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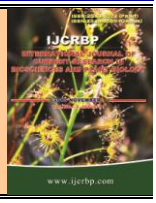

Original Research Article

The Effects of Nicorandil and Adenophostin on Midazolam Induced Modulation of the Loss of Righting Reflex in Mice

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Abstract

This study was conducted to evaluate the effect of the Benzodiazepine drug Midazolam on the Loss of righting reflex (LORR) in mice. The animals were pretreated with potassium channel modulators, and phospholipase C inhibitors and IP3 agonist. Nicorandil increased Ca+ insensitive K+ conductance and hyperpolarized the membrane and thus prevented the activation of the voltage-dependent Ca+ channel. The hyper polarization occurred to a greater extent in venous tissue than in arterial tissue. Nicorandil stimulated the synthesis of cyclic guanosine monophosphate (cGMP) in the polarized and depolarized muscle tissues. The results suggests that there is interactive effect of these modulators on the effects of Midazolam in mice.

Keywords

GABA
Loss of righting reflex
Midazolam
Nicorandil
Potassium

Introduction

The general anesthetic state comprises behavioral and perceptual components, including amnesia, sedation, unconsciousness or hypnosis, analgesia, immobility in response to noxious stimulation, and attenuation of autonomic responses to noxious stimulation (Evers and Crowder, 2001; Antognini and Carstens, 2002). Benzodiazepines are the prime class of compounds used in anxiety and they have remained the most commonly prescribed treatment for anxiety, but they can cause deterioration of cognitive functioning, sedation, muscle relaxation, ataxia, psychomotor impairment, confusion, anterograde amnesia, physical dependence and tolerance (Lader, and Morton, 1991; Suresh and Anupam, 2006). They act specifically on the benzodiazepine receptor of the gamma-aminobutyric acid $GABA_A$ receptor complex and thus modify the GABA-induced chloride flux.

Midazolam has the specific anxiolytic, anticonvulsant, sedative, muscle relaxant, and amnestic properties characteristic of the benzodiazepines. Its lipid solubility is among the highest in this class, making it one of the most potent benzodiazepines. Midazolam is approximately 3-4 times as potent as diazepam. Specific receptors for the benzodiazepines have been identified in the brain and spinal cord (Richter, 1981). The location of these receptors parallels that of the major inhibitory neurotransmitter in the brain, gamma-amino butyric acid (GABA), and the major inhibitory neurotransmitter in

the spinal cord, glycine. These sites are predominately located in neuronal surface membranes and are distributed widely throughout the central nervous system (Study and Barker, 1982).

Benzodiazepines combine in a selective, stereo specific manner with these receptor sites. They appear to intensify the physiological inhibitory effects of GABA via interference with GABA reuptake. The resultant accumulation of GABA produces neuronal membrane hyper polarization (Cheng and Brunner, 1981). Once midazolam binds to $GABA_A$ receptors, chloride ions enter the cells via the receptors, which results in hyper polarization of the neurons, making them more refractory to other stimuli (Nishimura et al., 1994; Thurmon et al., 1996).

While midazolam is similar to diazepam, it has a shorter duration of action, and a more rapid elimination half-life and total body clearance than diazepam. Diazepam is incompletely absorbed if given intramuscularly, and intravenous injections must be given carefully and slowly to prevent thrombophlebitis or cardio toxicity (secondary to the propylene glycol carrier) (Plumb, 2007a, b, c). Midazolam has been shown to have a hyperalgesic effect when given with opiates (Pakulska and Czarnecka, 2001; Rosland and Hole, 1990). Barbiturates (Tatsuo et al., 1999), dissociative agents (Okulicz-Kozaryn et al., 2000) and when given alone as treatment (Kojima et al., 1999).

Most general anesthetics have been found to modulate the activity of a variety of neuronal ion channels, in particular ligand-gated ion channels (Krasowski and Harrison, 1999; Yamakura et al., 2001). The GABA-induced chloride current can be potentiated by allosteric modulators such as benzodiazepines and most general anesthetics (Mohler, 2001).

