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In vivo rat brain opioid receptor binding of LY255582 assessed with a novel method using LC/MS/MS and the administration of three tracers simultaneously

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Abstract

LY255582 is a pan opioid selective receptor antagonist that has been shown to have high affinity for mu, delta, and kappa receptors *in vitro*. In order to better understand the *in vivo* opioid receptor selectivity of LY255582, we developed *in vivo* receptor occupancy assays in the rat for the opioid mu, kappa and delta receptors using the occupancy tracers naltrexone, GR103545 and naltriben respectively. Individual assays for each target were established and then a "triple tracer" assay was created where all three tracers were injected simultaneously, taking advantage of LC/MS/MS technology to selectively monitor brain tracer levels. This is the first report of a technique to concurrently measure receptor specific occupancy at three opioid receptors in the same animal. The opioid subtype selective antagonists cyprodime, JDTic and naltrindole were used to validate selectivity of the assay. Examination of LY255582 in dose-occupancy experiments demonstrated a relative order of potency of mu>kappa>delta, reproducing the previously reported order determined with *in vitro* binding.

Keywords: Opioid; Receptor occupancy; LY255582; *In vivo* binding; Rat

Introduction

Obesity is increasingly recognized as a serious issue in modern western societies. Poor diet and inadequate physical activity was the second highest cause of death in the United States, accounting for 14 and 16.6% of all deaths in the years 1990 and 2000 respectively (Mokdad et al., 2004, 2005) A number of pharmacological approaches to treating obesity have been proposed, some of which are now marketed in the United States (sibutramine and orlistat). While it is difficult to predict the impact of drugs not yet on the market, those currently available have not had a major effect on the prevalence of obesity. An additional pharmacological approach to treating obesity, currently under investigation, is the use of opioid antagonists that have been designed for selective activities at opioid receptor subtypes, as nonselective opioid antagonists such as naloxone are known to decrease food intake (Holtzman, 1974). LY255582 is an opioid receptor antagonist that exhibits high *in vitro* affinity for mu, kappa, and delta receptors (Mitch et al., 1993). Interestingly, LY255582 has been shown to be effective in multiple animal models of obesity following parenteral (s.c.), icv, or oral administration (Shaw et al., 1991; Levine et al., 1991; Shaw, 1993; Statnick et al., 2003). The *in vivo* effects in feeding models seen with LY255582 administration differ from those produced by naloxone, an observation that would not be predicted by their *in vitro* opioid affinity profiles (Levine et al., 1991). In order to better understand its effects on food consumption and body weight, we examined the *in vivo* binding selectivity of LY255582. To accomplish this, we established individual *in vivo* receptor occupancy (RO) assays for the mu, kappa and delta opioid receptors. In addition, because these assays employ liquid chromatography coupled to tandem mass spectral detection (LC/MS/MS) rather than scintillation spectroscopy to measure tracer distribution in the brain (Chernet et al., 2005), multiple tracers can be used simultaneously in the same animal. Therefore, we have also generated a "triple tracer" method for rat brain opioid RO and

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applied this assay to understanding the *in vivo* opioid receptor selectivity of LY255582.

Materials and methods

Test compounds

Naltrexone hydrochloride, naltrindole hydrochloride, naltriben methanesulfonate and cyprodime hydrobromide were purchased from Sigma-Aldrich (St Louis, MO, USA). Dosing solutions were prepared using salt weights, but for brevity, these compounds will subsequently be referred to without salts. The phenylpiperidines, LY255582 and JDTic, and the phenylacetylpiperazine GR103545 were synthesized at Eli Lilly and Company following published procedures (Werner et al., 1996; Thomas et al., 2003; Ravert et al., 2002). JDTic and LY255582 were dissolved in 1% lactic acid/saline. Naltrindole was prepared in 10% (2-hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich). Cyprodime, naltrexone, naltriben, and GR103545 were dissolved in saline. All drugs were administered intravenously in a volume of 0.5 ml/kg.

Animals

One study was conducted with mu receptor knockout mice (30–40 g; Taconic, Hudson, NY). These mice were prepared on a 129S6 background strain and backcrossed to ten generations. Knockout mice were housed individually in standard mouse shoebox cages, as some fighting was observed when they were group housed. Wild type 129S6 mice were housed ten per standard rat shoebox cage. Adult male Sprague–Dawley rats (240–300 g; HSD, Indianapolis, IN, USA) were used for all other experiments. They were housed 6 per wire bottom cage. All animals were housed in rooms using a 12-hour light/dark cycle (lights on at 6 AM) and had *ad libitum* access to normal rat chow and water. All studies were performed in accordance with the National Research Council Guide under protocols approved by the Animal Care and Use Committee of Eli Lilly and Company.

