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INHIBITION OF CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE (CAMP) IN THE GENERATION OF SEIZURE THRESHOLD BY POTENT SPECIFIC PHOSPHODIESTERASE (PDE-3) INHIBITORS

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ABSTRACT

Objective: To investigate the role of specific phosphodiesterase (PDE-3) inhibitors like cilostazol and milrinone with the cellular level accumulation of cAMP in the generation of seizures.

Materials & Methods: Generation of seizures were carried out in the animals by subjecting them to injection of chemical convulsants like pentylenetetrazole (PTZ) at the dose of 60 mg/kg, i.p and Isoniazid (INH) at the dose of 300mg/kg, s.c and by maximal electroshock (MES) at 45 mA for 0.2 sec. The animals were pre-treated with various dose levels of cilostazol (0.5 mg/kg, 0.6 mg/kg and 0.7 mg/kg) and milrinone (50 µg/kg, 100 µg/kg, 200 µg/kg, 300 µg/kg) 15 mins prior to the PTZ, INH and MES. The control animals received normal saline (5 ml/kg, i.p) 15 mins prior to the injection of PTZ and INH (or) MES.

Results: PDE-3 inhibitors significantly enhanced the onset of seizures induced by PTZ, INH and MES. In particularly milrinone potentiated the convulsive phenomenon more significantly ($p < 0.05$ and $p < 0.001$) when compare with cilostazol.

Conclusion: This study demonstrates the vital role of specific PDE-3 inhibitors like cilostazol and milrinone in the generation of seizure threshold through the increased cellular concentration cAMP results in the activation of protein kinase A and its phosphorylation processes.

KEYWORDS : PDE-3 inhibitors, cAMP, Cilostazol, Milrinone, Seizures.

INTRODUCTION

Epilepsy is one of the most common afflictions of human with a prevalence of approximately 1 % of the total population[1]. Seizure is a characteristic feature in epilepsy and is associated with disordered and rhythmic high frequency discharge of impulses by a group of neurons in the brain[2]. The cyclic adenosine 3',5'-monophosphate (cAMP) plays a major role in the generation of seizure activity. An elevation in cAMP content has been reported in the cerebral cortex accompanying chemically induced epileptic activity [3,4,5]. In epileptic patients, the cAMP concentration in the cerebrospinal fluid is also elevated after an attack[6]. Cyclic AMP plays a key function by controlling a wide variety of cellular processes[7,8], also which acts as a ubiquitous second messenger and modulator of signal transduction processes[9]. This cAMP is generated by the action of adenylyl cyclase[10] and degraded by hydrolysis process, which is regulated by a family of cyclic nucleotide phosphodiesterases (PDEs)[11,12]. Twelve members of the family have been identified and these can be further divided into a number of subtypes and splice variants. The PDE types differ in their amino acid sequence, substrate specificities, kinetic properties, allosteric regulators, inhibitor sensitivities and in their organ, tissue and sub cellular distribution[13-19]. Out of which PDE-3 enzyme was initially found mainly in the heart, liver, platelet and adipocyte.[20-22]. PDE-3 is characterized by its high affinity for cAMP and its capacity to hydrolyze both cAMP and cGMP. PDE-3A is mainly present in the heart, platelet, vascular smooth muscle and oocyte, whereas PDE-3B is mainly associated to adipocytes, hepatocytes and spermatocytes.

Secondly, it has been shown by various studies that cAMP and Ca^{2+}/calmodulin dependent protein kinases can phosphorylate key intracellular proteins such as ion channels, receptors, enzymes, transcriptions factors and regulate thus, neuronal excitability[23]. Abnormalities in this kinase mediated protein phosphorylation can be involved in the etiology of distinct forms of epilepsies. Alpha subunit of Ca^{2+}/calmodulin dependent protein kinase II have been shown to exhibit significant neurons excitability and epileptic seizures arising from limbic structures[24]. Also the repeated injection of initially subconvulsive dose of cAMP into the rat amygdala produced progressive seizure development similar to that of electrical kindling[25]. The elevated cAMP levels found in cortical structures in some experimental models of epilepsy[26].

