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Actions of tilidine and nortilidine on cloned opioid receptors

Christophe Thierry[a], Jean-Marie Boeynaems[b,c,*], Meoni Paolo[a]

[a]*Euroscreen Inc, 6041 Gosselies, Belgium*
[b]*Institute of Interdisciplinary Research, School of Medicine*
[c]*Department of Clinical Chemistry, Erasme Hospital, Free University of Brussels, 1070 Brussels, Belgium*

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Abstract

Tilidine, alone or combined with naloxone to prevent drug abuse, is used as an oral opioid analgesic. Although the analgesic action of tilidine and its active metabolite nortilidine is reversed by naloxone and therefore believed to involve the activation of the Mu opioid (MOP, OP_3, μ) receptor, this has never been studied in recombinant systems. We have measured the selectivity of tilidine and nortilidine for human opioid and opioid-like receptors stably expressed in CHO-K1 cells, using the inhibition of the forskolin (FK)-induced accumulation of cAMP as endpoint. In cells expressing the MOP receptor, tilidine and nortilidine inhibited cAMP accumulation with IC_{50} of 11 μM and 110 nM, respectively. The agonist effects of nortilidine and [D-Ala^2-MePhe^4-Gly^5-ol]enkephalin (DAMGO) on the MOP receptor were reversed by naloxone with very similar IC_{50} (1.2 versus 1.8 nM). At concentrations up to 100 μM, tilidine and nortilidine had no agonist effect on the DOP, KOP and NOP receptors. In conclusion, this study on cloned human receptors demonstrates that nortilidine is a selective agonist of the MOP receptor.

Keywords: Tilidine; Nortilidine; Opioid; Receptor; Nociceptin

1. Introduction

Tilidine is a cyclohexane derivative developed in the 70s as an opioid analgesic (Mauro and Shapiro, 1974; Bromm and Seide, 1982) and is marketed in some European countries either alone (Valoron) or in combination with naloxone (Valtran). Tilidine is considered to be a prodrug, from which the active metabolite nortilidine is formed by demethylation in the liver (Dubinsky et al., 1975; Schulz et al., 1978). According to recent studies, approximately two-thirds of the dose of tilidine is metabolized to nortilidine, although only one-third of the dose is available systemically as nortilidine for interaction with the opiate receptors after both intravenous and oral dosing of tilidine (Hajda et al., 2002). The rationale of the association between tilidine and naloxone in the clinic is to prevent the abuse of the analgesic by opiate addicts, and is based on the strong hepatic first-pass effect and non-linear kinetics of naloxone, so that inhibition of tilidine action will only be observed at supratherapeutic doses (Vollmer, 1988).

Although the molecular pharmacology of tilidine has been little studied, it was believed to act through the Mu opioid (MOP, OP3, μ) receptor (Herrmann, 1985), and its analgesic action was indeed shown to be inhibited by naloxone (Bromm et al., 1983). In order to better characterize the pharmacology of tilidine and its main metabolite nortilidine (Vollmer et al., 1989) on the different opioid and opioid-like receptors (Alexander et al., 2004), we have investigated the action of these compounds on cloned human Delta opioid (DOP, OP1, δ), Kappa opioid (KOP, OP2, κ), Mu opioid (MOP, OP3, μ), and nociceptin/orphanin (NOP, ORL1) receptors stably expressed in mammalian cells.

* Corresponding author. Chimie Médicale Hôpitale Erasme 808, Route de Lennik 1070 Brussels, Belgium. Tel.: +32 2 5553922; fax: 32 2 5556655.
E-mail address: jmboeyna@ulb.ac.be (J.-M. Boeynaems).

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2. Materials and methods

2.1. Materials

Tilidine and nortilidine were a gift from Pfizer, while morphine hydrochloride was obtained from the pharmacy of Erasme Hospital. SNC80 ((+)-4-[(alpha*R*)-alpha-((2*S*,5*R*)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide), U50488 (3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide), DAMGO ([D-Ala^2-MePhe^4-Gly^5-ol]enkephalin), nociceptin and naloxone were purchased from Tocris Cookson, UK.

Experiments were performed with recombinant CHO-K1 cell lines stably expressing the DOP (GenBank U07882), KOP (GenBank U17298), MOP (GenBank L29301), or NOP (GenBank X77130) receptor. All the cell lines used in this study were developed by Euroscreen.

