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Assessing the emetic potential of PDE4 inhibitors in rats

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1 Type 4 phosphodiesterase (PDE4) inhibitors mimic the pharmacological actions of alpha$_2$-adrenoceptor antagonists. This has been postulated as the mechanism by which PDE4 inhibitors induce emesis and was also demonstrated by their ability to reverse xylazine/ketamine-induced anaesthesia. We further characterized this latter effect since it appears to reflect the emetic potential of PDE4 inhibitors.

2 Selective inhibitors of PDE 1, 2, 3, 4 and 5 were studied in rats, on the duration of anaesthesia induced by the combination of xylazine (10 mg kg^{-1}, i.m.) and ketamine (10 mg kg^{-1}, i.m.). PMNPQ (i.e. 6-(4-pyridylmethyl)-8-(3-nitrophenyl)quinoline) – PDE4 inhibitor: 0.01–3 mg kg^{-1}), like MK-912 (alpha$_2$-adrenoceptor antagonist: 0.01–3 mg kg^{-1}), dose-dependently reduced the duration of anaesthesia. In contrast, vinpocetine (PDE1 inhibitor), EHNA (PDE2 inhibitor), milrinone (PDE3 inhibitor) and zaprinast (PDE5 inhibitor) had no significant effect at the doses tested (1–10 mg kg^{-1}). Analysis of plasma and cerebrospinal fluid (CSF) of treated animals confirmed the absorption and distribution to the brain of the inactive inhibitors.

3 Neither MK-912 (3 mg kg^{-1}) nor PMNPQ (0.1–1 mg kg^{-1}) altered the duration of anaesthesia induced via a non-alpha$_2$-adrenoceptor pathway (sodium pentobarbitone 50 mg kg^{-1}, i.p.).

4 Central NK$_1$ receptors are involved in PDE4 inhibitor-induced emesis. Consistently, [sar^9, Met(O$_2$)^{11}]-substance P (NK$_1$ receptor agonist, 6 μg i.c.v.) reduced the duration of anaesthesia induced by xylazine/ketamine.

5 In summary, this model is functionally coupled to PDE4, specific to alpha$_2$-adrenoceptors and relevant to PDE4 inhibitor-induced emesis. It therefore provides a novel way of evaluating the emetic potential of PDE4 inhibitors in rats.

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Abbreviations: CSF, cerebrospinal fluid; PDE, cyclic nucleotide phosphodiesterase; PDE4, Type 4 cyclic AMP-specific nucleotide phosphodiesterase; PMNPQ, 6-(4-pyridylmethyl)-8-(3-nitrophenyl)quinoline)

Introduction

Emesis is a major side effect associated with inhibitors of type 4 cyclic nucleotide phosphodiesterase (PDE4). It has been reported in man and in various animal species endowed with a vomiting reflex (Horowski & Sastre-y-Hernandez, 1985; Humpel *et al*., 1986; Heaslip & Evans, 1995; Silvestre *et al*., 1998; Murdoch *et al*., 1998; Robichaud *et al*., 1999). Recently, our group postulated that PDE4 inhibitors were able to trigger the emetic reflex through a sympathetic pathway, by mimicking the pharmacological actions of a pre-synaptic alpha$_2$-adrenoceptor inhibition (Robichaud *et al*., 2001).

This hypothesis was supported by the observations that clonidine (alpha$_2$-adrenoceptor agonist) prevented emesis induced by PDE4 inhibitors and that alpha$_2$-adrenoceptor antagonists (yohimbine, MK-192) triggered vomiting in ferrets (Robichaud *et al*., 2001). The ability of PDE4 inhibitors to reproduce the pharmacological effects of alpha$_2$-adrenoceptor antagonists was further demonstrated in rats and in ferrets against anaesthesia mediated through the activation of the alpha$_2$-adrenoceptor (Correa-Sales *et al*., 1992a; Robichaud *et al*., 2001). In these studies, a reduction in the percentage of animals exhibiting loss of righting reflex or a reduction in the duration of anaesthesia was reported in presence of a PDE4 inhibitor (Correa-Sales *et al*., 1992a; Robichaud *et al*., 2001).