On the other hand the cellular levels of cAMP hydrolyze into 5'-nucleotide monophosphates by PDEs. By blocking phosphodiesterase hydrolysis, PDE inhibition results in higher levels of cyclic AMP. Therefore, PDE inhibitors may have considerable therapeutic utility as anti-inflammatory agents, anti-asthmatics, vasodilators, smooth muscle relaxants, cardiotonic agents, antidepressants, antithrombotics and agents for improving memory and other cognitive functions.[27-30]. In view of these findings this study was designed to examine and investigate the possible roles of specific inhibitors of PDE-3 like cilostazol and milrinone in the generation of convulsive seizures. Cilostazol and milrinone both are well known non-glycosidic cardiotonic agents, which possess selective PDE-3 inhibitor action[31,32].

MATERIALS AND METHODS

Animals:

Swiss Albino mice of either sex weighing between 22-25 g were obtained from Karpaga Vinayaga Institute of Medical Sciences & Research Centre, Maduranthagam, Chennai- 603 308. The animals were kept under standard laboratory conditions. A 12:12 dark:light cycle was followed during the experiments. Animals had free access to food and water *ad libitum*. The Institutional Animal Ethical Committee approved the protocol of this study.

Drugs and Chemicals:

The following drugs and chemicals were used for conducting this study. Normal saline (0.9 %), Karpaga Vinayaga Institute of Medical Sciences & Research Centre, Maduranthagam, Pentylenetetrazole (Sigma, USA), Isoniazid (Fourts India Ltd, Chennai, India), Cilostazol (Cadila Pharmaceuticals, India), Milrinone (Sanofi Synthelabo Ltd, Mumbai, India). Both cilostazol and milrinone were diluted with sterile water for injection. Normal saline was administered in a volume of 5 ml/kg, i.p.

Chemoshock Method:

Albino mice divided into different groups each containing six animals (n=6). Seizures were induced in the animals by using chemical convulsant like pentylenetetrazole (PTZ) and Isoniazid (INH).

(I). Pentylenetetrazole (PTZ) induced seizures:

To elicit chemically induced seizures, a potent CNS stimulant PTZ at

the dose of 60 mg/100g i.p. was injected in mice[33]. 15 mins before the injection of the chemical convulsant the animals were pre-treated with varying doses of cilostazol (0.5mg/kg, 0.6 mg/kg and 0.7 mg/kg) and milrinone (50 µg/kg, 100 µg/kg, 200 µg/kg and 300 µg/kg). Onset of action, myoclonic jerks, clonus, tonic flexion and mortality were observed and tabulated.

(ii). Isoniazid (INH) induced seizures:

INH is a GABA synthesis inhibitor, which was injected to induce seizures at the dose of 300 mg/kg, s.c as described earlier[34].15 mins prior to the injection of INH the animals were pre-treated with varying doses of cilostazol (0.5mg/kg, 0.6 mg/kg and 0.7 mg/kg) and milrinone (50 µg/kg, 100 µg/kg, 200 µg/kg and 300 µg/kg). Onset of action, myoclonic jerks, clonus, tonic flexion and mortality were noted and tabulated.

Maximal Electroshock (MES) Method:

MES were induced in the animals using a technique described earlier[35]. The animals were pre-treated with aminone and milrinone in the same dose as mentioned in chemoshock method. The animals were subjected to electroshock (45 mA, 0.2 sec) via the corneal electrodes. After induction of seizures, tonic limb flextion, tonic extensor, clonus, stupor and recovery/mortality of the animals were observed and tabulated.

Statistical Analysis:

The data is represented as mean ± SEM, which were analysed using one-way ANOVA followed by Dunnett's test. Statistically significant difference was ascertained by 'P' value which is considered significant at the level of $p<0.05$ and highly significant at $p<0.001$.

