Determination of Mitragynine Bound Opioid Receptors

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ABSTRACT

Mitragyna Speciosa Korth from Rubiaceae family is a tropical plant indigenous to Southeast Asia particularly in Thailand, Peninsular of Malaysia and Indonesia. The leaves have been used by natives for their opium-like effect and cocaine-like stimulant ability to combat fatigue and enhance tolerance to hard work. This study is designed to examine the binding affinity and selectivity of mitragynine towards opioid receptors namely µ-, δ-, and κ-receptors and the density of those opioid receptors in brain tissues, spinal cord, duodenum, ileum and vas deferens. Our results suggest that mitragynine has a high affinity to the κ-opioid receptors and brain has the highest density of opioid receptors.

Key words: Mitragyna Speciosa, mitragynine, opioid, receptors, binding affinity.

Introduction

Opium has been used widely for medical purposes since the beginning of centuries. Opium is a crude extract of the seed capsules of the poppy, Papaver somniferum, which contains the opiate alkaloids morphine and codeine. Studies of the biological effects of opium and opioid have shown that the opioid possess the analgesia, myosis, bradycardia, sedation, hypothermia and lost of sensitivity. There are three major opioid receptors types, which is identified using molecular cloning techniques. These µ-, δ- and κ-opioid receptors are members of the G, (inhibitory) protein-coupled seven transmembrane receptors (GPCR) family [13,4,1,19]. Furthermore, the different receptor types of interact, such that pharmacological action at one opioid receptor type may modify the biochemical and /or functional properties of another [7,16,19].

The leaves of Mitragyna Speciosa (M.S) have been traditionally used as a substitute for opium. M.S was widely cultivate in Malaysia and known as “ketum” or “biak-biak”. Mature leaves of M.S are recognize as a rich source of alkaloids which is mitragynine, the major alkaloid that found in the leaves [21,23]. Jansen and Prast (1988) reported that the alkaloid from M.S had opiate-like effects such as analgesia, antitussive and also cause hypothermia in animals. However differences chemical structures exist between mitragynine and morphine, the opioid prototype. Therefore these preliminary studies were conducted to measure the density of opioid receptors in brain tissues, spinal cord, duodenum, ileum and vas deferens. In addition, we studied binding affinity and selectivity of mitragynine towards specific opioid receptors such as µ-, δ-, and κ-receptors.

Materials and Methods

Drugs:

Trizma hydrochloride, dimethyl sulfoxide, (D-Ala2, N-Me-Phe4, glisinol5)-encephalin or DAMGO and (5,7,8β)- N- methyl- N – [7-(1-pyrolidinyl)]l –
oxaspyro [4,5] dek- 8- il benzenacetamide or U-69,593 (Sigma St.Louis, USA), Naltrenib (NTB), 7-benzilidinmaltrexone or BNTX and nor-binaltorfimin dihydrochloride or BNI (Tocris Bristol, UK), Soluene-350 (Packard-Becker, USA), scintillation cocktail (J.T Baker, USA), [N-allil-2.3-H3]Naloxone (Amersham, UK), [5',7'-H3]Naltrindole and [15,16(a)-H3] Diphrenorphin (NEN, Product USA). Mitragynine was isolated from extract of leaves Mitragyna speciosa Korth. The cloned of μ-, δ-, and κ-opioid receptors from human opioid receptors was purchased from Perkin- Elmer ( Boston USA), and protein assay kit from BioRad-Enzyme (USA).

Isolation of Mitragynine:

Mature leaves of M. speciosa Korth were collected from natural sources in Malaysia. Authentication of plants was carried out at the Faculty of Forestry, University Putra Malaysia where the herbarium vouchers (ATS 001) have been deposited.

Plant Material:

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Isolation of Mitragynine:

Mature leaves were dried at 45-50°C, powdered and macerated with methanol for 2 days (repeated 3 times). The methanol filtrate were combined and evaporated in evaporator. The methanol extract was dissolved in 10% acetic acid solution, well shaken and left to stand overnight. The acidic filtrate was washed with petroleum ether, made alkali to pH 9 with 25% ammonia solution and extracted with chloroform. The combined chloroform extract was washed with distilled water, dried over anhydrous sodium sulphate and evaporated to yield dry crude alkaloid extract. The major alkaloid, isolated by silica gel CC eluting with diethyl ether was identified as mitragynine with NMR.

