Pergamon

Biochemical Pharmacology, Vol. 47, No. 6, pp. 939–945, 1994.

0006-2952/94 $6.00 + 0.00

0006-2952(93)E0072-F

HALOTHANE AND ISOFLURANE ENHANCE BASAL AND CARBACHOL-STIMULATED INOSITOL(1,4,5)TRIPHOSPHATE FORMATION IN SH-SY5Y HUMAN NEUROBLASTOMA CELLS

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(*Received* 30 *September* 1993; *accepted* 19 *November* 1993)

Abstract—The cellular mechanisms underlying the clinical effects of volatile anaesthetics remain unknown, although the plasma membrane and its associated proteins are likely targets. One such protein is the enzyme phospholipase C (PLC), which catalyses the formation of the second messenger inositol(1,4,5)triphosphate [Ins(1,4,5)P_3]. Using SH-SY5Y human neuroblastoma cells we have demonstrated that halothane (0.50, 0.75 and 1.00%) enhances basal Ins(1,4,5)P_3 mass formation approximately 1.8-fold. Halothane also caused a dose-dependent enhancement of carbachol-stimulated biphasic Ins(1,4,5)P_3 formation at both the peak (half-maximal stimulation, EC_{50} = 0.76%) and plateau (EC_{50} = 0.74%) phases. At 1%, halothane did not alter the affinity for carbachol at either the peak (IC_{50}: air = 9.4 ± 1.5, halothane = 12.7 ± 1.0 μM) or plateau (EC_{50}: air = 11.7 ± 1.2, halothane = 11.6 ± 1.0 μM) phase, but did increase the maximum Ins(1,4,5)P_3 response at both phases (air vs halothane: peak, 79.9 ± 0.5 vs 124.8 ± 2.5; plateau, 33.2 ± 0.5 vs 47.9 ± 0.6 pmol/mg protein). Isoflurane (2%) also enhanced basal and carbachol-stimulated Ins(1,4,5)P_3 formation 2-fold and 1.5-fold, respectively. In summary, clinically relevant doses of the volatile anaesthetics halothane and isoflurane enhance basal and carbachol-stimulated Ins(1,4,5)P_3 formation. Thus, activation of PLC, and subsequent potential Ins(1,4,5)P_3-mediated rises in intracellular calcium, could play a part in the cellular mechanisms of volatile agent-induced anaesthesia.

Key words: SH-SY5Y neuroblastoma cells; volatile anaesthetics; phospholipase C; halothane; isoflurane; inositol(1,4,5)triphosphate

The process of general anaesthesia is thought to involve the depression of synaptic transmission [1], and indeed, volatile anaesthetics do inhibit neurotransmitter release [2, 3], and potentially could inhibit post-synaptic signal transduction. However, the cellular mechanisms by which volatile anaesthetics cause their clinical effects remain unknown, although the plasma membrane and its associated proteins are likely targets [3, 4]. These cellular mechanisms may involve the regulation of cAMP formation [1, 3], and the closing of both voltage-sensitive and receptor-operated ion channels [3, 5, 6], including certain types of calcium channel [2, 7–9]. These changes may modulate neurotransmission [1, 4].

Another possible cellular target protein which plays a pivotal role in cellular calcium homeostasis is PLC†, a membrane-bound enzyme whose activation causes the formation of Ins(1,4,5)P_3, and the subsequent release of calcium from intracellular stores [10]. Indeed, volatile anaesthetics are known to alter calcium homeostasis by affecting both calcium entry and the release of calcium from internal stores [9, 11]. However, both stimulatory [12–14] and inhibitory [8, 15] effects of volatile anaesthetics on agonist-induced calcium release have been reported for systems known to be mediated by Ins(1,4,5)P_3. Furthermore, what little direct evidence for the actions of volatile anaesthetics on PLC activity there is, is contradictory. In GH_3 rat pituitary cells [16] and rat brain slices [17] volatile anaesthetics had no effect on, whilst in A7r5 vascular smooth muscle cells they inhibited [18, 19], total [^{3}H]inositol polyphosphate accumulation in the presence of lithium.

In this study we have used the SH-SY5Y human neuroblastoma cell line, which has a well-characterized muscarinic receptor-coupled polyphosphoinositide-Ca^{2+} signalling system, to examine the effects of the volatile anaesthetic agents halothane and isoflurane in Ins(1,4,5)P_3 formation, and report for the first time in a neuronal preparation that volatile anaesthetics enhance both basal and muscarinic-stimulated formation of this polyphosphate.