In addition, some general anesthetics also directly activate the $GABA_A$ receptor in the absence of GABA. Insensitive to the benzodiazepine diazepam (Benson et al., 1998). Whereas α1-containing $GABA_A$ receptors mediate the sedative and anterograde amnesic actions of diazepam (Rudolph et al., 1999; McKernan et al., 2000). Its anxiolytic action is mediated by α2-containing $GABA_A$ receptors (Low et al., 2000) general anesthesia arise from the effects of a single drug in different parts of the CNS (Kendig, 1993; Eger et al., 1997; Kissin, 1997) and it is now widely accepted that general anesthetics cause immobility by depressing spinal neurons, and amnesia and hypnosis by acting on neurons in the brain (Campagna et al., 2003; Urban and Bleckwenn, 2002; Sonner, et al., 2003). Nicorandil acts by enhancing the ATP activity of SUR1 subunit and the resultant channel opening causes hyper polarization) (Glab et al., 2006; Schwanstecher et al., 1998). These drugs open K_{ATP} channels in pancreas, heart, vascular tissue and intestine. Hence they have wider application in cardiac ischemia, urinary incontinence, neuro degeneration, obesity and autoimmune diseases. The K^+ channel openers like Nicorandil, pinacidil, diazoxide and Minoxidil are a group of drugs which produce stabilizing action on cell membranes through membrane hyperpolarisation via increased Trans membrane K^+ conductance (Quayle Nelson, 1997).

The purpose of these studies were to we investigate the effects of ATP sensitive channels opener Nicorandil, and DAG lipase inhibitor on Midazolam induced effects of behavioral hypnosis in the loss of righting reflex (LORR), in the mice measured by latency period technique.

Materials and methods

Animals

Thirty six Swiss *Albino mice* were included in the study in 6 groups of 6 animals in each (n=6) weighing between 22-40g. The animals were obtained from central animal house of JKKMMRFs College of Pharmacy. They were housed in standard polypropylene cages. Animals were quarantined for 7 days before use. The animals were maintained under standard laboratory conditions (12:12 hr light: dark cycles and temperature 27°C±1°C with free access to food and water *ad libitum*. All the experiments were carried out around the same time each day. All the experimental procedures and protocols were viewed and approved by the Institutional Animal Ethics Committee (IAEC) of the institute, with protocol number. (Registration No. JKKMMRFCP/ IAEC/ 2012/ 014).

Behavioral study

Anesthetic states were evaluated using two endpoints: loss of rolling response (as a measure of unconsciousness) and loss of response to noxious stimulation (as a measure of immobility). In this study, righting reflex was used to indicate the rolling response, and clamping the mice tail was the noxious stimulation. The mice were examined

individually in a circular glass beaker (13.5 cm diameter 19 cm high).

To examine the righting reflex, we tilted the beaker by hand to an angle of approximately 45° from a horizontal plane. The beaker was tilted three times at each recording time after IV administration of Midazolam. Righting reflex was assessed and recorded every 2 min for the first 1 h and every 10 min for the following 6 h after administration of Pretreated with K^+_{ATP} channel opener Nicorandil, PLC inhibitor U73122, IP_3 agonist Adenophastin A, potassium channel blocker 4-Aminopyridine and every 1 h for 24 h after administration of Midazolam, by a blinded observer. Righting reflex scores were evaluated according to the rating scale of (Irifune et al., 2003; Bianchi and Franceschini, 1954; Litchfield and Wilcoxon, 1949) a score of 0 indicated a normal righting reflex;+1 indicated that the mouse righted itself within 2 s on all three trials (slightly impaired righting reflex); +2- indicated that the latency to righting was 2 s, but10 s at the best response in three trials (moderately or severely impaired righting reflex); +3 - corresponded to absence of righting reflex (no righting within 10 s on all three trials).

To determine immobility, a tail clamp was applied with arterial forceps close to the base of the tail for 1 min or until the animal moved at the time when each drug groups produced a peak effect on the righting reflex. Purpose full movement of head and/or legs after tail clamp stimulation was considered a response.

Chemicals and drugs

All standard chemicals used in this study were of analytical grade. Midazolam (Shreeji Pharma, International, Vadodara, India), Nicorandil Ultra-Tech Specialty Chemicals Pvt. Ltd., Mumbai, Maharashtra, U73122 (Pro Lab Marketing Pvt. Ltd., New Delhi, India) U73122 Soluble to 100 mM in DMSO and to 100 mM in ethanol Stock solutions in DMSO stable for 2 months when stored at -20°C. Preferably dissolve just before use. Use caution in reusing stored DMSO solutions. Discard solutions that have turned to a pink color, which indicates a loss of inhibitor activity. Dried aliquots prepared from chloroform solutions are stable for up to 1 month when stored at -20°C., Adenophastin A was bought from Merck Millipore India. Pvt. Ltd. (Bangalore, Karnataka, India) soluble sterile water following reconstitution, aliquot and freeze (-20°C). Stock solutions are stable up to 3 months at -20°C 4-Aminopyridine ((4-AP, BI Biotech India PVT. Ltd. New Delhi, India**)** 4-Aminopyridine Soluble in water to 100 mM, peptide solutions should be aliquoted and kept frozen at -20°C.

