Opioid peptides are the most effective drugs in controlling pain; their action is elicited by binding to specific membrane receptors. The gastrointestinal tract represents, after the nervous system, the site in which the opioid receptors are expressed at high levels. The opioid agonist morphine has a significant inhibitory effect on intestinal motility, this action is blocked by naloxone an opioid antagonist mainly active at mu and kappa receptors. In this study the presence of mu opioid receptor on rabbit jejunum was investigated by western blot. The effects of beta-endorphin, the endogenous opioid peptide with the highest affinity to the mu opioid receptor and those of naloxone on spontaneous rabbit jejunum contractions were evaluated. Beta-endorphin (10⁻⁶M) showed a relaxant effect on jejunum contractility while naloxone showed a dual effect inducing an increase of spontaneous contractility at low concentrations (10⁻⁶M, 10⁻⁷M, 10⁻⁸M) and a decrease when high concentrations (10⁻³M, 10⁻⁴M, 10⁻⁵M) were utilized. The obtained results demonstrate that mu opioid receptor is expressed in rabbit jejunum and suggest that this receptor may be involved in mediating the effects of both opioid agonist and antagonist on jejunum contractions.

**Key words:** rabbit jejunum, opioids, mu-opioid receptor, beta-endorphin, naloxone.

**INTRODUCTION**

Opioid peptides and some alkaloids are among the most effective pain-relieving drugs; they also affect a number of physiological functions including hormone secretion, neurotransmitter release, feeding, gastrointestinal motility and respiratory activity (1). Their effects are mediated via cell surface receptors
defined upon pharmacological studies into three classes: mu, delta, kappa (2). Because of the multiple functions exerted by opioid peptides, until today no sufficient information are available on the mechanism by which they modulate animal life. Opioid receptors, firstly detected in the central and peripheral nervous system of mammals (2), have now been found to be widely expressed in several peripheral tissues including the small and large intestine, adrenal, kidney, lung, spleen, testis, ovary, uterus (3), and gametes (4, 5).

The presence of opioid receptors on the gastrointestinal apparatus, has been evidenced in nerve and smooth muscle (6) and on stomach, small and large intestine (3).

The gastrointestinal tract represents, after the nervous system, the site in which the opioid receptors are expressed at high levels (6). Until today the activity of the three classes of opioid receptors, in rabbit jejunum, has been demonstrated by pharmacological studies (7, 8) so that the presence of opioid receptors has been only indirectly evidenced. Endogenous opioids participate in the regulation of nervous visceral afference and sensitivity as well as of several visceral motor function induced by the CNS and through the enteroenteric and the myoenteric reflexes. The final effect of opioids on gut physiology is obtained by the net and harmonically balanced binding to mu and kappa opioid receptors subtypes (9).

Moreover it has been demonstrated that the opioid agonist morphine has a significant inhibitory effect both on the motility and on the intestinal immune response capacity. The effect is mediated by opiate receptors while it is blocked by naloxone (Nx) (10), a non selective opioid antagonist mainly active on mu and kappa receptors (11).

In rats, morphine reduces villus height, mucosal weight and protein content in jejunum and all these effects are antagonized by Nx (12). Pol et al., 1994 (13) have observed that a sensitization of opioid receptors in peripheral and/or central terminals of myenteric and submucosal plexus neurons occurs during acute inflammation of the gut and that the effect is mediated by the same type of opioid receptors present in non inflamed tissue.

It has been found that an activation of mu-opioid receptors in the gastrointestinal tract is responsible for inhibition of gut motility (14). In particular, gut contractility is controlled by the internal concentration of calcium ions in the smooth muscle cells that line the gut, in connection with the neuronal circuits in the gut wall, and those of non-neuronal "pacemaker" cells (15). Natural and synthetic opioids acting to specific receptors inhibit voltage-dependent L type calcium channels activity (16). It has been previously demonstrated, that Nx may modulate calcium handling through an agonist action at voltage-dependent L type calcium channels (17). Moreover it has been demonstrated that Nx induces an increase of calcium levels by mobilization of intracellular stores via activation of phospholipase C (18). Naloxone has been shown to have opposite effects depending on concentration, suggesting that it may function as a partial agonist at high concentration or as antagonist at lower concentrations, reverting the opioid
agonist-dependent modulatory action of the mu-opioid receptor (MOR) on ion channels and second messenger effectors (4, 18).