Animals:

Male Spraque Dawley rats (180-200 g) were obtained from Animal center Faculty of Medicine and Health Sciences (FMHS), University Putra Malaysia. Animals were housed in group of six in a temperature-controlled room. They are maintained under standard laboratory conditions with natural dark and light cycle (24h) and fed with standard commercial food pellets and water ad libitum. Animals were acclimatized for 1 week prior to the experimental use. Were approved and guided by the Animals Ethical Committee of the FMHS, UPM.

Single Saturation Assay:

For membrane preparation, the mouse brain (cortex, hypothalamus, hippocampus), spinal cord, duodenum, ileum and vas deferens tissue were quickly removed after decapitation and rapidly transferred to a tube filled with an ice-cold buffer. Membrane were homogenized (while still frozen), using a polytron in 10 ml/gm wet weigh of ice-cold mM potassium phosphate, pH 7.4. Following centrifugation at 1000g for 10 min (all centrifugations took place at 4°C) and the supernatant was recentrifuged. The pellet was resuspended at 4°C for 20 min at 48,000 g. The final pellet was resuspended and retained as membrane fraction. The receptor binding assays were performed in duplicate with mitragynine and naloxone 200 µl in a final volume of 1.0 ml that contained 50 mM tris-HCl buffer, pH 7.4 and 0.1 ml of the membrane fraction. The amount of membrane proteins used in each assay was in the range of 0.8-1.0 mg/ml. The test tubes were incubated for 1h at 25°C. Specific binding was defined as the difference in bindings observed in the absence and presence of 1µM unlabeled naloxone or mitragynine. Incubation was terminated by increasing the speed 10,000 rpm for 10 min at 4°C. The filters then were washed and transferred to scintillation counting vials containing scintillation mixture (0.5 ml of Soluene-350 and 4 ml of Hionic Fluor mixture, Packard Instrument Co., Inc., Meriden, CT). The radioactivity in the samples was determined with a liquid scintillation analyzer.

Competitive Binding Assay:

Competitive binding experiments were performed using [3H]diphrenorphin and the competitive ligand are mitragynine, naltrenib, U-69593 and DAMGO with increasing concentration. [3H]naltrindole was used as a radioligand for δ opioid receptor while for competitive ligands were used mitragynine, naltrenib, U-69593 and 7-benzilidinmaltrexone (BNTX). For kappa opioid receptor [3H]diphenorphin was used as a radioligand and the competitive ligands were mitragynine, nor-binaltorfimin dihydrochloride (BNI), U-69593 and DAMGO. Tissue homogenates, were incubated with seven increasing concentrations of unlabelled opioid drugs [(10-10 to 10-6 M). The reaction was initiated by the addition of membrane suspension (3-8 µg of membrane proteins/assay) into the assay buffer together with the opioid receptor agonist. The suspensions were incubated at 25 °C for 2 h, and the reaction was terminated by increasing the speed to 10,000 rpm for 10 min at 4°C. The filters then were washed and transferred to scintillation counting vials containing scintillation mixture (0.5 ml of Soluene-350 and 4 ml of Hionic Fluor mixture, Packard Instrument Co., Inc., Meriden, CT). These sections were dried overnight at room temperature and stored until counting. Radioligands binding without antagonist is considered as 100%.
Fig. 1: Schematic representation of opioid receptors in five tissue.

Fig. 2: Competitive binding of MG, BNI, U-69593 and DAMGO to k opioid receptors rats. Specific binding at each concentration of radioligand is shown in the panel.

Fig. 3: Competitive binding of MG, BNI, U-69593 and DAMGO to µ opioid receptors rats. Specific binding at each concentration of radioligand is shown in the panel.

Fig. 4: Competitive binding of MG, BNI, U-69593 and DAMGO to δ opioid receptors rats. Specific binding at each concentration of radioligand is shown in the panel.
Fig. 5: MG bound in three opioid receptors.

