_f+I_s)=0.72$ (Fig. 7C), the magnitudes of inhibition produced by endomorphin-1 were 24% and 3%, respectively. The amplitudes of partially inhibited currents from control and co-cultured neurons did not change when the time between successive ATP applications was increased twice, from 30 s to 1 min for 2.5 s ATP pulses and from 2.5 min to 5 min for 10 s pulses. This observation excludes a hypothetic possibility that the inhibitory action of endomorphin-1 on ATP-activated currents is determined by delayed recovery from desensitization. There were no differences in the kinetics of ATP-activated currents before endomorphin-1 application and currents partially inhibited by endomorphin-1 (Fig. 7, right panel).

The time constants of the fast and slow desensitization components did not change in culture. For control neurons cultured alone for 2–3 days, time constants for the fast and slow desensitizing components were $\tau_f=1264\pm295$ ms and $\tau_s=34332\pm18845$ ms (n=11), respectively. When the neurons were co-cultured with fibrosarcoma cells for 2–3 days, the time constants were $\tau_f=1325\pm528$ ms and $\tau_s=30023\pm18097$ ms (n=10) for the fast and slow desensitizing components, respectively. Comparison of 2–3 days control and co-cultured nodose neurons shows that both time constants are not significantly different (p=0.69). For control neurons cultured for 4–5 days the time constants were: $\tau_f=1305\pm346$ ms and $\tau_s=34332\pm19340$ ms (n=13), correspondingly. When neurons were co-cultured for 4–5 days, these time constants were $\tau_f=1343\pm733$ ms and $\tau_s=29684\pm11606$ ms (n=14), respectively. There was no difference between reciprocal time constants (p=0.78).

We assumed that the changes in desensitization kinetics expected from initial observations on co-cultured neurons (Fig. 1) were due to the increase in the number of cells with a predominantly slow desensitization component. We compared the mean values of $I_s/(I_f+I_s)$ for control and co-cultured neurons. After 2–3 days and 4–5 days in culture (control neurons) $I_s/(I_f+I_s)$ was 0.49 ± 0.1 (n=11) and 0.48 ± 0.25 (n=13) respectively in control condition. These data indicate that in nodose neurons cultured for 2–5 days, the contribution of the slow desensitization component to the total ATP-activated current desensitization is

about 50% and does not change with time in the culture (p=0.88). After 2–3 days in co-culture, the mean value of $I_s/(I_f+I_s)$ was 0.53±0.16 (n=10), and after 4–5 days it was 0.64±0.19 (n=14). Comparison of 2–3 days control and co-cultured neurons showed no significant differences in values of $I_s/(I_f+I_s)$ (p=0.44), while after 4–5 days the increase in the contribution of the slow component in the co-culture condition was significant ($p<0.05$). These data correspond with the initial data demonstrated in Fig. 1: the differences in $I_{2.5}/I_{max}$ were significant only after 4–5 days in co-culture.

The shift to slower desensitization kinetics after more time in co-culture can be clearly seen when comparing the distribution of control and co-cultured cells to the contribution of the slow desensitization component (Fig. 8). All control and co-cultured neurons analyzed for I_s contribution (that is, subjected to 10 s long agonist application) were separated into several groups in accordance with the value of $I_s/(I_f+I_s)$. The relative number of cells was calculated for each group (n/N).

Fig. 8 shows that for the control cells the most frequent values of $I_s/(I_f+I_s)$ (largest open bar) were within the interval of 0.4–0.6 (almost equal contribution of fast and slow desensitization components to the total current decay). For the co-cultured neurons, the most frequent value of $I_s/(I_f+I_s)$ was within the interval of 0.6–0.8 (largest shaded bar), reflecting the prevalence of the slow desensitization component of ATP-activated currents over the fast one (see Fig. 7C). Moreover, the fraction of cells with extremely slow desensitization kinetics ($I_s/(I_f+I_s)>0.8$) is considerably larger in co-cultured neurons. As demonstrated above (Figs. 3, 4 and 7C), such cells are almost insensitive to endomorphin-1. Thus, co-culture of nodose neurons with fibrosarcoma cells leads to the increased proportion of neurons with a prevalent contribution of the slow desensitization component of ATP-induced current which is insensitive to opioids.

Discussion

The novel in vitro co-culture model used in the present study to investigate functional interactions between fibrosarcoma cells and nodose neurons was originally developed for DRG neurons. The results obtained for co-cultured DRG neurons indicated that factors released from the fibrosarcoma cells increased neuronal excitability, and up-regulated calcium channel subunits and TRPV1 receptor expression. Notably, changes in neurons co-cultured with cancer cells were similar to those found in DRG neurons obtained from tumor-bearing mice that exhibited hyperalgesia (Khasabova *et al.*, 2007). Therefore, the co-culture of sensory neurons with cancer cells is a valuable model for studying the underlying cellular mechanisms that mediate functional interactions between cancer cells and sensory neurons that contribute to cancer pain.