RESULTS

Assessment of onset of seizures:

(I). PTZ induced seizures:

Table 1 and 3 summarizes the data obtained from experiments conducted with PTZ induced seizures. In animals treated with normal saline onset of action were observed 89.5 ± 1.41 sec after PTZ and convulsions appeared 182.5 ± 1.61 sec after PTZ. Cilostazol in a dose of 0.6 mg/kg significantly enhanced onset of action ($p<0.05$) and stimulate the convulsions ($p<0.001$). The results show that there was a significant increase in onset of action of seizure activity when increased the dose (0.7 mg/kg) of cilostazol ($p<0.001$). At the same time milrinone in a dose of 300 µg/kg significantly potentiated onset of action ($p<0.001$) and produce the convulsion phenomenon much faster ($p<0.001$) than cilostazol. Even at a very low dose 33% of morality was observed while using cilostazol (0.6 mg/kg and 0.7 mg/kg) and milrinone (200 µg/kg and 300 µg/kg).

TABLE- 1

Action of various dose levels of cilostazol on chemoshock seizures in mice (n=6)

Treatment (mg/kg, i.p)	CHEMO-CONVULSANT					
	PENTYLENETETRAZOLE (PTZ)			ISONIAZID (INH)		
	Onset time of various phases of convulsions (in sec)					
	Action	Jerky Movements	Convulsions	Action	Jerky Movements	Convulsions
Normal saline (5 mg/kg, i.p)	89.5 ± 1.41	140.33 ± 1.36	182.5 ± 1.61	2950 ± 54.33	3000 ± 46.47	3105 ± 46.43
Cilostazol (0.5 mg/kg, i.p)	66 ± 1.24	97 ± 1.07*	125 ± 1.91*	2190 ± 45.82*	2393.33 ± 44.24*	2320 ± 43.42*
Cilostazol (0.6 mg/kg, i.p)	58 ± 0.93*	76.83 ± 1.33**	93.83 ± 0.87**	1938 ± 37.62**	2025 ± 39.30**	2063.33± 34.79**
Cilostazol (0.7 mg/kg, i.p)	48.5 ± 0.92**	56.83 ± 2.24**	75.33 ± 1.82**	1601.67 ± 17.78**	1681.67 ± 20.56**	1730 ± 15.27**

Values are mean ± SEM, represents onset time of various phases of convulsion in seconds. Treatments were given 15 mins prior to chemical convulsant injection like PTZ (60 mg/kg, i.p) and INH (300 mg/kg, s.c). The data were analysed by one-way ANOVA followed by Dunnett's test. * $p<0.05$ and ** $p<0.001$, compared to the normal saline treated group.

TABLE- 3

Action of various dose levels of milrinone on chemoshock seizures in mice (n=6)

Treatment (mg/kg, i.p)	CHEMO-CONVULSANT					
	PENTYLENETETRAZOLE (PTZ)			ISONIAZID (INH)		
	Onset time of various phases of convulsions (in sec)					
	Action	Jerky Movements	Convulsions	Action	Jerky Movements	Convulsions
Normal saline (5 mg/kg, i.p)	87.5 ± 1.29	141.33 ± 1.36	172.5 ± 1.61	2730 ± 52.33	3000 ± 46.47	3065 ± 45.43
Milrinone (50 µg/kg, i.p)	103.8 ± 4.37	138 ± 4.41	128.3 ± 5.88*	2520 ± 34.65	2860 ± 25.31	3060 ± 21.92
Milrinone (100 µg/kg, i.p)	63.7 ± 1.92	76.3 ± 2.78*	79.8 ± 2.65**	2220 ± 34.65*	2583.3 ± 26.04*	2710 ± 25.71*
Milrinone (200 µg/kg, i.p)	54.5 ± 1.35*	60.8 ± 1.26**	63.8 ± 1.38**	1870 ± 36.04**	2100 ± 30.98**	2270 ± 36.04**
Milrinone (300 µg/kg, i.p)	47.3 ± 1.18**	49.8 ± 1.43**	52.2 ± 1.51**	1670 ± 21.91**	1890 ± 25.71**	2010 ± 33.76**