Interestingly, the ability of PDE4 inhibitors to reverse anaesthesia appears to reflect the emetic potential of these agents (Robichaud *et al*., 1999; 2001). Therefore, assessing the anaesthetic reversal effect of PDE4 inhibitors could be an efficient way to evaluate the emetic potential of this class of compounds in rats, since rodents do not have a vomiting reflex (Andrews & Davis, 1993). Hence, the objective of the present study was to further characterize the anaesthetic reversing property of PDE4 inhibitors in rats.

Methods

Male Sprague-Dawley rats (366±4 g; Charles River, St-Constant, Qc, Canada) were used. The animals were housed in a humidity and temperature controlled environment with food and water provided *ad libitum*. A period of at least 2

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days of acclimatization was allowed prior to experimentation. Experimental protocols were approved by the Animal Care Committee at Merck Frosst Centre for Therapeutic Research in accordance with the guidelines established by the Canadian Council on Animal Care.

Experiments were conducted according to procedures described previously (Robichaud *et al*., 2001). Briefly, rats were anaesthetized with a combination of xylazine (10 mg kg^{-1}) and ketamine (10 mg kg^{-1}) administered in a single intramuscular injection in the back hindlimb or with sodium pentobarbitone (50 mg kg^{-1} i.p.). Fifteen minutes later, the test compound or its vehicle was injected subcutaneously and the animals were placed in dorsal recumbency. The restoration of the righting reflex, i.e. when the animal no longer remained on its back and turned itself spontaneously to the prone position, was used as an endpoint to determine the duration of anaesthesia. Unless stated otherwise, test compounds were dissolved in polyethylene glycol (PEG; MW 200) immediately before use and injected in a dosing volume of 1 ml kg^{-1}.

In certain experiments, the test compound or its vehicle was injected directly into the lateral ventricle of the rat brain (i.e. intracerebroventricular (i.c.v.) administration). Rats were anaesthetized with the combination of xylazine (5 mg kg^{-1}) and ketamine (70 mg kg^{-1}) and their left lateral ventricle was stereotaxically cannulated. A 22 gauge stainless steel guide cannula was inserted 1.4 mm lateral and 0.9 mm posterior of the bregma reference point. Following cannulation, the animals received post-operative analgesic (Buprenex, 5 μg kg^{-1} i.m.) and antibiotic (Penlong XL, 0.1 ml, i.m.) injections and were allowed 2–4 days to recover. I.c.v. injections were made at 4 mm below the skull surface, using a 28 gauge injection unit. Test compounds were dissolved in dimethylsulphoxide (DMSO) or sterile water and were injected in a total volume of 2–3 μl. At the end of the experiment, the position of the cannula was confirmed histologically with the injection of 2 μl of blue dye.

Drug determination

At the end of the experiment, plasma and cerebrospinal fluid (CSF) samples were collected for quantification of drug concentrations. Sampling was done at 1 h post-dosing, from the group treated with the highest dose of the test compound. The samples were stored at −80°C until they were analysed by high performance liquid chromatography (HPLC) (Alliance Waters 960, Waters, Milford, MA, U.S.A.) or by liquid chromatography coupled to mass spectrometry (LC-MS) (Sciex API 100 and API-2000, Perkin-Elmer, Norwalk, CT, U.S.A.).