MATERIALS AND METHODS

Cell culture and harvesting. SH-SY5Y human neuroblastoma cells (passages 60–90) were cultured in minimum essential medium with Earle's salts supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone and 10% foetal calf serum (Gibco, Uxbridge, U.K.). For [^{3}H]inositol phosphate accumulation studies the media was also sup-

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† Abbreviations: PLC, phospholipase C; Ins(1,4,5)P_3, inositol(1,4,5)triphosphate; AVP, arginine vasopressin; TRH, thyrotropin-releasing hormone; PIP_2, phosphatidylinositol 4,5-biphosphate.

plemented with 4 μCi/mL *myo*-[^{3}H]inositol (20 Ci/mmol; Amersham, U.K.) for 72 hr prior to harvesting..

Cells were harvested with 10 mM HEPES-buffered saline/0.02% EDTA pH 7.4, washed twice with, and then resuspended in, Krebs/HEPES buffer pH 7.4, of the following composition (mM): Na^+ (143.3), K^+ (4.7), Ca^{2+} (2.5), Mg^{2+} (1.2), Cl^- (125.6), HCO_3^- (25), $H_2PO_4^{2-}$ (1.2), SO_4^{2-} (1.2), glucose (11.7) and HEPES (10). For [^{3}H]inositol phosphate accumulation studies the buffer was supplemented with 4 μCi/mL [^{3}H]inositol (which had previously been cleaned by passing through a small DOWEX chloride 100–200 mesh column).

Measurement of inositol phosphates. Whole cell suspensions ([^{3}H]inositol-labelled, in the presence of 5 mM Li^+, or unlabelled: final volume 0.3 mL) were pre-incubated at 37° for 15 min. The cell suspensions were then incubated at 37° in the presence or absence of carbachol (0.3–1000 μM) for 0–300 sec. Some of the cell suspensions were gassed throughout the pre-incubation and incubation periods with humidifed air (1.5 L/min) or humidified air containing halothane (0.25–5% v/v) or isoflurane (2% v/v), delivered via a precalibrated Fluotec 3 vaporiser. Reactions were terminated by the addition of 0.3 mL 1 M trichloroacetic acid. Inositol phosphates were extracted with Freon/octylamine (1:1 v/v) and neutralized with 25 mM $NaHCO_3$. The amount of volatile anaesthetic agent delivered was checked regularly using a Capnomac [20]. In addition, measured using gas chromatography [21] the buffer concentration of halothane reached equilibrium by 5–10 min. After a 15 min pre-incubation with 1, 2 and 3% halothane the aqueous concentrations were 0.238, 0.484 and 0.876 mM [3]. Total [^{3}H]inositol phosphate accumulation was quantified using DOWEX chloride (200–400 mesh) columns, as described previously [22].

Ins(1,4,5)P_3 was assayed using bovine adrenocortical binding protein and [^{3}H]Ins(1,4,5)P_3 (41 Ci/mmol; Amersham) at 4°. Authentic Ins(1,4,5)P_3 (0.036–12 pmol; Siemat, U.K.) in buffer, taken through an identical extraction process, was used as a standard. Non-specific binding was defined in the presence of excess Ins(1,4,5)P_3 (0.3 nmol). Bound [^{3}H]Ins(1,4,5)P_3 was separated by rapid vacuum filtration [23].

Data analysis. All data are given as means ± SEM unless otherwise stated. EC_{50} (half-maximal stimulation) values were obtained using unweighted least squares linear regression with GRAPHPAD. Curves were fit to four parameters, max, min, EC_{50} and slope factor. Statistical comparisons were made where appropriate by Student's *t*-test, and/or ANOVA, and considered significant when $P < 0.05$.

RESULTS

Carbachol caused a biphasic stimulation of Ins(1,4,5)P_3 formation (Fig. 1), which rose from basal (6.25 ± 0.17 pmol/mg protein) to a peak (12.8-fold) at 10 sec and then declined until 60 sec to a steady plateau phase (3-fold), which was maintained until 300 sec when sampling ended. This stimulation of Ins(1,4,5)P_3 formation by carbachol was dose dependent, being significantly increased from basal formation at 1 μM and above, at both the peak and plateau phases (Fig. 1 insert), with an EC_{50} of 21.4 ± 1.0 and 14.1 ± 1.0 μM, respectively. The peak phase, which releases calcium from the intracellular stores, is calcium independent, whilst the plateau phase, which may or may not release calcium, is dependent on the influx of extracellular calcium [24].