The results of the present study demonstrate that co-culture of nodose neurons with fibrosarcoma cells leads to changes in characteristics of ATP-evoked currents as well as alterations in the modulation of ATP currents by endomorphin-1. The data indicate that tumorogenic factors from cancer cells alter the phenotype of rat nodose neurons with respect to ATP currents and are consistent with earlier observations showing changes in DRG neuron excitability when co-cultured with fibrosarcoma cells (Khasabova *et al.*, 2007).

Contrary to the control condition, co-cultured nodose neurons demonstrated a significant correlation between the kinetics of desensitization of P2X currents and the magnitude of inhibition by opioids. Namely, slower desensitization kinetics of initial currents resulted in lower opioid inhibition. The fraction of neurons with ultra-slow desensitization kinetics of ATP-activated currents was practically insensitive to endomorphin-1. The proportion of nodose neurons with lower sensitivity to opioid inhibition increased when co-cultured with cancer cells. Also, the initial opioid-induced potentiation of P2X responses observed in control neurons (Chizhmakov *et al.*, 2005) was absent in co-cultured conditions.

The mechanisms which cause slowly desensitizing ATP-induced currents in co-cultured nodose neurons to be less sensitive to endomorphin-1 are unclear. Recent studies suggest that heterooligomeric $P2X_{2/3}$ receptors contain one $P2X_2$ subunit and two $P2X_3$ subunits ($P2X_2(P2X_3)_2$), though molecular biology of P2X receptors does not exclude alternative subunit composition, $(P2X_2)_2P2X_3$ (Jiang *et al.*, 2003). Hypothetically, fast desensitization component can be due to the activity of $P2X_2(P2X_3)_2$ receptors, while $(P2X_2)_2P2X_3$ receptors demonstrate slow desensitization. Variable contribution of these receptor subpopulations into integral ATP current could contribute to the heterogeneity of desensitization kinetics. A similar assumption was previously made suggesting a variable contribution of kinetically distinct P2X receptor subpopulations to explain heterogeneous (fast and slow) desensitization kinetics of ATP-induced currents in different preparations (Liu *et al.*, 2001; Taschenberger *et al.*, 1999). To account for our data, we can further assume that both receptor subpopulations are equally affected by endomorphin-1 under normal conditions, while $(P2X_2)_2P2X_3$ subpopulation escapes from opioid control after co-culturing with tumor cells. There is at least one case reported for DRG neurons which indicates that the modulation of $P2X_3$ subunit is related to the receptor subunit arrangement: activation of $GABA_B$ metabotropic receptor inhibited $P2X_3$-mediated currents without affecting $P2X_{2/3}$-mediated currents (Sokolova *et al.*, 2003). Changes in the signal transduction pathways that mediate μ-opioid modulation of P2X receptors in the course of co-culturing are indirectly supported by the following observations. First, inhibition of ATP-activated currents by endomorphin-1 becomes dependent on desensitization kinetics of the current. Second, the effect of opioid becomes monophasic (loss of potentiation phase).

Attenuation of μ-opioid receptor signaling can change the properties of $P2X_3$ and/or $P2X_{2/3}$ receptors through modulation of the activity of different protein kinases (Belcheva *et al.*, 2005; Chen & Huang, 1991; Kaminski, 2004). For example, $P2X_3$ subunit can be sensitized by enhancement of Ca^{2+}/calmodulin protein kinase II mechanism (Xu & Huang, 2002). Moreover, PKC controls the desensitization kinetics of $P2X_2$-mediated currents (Boue-Grabot *et al.*, 2000) and indirectly regulates currents through $P2X_3$ receptor (Brown & Yule, 2007). Thus, diffusible substances, like chemokines or metalloproteases, released by fibrosarcoma cells may affect the liability P2X receptors to opioid control using various modulatory pathways. Both, the nature and signaling of tumorogenic substances remain to be determined.

There are several tumorogenic substances that may change μ-opioid signaling in sensory neurons. One of the more likely candidates is CC chemokine ligand 2 (CCL2). Previously it was shown that chemokine and μ-opioid receptors morphologically co-localize on small to

medium diameter peripheral sensory neurons, and pretreatment of sensory neurons with proinflammatory chemokines, including CCL2, induced internalization and severely impaired the μ-opioid receptor-mediated inhibition of cAMP accumulation (Zhang *et al.*, 2004). The presence of CCL2 immunoreactivity and peptide in fibrosarcoma tissue and microperfusates from tumor as well as contribution of CCL2 to the tumor cell-induced sensitization of sensory neurons (Khasabova *et al.*, 2007) suggest that CCL2 may induce the changes in μ-opioid receptor signaling in nodose neurons co-cultured with fibrosarcoma cells. Variations in the uptake level of CCL2 and/or some other, still unknown, factor(s) by different sensory neurons (Gatzinsky *et al.*, 2004) could account for predominant action of these factors on the neurons expressing slowly desensitizing ATP-activated current.