The aim of this study was to investigate on the presence of MOR on rabbit jejunum. Moreover in an in vitro model, the effects of beta-endorphin (b-end), the endogenous opioid peptide with the highest affinity to MOR (19) and different concentrations of Nx on gut motility were evaluated.

MATERIALS AND METHODS

Preparation of rabbit isolated jejunum: The abdominal cavity of rabbits slaughtered at the local slaughterhouse was opened by midline incision and segments of jejunum 6 cm long were quickly excised (1-6 cm from the ligament of Treitz) and placed in ice cold modified Krebs' solution at pH 7.4, containing: NaCl 113mM, KCl 4.8mM, CaCl$_2$·H$_2$O 2.2mM, MgSO$_4$ 1.2mM, NaH$_2$PO$_4$ 1.2mM, NaHCO$_3$ 25mM, glucose 5.5mM, sodium-ascorbate 5.5mM. The jejunum was cut along the longitudinal axis, cleaned of all intestinal contents and divided into longitudinal strips 3 cm long immediately refrigerated at 4°C.

Western blot analysis: Crude plasma membranes from rabbit jejunum were prepared by a modification of the procedure reported by Albrizio and coworkers (5). Briefly, 10mg of rabbit jejunum, after the excision procedure described above, were frozen in liquid nitrogen and successively fine pulverized into a mortar and suspended in ice-cold homogenizing buffer (0.25M sucrose, 10mM Tris-HCl, pH 7.5) containing protease inhibitors (1mM PMSF, 1µg/ml leupeptin and 1µg/ml pepstatin). The suspension was homogenized in a motor-driven homogenizer and centrifuged at 10000xg for 30 min at 4°C. The resulting supernatant was recovered and protein concentration was spectrophotometrically assessed by the BCA assay (Pierce, Rockford, IL, USA).

Western blot analysis was performed using a polyclonal anti-MOR antibody against the third extracellular loop of the receptor that selectively binds mu-agonists (20). Before loading, 30µg isolated protein were denatured for 4 min at 90°C and run on a 12% (w/v) precasted polyacrylamide gel (BioRad, Milano, Italy). Separated proteins were transferred by means of a Trans-Blot semidry apparatus (BioRad, Milano, Italy) onto an Immobilon-P membrane (Millipore, Bedford, MA, USA). After transfer, the membrane was blocked with Blotto (20mM Tris-HCl pH 7.5, 0.15M NaCl, 1% (v/v) Triton X-100) containing 5% (w/v) non fat dry milk (blocking buffer) for 1h and then incubated with the rabbit polyclonal anti-MOR antibody (Chemicon Int. Temecula, CA, USA) diluted 1:7500 in blocking buffer. After washing, the membrane was incubated for 1 h with peroxidase-conjugated goat anti-rabbit IgG antibody (Sigma, Milano, Italy) diluted 1:10000 and then revealed for horseradish peroxidase activity by SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

Pharmacological test: Three experiments were performed each repeated three times on jejunum strips of two different subjects. A 3 cm long segment of jejunum was placed in an isolated organ bath (mod. 4050 Ugo Basile, Milano, Italy) containing 30 ml of a modified Krebs'solution. The tissue was suspended between parallel hooks and connected to an isometric force transducer (mod. 7003, Ugo Basile, Milano, Italy) balanced by 1g loading and allowed to equilibrate for 105 minutes. The bath was maintained at 37°C and continuously bubbled with a mixture of 95% O$_2$ and 5% CO$_2$. During the equilibration period, regular spontaneous activity was recorded with an unirecord polygraph (mod. 7050, Ugo Basile, Milano, Italy).

In the first set of experiments, after the equilibration period, spontaneous contractions were recorded for 10 min. To determine the effects of the MOR agonist, single strips were exposed to 10$^{-6}$, 10$^{-7}$ and 10$^{-8}$M b-end (beta-endorphin, Sigma, Milano, Italy) for 5-10 min.
In the second set of experiments, the effects of an opioid antagonist were determined exposing strips to different concentrations, ranging from $10^{-3}$ to $10^{-8}$M, of Nx hydrochloride (Sigma, Milano, Italy). Each administration of b-end or Nx was followed by a washout with 30 ml of Krebs'solution.

