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ORIGINAL INVESTIGATION

# Role of calcium-independent phospholipase $A_2$ in cortex striatum thalamus cortex circuitry–enzyme inhibition causes vacuous chewing movements in rats

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**Abstract**
*Rationale* High levels of calcium independent phospholipase $A_2$ ($iPLA_2$) are present in certain regions of the brain, including the cerebral cortex, striatum, and cerebellum (Ong et al. 2005).
*Objectives* The present study was carried out to elucidate a possible role of the enzyme in the motor system.
*Methods* The selective $iPLA_2$ inhibitor bromoenol lactone (BEL), the nonselective $PLA_2$ inhibitor methyl arachidonyl fluorophosphonate (MAFP), and an antisense oligonucleotide were used to interfere with $iPLA_2$ activity in various components of the motor system. Control animals received injections of carrier (phosphate buffered saline, PBS) at the same locations. The number of vacuous chewing movements (VCM) was counted from 1 to 14 days after injection.
*Results* Rats that received BEL and high-dose MAFP injections in the striatum, thalamus, and motor cortex, but not the cerebellum, showed significant increase in VCM, compared to those injected with PBS at these locations. BEL-induced VCM were blocked by intramuscular injections of the anticholinergic drug, benztropine. Increased VCM was also observed after intrastriatal injection of antisense oligonucleotide to $iPLA_2$. The latter caused a decrease in striatal $iPLA_2$ levels, confirming a role of decreased enzyme activity in the appearance of VCM.
*Conclusions* These results suggest an important role for $iPLA_2$ in the cortex–striatum–thalamus–cortex circuitry. It is postulated that VCM induced by $iPLA_2$ inhibition may be a model of human parkinsonian tremor.

**Keywords** Calcium independent phospholipase $A_2$ · Arachidonic acid · Basal ganglia · Parkinson's disease · Tremor

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## Introduction

Phospholipases $A_2$ ($PLA_2$) constitute a superfamily of enzymes that hydrolyze fatty acids at the *sn*-2 position of phospholipids. They include the intracellular phospholipases $A_2$, i.e., calcium-dependent $PLA_2$ ($cPLA_2$) and calcium-independent $PLA_2$ ($iPLA_2$) and secretory $PLA_2$ ($sPLA_2$). The enzymes differ in their substrate specificities. $cPLA_2$ prefers arachidonic acid, whereas $iPLA_2$ has preference for linoleic acid (Yang et al. 1999) and docosahexaenoic acid (Strokin et al. 2003, 2006) at the *sn*-2 position of phospholipids. Docosahexaenoic acid containing phospholipids may be the actual substrate of

$iPLA_2$ in the brain because very little linoleic acid is present in brain phospholipids. $sPLA_2$ has no fatty acid substrate specificity. The $PLA_2$ isoforms also differ in their distribution in the brain (Yang et al. 1999). Generally, low levels of $cPLA_2$ are present in the forebrain while higher levels are present in the hindbrain and cerebellum. In contrast, high levels of $iPLA_2$ are present in the forebrain, cerebellum, and certain brainstem nuclei (Ong et al. 1999, 2005).

Elevated $PLA_2$ activity has been reported in the plasma of drug-free schizophrenic patients compared to nonschizophrenic psychiatric patients and healthy controls (Gattaz et al. 1987, 1990; Ross et al. 1997). Increased $iPLA_2$ activity is also present in a subset of bipolar I disorder patients with history of psychosis (Ross et al. 2006). Mutations in PLA2G6, encoding a calcium-independent group VI phospholipase $A_2$ were identified in infantile neuroaxonal dystrophy (INAD) and neurodegeneration with brain iron accumulation (NBIA). This discovery implicates phospholipases in the pathogenesis of neurodegenerative disorders with iron dyshomeostasis (Morgan et al. 2006).

$iPLA_2$ has been shown to affect neuronal function in culture. The $iPLA_2$ inhibitor bromoenol lactone (BEL) was found to affect long-term potentiation in hippocampal neurons (Wolf et al. 1995; Martel et al. 2006). BEL also affected AMPA receptor phosphorylation in cultured neurons (St-Gelais et al. 2004). Little, however, is known about possible functions of $iPLA_2$ in the brain. Intracerebroventricular injection of BEL inhibited nociceptive responses after facial carrageenan injection in mice (Yeo et al. 2004), but whether $iPLA_2$ could have effects on other systems is unknown. The enzyme has been localized to the nuclei of Purkinje neurons in the cerebellar cortex (Shirai and Ito 2004) and neuronal nuclei and processes in the cerebral cortex and striatum (Ong et al. 2005). The finding of high levels of expression of $iPLA_2$ in the above regions suggests that the enzyme may play a specific role the regulation of the subroutines underlying motor behavior.