Currently, opioid therapy remains a major analgesic approach in patients with cancer pain (Lesage & Portenoy, 1999; Levy *et al.*, 2006; Portenoy & Lesage, 1999). However, the decreased analgesic potency of opioids leads to the need for increasing doses of opioids in patients (Breivik, 2001; Luger *et al.*, 2002; Peters *et al.*, 2005; Portenoy, 1999). Since ATP may play a significant role in cancer pain (Burnstock & Wood, 1996; Burnstock, 2006), it is relevant to study interactions between cancer cells and P2X receptor signaling. Our results indicate that a population of neurons insensitive to opioid exists and its contribution increases with the time in co-culture conditions. Decreased opioid sensitivity of ATP-evoked currents may contribute to the attenuated analgesic potency of opioids in managing cancer pain.

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Abbreviations

CCL2	chemokine (C-C motif) ligand 2
DRG	dorsal root ganglion
GABA	gamma-aminobutyric acid
NCTC	National Collection of Type Cultures
PKC	protein kinase C
TRPV1	transient receptor potential vanilloid 1

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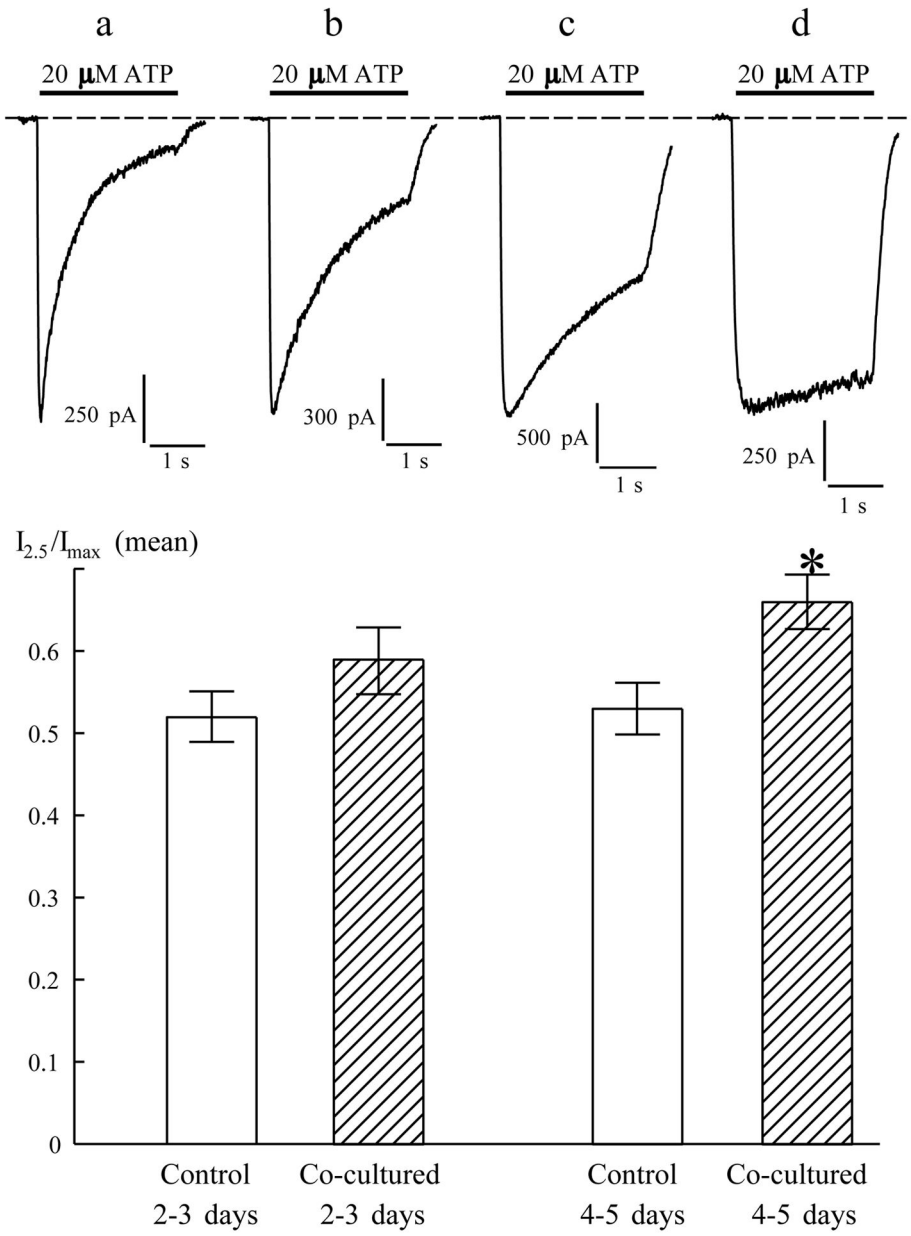

Fig. 1.