In the third set of experiments the ability of Nx in reverting the effects of b-end was tested, so that strips were exposed for 10 min to $10^{-6}$M b-end followed by an incubation of 10 min with $10^{-6}$M Nx.

**Analysis of data:** Most segments showed spontaneous contractions. Those that did not exhibit contractions were discarded. Contractile responses were expressed as percentage of the observed average differences in tension-increase or tension-decrease in respect to the spontaneous contractile activity moreover changes of basal tone were also reported in grams of tension. Values were expressed as mean ± SEM. The results were evaluated for statistical significance by ANOVA and t-Student tests for paired values and were considered statistically significant for $p<0.05$.

**RESULTS**

**Immunoblotting:** A rabbit anti-MOR polyclonal antibody was employed in the immunoblotting analysis. A clear band corresponding to MOR protein with a molecular weight around 65 kDa was observed in the crude plasma membrane fraction obtained from rabbit jejunum (Fig. 1). A faint band of around 45 kDa was also visible. No immunoreactivity was noted in control blots incubated with the antiserum depleted of anti-MOR antibodies by preadsorption with a molar excess of the immunizing peptide and in blots incubated with rabbit preimmune serum (Fig. 1b, c).

**Concentration-related effects of β-endorphin on contraction:** We tested b-end, at concentration of $10^{-8}$M, $10^{-7}$M and $10^{-6}$M, on spontaneous contractile activity of rabbit jejunum. In a previous paper Dell'Aquila et al. 2002 (4), showed that β-end

![Fig. 1. Immunoblotting analysis of MOR in plasma membranes from rabbit jejunum.](image)
at $10^{-8}$M has effects on bovine oocytes in vitro maturation so that we firstly tested the $10^{-8}$M concentration. Because this concentration didn't give any statistically significant effect, we tested the concentration of $10^{-7}$M and $10^{-6}$M.

The first statistically significant effect, on spontaneous contractility, was observed at $10^{-6}$M, therefore we focused on this value that was required to produce a response (Fig. 2). Beta-endorphin at $10^{-6}$M had relaxant effect on rabbit jejenum contractility and decreased the tension of basal tone in a highly statistically significant manner (p<0.001).

**Concentration-related effect of Nx on jejenum contractility:** We tested the effects of different concentrations (from $10^{-8}$M to $10^{-3}$M) of the mu-opioid receptor antagonist Nx on rabbit spontaneous jejenum contractility (Fig. 3). As shown in Fig 3a, Nx at low concentrations increases immediately the spontaneous

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**Fig. 2. Tracing of rabbit jejenum contraction** Typical tracing of a rabbit jejenum preparation showing the effects induced by b-end ($10^{-8}$, $10^{-7}$, $10^{-6}$M) on spontaneous contractile activity in respect to the basal tone. The decrease of the amplitude is obtained with $10^{-6}$M b-end.
contractile activity in respect to the basal tone; at all the concentrations tested Nx showed statistically significant effects: p<0.001 (Fig. 4a).

On the contrary Nx at highest concentrations (10^{-5}, 10^{-4}, 10^{-3}M) gradually decreases the spontaneous contractile activity (Fig. 3b) in a 5 min period. Each concentration induced a significant inhibition of the contractile response in respect to the basal tone (at 10^{-4}M, p<0.05; at 10^{-5} and 10^{-3}M p<0.001) (Fig. 4b).

Combined effects of beta-endorphin and Nx: In order to evaluate a possible reverting effect of Nx on relaxation induced by 10^{-6}M b-end, we tested the mu opioid receptor antagonist at the same concentration. As shown in Fig. 5a, b-end after 5-10 minutes determined a decrease in the amplitude of spontaneous contractile activity of rabbit jejunum and the subsequent administration of Nx
completely reversed in a statistically significant manner (p<0.001) this effect determining an increase of the contractility that overcame the baseline value.