Drugs

Xylazine (*Rompun*) was purchased from Bayer (Etobicoke, Ont., Canada), ketamine (*Ketaset*) from Ayerst (Montreal, Qc, Canada), sodium pentobarbitone (*Somnotol*) from MTC Pharmaceuticals (Cambridge, Ont., Canada), vinpocetine and zaprinast from Tocris (Ballwin, MO, U.S.A.), milrinone and [sar^9, $Met(O_2)^{11}$]-substance P from Sigma-Aldrich Canada Ltd (Oakville, Ont., Canada) and erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA) from Biomol (Plymouth, PA, U.S.A.). MK-912 was obtained from Merck Research Laboratories (Rahway, NJ, U.S.A.) and PMNPQ [i.e. 6-(4-pyridylmethyl)-8-(3-nitrophenyl)quinoline] was synthesized by the Department of Medicinal Chemistry at Merck Research Laboratory (Montreal, Qc, Canada). The biochemical characterization of PMNPQ has been published under the incorrect name of RS14203 following a mistake in the identification of the compound (Brideau *et al*., 1999; Laliberté *et al*., 2000).

Data analysis and statistics

Results are presented as mean ± s.e.m. Differences between means of treated and control groups were compared using an analysis of variance (ANOVA) with multiple comparisons (Bonferroni). A value of $P<0.05$ was considered statistically significant.

Results

PDE inhibitors

Selective inhibitors of PDE 1, 2, 3, 4 and 5 were studied for their effects on the duration of xylazine/ketamine-induced anaes-

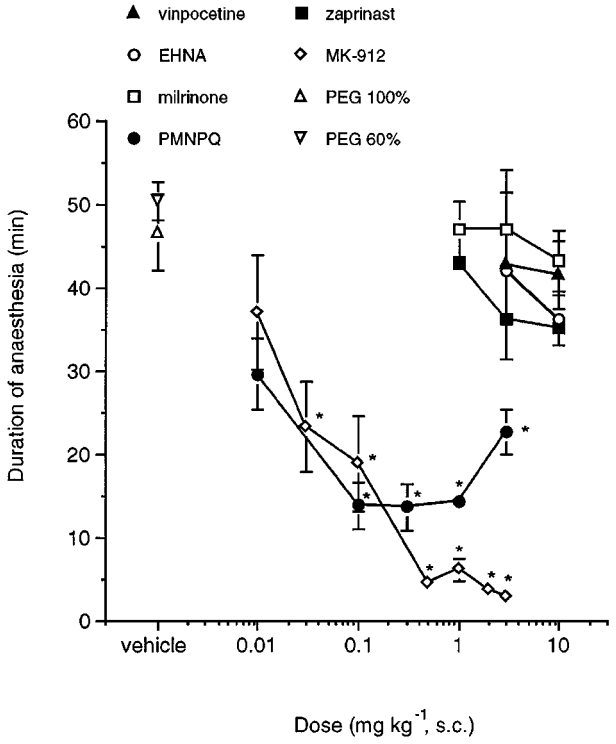

Figure 1 Effect of PDE inhibitors on the duration of anaesthesia induced by the combination of xylazine (10 mg kg^{-1}) and ketamine (10 mg kg^{-1}) in rats. Fifteen minutes following the induction of anaesthesia, rats were injected subcutaneously with increasing doses of: vinpocetine (PDE1 inhibitor), EHNA (PDE2 inhibitor), milrinone (PDE3 inhibitor), PMNPQ (PDE4 inhibitor), zaprinast (PDE5 inhibitor) or MK-912 (alpha$_2$-adrenoceptor antagonist). The duration of anaesthesia was assessed by the return of righting reflex. Polyethylene glycol (PEG) 60% ($n=28$) was the vehicle used, with the exception of vinpocetine where PEG 100% ($n=4$) was used. Data is expressed as mean ± s.e.m., with 3–9 animals/dose tested. *Statistical difference from vehicle group at $P<0.05$.

thesia in rats. PMNPQ (PDE4 inhibitor; 0.01 – 3 mg kg^{-1}, s.c.), like the potent and brain penetrant alpha$_2$-adrenoceptor antagonist, MK-912 (0.01 – 3 mg kg^{-1}, s.c.), dose-dependently reduced the duration of anaesthesia (Figure 1). In contrast, vinpocetine (PDE1 inhibitor), EHNA (PDE2 inhibitor), milrinone (PDE3 inhibitor) and zaprinast (PDE5 inhibitor) had no significant effect on the duration of anaesthesia at the doses tested (1 – 10 mg kg^{-1}, s.c.) (Figure 1).