Desensitization rate of ATP-activated currents depends on the time in co-culture. Upper panel: Representative current traces recorded from different neurons in control. Here and below: bars indicate application intervals, agonist concentrations are given above each bar. Holding potential was −70 mV. Within 2.5 s of agonist application the currents desensitized down to 11%, 28%, 54% and 88% of their initial amplitudes (a, b, c, d correspondingly). Lower panel: The mean values of $I_{2.5}/I_{max}$ (mean ± SD) for ATP-activated currents in control (open bars) and in co-cultured (shaded bars) nodose neurons. Time in culture/co-culture is indicated below the bars. Asterisk indicates $p<0.05$, when compared to control cells, cultured for 4–5 days.

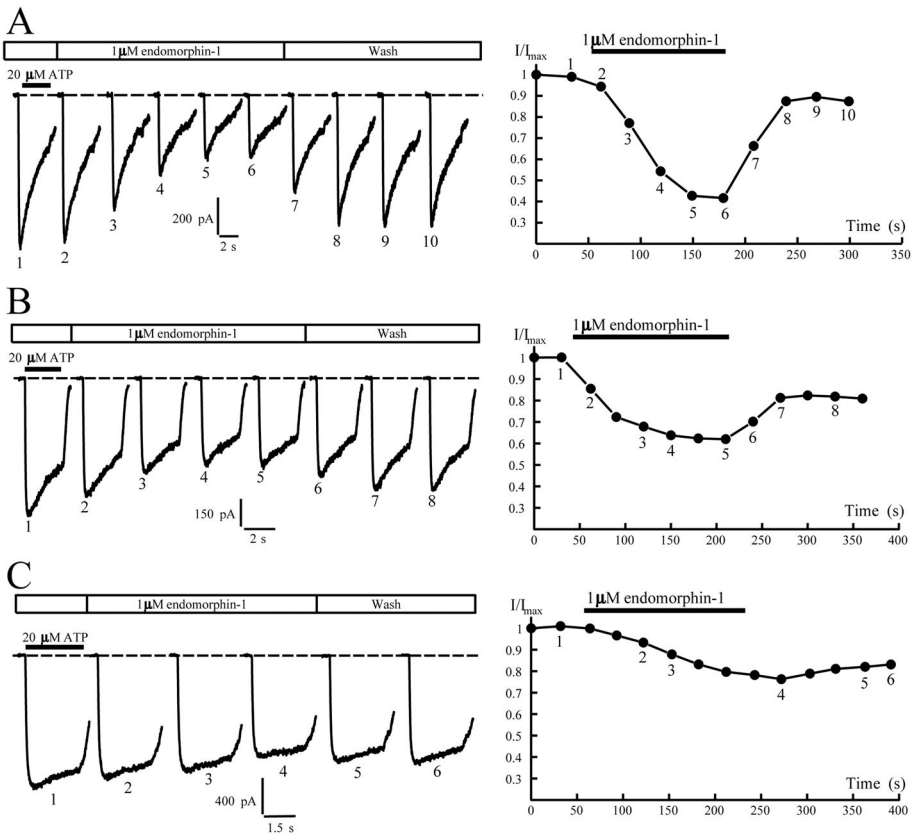

Fig. 2.

Effect of endomorphin-1 on ATP-activated currents in rat nodose neurons co-cultured with fibrosacroma cells. Extracellular application of endomorphin-1 (1 μM) effectively inhibits ATP-activated current in nodose neurons co-cultured with fibrosarcoma cells that demonstrate fast desensitizing kinetics (A, left panel) and has much smaller effects in neurons with slow desensitizing kinetics (B, C, left panel). The normalized amplitudes of peak ATP currents (I/I_{max}) demonstrate the time course of endomorphin-1 blocking action and washout (Fig. 2A, B, C, right panel). The numbers below the current traces in the left panel correspond to the times indicated in the right panel. Here and below: the protocol of drug application is presented above the current traces.