Repeated administration of dopamine antagonists or cholinomimetics to rats may induce rapid vertical deflections of the lower jaw that resembles chewing but is not directed at any particular stimulus, known as vacuous chewing movements (VCM) (Iversen et al. 1980; Salamone et al. 1986). The present study was carried out using brain injections of $iPLA_2$ inhibitors and antisense oligonucleotide, to test the hypothesis that such inhibition would induce VCM in Wistar rats.

## Materials and methods

### Chemicals

BEL was purchased from Sigma-Aldrich (St. Louis, USA), methyl arachidonyl fluorophosphonate (MAFP) was purchased from Cayman Chemical Company (Michigan, USA), and benztropine was purchased from National University Hospital, Singapore. BEL is an irreversible and selective $iPLA_2$ inhibitor (Ackermann et al. 1995; Balsinde and Dennis 1997). MAFP is an irreversible inhibitor of $cPLA_2$ at low concentration, and $iPLA_2$ at high concentration (Balsinde et al. 2002; Mendes et al. 2005). Benztropine is an anticholinergic drug used to treat extrapyramidal side effects in humans.

### Oligonucleotides

A 20-base long antisense oligonucleotide corresponding to nucleotides 230–249 of the rat group VI $iPLA_2$ sequence (5′CTCCTTTACCCGGAATGGGT-3′) was used to reduce the expression of $iPLA_2$ in the rat brain. This sequence is identical to that which had previously been shown to be effective in reducing group VI $iPLA_2$ expression in the mouse (Balsinde et al. 1997), except for a single base (from C to T in position 243 underlined). The scrambled version of the sense sequence 5′-ACCCATTCCGGG TAAAGGAG-3′ was used as control as previously described (Balsinde et al. 1997), with appropriate modification. Both sense and antisense oligonucleotides contained phosphorothioate linkages to prevent nuclease degradation.

### Rats and treatment

Male Wistar rats weighing approximately 250 g each were obtained from Sembawang Animal Center and housed at a constant temperature of 25°C and a 12 h light/12 h dark cycle. They were divided into groups of four or five rats each, and treated as follows: (1) stereotaxic injections of BEL (5 μl in 0.5 nmol solution in ethanol + phosphate-buffered saline (PBS)), or vehicle to the striatum, thalamus, motor cortex, and cerebellum; (2) stereotaxic injection of MAFP (5 μl of a 0.5 or 5 nmol solution in ethanol + PBS), or vehicle to the striatum; (3) stereotaxic injection of BEL (5 μl of 0.5 or 5 nmol solution in ethanol + PBS) to the striatum plus intramuscular injection of benztropine 413 μl of a 4 mg/ml solution. Control rats were injected with BEL and intramuscular injection of PBS. (4) Stereotaxic injection of MAFP (5 μl of a 0.5 or 5 nmol solution in ethanol + PBS) to the striatum plus intramuscular injection of benztropine 413 μl of a 4 mg/ml solution. (5) Stereotaxic injection of antisense oligonucleotide to $iPLA_2$ (30 μg) in 5-μl vehicle to the striatum (or scrambled sense sequence control). Behavioral assessments were then carried out on the rats, over several days (see below). All procedures involving animals adhered to the Principles of Laboratory Animal Care, and were approved by the Institutional Animal Care and Use Committee.

### Stereotaxic injections

Rats were anesthetized by intraperitoneal injection of 50 mg/kg Nembutal and placed on a stereotaxic apparatus. A midline incision was made on the scalp and a small craniotomy was created over one of the following injection sites: motor cortex (AP 3.2 mm, L 3.5 mm, V 3.0 mm from the surface of the cortex), striatum (AP 1.0 mm, L 3.0 mm, V 5.0 mm from the surface of the cortex), thalamus (AP −2.3 mm, L 1.5 mm, V 6.0 mm from the surface of the cortex), and cerebellum (AP −11.60 mm, L 3.0 mm, V 4.0 mm from the surface of the cortex). The coordinates were chosen based on the atlas of Paxinos and Watson (1998). Compounds ($PLA_2$ inhibitors or oligonucleotides) were injected using a microliter syringe over 5 min and the needle was slowly withdrawn. The wound was closed and the animals were allowed to recover before they were returned to the cages.