To rule out a possible failure of absorption or distribution to the brain of the inactive PDE inhibitors tested, samples of plasma and cerebrospinal fluid (CSF) were collected at the end of the experiment from the animals treated with the highest dose of vinpocetine, EHNA, milrinone and zaprinast. Analysis of these samples revealed the presence of the PDE inhibitors in question both in the plasma and in the CSF (Table 1). Treatment with vinpocetine and zaprinast resulted, however, in low concentrations of these inhibitors in the CSF. These compounds were therefore re-tested for their effects on the duration of xylazine/ketamine-induced anaesthesia following an i.c.v. administration. Delivering 6 μg of vinpocetine or zaprinast directly into the lateral ventricle had no significant effect on the duration of anaesthesia compared to the vehicle-treated group (Figure 2). In contrast the i.c.v. administration of a similar dose of the PDE4 inhibitor, PMNPQ, significantly reduced the sleeping time (Figure 2).

Pentobarbitone-induced anaesthesia

In order to assess the specificity of the effect of PDE4 inhibitors on the duration of anaesthesia, the PDE4 inhibitor, PMNPQ (0.01 – 1 mg kg^{-1}, s.c.), or the alpha$_2$-adrenoceptor antagonist, MK-912 (3 mg kg^{-1}, s.c.), were administered to rats anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.). Compared to the vehicle-treated group, neither MK-912 nor PMNPQ altered the duration of anaesthesia induced by sodium pentobarbitone (Figure 3).

NK$_1$ receptor agonist

Central NK$_1$ receptors have been shown to be involved in the induction of emesis induced by PDE4 inhibitors (Robichaud *et al*., 1999). To evaluate the relevance of this model to emesis induced by PDE4 inhibitors, the tachykinin NK$_1$ receptor agonist, [sar^9, Met(O$_2$)^{11}]-substance P, was studied for its effect on the duration of anaesthesia induced by xylazine/ketamine in rats following an i.c.v. administration. Compared to the vehicle-treated group, the administration of 6 μg of [sar^9, Met(O$_2$)^{11}]-substance P directly into the lateral ventricle significantly reduced the duration of anaesthesia (Figure 4).

Table 1 Plasma & CSF concentrations of inactive PDE inhibitors

Treatment	*PDE inhibitor*	*Dose* (mg kg^{-1})	*Plasma concentration* (μM)	*CSF concentration* (nM)
vinpocetine	1	10	0.53±0.17	4±2
EHNA	2	10	1.16±0.53	190±61
milrinone	3	10	34.3±2.9	941±176
zaprinast	5	10	7.3±3	3.8±1.8