2.2. Measurement of cAMP accumulation

The activation of the opioid receptors was evaluated by the inhibition of cAMP accumulation induced by forskolin (FK: 10 μM). cAMP was measured by Homogeneous Time-Resolved Fluorescence (HTRF) using a kit from CIS Bio International (Marcoule, France, Cat#62AM2PEB). The readout of the test is represented by the 665/620 intensity ratio, which is inversely proportional to the amount of cAMP produced by the cells. Stable, recombinant CHO-K1 cell lines expressing the receptors of interest were cultured in HamF12 with 10% fetal bovine serum, harvested with phosphate-buffered saline–EDTA (PBS-EDTA), washed, and resuspended in Krebs–Ringer HEPES–IBMX (KRH-IBMX) at a density of 5000 cells/12 μl. For agonist testing, 12 μl of cell suspension were mixed in each well of a Costar 96-well plate with 12 μl of KRH-IBMX buffer containing forskolin 20 μM with or without the test or reference agonist at the desired concentration. Following 30 min of incubation at room temperature, 12 μl each of reagent A and reagent B of the HTRF kit were added to the plates, incubated for a further 60 min at room temperature and measured on a RUBYstar+ (BMG Labtechnologies) time-resolved fluorescence reader. For antagonist testing, 12 μl of cell suspension were mixed in each well of a Costar 96-well plate with 6 μl of KRH-IBMX buffer containing the requested concentration of the test or reference antagonist compound and incubated for 10 min at room temperature. Following this incubation, 6 μl of KRH-IBMX buffer containing the requested forskolin concentration and an EC80 concentration of the reference agonist for the receptor were added to cells. Following 30 min of incubation at room temperature, 12 μl each of reagent A and reagent B of the HTRF kit were added to the plates, incubated for a further 60 min at room temperature and measured on a RUBYstar+ (BMG Labtechnologies) time-resolved fluorescence reader. The results are expressed as cAMP concentrations (nM) for 5000 cells, based on a standard curve carried out for each experiment.

3. Results

3.1. Activity of test compounds on parental cell lines

The compounds tested (DAMGO (10 μM), SNC80 (100 μM), U50488 (10 μM), nociceptin (10 μM), morphine (100 μM), tilidine (1 mM), nortilidine (1 mM), and naloxone (1 mM)) failed to significantly reduce cAMP levels stimulated by forskolin (10 μM) in the parental CHO-K1 cell line used to develop the recombinant cell

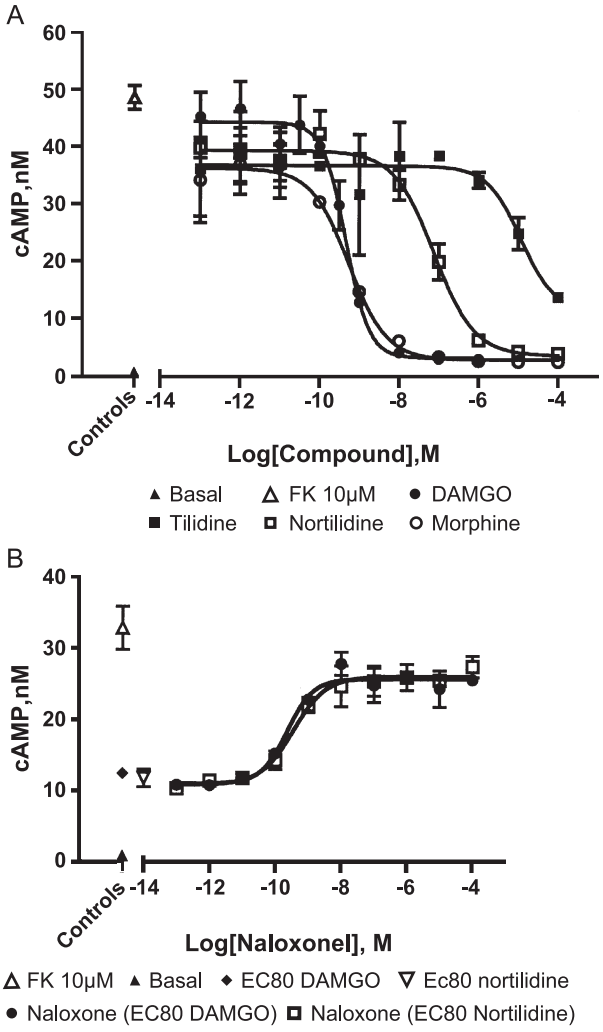

Fig. 1. (A) Inhibitory effect of reference agonists, nortilidine and tilidine, on the forskolin-induced accumulation of cAMP in CHO-K1 cells stably expressing the MOP receptor. Each point is the mean±S.D. of triplicate determinations in one representative experiment out of two. (B) Reversal by naloxone of the cAMP inhibitory effect of DAMGO and nortilidine in CHO-K1 cells stably expressing the MOP receptor. The cells were exposed to a DAMGO or nortilidine concentration producing 80% inhibition of cAMP accumulation in response to forskolin. Each point is the mean±S.D. of triplicate determinations in one representative experiment out of two.

Table 1
Inhibition of forskolin-induced cAMP accumulation by reference agonists, tilidine and nortilidine, in CHO-K1 cells expressing opioid and opioid-like receptors

Receptor	Reference agonist	Tilidine	Nortilidine
DOP	SNC80: 0.3±0.2 nM	no effect	no effect
KOP	U50488: 1.6±0.3 nM	no effect	no effect
MOP	DAMGO: 0.7±0.2 nM Morphine: 1.6±1.5 nM	10.5±1.1 μM	110±49 nM
NOP	Nociceptin: 0.08±0.04 nM	no effect	no effect

Results are expressed by the IC_{50}. Each value is the mean±S.D. of at least three independent experiments.

lines expressing opioid and nociceptin receptors used in this study (data not shown).