### Behavioral assessment

This was carried out 1, 2, 3, 5, 7, and 14 days after injection for treatment group 1 above, and all except 14 days after injection for the other groups. The assessments were carried out at approximately the same time every morning by the same observer. The animals were placed in an empty glass tank and allowed a 2-min acclimatization period. Vacuous chewing movements (VCM) were defined as rapid, single, purposeless deflections of the lower jaw resembling normal chewing, with or without tongue protrusion but not directed at any stimuli (Andreassen and Jorgensen 2000; Andreassen et al. 2003). These were counted over a period of 20 min for each rat. Only jaw movements occurring independently of grooming were counted. Other movements associated with VCM, like, episodes of jaw tremors, yawning, hiccups, and sniffing (own observations) were also observed but not further analyzed. The mean and standard deviation for each group were analyzed by independent samples *t* test or one-way ANOVA with Bonferroni's multiple comparison post hoc test. $P<0.05$ was considered significant.

### Western blot analysis

Rats that had been injected with oligonucleotides were deeply anaesthetized with intraperitoneal injection of Nembutal (60 mg/kg), and decapitated. Blocks consisting of the striatum were dissected out and snap frozen in liquid nitrogen. Total cell extracts were prepared by homogenizing brain tissues in 10 volumes of ice-cold buffer (M-Per mammalian protein extraction kit, 1 mM EDTA and 0.25 mM DTT), centrifuged at 13,000 rpm for 20 min, and the supernatant collected. Protein concentration was measured using the Bio-Rad protein assay kit. Proteins (90 μg) injected striatum were resolved in 10% sodium dodecyl sulfate-polyacrylamide gel under reducing conditions and electrotransfered to polyvinylidine difluoride (PVDF) membrane. The molecular weight of proteins was determined using the Bio-Rad Prestained Protein Ladder (Bio-Rad, USA). Nonspecific binding sites on the PVDF membrane were blocked by incubation with 5% nonfat milk for 1 h. The PVDF membrane was then incubated overnight in an affinity-purified goat polyclonal antibody to $iPLA_2$ (Santa Cruz Biotechnology, Santa Cruz, USA). The membrane was then washed in 0.1% Tween-20 in Tris-buffered saline (TBS) and incubated with horseradish peroxidase conjugated horse anti-goat IgG (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Immunoreactivity was visualized using the Supersignal West Pico chemiluminescent substrate (Pierce Biotechnology, USA).

## Results

### Rats treated with BEL

Rats that had been injected with BEL showed vacuous chewing movements, defined as rapid deflections of the lower jaw that resembled normal chewing but not directed at any stimuli (Andreassen and Jorgensen 2000; Andreassen et al. 2003) and tongue protrusions, over several days after injection. Significantly more VCM were observed in rats that received intrastriatal injections of BEL, compared to control animals that received intrastriatal injections of PBS at 2 and 5 days after surgery (Fig. 1a).

Rats that received intrathalamic injections of BEL showed significantly more VCM compared to controls that received intrathalamic PBS injections from 1 till 7 days postinjection (Fig. 1b).

More VCM were also observed in rats that received intracortical injections of BEL compared to controls. The effect of BEL appeared to be greater than after either striatal or thalamic injections, and lasted from 2 till 14 days postinjection (Fig. 1c).

No significant difference in vacuous chewing movement was observed after injection of BEL into the cerebellum compared to controls (Fig. 1d).

### Rats treated with MAFP

No significant difference in VCM was observed in rats that received 0.5 nmol MAFP, compared to controls that had been injected with PBS (Fig. 2a). In contrast, significantly more VCM were observed in rats that received 5 nmol MAFP compared to controls at 1 and 3 days after surgery