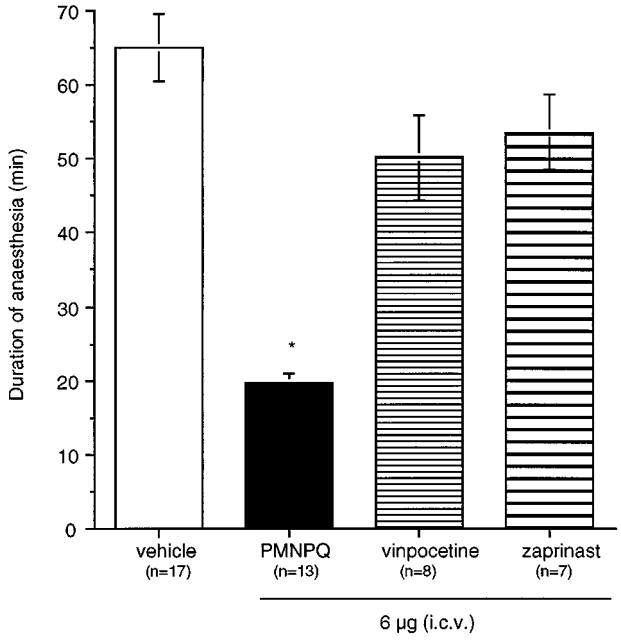

Figure 2 Effect of i.c.v. administered PMNPQ, vinpocetine and zaprinast on the duration of anaesthesia induced by the combination of xylazine (10 mg kg^{-1}) and ketamine (10 mg kg^{-1}) in rats. Fifteen minutes following the induction of anaesthesia, rats received an i.c.v. injection of PMNPQ (6 μg), vinpocetine (6 μg), zaprinast (6 μg) or vehicle (DMSO, 2 μl). The duration of anaesthesia was assessed by the return of righting reflex. Data is expressed as mean±s.e.m. *Statistical difference from vehicle group at $P<0.05$.

Discussion

PDE4 inhibitors are thought to trigger the emetic reflex *via* a sympathetic pathway by mimicking the pharmacological effect of a pre-synaptic alpha$_2$-adrenoceptor inhibition (Robichaud *et al*., 2001). The hypothesis that PDE4 inhibitors reproduce the actions of alpha$_2$-adrenoceptor antagonists was strengthened by the demonstration of their ability to reverse anaesthesia mediated through the activation of the alpha$_2$-adrenoceptor in rats and ferrets (Correa-Sales *et al*., 1992a; Robichaud *et al*., 2001). The reversal of the hypnotic effect of alpha$_2$-adrenoceptor mediated anaesthesia has been described with the prototypic PDE4 inhibitor rolipram but also with some more recent structurally diverse PDE4 inhibitors such as PMNPQ (Correa-Sales *et al*., 1992a; Robichaud *et al*., 2001). Because the emetic potential of PDE4 inhibitors appears to be in relation with the ability of these inhibitors to reduce the duration of anaesthesia in rats (Robichaud *et al*., 1999; 2001), the objective of the present work was to further characterize this effect.

Using selective inhibitors, our results suggest that PDE4 is a key phosphodiesterase involved in the hypnotic response of alpha$_2$-adrenoceptor agonist-mediated anaesthesia in rats. In contrast, PDE1, 2, 3 and 5 do not appear to play any significant functional role in this response. The absence of effect seen by these latter inhibitors can not be explained by a lack of absorption or distribution to the brain. The presence of inhibitors of PDE1, 2, 3, or 5 was detected both in the plasma and in the CSF of treated animals. Moreover, when the inhibitors were delivered directly into the lateral ventricle

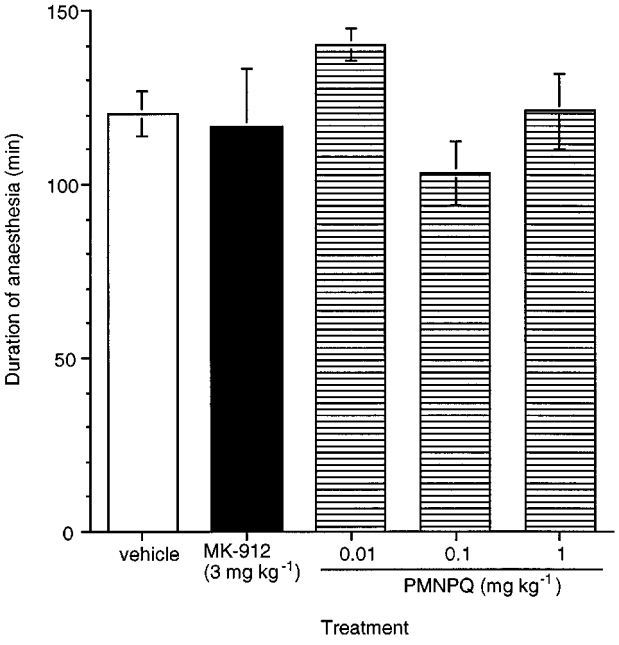

Figure 3 Effect of MK-912 and PMNPQ on the duration of anaesthesia induced by sodium pentobarbitone (50 mg kg^{-1}, i.p.) in rats. MK-912 (3 mg kg^{-1} s.c.; $n=4$) or PMNPQ (0.01–1 mg kg^{-1} s.c.; $n=3-7$ animals/dose) were injected 15 min after the induction of anaesthesia. Polyethylene glycol (PEG) 60% was the vehicle used to dissolve the test compounds ($n=7$). The duration of anaesthesia was assessed by the return of righting reflex. The experiment was terminated at 145 min post-drug injection. The animals that did not restore their righting reflex by this time were given a recording of the maximum amount of time allowed. Data is expressed as mean ± s.e.m.