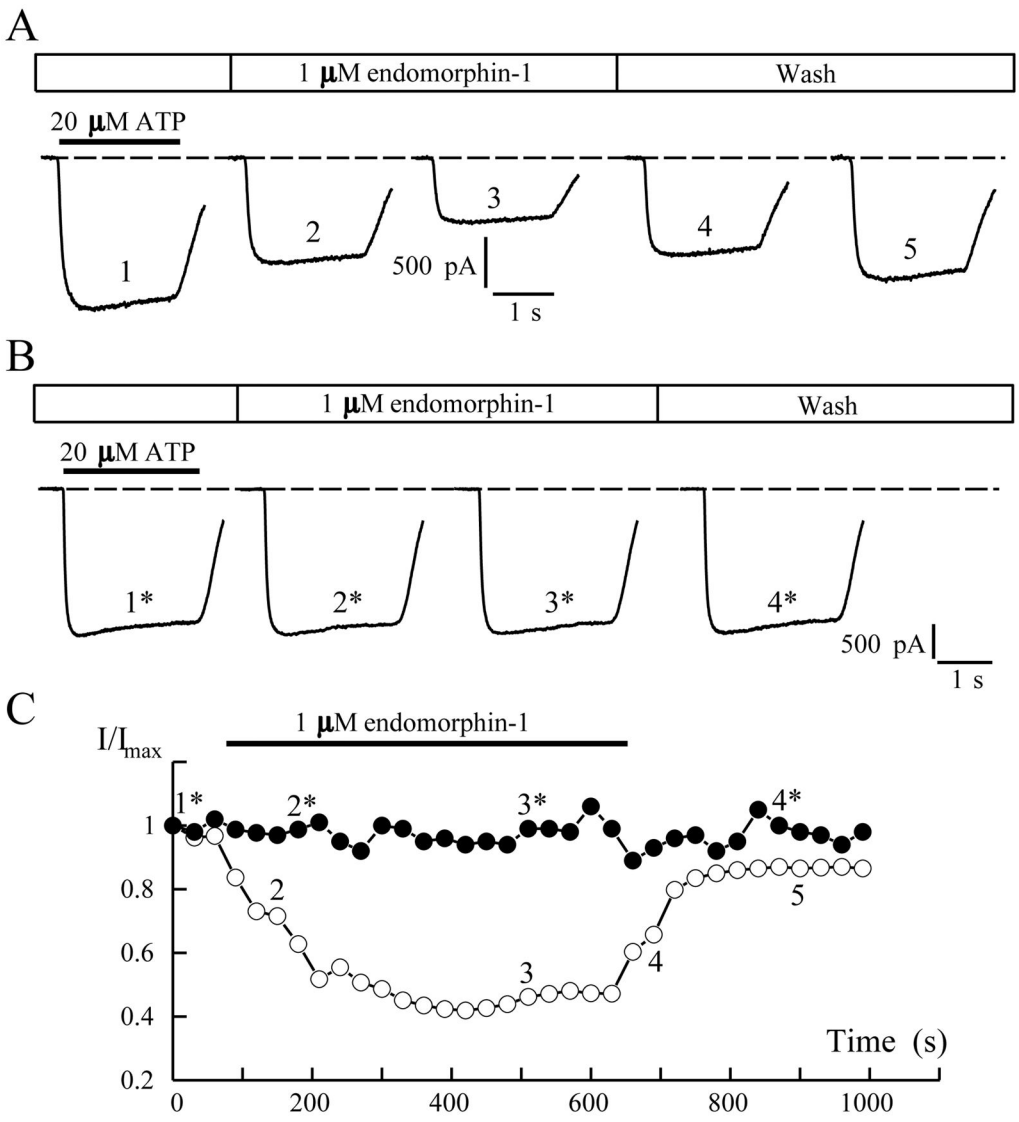

Fig. 3.

The effects of endomorphin-1 on ATP-activated currents with “ultra-slow” desensitization kinetics in control and co-cultured neurons. (A) – Endomorphin-1 inhibits the ATP-activated current in a control neuron with slow desensitization kinetics ($I_{2.5}/I_{max}$ close to 1); (B) – endomorphin-1 does not affect ATP-activated current in co-cultured nodose neuron with similar kinetics; (C) – Timecourse of endomorphin-1 action on ATP currents in control (open circles) and co-cultured (closed circles) nodose neurons. The numbers above current traces in A and B correspond to the times indicated in C.

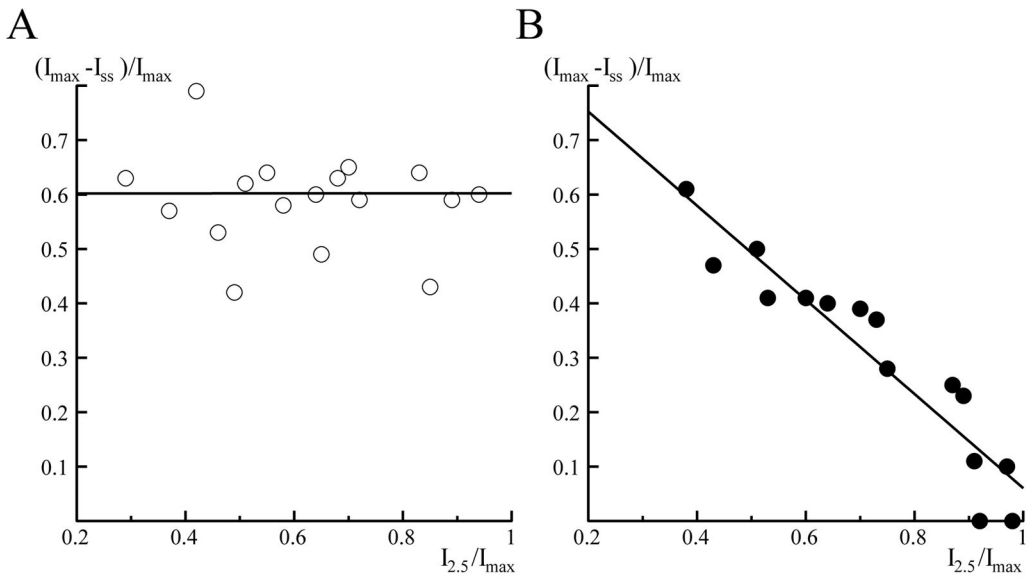

Fig. 4.