In preliminary studies, carbachol had stimulated [^{3}H]inositol phosphate accumulation in Li^+-treated cells by 3.2-fold after 5 min, but halothane (1% v/v) had no effect on this, or basal, turnover (Table 1). In contrast, halothane enhanced basal Ins(1,4,5)P_3 mass formation in a time- and dose-dependent manner (Table 2), with 0.75% and 1% enhancing Ins(1,4,5)P_3 formation 1.8-fold, with an estimated $T_{1/2}$ of 115 and 75 sec, respectively. Furthermore, halothane enhanced 1 mM carbachol-stimulated Ins(1,4,5)P_3 formation in a dose-dependent manner at both the peak and plateau phases by 1.6-fold (Fig. 2), with an EC_{50} of 0.76% and 0.74% (v/v), respectively.

Halothane (1% v/v) increased the maximum Ins(1,4,5)P_3 response to, without altering the EC_{50} for, carbachol at both the peak and plateau phases (Fig. 3, Table 3). Gassing with air alone increased the maximum response by 17% at the peak and 23% at the plateau, probably due to increased agitation. However, halothane in air produced a greater enhancement (56% at the peak and 54% at the plateau) than air alone (Table 3).

Isoflurane (2% v/v) also enhanced basal and carbachol-stimulated Ins(1,4,5)P_3 formation, by 2-fold and 1.5-fold (at both the peak and the plateau), respectively (Fig. 4). This degree of enhancement is similar to that seen with halothane.

DISCUSSION

We report here for the first time in a neuronal preparation that the volatile anaesthetic agents halothane and isoflurane enhance both basal and muscarinic receptor-stimulated PLC activity, and the subsequent formation of the second messenger Ins(1,4,5)P_3, at clinically relevant doses [25]. Moreover, increased concentrations of Ins(1,4,5)P_3 have been implicated in a number of cellular processes, most notably the control of calcium homeostasis by the release from intracellular stores and possibly regulation of calcium entry [10].

In the current study both halothane and isoflurane enhanced basal Ins(1,4,5)P_3 formation, at clinically relevant concentrations [25]. Volatile anaesthetics do increase intracellular calcium in several cell types, although it is often hard to discern whether this is due to release from intracellular stores or calcium influx [9]. However, two recent studies have shown that halothane causes a transient rise in intracellular calcium which may be Ins(1,4,5)P_3 mediated, as it is extracellular calcium independent [11, 18]. However, in the earlier study [18] halothane had no effect on basal total [^{3}H]inositol polyphosphate accumulation, although this was measured at a different time to the calcium concentrations. Furthermore, we have shown in this study that the relatively small rises in basal Ins(1,4,5)P_3 formation