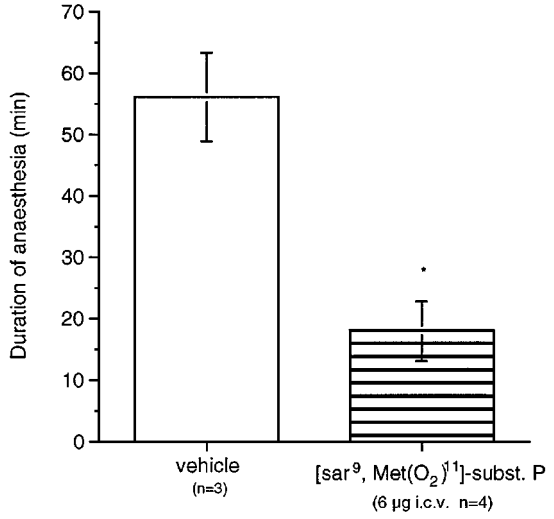

Figure 4 Effect of a central administration of the tachykinin NK_1 receptor agonist, [sar^9, $Met(O_2)^{11}$]-substance P, on the duration of anaesthesia induced by the combination of xylazine (10 mg kg^{-1}) and ketamine (10 mg kg^{-1}) in rats. Fifteen minutes following the induction of anaesthesia, rats were injected with [sar^9, $Met(O_2)^{11}$]-substance P (6 μg i.c.v.) or vehicle (sterile water, 3 μl). The duration of anaesthesia was assessed by the return of righting reflex. Data is expressed as mean ± s.e.m. *Statistical difference from vehicle group at $P<0.05$.

of the rat brain, no significant effect was observed on the duration of anaesthesia compared to vehicle treatment. In contrast, when the same dose of the PDE4 inhibitor, PMNPQ, was injected in the lateral ventricle, it significantly reduced the sleeping time. Finally, significant pharmacological effects have been reported in the central nervous system (such as reversing memory deficit, preventing cerebral vasospasm, increasing adenosine formation, or protecting against ischaemic damage) with inhibitors of PDE1, 2, 3, or 5 at doses similar to those used in the present study (Rischke & Krieglstein, 1990; 1991; Yao *et al*., 1994; Hirai & Okada, 1995; Kobayashi *et al*., 1998; Khajavi *et al*., 1997; Prickaerts *et al*., 1997).

The ability of PDE4 inhibitors to reverse anaesthesia appears to be specific to the $alpha_2$-adrenoceptor mediated anaesthetic regimen. The PDE4 inhibitor, PMNPQ, had no effect on the duration of the anaesthesia induced by sodium pentobarbitone. The anaesthetic effect of sodium pentobarbitone is mediated through an enhancement of the γ-aminobutyric acid (GABA)-mediated inhibition of synaptic transmission (Fish, 1997) and consistently, the $alpha_2$-adrenoceptor antagonist, MK-912, was devoid of effect. Similar results have been reported by others with rolipram (Correa-Sales *et al*., 1992a).