3.2. Effect of tilidine and nortilidine on MOP, DOP, and KOP opioid receptors

Nortilidine, and with a much lower potency tilidine, inhibited the forskolin-induced accumulation of cAMP in CHO-K1 cells expressing the MOP receptor (Fig. 1A). The maximal inhibitory effect of nortilidine was comparable to that of the reference agonists, DAMGO and morphine, while the IC_{50} for nortilidine is 2 log higher than the IC_{50} obtained for the two reference agonists (Table 1). Tilidine only showed approximately 67% of the maximum response elicited by DAMGO at the highest concentration of 0.1 mM. Interestingly, at the concentration of 0.01 and 0.1 mM, tilidine also showed a small antagonist effect on DAMGO (approximately 10% reduction of DAMGO EC_{80}, data not shown), suggesting that it might act as a partial agonist at this receptor. The agonistic effects of nortilidine and DAMGO at the MOP receptor were reversed with a similar potency by the reference antagonist naloxone (Fig. 1B).

At concentrations up to 0.1 mM, neither nortilidine nor tilidine had any agonist effect on forskolin-induced cAMP accumulation in the DOP- or KOP-expressing CHO cell lines (Table 1). At 0.1 mM, but not lower concentrations, nortilidine partially antagonized the effects of SNC80 on the DOP receptor (−49%) and U50488 on the KOP receptor (−45%). Tilidine only showed a partial inhibition of the agonist effect of SNC80 on the DOP at the highest concentration of 0.1 mM (−25%; Table 2).

3.3. Effect of Tilidine and Nortilidine on NOP nociceptin receptor

Neither tilidine nor nortilidine showed any agonistic effect on the NOP receptor at concentrations up to 0.1 mM (Table 1). Nortilidine showed a slight antagonistic effect (−14%) against nociceptin at the highest concentration tested (0.1 mM; Table 2).

4. Discussion

The activity of many clinically used opioid analgesics has been tested on recombinant human opioid and opioid-like receptors expressed in CHO or HEK cells, using either radioligand binding or cAMP inhibition assays (Gharagozlou et al., 2003a,b; Gillen et al., 2000; Lai et al., 1996; Seki et al., 1999; Zaveri et al., 2001). Tilidine and its metabolite nortilidine were conspicuously absent from the list of tested compounds, and their pharmacology on the different opioid receptors remains largely unelucidated. Although the analgesic action of tilidine in humans is inhibited by naloxone (Bromm et al., 1983), the activity of this compound on the MOP receptor and the relative activities of tilidine and nortilidine at a molecular level remained unknown, so that the possibility of an action on other opioid and opioid-like receptors could not be excluded. Therefore, we made a systematic study of the agonist and antagonist action of tilidine and nortilidine on recombinant human DOP, KOP, MOP, and NOP receptors stably expressed in CHO cells. Nortilidine proved to be a selective MOP agonist displaying a potency about 100-fold higher than that of the parent molecule tilidine. Its maximal effect was comparable to those of DAMGO and morphine and consistent with full agonist property. In comparison, the action of tilidine on this receptor was more in line with that of a partial agonist at the MOP receptor and agrees with previous suggestions of an agonist–antagonist activity of this compound on opioid receptors (Herrmann et al., 1984). The agonist action of tilidine and nortilidine proved to be selective for the MOP receptor, as at concentrations below 0.1 mM, no agonist effect was observed on any of the other opioid or nociceptin receptors tested. It is therefore likely that all the analgesic and other activities of tilidine in patients reported in the literature are mediated through this

Table 2
Reversal of the effect of agonists on the respective opioid and opioid-like receptors expressed in CHO-K1 cells by naloxone, tilidine, and nortilidine

Receptor	Agonist	Naloxone	Tilidine	Nortilidine
DOP	SNC80	272±83 nM	25±5% at 0.1 mM	49±6% at 0.1 mM
KOP	U50488	125±2 nM	<5% at 0.1 mM	45±2% at 0.1 mM
MOP	DAMGO	1.2±0.9 nM	7±14% at 0.1 mM	–
MOP	Nortilidine	1.8±1.9 nM	–	–
NOP	Nociceptin	<5% at 0.1 mM	<5% at 0.1mM	14±2% at 0.1 mM

The agonists were added at a concentration producing a 80% inhibition of cAMP accumulation. Results are expressed by the IC_{50} or by the percent (%) reversal at the maximal concentration tested. Each value is the mean±S.D. of at least three independent experiments.

receptor. The small antagonist effect by tilidine or nortilidine that could be observed on some of the tested receptors at the concentration of 0.1 mM is irrelevant at the doses recommended for analgesia. Indeed, pharmacokinetic studies indicate that the maximal concentration of nortilidine in plasma (C_{max}) following the maximal therapeutic dose of tilidine (300 mg) is about 2.5 μM (Martin et al., 1999; Brennscheidt et al., 2000). In conclusion, the present study has shown that tilidine and nortilidine are specific agonists of the MOP receptor and that the hepatic metabolism of tilidine into nortilidine increases the potency at this receptor by a 2-log factor.

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