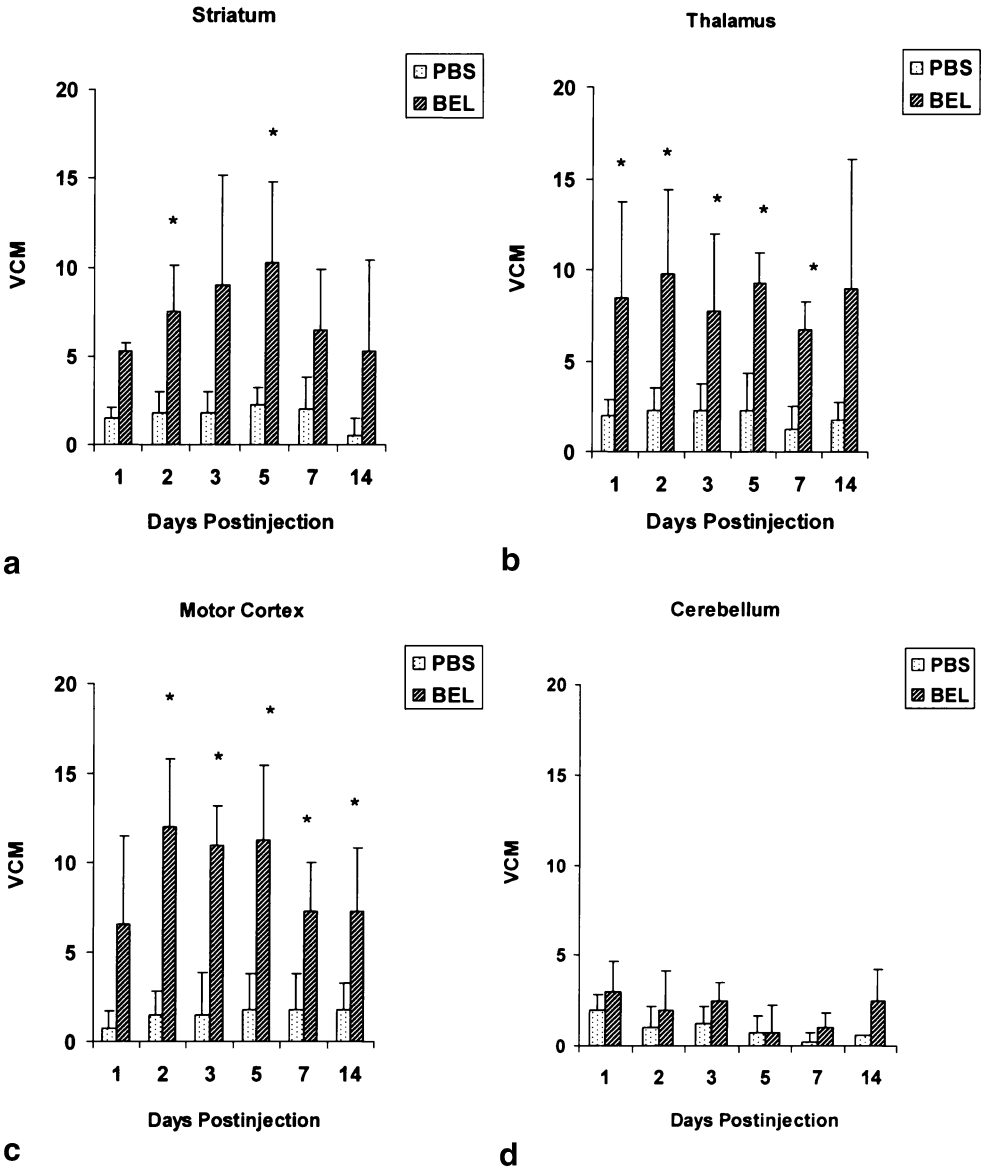


**Fig. 1** Effects of intracerebral BEL injections on VCM. **a** Intrastriatal injection, **b** intrathalamic injection, **c** intracortical injection, and **d** intracerebellar injection. An increase in vacuous chewing was observed between 2 and 5 days after injection in rats that received intrastriatal injection, intrathalamic, and intracortical, but not intracerebelllar injections of BEL. $N$=4 in each group. Data were analyzed using independent samples $t$ test. *Asterisks* indicate significant differences ($P$<0.05)

(Fig. 2b). The inhibitor MAFP inhibits calcium-dependent $PLA_2$ at low concentration and calcium-independent $PLA_2$ at higher concentration (Balsinde et al. 2002; Mendes et al. 2005). The above results therefore suggest that MAFP is causing vacuous chewing movements by inhibiting against $iPLA_2$ and not $cPLA_2$.