Central NK_1 receptors have been shown to play a role in the induction of emesis by PDE4 inhibitors in ferrets (Robichaud *et al*., 1999). Indeed, emesis induced by various PDE4 inhibitors in ferrets was prevented by the human type selective NK_1 receptor antagonist, CP-99,994 but not by the novel quaternized non-peptide NK_1 receptor antagonist L-743,310, which is much less brain penetrant than CP-99,994 but has equivalent peripheral activity *in vivo* (Robichaud *et al*., 1999). The relevance of the model described in this study to emesis induced by PDE4 inhibitors was evaluated by studying the effect of a central administration of a tachykinin NK_1 receptor agonist, [sar^9, $Met(O_2)^{11}$]-substance P, on the duration of anaesthesia induced by xylazine/ketamine in rats. The NK_1 receptor agonist was studied at the dose of 6 μg i.c.v. since a similar dose of the PDE4 inhibitor PMNPQ produced emesis in all ferrets tested and was effective at reducing the duration of anaesthesia induced by xylazine/ketamine in rats. Consistently, when [sar^9, $Met(O_2)^{11}$]-substance P was injected in the lateral ventricle of the brain, it significantly reduced the duration of anaesthesia. Taken together, these results therefore suggest that assessing the anaesthetic reversing effect of PDE4 inhibitors in rats is a valid approach to evaluate the emetic potential of these inhibitors.

$Alpha_2$-adrenoceptor agonists, such as xylazine, are commonly used in laboratory animals alone or in combination with other agents to induce sedation, immobilization or anaesthesia (Flecknell, 1996; Fish, 1997). $Alpha_2$-adrenoceptor antagonists are known to reverse all anaesthetic regimens using xylazine (Flecknell, 1996; Sylvina *et al*., 1990; Robichaud *et al*., 2001). The hypnotic action of $alpha_2$-adrenoceptor agonists is believed to be mediated at the locus coeruleus (LC); a brain stem nucleus from which both ascending and descending noradrenergic fibres originate to innervate the central nervous system (Correa-Sales *et al*., 1992b; MacDonald & Scheinin, 1995). Using antisense technology, Mizobe *et al*. (1996) demonstrated that among the three different subtypes of $alpha_2$-adrenoceptors that are

known to exist (2A,B,C), it is the alpha$_{2A}$ subtype that is mediating the hypnotic effect in rats. In agreement with this result, mRNA coding for the alpha$_{2A}$-adrenoceptor was found to be particularly abundant in the LC (Scheinin *et al*., 1994; MacDonald & Scheinin, 1995).

Inhibition of adenylate cyclase activity is believed to play a pivotal role in the hypnotic response to alpha$_2$-adrenoceptor agonists. Correa-Sales *et al*. (1992a) have shown a dose-dependent reduction in the percentage of rats exhibiting loss of righting reflex to dexmedetomidine (an alpha$_2$-adrenoceptor agonist) following a pre-treatment with the non-hydrolysable permeant analogue of cyclic AMP, dibutyryl cyclic AMP, administered directly in the LC. Consistently, similar results were obtained in rats and in ferrets using structurally diverse PDE4 inhibitors (Correa-Sales *et al*., 1992a; Robichaud *et al*., 2001). Moreover, a significant elevation in the frequency of discharge of LC neurons and a near doubling of the cyclic AMP content in that nucleus have been reported in rats following a treatment with rolipram (Scuvée-Moreau *et al*., 1987; Correa-Sales *et al*., 1992a). Based on these results, we postulate that PDE4 is functionally coupled to the alpha$_{2A}$-adrenoceptor in the rat brain.

In summary, we characterized the anaesthetic reversing property of PDE4 inhibitors in rats. Our findings showed that this model is functionally coupled to PDE4, specific to alpha$_2$-adrenoceptor agonist-mediated anaesthesia and relevant to emesis induced by PDE4 inhibitors. Thus, we believe this model provides a novel and valid approach to evaluate the emetic potential of PDE4 inhibitors in rats. It has the advantages of being simple and rapid and it is also less expensive than the traditional emesis models (e.g. ferret, dog). Nevertheless, it should be taken into account that a reduced duration of anaesthesia is likely to be seen in this model with agents that have a general stimulatory effect on the central nervous system activity such as amphetamine or cocaine. Therefore, an additional assessment on central nervous system activity with compounds that reduce the duration of anaesthesia should be considered.

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