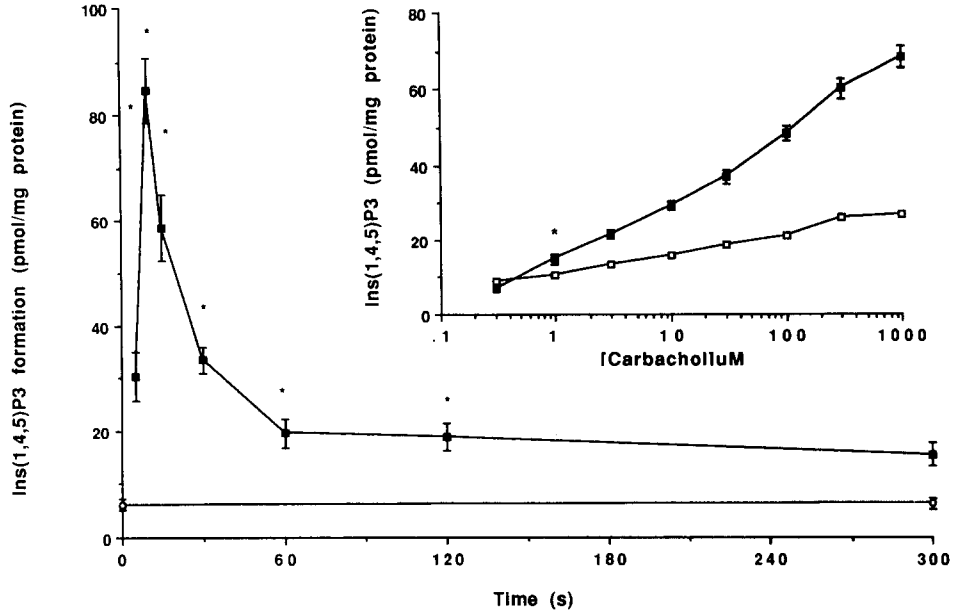

Fig. 1. Time course of the biphasic stimulation of Ins(1,4,5)P_3 formation by 1 mM carbachol (■), compared to basal (○). The insert shows the carbachol dose–response curve at the peak (■) and the plateau (□) phases. Whole cell suspensions (final volume 0.3 mL) were pre-incubated at 37° for 15 min, and then incubated with carbachol (0.3–1000 μM) for 0–300 sec. Ins(1,4,5)P_3 was measured by a specific radio-receptor mass assay. All results are means ± SEM, where N = 4–5. Time course from 5 to 120 sec, and both dose–response curves, $P < 0.05$ by ANOVA. *Denotes $P < 0.05$ significantly increased compared to basal (only first significant point shown on dose–response curves).

Table 1. Halothane has no effect on total [^{3}H]inositol phosphate accumulation in Li^+-treated SH-SY5Y cells

	dpm/mg protein	
	Air	1% Halothane
Basal, 0 sec	1463 ± 38	1535 ± 85
Basal, 300 sec	1475 ± 36	1547 ± 54
1 mM Carbachol, 300 sec	4705 ± 228*	4331 ± 136*

Values are means ± SEM, N = 3. * $P < 0.05$ increased compared to basal.

Table 2. Halothane causes a time- and dose-dependent enhancement of basal Ins(1,4,5)P_3 formation in SH-SY5Y cells following a 15 min pre-incubation

Time (sec)	% Halothane (v/v)		
	0.5	0.75	1.0
0	102.7 ± 9.9	90.0 ± 10.9	108.4 ± 5.3
75	103.6 ± 10.0	123.4 ± 22.4	147.1 ± 11.0*
150	104.4 ± 10.3	166.3 ± 20.2*	173.9 ± 15.3*
225	148.5 ± 9.6*	174.4 ± 15.3*	186.2 ± 7.5*
300	176.0 ± 8.1*	194.7 ± 8.2*	178.1 ± 6.9*

Values, % of air control (=100%), are means ± SEM (N = 5). * $P < 0.05$ compared to air.

caused by halothane, seen with the specific radio-receptor mass assay, were not detected by the measurement of total inositol polyphosphate accumulation in the presence of lithium. This disparity between total [^{3}H]inositol polyphosphate turnover and Ins(1,4,5)P_3 mass formation may be due to both the relatively small stimulation caused by volatile anaesthetics and the minor contribution of Ins(1,4,5)P_3 to total polyphosphate turnover. It is worth noting that halothane has also been linked to rises in basal Ins(1,4,5)P_3 formation in hepatocytes [9, 12]. Moreover, if Ins(1,4,5)P_3 is increased, then diacylglycerol will also be increased, implying activation of protein kinase C [10] by volatile anaesthetic agents. However, halothane has been shown to inhibit protein kinase C [26].

Carbachol caused a biphasic increase in Ins(1,4,5)P_3 formation, with a peak at 10 sec and a plateau phase extending to 300 sec (when sampling ended). This response was dose dependent at both the peak and plateau phase. Moreover, the EC_{50} values were <2-fold different, surprisingly less than the approximately 10-fold difference in the EC_{50} values between the two phases for carbachol-induced rises in intracellular calcium concentrations [27].