Inhibition of ATP-induced currents by endomorphin-1 depends on desensitization kinetics in co-cultured, but not in control nodose neurons. Degree of inhibition was described as $(I_{max}-I_{ss})/I_{max}$, where I_{max} is the peak current prior to drug application and I_{ss} is the current value at the steady-state level of opioid-induced inhibition. In control nodose neurons, endomorphin-1 attenuates ATP-activated currents irrespective of their desensitization kinetics (A, open circles). In the neurons co-cultured with fibrosarcoma cells ATP-activated currents with fast desensitization kinetics ($I_{2.5}/I_{max}<0.5$) maintain sensitivity to endomorphin-1, while currents with slow desensitization kinetics ($I_{2.5}/I_{max}$ close to 1) are resistant to the drug's inhibitory action (B, closed circles). Linear approximations of the datapoints are represented by the lines (see Methods).

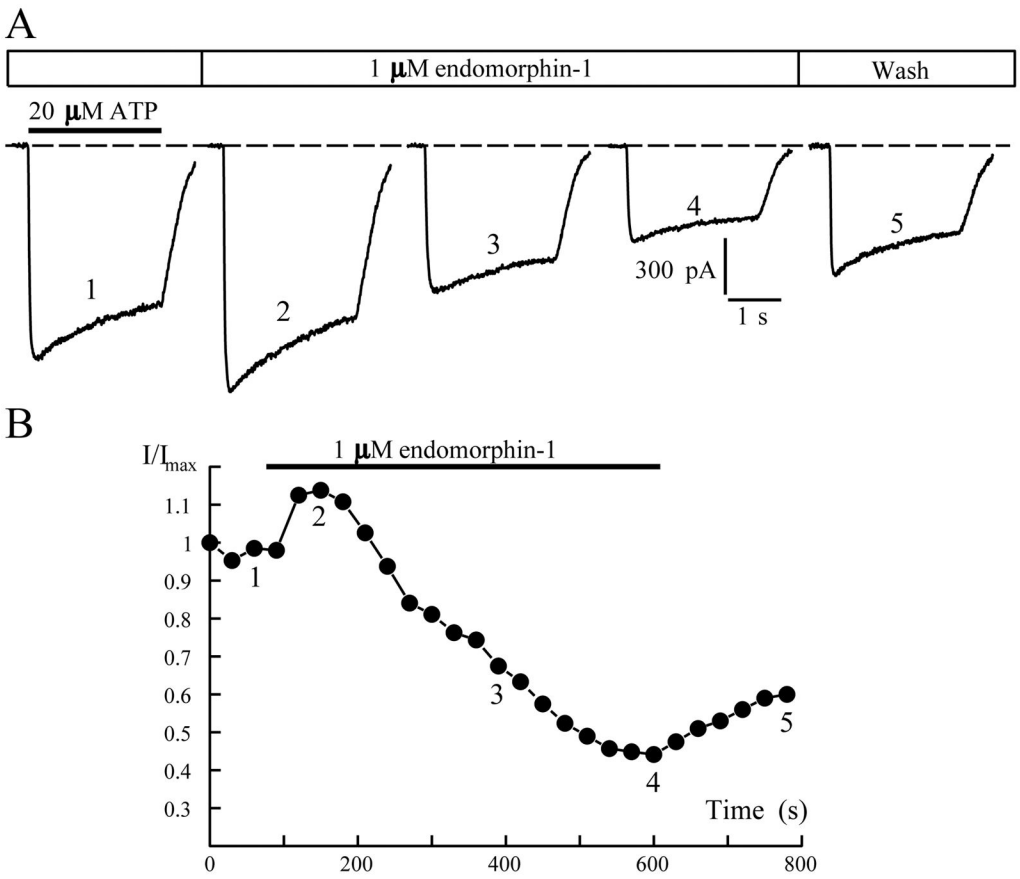

Fig. 5.

Effect of endomorphin-1 on ATP-activated currents in rat nodose neuron co-cultured with fibroblasts (4 days in co-culture). A: The neuron demonstrated biphasic reaction to endomorphin-1: inhibition of the current is preceded by its initial potentiation like in the majority of control neurons. B: Time course of endomorphin-1 action and washout. The numbers below the current traces in A correspond to the times indicated in B.