Treatment groups

Groups are divided as follows:

Groups	Treatment Group
Group I	Nicorandil 10 µM
Group II	Test dose- (Midazolam12.5-16.6 mg/kg (i.v) (Bleasdale et al.,1990)
Group III	Pretreated with ATP Sensitive potassium channels opener with test dose (Nicorandil 1.3 and 2.6 µmol (i.p).+ (Midazolam 12.5-16.6 mg/kg (i.v).
Group IV	Pretreated with PLC inhibitor With test dose (U73122 (10 µmol/kg i.v) (Smith et al., 1990; Gonzalez-Reyes et al., 2013) +(Midazolam12.5-16.6 mg/kg (i.v)
Group V	Pretreated with IP3 agonist Adenophastin A,with test dose Adenophastin A, (2.5 µM, i.p). (Ben-Shlomo et al., 2001)+(Midazolam12.5-16.6 mg/kg (i.v)
Group VI	Pretreated with potassium channel blocker along with test dose. 4- Aminopyridine (1.5 mg/kg s.c) (Han et al., 2015) + (Midazolam 12.5-16.6 mg/kg i.v)

Results

The results of studies are shown in Table 1, i.e., the investigation of the effects of Midazolam general anesthetic drug which may modulate the ATP sensitive channels. Nicorandil, and PLC inhibitor U73122 were used for this study and the behavioral hypnosis in the loss of righting reflex (LORR), in the mice measured by latency. The following observation can be drawn based on the 6 groups of mice results of the studies: Midazolam produced complete immobility and loss of righting reflex within 12 min after IV injection. Induction of an aesthesia was very fast with midazolam treated alone lost the pedal withdrawal reflex. (29.6±1.3min). Loss of the righting reflex was significantly longer following midazolam with

nicorandil (87.2 ± 4.32). The addition of midazolam with PLC inhibitor U73122 sleep time was (48.29± 2.48) significantly longer Compare to Control ($p \leq 0.05$). Adenophostin A, a potent IP_3 receptor agonist with midazolam increases the duration of LORR compared to control (68.36± 3.24*). 4-aminopyridine (4AP) is a potassium channel blocker could significantly decrease loss of the righting reflex with midazolam (35.09 ± 4.3).

Table 1. Effects of Different Drugs Groups a Loss of Righting Reflex (LORR), in the mice.

Group	Drug Name	Dose	(LORR; as a measure of unconsciousness)Tail-clamp stimulation loss of Movement 60 minutes post-administration (Latency in seconds (Mean ± SEM)
Group I	Nicorandil	10 µM	34.4 ± 3.57
Group II	30 min before Midazolam(Test dose)	12.5-16.6 mg/kg (i.v)	29.6 ± 1.3 min*
Group III	Pretreated with Nicorandil +Midazolam	10 μM. (i.p)	87.2 ± 3.6***
Group IV	Pretreated with U73122+Midazolam	10 µmol/kg, (i.v)	48.29 ± 2.48**
Group V	Pretreated with Adenophastin A, with +Midazolam	2.5 µM, (i.p)	68.36 ± 3.24*
Group VI	Pretreated with 4- Aminopyridine + Midazolam	1.5 mg/kg (s.c)	35.09 ± 4.3*

Statistical analysis of parametric data (induction and recovery times) was performed using one-way analysis of variance (ANOVA) Asterisks (**) denote the significant level P values= p <0.01. (***) Extremely significant p <0.001. A repeated measure analysis of variance with time and treatment as factor was used to compare latency. All results are expressed as mean ±SEM and differences were considered significant at Group III (***) p <0.001 significant, Group IV-** p <0.01, Group V-* p <0.05, Group VI-* p <0.05.

These findings suggest that effects of the potassium channel opener Nicorandil, Phospholipase C- IP_3 receptor mediated actions by using the agent Adenophostin and the effects of the phospholipase C inhibitor U73122 on the induce LORR treated with midazolam.

Discussion

The mammalian gamma-aminobutyric acid type $GABA_A$ receptor, a likely target of anesthetic action, receptor appears to be important in the mediation of the immobilizing (tail clamp) effect of midazolam anesthetic agent and produces hypnosis by different specific molecular mechanisms. Mice showed markedly increased sleep time induced by benzodiazepine, midazolam.

GABA, acting via $GABA_A$ receptors, is the brain's major inhibitory neurotransmitter system in the central nervous system. Because the ability to enhance the ion channel activation of $GABA_A$ receptors is a common feature of many sedative and hypnotic drugs, it is probable that the action on the $GABA_A$ receptor complex is a molecular target for these drugs in the mammalian central nervous system.

Nevertheless, many of these drugs, including the family of potassium channel openers Nicorandil, drugs that share the property of activating adenosine triphosphate-sensitive potassium K_{ATP} channels, metabolic sensors responsible for adjusting membrane potential-dependent functions to match cellular energetic demands. K_{ATP} channels, widely represented in metabolically-active tissue, are heteromultimers composed of an inwardly rectifying potassium channel pore and a regulatory sulfonylurea receptor subunit, the site of action of potassium channel opening drugs that promote channel activity by antagonizing ATP-induced pore inhibition.