Determination of tracer dose and post-tracer survival interval

Doses of the occupancy tracers naltrexone (10 μg/kg), naltriben (10 μg/kg) or GR103545 (1.5 μg/kg) were selected to be low and still allow accurate measurement by LC/MS/MS 1 h after injection. In order to select the appropriate post-tracer survival interval, a time course of brain tracer levels was examined. Groups of 4 rats were injected with an individual tracer and sacrificed 5, 15, 30, 45 or 60 min later. Striatal, thalamic and cerebellar levels of tracer were measured by LC/MS/MS as described below. In this and all subsequent experiments, striatal GR103545 and naltriben levels represent total binding to the kappa and delta receptors respectively. For the mu receptor, thalamic naltrexone levels represent total binding. Cerebellar levels represent non-specific binding for all 3 targets, as this is a region with little or no expression of mu, kappa, or delta opioid receptors (Mansour et al., 1994). Specific binding is the difference between the tracer level measured in the total binding region, the striatum or thalamus, and the tracer level measured in the non-specific binding region, the cerebellum. A post-tracer survival interval was chosen at the earliest time point where specific binding was maximal or near maximal.

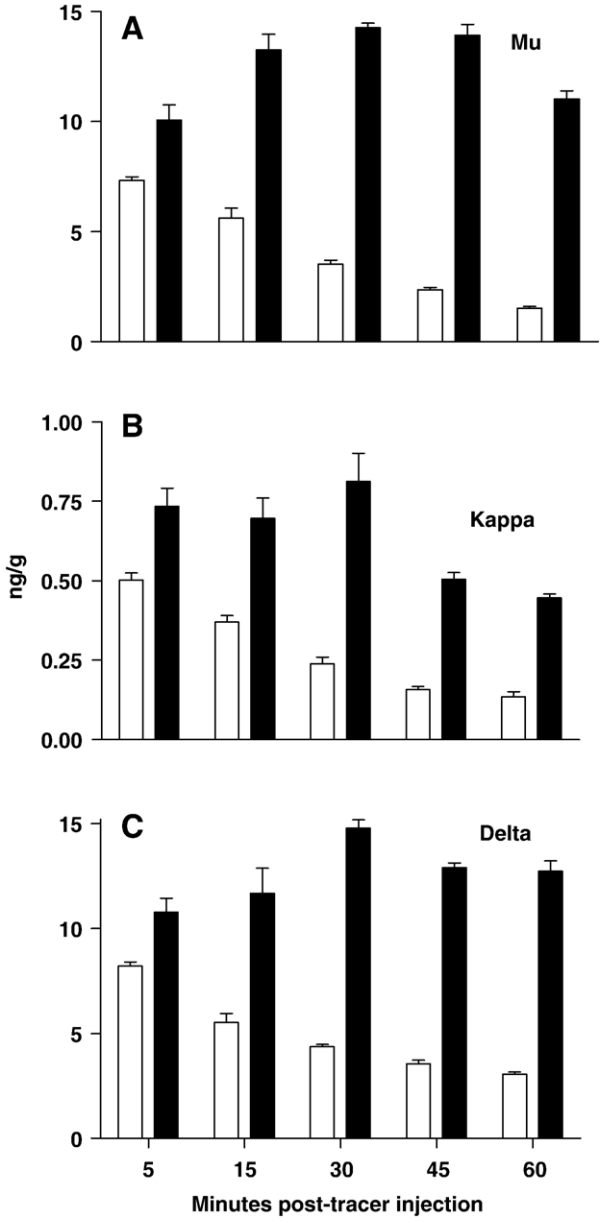

Fig. 1. Time course for tracer levels measured in total and non-specific brain binding areas following intravenous tracer administration, expressed as ng/g of brain tissue. Panel A shows the level of naltrexone (mu tracer) in the thalamus (dark bars) and cerebellum (light bars) after an intravenous dose of 10 μg/kg. Panel B represents the levels of GR103545 (kappa tracer) in the brain striatum (dark) and cerebellum (light) after 1.5 μg/kg, iv. Panel C contains levels of the delta tracer, naltriben, in the striatum (dark) and cerebellum (light) after a dose of 10 μg/kg. Bars represent means ± SEM, $N=3$.

Opioid receptor occupancy of LY255582

The *in vivo* mu, delta, and kappa opioid receptor occupancy produced by LY255582, 30 min after its intravenous administration, was determined using the occupancy tracers naltrexone