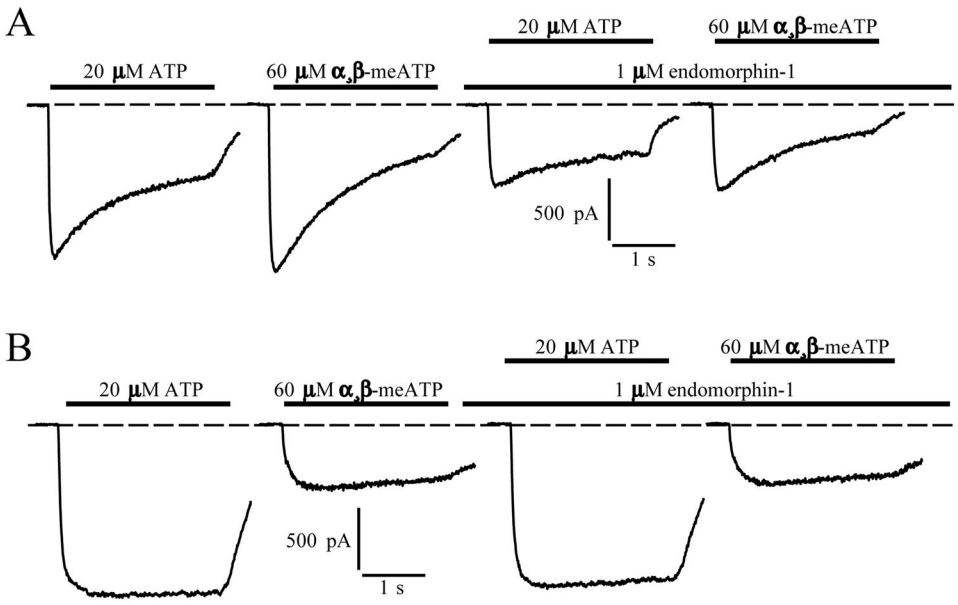

Fig. 6.

Effect of endomorphin-1 on P2X receptor-mediated currents with fast (A) and slow (B) desensitization kinetics, activated by ATP and α,β-methylene ATP (α,β-meATP) in co-cultured neurons. To observe the action of endomorphin-1 we used the protocol described above. Represented current traces were recorded before the application of endomorphin-1 and when opioid-induced inhibition reached its steady-state level. The currents partially recovered after washout of endomorphin-1 (not shown).

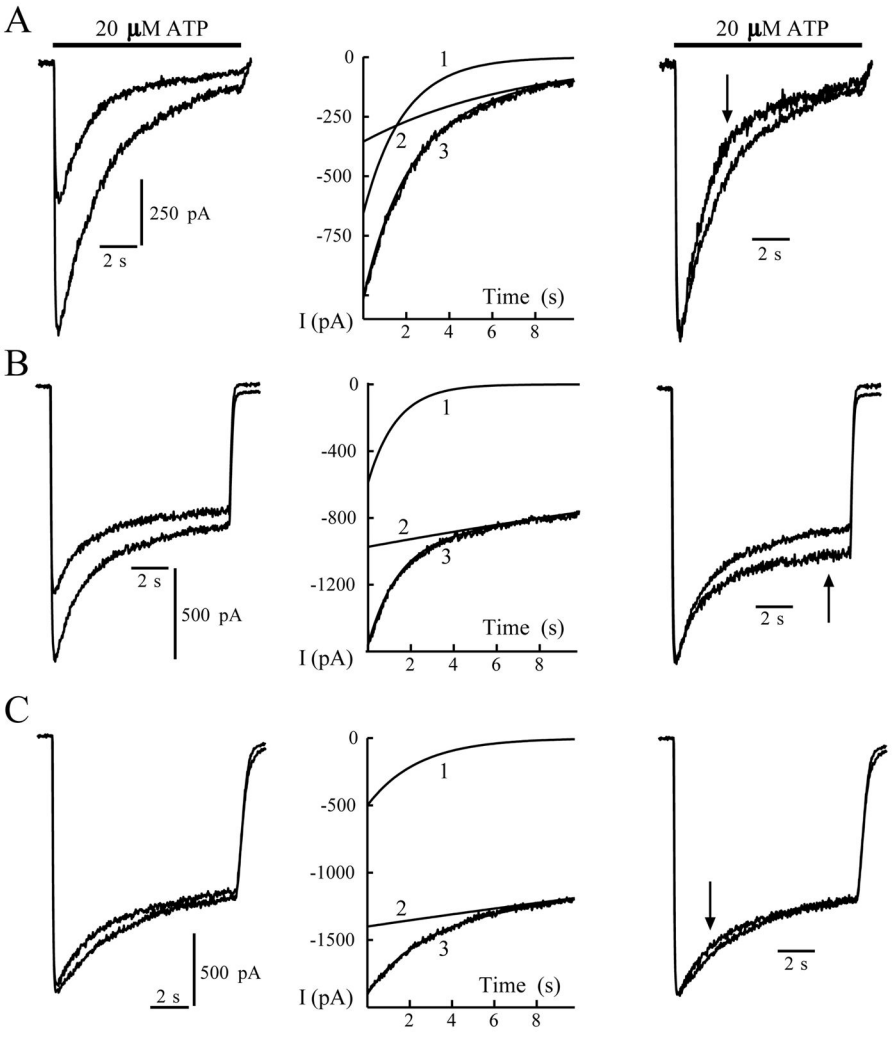

Fig. 7.