Rats treated with $iPLA_2$ inhibitors plus benztropine

Treatment with benztropine significantly decreased VCM in rats that have been injected with $iPLA_2$ inhibitors. Significantly fewer VCM were observed in rats that received intrastriatal injections of high concentration of MAFP plus intramuscular administration of benztropine compared to rats that have been injected with MAFP plus intramuscular injection of PBS (Fig. 3a). Similarly, fewer VCM were observed in rats that received intrastriatal injections of MAFP plus intramuscular administration of benztropine compared to rats that have been injected with MAFP plus intramuscular injection of PBS (Fig. 3b).

Rats treated with $iPLA_2$ antisense oligonucleotides

The antibody to $iPLA_2$ detected a denser 85 kDa band in total extracts of control rats and sense sequence injected rats. The intensity of this 85-kDa band was significantly decreased in striatal extracts of rats, 4 days after intrastriatal injection of antisense oligonucleotide to $iPLA_2$ (Fig. 4a). The decrease in $iPLA_2$ protein expression was correlated with increased VCM in the same animals at this time (Fig. 4b). Similar increases in VCM were also observed at 5 days postinjection in another set of animals that were monitored up to 7 days postinjection (Fig. 4c).

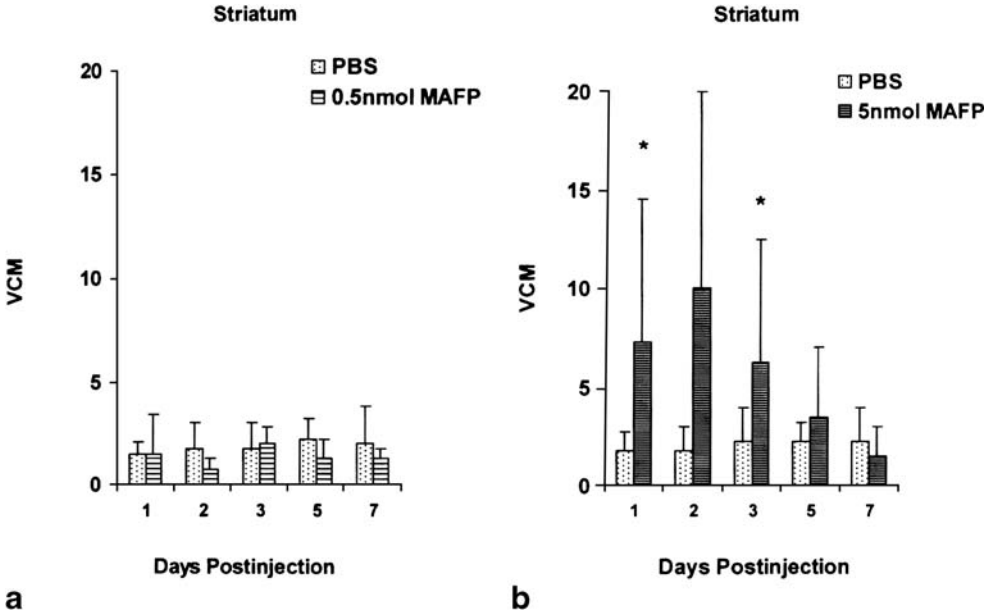


**Fig. 2** Effects of intrastriatal MAFP injection on VCM. **a** Low concentration of MAFP; **b** high concentration of MAFP. No increase in VCM was observed after injection of low concentration of MAFP, whereas significant increase in VCM was observed after high concentration of MAFP at 1 and 3 days after injection. $N$=4 in each group. Data were analyzed using independent samples $t$ test. *Asterisks* indicate significant differences ($P$<0.05)

## Discussion

High levels of expression of $iPLA_2$ are present in certain regions of the brain, including the cerebral cortex, striatum, and cerebellum (Ong et al. 2005), and the present study was carried out to elucidate behavioral effects of intracerebellar injection of $iPLA_2$ inhibitors in an attempt to better understand a possible role of the enzyme in these regions. Striatal injections of the $iPLA_2$ inhibitor, BEL, resulted in significantly increased VCM in Wistar rats from 2 to 5 days after injection. The number of VCM diminished at 1 and 2 weeks postinjection. Besides the striatum, significantly increased VCM was also observed after intrathalamic injections, after injection of BEL. The injections were directed at the approximate center of the ventral tier of thalamic nuclei, and would have affected the ventral lateral and possibly the ventral anterior and ventral posterior nuclei. The effect of intrathalamic injection appeared earlier