The data presented in this study showed that the LORR of the mice produced an onset of sleep and prolongation of the total sleep duration induced by

Midazolam. Onset of sleep and the duration of sleep induced by midazolam (p <0.01) (29.6 ± 1.3). The mean sedation time was significantly longer and provided adequate muscle relaxation as judged by complete loss of skeletal muscle tone. Recent data suggests that Midazolam-induced hERG potassium channel could hitherto be a novel mechanism of action for this bezodiazepine class of drug. The blockade was state-dependent, with inhibition occurring in the open and inactivated but not closed states. State dependence of block is a common finding among hERG channel inhibitors. Further, it has been shown by Mitcheson et al that most hERG inhibitors bind to distinct aromatic residues located within the pore cavity of hERG (Zitron et al., 2002; Mitcheson et al., 2000; Fischer et al., 2013; Gottesmann, 2002).

Potentiating of Midazolam induced sleep by the fractions suggests that they possess sedative property. GABA (γ-amino butyric acid) systems are known to play an important role in sleep and positive allosteric modulators of $GABA_A$ receptors (e.g. Benzodiazepines) are widely used to promote restful sleep (Tobler et al., 2001). Diazepam binds to $GABA_A$ receptor and potentiates its activation. This enhancement of neuronal inhibition by GABA produces sedation (reduction of motor activity) which is mediated via α1 $GABA_A$ receptors (Haefely, 1989).

The time of loss of the righting reflex was significantly longer .midazolam given alone can induce hyperalgesia in mice investigation, we observed tail-flick latencies suggestive of hyperalgesia in rats administered Midazolam. Because the tail-flick test is a reflex-based test requiring some level of muscle function, This potentiating, rather than attenuation, of the antinociceptive effect may be attributed to the difference in receptors. Benzodiazepines, ethanol, and barbiturates all potentiate the actions of the neurotransmitter GABA by prolonging the open state of the chloride-associated channels.

The effects of the benzodiazepines are thus limited by the action of endogenous GABA, and they are not known to produce nonspecific effects related to its lipid solubility even at very large doses (Haefely, 1989). Midazolam with Nicorandil, p<0.001 (87.2± 3.6).effect of lost the pedal withdrawal reflex Opening of K^+ channels in cell membranes with resulting increase in K^+ conductance, shifts the membrane potential in a hyperpolarizing direction towards the K^+ equilibrium potential. Hyper polarization reduces the opening probability of ion channels involved in membrane depolarization and excitation is reduced. K^+ channel openers are believed to hyperpolarize smooth muscle cells by a direct action on the cell membrane.

Midazolam alongwith phospholipase C (PLC-inhibitor) p<0.05 (48.29 ±2.48) can potently inhibit Ca^{2+} release from smooth muscle cells independent of its effect on U73122 as the main pharmacological tool to assess the role of the phosphotidyl inositol-PLC pathway in cellular signaling (Feisst et al., 2005; Smith et al., 1996; Wang et al., 1994). Respectively, interaction between Adenophostin A, a potent IP_3 receptor agonist with midazolam increases the duration of LORR compared to control (68.36± 3.24*). Completely blocked by 4-aminopyridine (4-AP) is a potassium channel blocker significant antagonist effect (35.09 ± 4.3 min). Inositol triphosphate may modulate the effects of the potassium channel and the chloride channel therefore the PLC inhibitor was able to produce a potentiating effect as observed in this study.

Conclusion

Nicorandil increased Ca^+ insensitive K^+ conductance and hyperpolarized the membrane and thus prevented the activation of the voltage-dependent Ca^+ channel. The hyper polarization occurred to a greater extent in venous tissue than in arterial tissue.

Nicorandil stimulated the synthesis of cyclic guanosine monophosphate (cGMP) in the polarized and depolarized muscle tissues; nicorandil reduces the concentrations of free Ca^+ in the myoplasm, due to acceleration of the Ca^+ pump at the sarcolemma, and may prevent the phosphorylation of myosin through phosphorylation of myosin light chain kinase. These actions of nicorandil may not contribute to the synthesis of inositol-1, 4, 5-trisphosphate hydrolyzed from phosphatidylinositol-4, 5-bisphosphate. The above actions of nicorandil--hyperpolarization and increase in the cyclic GMP may cause relaxation of the tissues pre contracted by various stimulants. The findings of the present study suggest that Nicorandil potentiates LORR treated with midazolam, immobility, and antinociception appear to be associated with its ability to inhibit potassium channel mediated actions in the central nervous system.

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