Halothane caused a dose-dependent enhancement of carbachol-stimulated Ins(1,4,5)P_3 formation at both the peak and plateau phases, with EC_{50} values of 0.76% and 0.74% (v/v), respectively. These values correspond very well with the MAC (minimum alveolar concentration which produces a lack of reflex response to a surgical incision in 50% of patients [25]) for halothane, of 0.75% [3]. Alterations

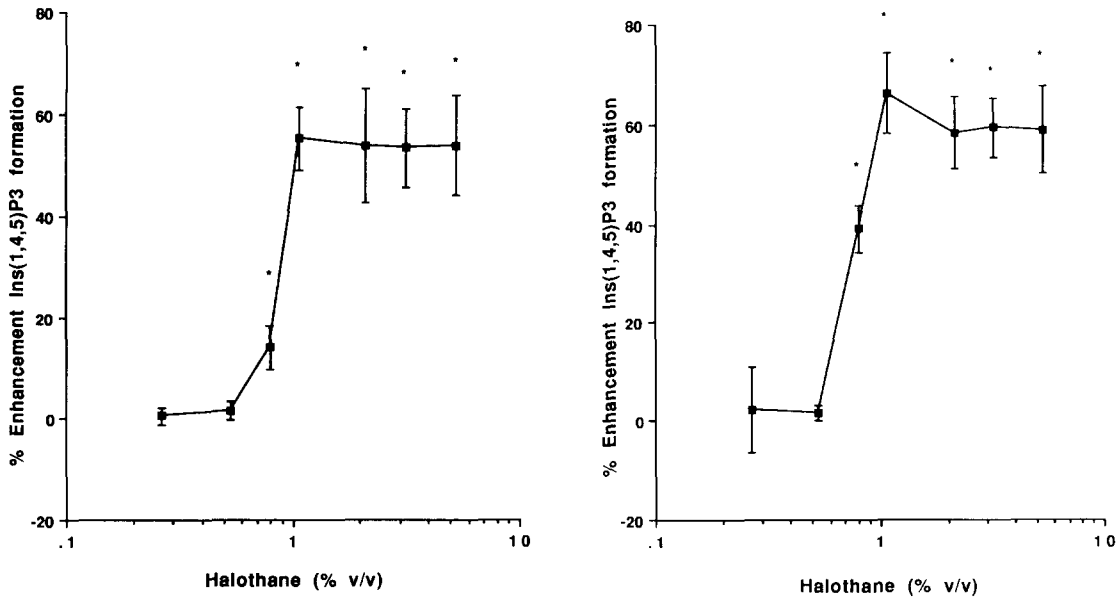

Fig. 2. Halothane causes a dose-dependent enhancement of carbachol-stimulated Ins(1,4,5)P_3 formation in SH-SY5Y cells. The left panel shows the dose–response curve at the peak phase (10 sec), whilst the right panel shows the dose–response curve at the plateau phase (300 sec). Whole cell suspensions (final volume 0.3 mL) were pre-incubated at 37° for 15 min, and then incubated with 1 mM carbachol for 10 or 300 sec, whilst being gassed with air or air containing halothane (0.25–5% v/v) throughout. Ins(1,4,5)P_3 was measured by a specific radio-receptor mass assay. All results are means ± SEM, where N = 5. Whole curve $P < 0.05$ by ANOVA. *Denotes $P < 0.05$ significantly increased compared to air control.