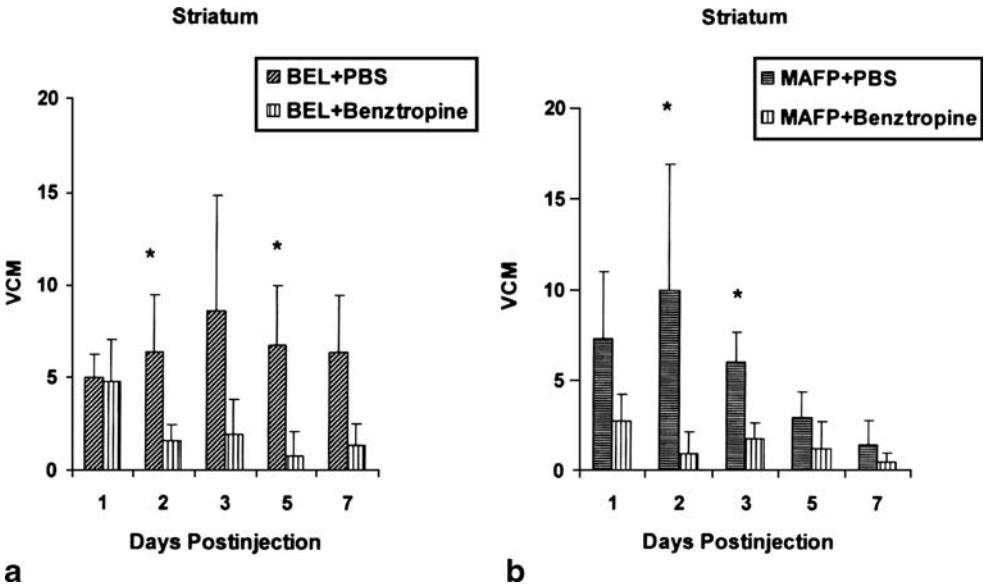


**Fig. 3** Effect of benztropine on BEL and MAFP induced VCM. **a** Significantly reduced VCM were observed in the rats that received intrastriatal injection of BEL + intramuscular injection of benztropine, compared to rats that received BEL + intramuscular injection of PBS on the second and fifth days after injection. **b** Rat that received intrastriatal injection of high concentration of MAFP + intramuscular injection of benztropine, also showed significantly reduced VCM compared to rats that received MAFP + PBS on the second and third days after injection. Injection of a lower concentration of MAFP had no effect in inducing VCM. $N$=4 in each group. Data were analyzed using independent samples $t$ test. *Asterisk* indicates significant differences ($P$<0.05)