Statistical Analysis:

Data were expressed as the mean ± SEM. IC_{50} value and K_i were calculated with the methods of Cheng and Prusoff (1973).

Result and Discussion

The results of this study indicate that the profile of receptor densities of μ opioid receptor, κ opioid receptor and δ opioid receptor depends on the region in the rats (Figure 1). The density of opioid receptors in the brain tissue (59.9 ± 3.5 fmol/mg tissue) is higher than in the spinal cord tissue (37.7 ± 2.8 fmol/mg tissue), ileum (32.8 ± 2.5 fmol/mg tissue), duodenum (19.1 ± 1.2 fmol/mg) and vas deferens (19.5 ± 2.7 fmol/mg) (Fig. 1).

Figure 2, 3 and 4 illustrated the standard curves of MG inhibition and various competitive ligands on the radioligands bound to μ-, δ-, and κ- opioid receptors. The concentration of MG gives 50% inhibition (IC_{50}) on the radioligands binding at specific opioid receptors and constant of affinity (K_i) MG shown in the Figure 5.

Discussion:

In the present study, saturation binding assay were conducted in order to investigate the density of opioid receptors in the brain, spinal cord, duodenum, ileum and vas deferens. The results of this study indicate that the profile of receptor densities of opioid receptors depends on the region in the rat tissue. The density of opioid receptor in the brain tissue is higher than other tissue. The selectivity of opioid receptor agonist is retained in the rat brain membranes. However, opioid receptor activation appears to be correlated closely with the anatomical location as well as expression levels of opioid receptors subtypes [14,2]. Brain has high densities of opioid receptors and opioid peptide. The distribution of μ-, κ- and δ-opioid receptor in the brain as evaluated is widespread, however expression is particularly high in cortico-limbic areas, hypothalamus, various brain stem nuclei, and spinal cord. The part of brain such as hypothalamus, cortex and hippocampus have been implicated in stress responses, including unconditional fear and anxiety, cardiovascular system and neurotransmitter release [3,18,12]. Furthermore, there is evidence of an interaction between endogenous opioid peptides and the dopaminergic mesocorticolimbic system in behavioral responses to stress. Vas deferens contains predominantly δ opioid receptors which stimulate its contraction while ileum contains κ- and μ- opioid receptors. The gastrointestinal tract is a major site of action for opioid and opium which have been used to reduce gut motility since ancient time [20].

Following the saturation assay, a set of competitive binding assay were performed in membrane expressing μ opioid receptors, δ opioid receptors and κ opioid receptors to determine the binding affinity of mitragynine, using the tracer [3H]diphenorphine and [3H]naltrindole which is a nonselective opioid ligand. Competitive binding study shown that MG bound to 3 types of opioid receptors with different affinity with the highest affinity in κ- followed by μ- and δ opioid receptors. This result suggested that mitragynine predominantly acts on κ opioid receptors. However, it have been found that there is chemical structure differences between mitragynine and morphine, the opioid prototype. Generally, all types of opioid drugs like morphine, derivatives of morphine or another synthetic opioids have a same structure called the ‘T bone shape’ [5,9]. This structure was established by a set of piperidine ring on the rings D opioid drugs which help opioid drug bound to the receptors.
In addition, from this study were also found the mechanism of action MG in mammals on the biological system is based more on competitive binding on opioid receptors. For example, MG inhibited the electrically stimulated ileum, in the other hand the inhibition action of MG in the ileum was antagonized by the non-selective opioid receptor antagonist naloxone. This condition happen because of the activation of specific opioid system and mitragynine binding to different opioid receptors [8,29,27]. Differences binding affinity of MG towards µ-, κ- and δ-opioid receptors maybe due to differences in the interaction between the polar structures of MG with a set of N termini and carboxyl (COOH) transmembrane 4 and extracellular loop 2 and 3 which distinguish µ-, κ- and δ- opioid receptors [14]. Further study will focus on activity of MG in µ- and κ-opioid receptors considering the high affinity of MG to both receptors.

**Conclusion:**

Expression of opioid receptors is more higher on the brain rats compared to other tissues. Mitragynine bound to those three opioid receptors but have the higher affinity towards κ opioid receptors.

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