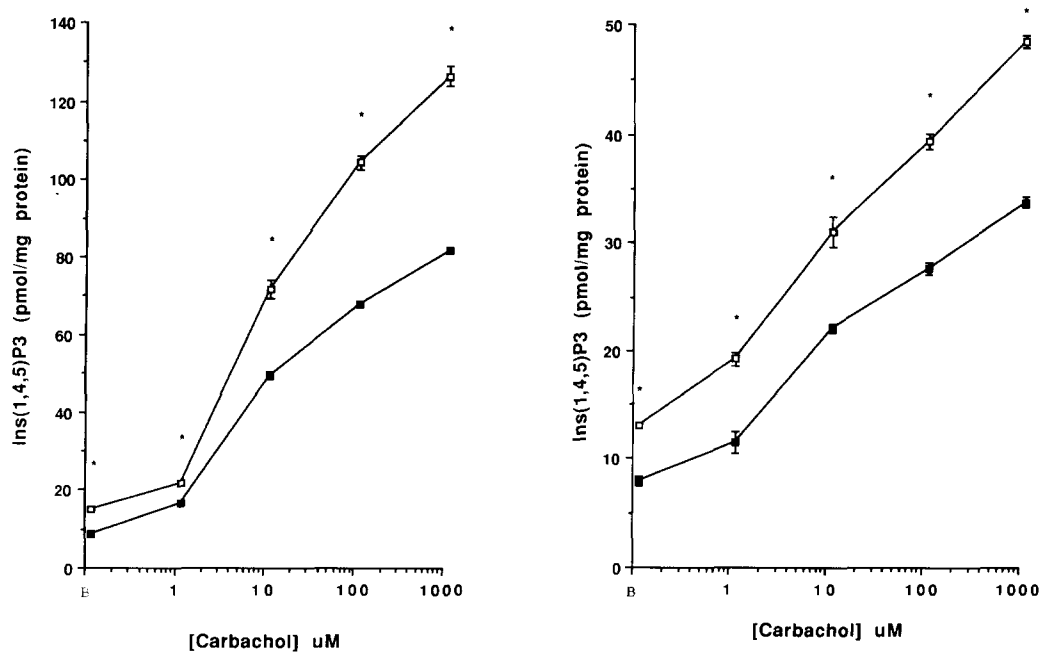

Fig. 3. Halothane enhances the dose-dependent stimulation of Ins(1,4,5)P_3 formation by carbachol in SH-SY5Y cells. The left panel depicts the carbachol dose–response curve with (□) or without (■) halothane at the peak phase (10 sec), whilst the right panel depicts the curves at the plateau phase (300 sec). B denotes Basal Ins(1,4,5)P_3 formation. Whole cell suspensions (final volume 0.3 mL) were pre-incubated at 37° for 15 min, and then incubated with or without carbachol (1–1000 μM) for 10 or 300 sec, whilst being gassed with air or air containing halothane (1% v/v) throughout. Ins(1,4,5)P_3 was measured by a specific radio-receptor mass assay. All results are means ± SEM, where N = 5. Whole halothane curve $P < 0.05$ by ANOVA. *Denotes $P < 0.05$ increased compared to air control.

Table 3. Halothane enhances the maximum Ins(1,4,5)P_3 response to carbachol in SH-SY5Y cells

	EC_{50} (μM)	Maximum response (pmol/mg protein)
Ungassed, peak	21.4 ± 1.0	68.3 ± 2.8
Ungassed, plateau	14.1 ± 1.0	26.9 ± 0.6
Air, peak	9.4 ± 1.5	79.9 ± 0.5*
Air, plateau	11.7 ± 1.2	33.2 ± 0.5*
Halothane, peak	12.7 ± 1.0	124.8 ± 2.5†
Halothane, plateau	11.6 ± 1.0	47.9 ± 0.6†

Values are means ± SEM, N = 4–5.
* Increased $P < 0.05$ compared to ungassed control.
† Increased compared to paired air control.

in Ins(1,4,5)P_3 formation could lead to changes in calcium homeostasis and thus might possibly affect neurotransmission [4, 10]. Isoflurane also enhanced carbachol-stimulated Ins(1,4,5)P_3 formation. In addition, in preliminary studies, halothane caused a dose-related enhancement of cAMP formation in SH-SY5Y cells [28].

These current findings are indirectly supported by data from PC12 variant cells (which lack nicotinic receptors), where halothane enhanced the Ins(1,4,5)P_3-mediated rise in intracellular calcium following carbachol stimulation [13]. Furthermore, in astrocytes both halothane and isoflurance caused the closure of gap junctions, an effect associated with increases in Ins(1,4,5)P_3 formation [14]. However, in A10 vascular smooth muscle cells [11] and endothelial cells [15] halothane and isoflurane inhibited the Ins(1,4,5)P_3-mediated rise in intracellular calcium following AVP or bradykinin stimulation. In addition, both halothane and isoflurane inhibited AVP-induced total inositol polyphosphate accumulation in the presence of lithium in A7r5 vascular smooth muscle cells [18, 19]. Stern *et al.* [16] also failed to show an effect of halothane on total polyphosphoinositide turnover in GH_3 cells stimulated with TRH. Indeed, we were unable to detect the halothane-induced enhancement in carbachol-stimulated Ins(1,4,5)P_3 formation (as seen using the radio-receptor mass assay) using total inositol phosphate accumulation in lithium-treated cells.

We have shown that this enhancement of carbachol-stimulated Ins(1,4,5)P_3 formation by halothane does not involve changes in the affinity for carbachol. Bazil and Minneman [17] also failed to show an effect of halothane on muscarinic receptor binding by carbachol. Nor did halothane have an effect on TRH receptor binding [16]. Indeed, in general, volatile anaesthetics seem to have little or no effect on agonist binding [3, 4].