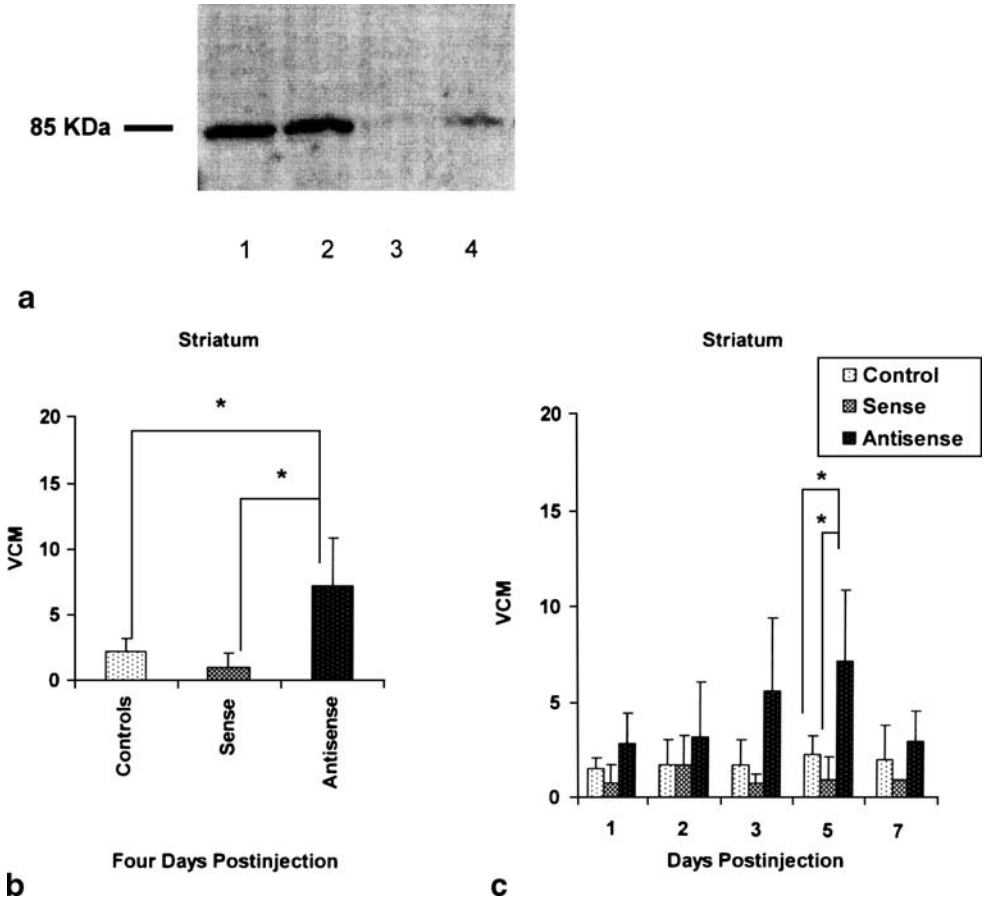


**Fig. 4** Effect of antisense and sense injection of oligonucleotides to the striatum. **a** Western blot analysis of $iPLA_2$. *Lanes 1* and *2* were loaded with total cell extracts from striatal homogenates of the control rat brains. *Lanes 3* and *4* were loaded with total cell extracts from striatal homogenates of the striatum after injection of antisense oligonucleotide to $iPLA_2$. The $iPLA_2$ antibody detects a 85-kDa band from antisense oligonucleotide extracts (*lanes 3* and *4*) but a denser 85-kDa band from control extracts (*lanes 1* and *2*). **b**, **c** An increase in VCM was observed in rats that received antisense oligonucleotide compared to those that received scrambled sense oligonucleotide or PBS, at day 5 after injection. $N=4$ in each group. Data were analyzed by one-way ANOVA with Bonferroni's multiple comparison post hoc test. *Asterisks* indicate significant differences ($P<0.05$)

and lasted longer than the intrastriatal injections, starting at 1 day after injection and lasted until 7 days after injection. Increased VCM was also detected after BEL injection into the primary motor cortex. In this instance, the effect was long lasting, and significantly increased VCM was observed even at 14 days after injection. In contrast to the above brain regions, no significant effect was observed after BEL injection into the cerebellum. Of these regions, the observation of increased VCM after BEL injection in the striatum and cortex is probably the most relevant, as they contained high levels of $iPLA_2$ immunoreactivity in the normal brain. In comparison, only low levels of $iPLA_2$ are present in the thalamus (Ong et al. 2005).

Although BEL is a potent inhibitor of $iPLA_2$, it also inhibits magnesium dependent phosphatidic acid phosphohydrolase (Balsinde et al. 2002) and diacylglycerol lipase (Moriyama et al. 1999). We therefore sought to replicate the effects of BEL using another $PLA_2$ inhibitor, MAFP. At low concentration, MAFP inhibits $cPLA_2$ but at high concentration, it inhibits both $cPLA_2$ and $iPLA_2$ (Balsinde et al. 2002; Mendes et al. 2005). Injection of low concentration of MAFP (5 μl of a 0.5 nmol solution) into the striatum resulted in no change in the number of VCM. In contrast, significantly increased VCM was observed after intrastriatal injection of high concentration of MAFP (5 μl of a 5-nmol solution). These findings suggest that increased VCM after MAFP injection was because of inhibition of $iPLA_2$ and not $cPLA_2$. The observations with BEL and MAFP point to a role for inhibition of $PLA_2$ enzymatic activity in VCM, i.e., the behaviors were not caused by the BEL or MAFP per se.

This was further confirmed using antisense oligonucleotides to $iPLA_2$. Intrastriatal injections of scrambled sense sequence of $iPLA_2$ showed no effect on VCM. In contrast, increased VCM was observed after intrastriatal injection of antisense oligonucleotides to $iPLA_2$. The latter resulted in decreased protein levels of $iPLA_2$ in the striatum in Western blots confirming a role of $iPLA_2$ inhibition in the appearance of VCM.

It is interesting to note that the VCM only appeared after injection into the striatum, thalamus, and the motor cortex and not into the cerebellum even though the latter also contained moderate levels of $iPLA_2$ (Shirai and Ito 2004; Ong et al. 2005). This suggests that VCM could be induced by specific alterations to all portions of the cortex–striatum–thalamus–cortex loop, and not only the striatum, as often assumed. No increase in VCM was observed after BEL injection into the cerebellum, which does not directly form part of the loop.