Values are mean ± SEM, represents onset time of various phases of convulsion in seconds. Treatments were given 15 mins prior to chemical convulsant injection like PTZ (60 mg/kg, i.p) and INH (300 mg/kg, s.c). The data were analysed by one-way ANOVA followed by Dunnett's test. * $p<0.05$ and ** $p<0.001$, compared to the normal saline treated group.

(ii). INH induced seizures:

Table 1 and 3 shows the data obtained from experiments conducted with INH induced seizures. In animals treated with normal saline onset of action were noticed 2950 ± 54.33 sec and convulsions appeared 3105 ± 46.43 sec after INH. Cilostazol in a dose of 0.5 mg/kg significantly potentiated the onset of action, jerky movements and convulsions ($p<0.05$). where as the rate of onset of action, jerky movements and convulsions time was reduced significantly in the doses like 0.6 mg/kg and 0.7 mg/kg of cilostazol ($p<0.001$). Simultaneously the rate of onset of action, jerky movements and convulsion time was reduced at the great extent even in the low doses like (200 µg/mg and 300 µg/mg) of milrinone ($p<0.001$) considerable mortality (67 %) was observed while using cilostazol (0.6 mg/kg and 0.7 mg/kg) and milrinone (100 µg/mg, 200µg/mg and 300µg/kg).

Maximal electroshock test:

Table 2 and 4 illustrates the action of various dose levels of cilostazol and milrinone against MES induced seizures. In which 0.6 mg/kg and 0.7 mg/kg of cilostazol produced a gradual reduction in tonic limb flextion significantly ($p<0.05$) when compare with normal saline. Significant ($p<0.001$) was observed in stupor phase of convulsion at the dose of 0.6 mg/kg and 0.7 mg/kg of cilostazol. Likewise milrinone treated animals showed a significant ($p<0.001$) reduction in tonic limb tonic extensor and stupor flexion, phases of convulsion in the 200 µg/kg and 300 µg/kg dose levels. Milrinone in the doses like 200 µg/kg and 300 µg/kg treated animals produced the significantly reduced the clonus phases of convulsion at the level of $p<0.05$ and $p<0.001$ respectively. Mortality (67 %) was observed in both doses like 200 µg/kg and 300 µg/kg of milrinone.

TABLE- 2

Action of various dose levels of cilostazol on maximal electroshock induced convulsions in mice (n=6)

Treatment (mg/kg, i.p)	Onset time (sec) in various phases of convulsion				
	Tonic limb flextion	Tonic Extensor	Clonus	Stupor	Recovery/Death
Normal saline (5 mg/kg, i.p)	5.57 ± 0.33	22.33 ± 0.67	35.83 ± 1.38	65.67 ± 1.23	196.25 ± 5.55
Cilostazol (0.5 mg/kg, i.p)	4.32 ± 0.21	18.83 ± 0.31	38 ± 0.73	59.83 ± 0.6*	226 ± 0.82
Cilostazol (0.6 mg/kg, i.p)	3.5 ± 0.22*	15.5 ± 0.43*	34.33 ± 0.42	53.83 ± 0.6**	207 ± 3.72
Cilostazol (0.7 mg/kg, i.p)	3.17 ± 0.17*	9.83 ± 0.31**	28 ± 0.51*	43.21 ± 0.86**	207.33 ± 0.42

Values are mean ± SEM, represents onset time of various phases of convulsion in seconds. Treatments were given 15 mins prior to maximal electroshock (45 mA, 0.2 sec). The data were analysed by one-way ANOVA followed by Dunnett's test. * $p<0.05$ and ** $p<0.001$, compared to the normal saline treated group.