Representative examples of inhibitory effects of endomorphin-1 in nodose co-cultured neurons with different kinetics of desensitization of ATP current (A, B, C). Left panel: the current traces recorded before endomorphin-1 application (initial currents) and when the inhibitory effect of opioid reached its maximum (partially inhibited currents). (A) endomorphin-1 demonstrated maximum inhibitory effect in co-cultured neurons with fast kinetics of desensitization of ATP current. (B–C) the inhibition decreased with elevated contribution of the slow component. Middle panel: desensitization time course of the current, recorded before drug application. Current desensitization was described as a sum of two exponentials (fast component, $1 - I_f \cdot \exp(-t/\tau_f)$ and slow component, $2 - I_s \cdot \exp(-t/\tau_s)$). 3 – Bi-exponential fit matching current trace. Right panel: comparison of desensitization kinetics of initial and partially inhibited ATP-activated currents. Partially inhibited currents were normalized to the amplitude of the initial current and marked by arrows.

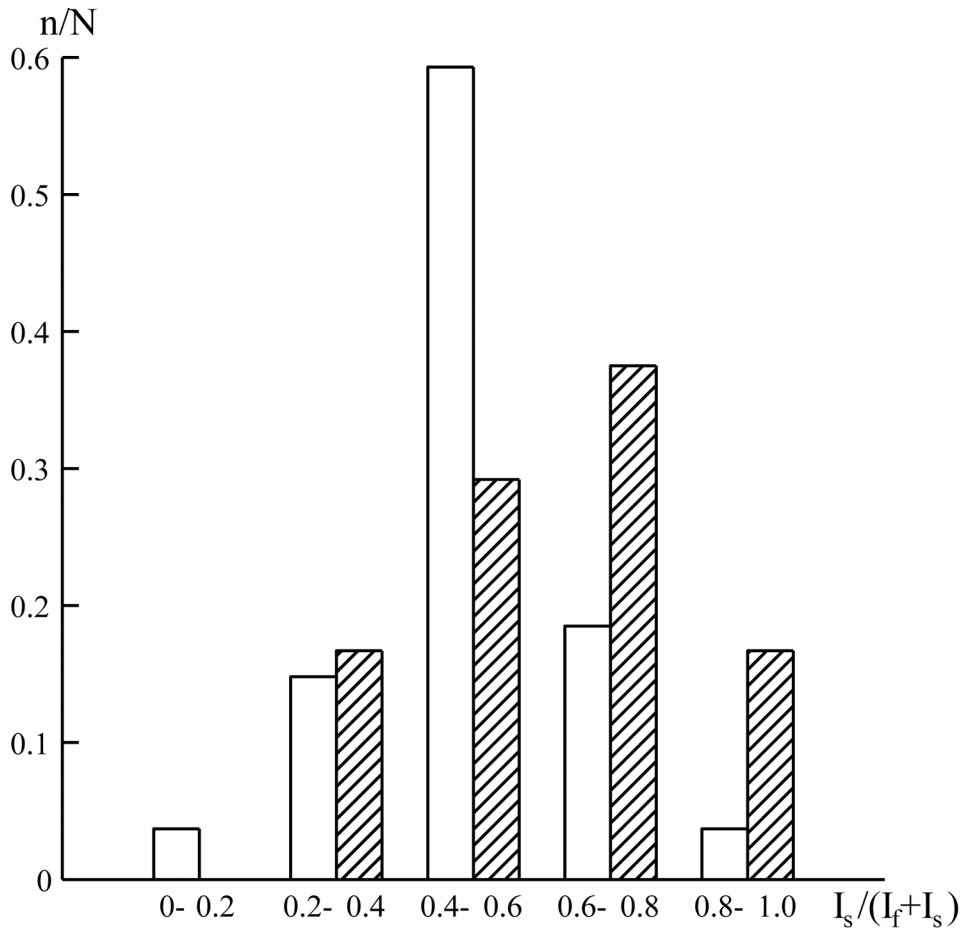

Fig. 8.

Distribution of control and co-cultured neurons according to the contribution of the slow desensitization component in total current decay. Control cells are represented by open bars, co-cultured – by shaded bars.