Because oral movements in rats have been observed to result from chronic administration of dopamine antagonists, it has been suggested that VCM represents an animal model of tardive dyskinesia (Ellison et al. 1987). The latter, however, is observed after chronic administration of dopamine antagonists, whereas VCM can be produced by acute or subchronic administration (Jicha and Salamone 1991; Steinpreis and Salamone 1993; Steinpreis et al. 1993; Egan et al. 1996). In this study, VCM was observed almost immediately in the days after the injection of $iPLA_2$ inhibitors. Drug-induced tremulous oral movements have been linked to human parkinsonian tremor (Salamone et al. 1998). Although the most common tremulous movements in parkinsonism typically involve the hand, evidence also indicates that tremors shown by people with idiopathic or neuroleptic induced parkinsonism can involve the jaw as an "up-and-down movement" (Victor and Ropper 2001). The observation that $iPLA_2$ inhibition-induced VCM in this study was blocked by an antimuscarinic agent (benztropine) also does not support the movement as a model of tardive dyskinesia. The latter is exacerbated by anticholinergic antiparkinsonian drugs and slightly improved by cholinomimetic drugs (Fahn et al. 1974; Burnett et al. 1980). In contrast, antiparkinsonian anticholinergic drugs and dopamine agonists suppress jaw movement activity (Steinpreis et al. 1993; Cousins et al. 1997; Salamone et al. 1998), and benztropine and other muscarinic antagonists are used to treat parkinsonian symptoms in humans (Katzenschlager et al. 2003; Lees 2005).

The molecular mechanisms by which $iPLA_2$ inhibition could lead to VCM are unknown. However, several recent studies have implicated $iPLA_2$ in neuronal function. (1) BEL has been shown to enhance in phosphorylation of the AMPA receptor subunit GluR1, but not GluR2/3, in hippocampal neurons (Menard et al. 2005a,b). Because GluR1 subunits have high calcium permeability compared to GluR2 subunits, this suggests that BEL may increase calcium influx in neurons. This might have an effect on neuronal excitability. (2) Postsynaptic injection of $iPLA_2$ inhibitors selectively increases AMPA receptor-mediated synaptic transmission (St-Gelais et al. 2004). (3) BEL has been shown to inhibit the induction of long-term potentiation in hippocampal slices (Wolf et al. 1995; Fujita et al. 2001). (4) BEL modulates intracellular membrane trafficking by inhibiting $iPLA_2$ activity in membrane tubule formation during reassembly of the Golgi complex (Brown et al. 2003). (5) In addition, BEL treatment interferes with membrane fusion events during endocytosis and exocytosis (Farooqui and Horrocks 2007). (6) Another possibility, in view of the observation of $iPLA_2$ immunoreactivity in the nucleus of neurons in the cortex and striatum (Ong et al. 2005), is that $iPLA_2$ inhibitors may affect nuclear signaling and gene expression. It has been shown that treatment of LA-N-1 neuroblastoma cells cultures with retinoic acid results in marked stimulation of $iPLA_2$, and arachidonic acid generated in the nucleus could affect neurite development in these cells (Antony et al. 2001; Farooqui et al. 2004).

Whether any of the above mechanisms could be operating to cause VCM after injection of BEL is unknown. It is, however, postulated that changes in components of the basal ganglia circuitry, e.g., the direct pathway, and/or the projection from output nuclei to thalamic nuclei and the cerebral cortex could cause the observed increase in VCM. The findings adds to previous studies, which showed VCM induction after treatment with antipsychotic drugs or dopamine D1 agonists (Ellison et al. 1988), dopamine depletors (reserpine) (Steinpreis and Salamone 1993), and cholinergic agents (e.g., pilocarpine) (Salamone et al. 1990) and points to the role of an enzyme ($iPLA_2$) and possibly its reaction products, i.e., free fatty acids and lysophospholipids, in VCM.

In summary, we have utilized the $PLA_2$ inhibitor, BEL, and $iPLA_2$ antisense oligonucleotide to obtain independent conclusive confirmation that inhibition of $iPLA_2$ induces VCM. Further studies are necessary to determine possible protein and lipid changes in the cortex–striatum–thalamus–cortex loop after $iPLA_2$ inhibition.

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