TABLE- 4

Action of various dose levels of milrinone on maximal electroshock induced convulsions in mice (n=6)

Treatment (mg/kg, i.p)	Onset time(sec) in various phases of convulsion				
	Tonic limb flextion	Tonic Extensor	Clonus	Stupor	Recovery/Death
Normal saline (5 mg/kg, i.p)	5.67 ± 0.33	24.33 ± 0.67	37.83 ± 1.38	66.67 ± 1.23	196.25 ± 5.55
Milrinone (50 µg/kg, i.p)	4.8 ± 0.33	19.3 ± 0.41	36.8 ± 0.41	58.5 ± 0.78*	217.3 ± 0.86
Milrinone (100 µg/kg, i.p)	3.3 ± 0.20*	16.2 ± 0.49*	32.7 ± 0.82	53.5 ± 0.65**	280 ± 3.14
Milrinone (200 µg/kg, i.p)	2.7 ± 0.20**	8.7 ± 0.33**	25.8 ± 0.33*	47.7 ± 0.65**	225.0 ± 1.71
Milrinone (300 µg/kg, i.p)	2.2 ± 0.17**	7.3 ± 0.33**	20 ± 0.57**	39.8 ± 0.69**	237.5 ± 1.43

Values are mean ± SEM, represents onset time of various phases of convulsion in seconds. Treatments were given 15 mins prior to maximal electroshock (45 mA, 0.2 sec). The data were analysed by one-way ANOVA followed by Dunnett's test. * $p<0.05$ and ** $p<0.001$, compared to the normal saline treated group.

DISCUSSION

Bipyridine derivative of selective PDE-3 inhibitors such as cilostazol and milrinone is a new class of positive inotropic drugs chemically and pharmacologically distinct from digitalis and catecholamines.[31,32,36]. The mechanism of the positive inotropic effect of PDE inhibitors is similar to that of β-adrenergic agents[37]. Milrinone has been the most studied and used extensively as PDE-3 inhibitor and it is currently used in the acute treatment of heart failure to diminish long term risk[38]. This study demonstrates the importance of the PDE-3 inhibitors such as cilostazol and milrinone in the generation of seizure activity with the accumulation of cellular levels of cAMP by inhibiting its metabolism.

The value obtained from this study show that pre-treatment with PDE-3 inhibitors potentiates the onset of action and various phases of convulsions against PTZ, INH and maximal electroshock induced convulsions as depicted in Table 1 to 4. Earlier studies suggest that the elevated levels of cAMP was found in cortical structure in some experimental models of epilepsy[26,39], repeated injection of cAMP produced progressive seizure development[25] and the neuronal excitability was regulated by cAMP and Ca^{2+}/ calmodulin dependent protein kinase and its phosphorylation process[23]. Apart from these findings, PDE-3 inhibitors possess transmembrane influx of Ca^{2+} [36]. This influx of Ca^{2+} is responsible for the phosphorylation process of intracellular proteins, such as ion channels, receptors, enzymes and transcription factors which exhibit significant neuronal excitability and epileptic seizures[24]. Our study results also clearly suggest that rate of onset of convulsive time was significantly ($p<0.05$ and $p<0.001$) reduced with increasing the dose levels of both cilostazol and milirinone against PTZ, INH and MES induced seizures.

On the other hand phosphorylation of variety of substrates regulates the myriad of physiological process, such as immune responses, cardiac and smooth muscle contraction, visual response, glycogenolysis, platelet aggregation, ion channel conductance, apoptosis and growth control[40]. The present study results also early correspond with the generation of seizure activity due to the breakdown of hydrolysis of cAMP which promotes protein kinase phosphorylation process.

Thus, in conclusion the study shows a definite relationship between the specific PDE-3 inhibitors and increase the cellular level of cAMP and Ca^{2+} ions with the generation of seizures.

Conflict of interest:
None

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