One potential mechanism for these stimulatory effects of halothane and isoflurane on both basal and agonist-induced PLC activity is the increase in membrane fluidity caused by volatile anaesthetic agents [29, 30]. Basal Ins(1,4,5)P_3 formation results from random interaction of PLC and the membrane-bound substrate PIP_2 [10], and an increase in fluidity would allow PLC and PIP_2 to move more freely and thus interact more often. Agonist-induced Ins(1,4,5)P_3 formation could also be enhanced by an increase in membrane fluidity, but this is unlikely to be due to changes in agonist–receptor coupling,

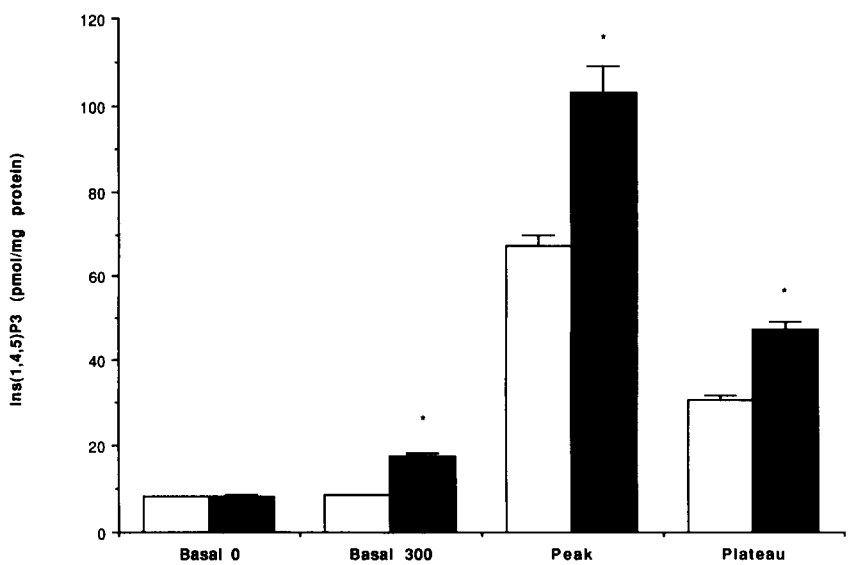

Fig. 4. Isoflurane enhances Ins(1,4,5)P_3 formation in SH-SY5Y cells. (□) Air control, (■) +2% isoflurane. Whole cell suspensions (final volume 0.3 mL) were pre-incubated at 37° for 15 min, and then incubated in the presence or absence of 1 mM carbachol for 0–300 sec, whilst being gassed with air or air containing isoflurane (2% v/v) throughout. Ins(1,4,5)P_3 was measured by a specific radio-receptor mass assay. All results are means ± SEM, where N = 5. *Denotes $P < 0.05$ increased compared to air control.

as the affinity for carbachol was not affected by halothane. However, the increase in membrane fluidity could enhance the interaction between the G-protein and PLC, and/or PLC and PIP_2. It is possible that the increased Ins(1,4,5)P_3 formation could result from volatile anaesthetic agent-induced reduction in protein kinase C activity [see 26] if this enzyme were exerting an inhibitory influence on receptor-derived PLC activity in these cells. Indeed, we have reported that acute phorbol ester treatment reduced carbachol-stimulated total inositol phosphate turnover in SH-SY5Y cells [31]. However, this hypothesis would not explain the observed enhancement of basal Ins(1,4,5)P_3 formation. In view of the fact that a decrease in awareness would be more likely to occur as a result of decreased Ins(1,4,5)P_3 formation, a general non-specific effect of volatile anaesthetic agents on membrane fluidity must also be considered. Yet, if this were the case these agents should cause a general increase in Ins(1,4,5)P_3 formation, inconsistent with the published literature.

In conclusion, this study reports for the first time that halothane and isoflurane enhance both basal and muscarinic-stimulated PLC activity in human neuroblastoma cells at concentrations required to produce clinical anaesthesia.

Acknowledgements—We thank R. A. Hirst for measurement of halothane concentration by gas chromatography. Financial support from Leicestershire Health Authority is also gratefully acknowledged.

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