**DISCUSSION**

In this study, we show, for the first time, that rabbit jejunum expresses MOR. Western blot analysis demonstrated the presence of a clear band of approximately 65 kDa, corresponding to the MOR protein expressed in nervous system of mammals (21). Moreover an additional faint band of approximately 45 kDa was also present likely corresponding to the non post-translational modified form of the protein, in accordance with the finding in rat immune cells (22).

We also show that b-end added at a concentration of $10^{-6}$M reduced the amplitude and tone of the rhythmic spontaneous contractions of rabbit jejunum segments as well as $10^{-5}$, $10^{-4}$, $10^{-3}$ M Nx did. While, Nx added at the
concentrations of $10^{-8}$, $10^{-7}$ and $10^{-6}$M affected jejunum spontaneous contractions, increasing significantly the amplitude and the tone.

An analogue result was obtained by Liu and coworkers in 2004 (23), they observed that exogenously added opioid agonists at concentration from 0.05 to 1 µM inhibited the contractility of rat cathartic colon strips and that the inhibitory effects were negatively correlated with concentrations. In contrast, mu receptor antagonists elevated contractions of cathartic colon in rats.

Moreover our results show that the pharmacological effects of Nx are strictly related to the concentration used showing two different and opposite dose-response curves. When used at high concentrations Nx behaved as an opioid agonist inducing a relaxing effect on the smooth muscle cells, while at lowest concentrations showed its classic antagonistic effect increasing amplitude and tone of the contractions. This opposite dose-dependent effect, exerted by Nx on MOR, has been previously observed on in vitro maturation of bovine oocytes (4).
In that work Nx at $10^{-8}$M increased the percentage of bovine oocytes that reached meiotic competence while at $10^{-3}$M Nx inhibited oocytes maturation mimicking, this latter, the inhibiting effect on maturation exerted by opioids.

Our results suggest that Nx at $10^{-8}$M could determine a complete functional activity of the opioid receptors inducing a maximal contractile response (Fig. 6); while at $10^{-7}$ and $10^{-6}$M, reduces its pharmacological effect (tension increase of basal tone) in a gradual manner, probably by a slight desensitization and down regulation of the opioid receptors.

Previous studies indicated that opioids and opioid receptor agonists could inhibit contractility of the gastrointestinal tract by suppression of excitatory neurotransmitter release (24, 25).

In our study, b-end showed a significantly inhibitory effect at the concentration of $10^{-6}$M, so that to evaluate if the effect exerted by Nx is due to an action that directly involves the receptor, we tested the antagonistic effect of Nx in reverting the contraction inhibition induced by b-end.

Therefore on the same strip, we tested $10^{-6}$M b-end for 10 min and successively $10^{-6}$M Nx. The results obtained demonstrated that Nx is able to completely revert the relaxation induced by b-end acting on the same receptor (Fig. 5b). Moreover the amplitude of the contractions after Nx administration was
higher than that of the basal tone suggesting that strips probably already contain a quote of linked b-end. These results let us to exclude a tolerance development of rabbit jejunum to the b-end used at 10^{-6}M, infact Nx reverts the agonist effect of b-end instead of precipitating contracture as previously observed in experiment on rabbit jejunum with the use of 1.3 \times 10^{-7} \text{ M} morphine instead of b-end (7).

The opioidergic system seems to be involved in modulating rabbit gut motility through the interactions with mu-type opioid receptor that regulates cytosolic Ca^{2+} by L-type calcium channels. It is well known that smooth muscle contractions are regulated by cytosolic Ca^{2+} concentrations and by sensitivity to Ca^{2+} of the contractile elements in response to its changes in the cell. Grasa et al., 2004 (26) showed that Ca^{2+} enters cytosol by L-type voltage-dependent calcium channels and that antagonist to those channels decreased rabbit intestinal motility.

The observed dual, agonist/antagonist effect showed by the utilized Nx concentrations, provides further support to the pharmacodynamic concept of relative antagonist and agonist action of Nx in relation to the dose, this well correlates with the clinical evidences of beneficial effects of low doses Nx (27). On the whole all the results obtained on rabbit jejunum could offer new therapeutic opportunities to treat anomalies in the peristaltic kinetic such as conditions of static ileum or spastic contractions characteristic of the "colic syndrome".

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