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HALOTHANE MEDIATED INHIBITION OF CALMODULIN-NEUROPEPTIDE BINDING

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ABSTRACT

Binding of volatile anesthetics by proteins has been proposed as a mechanism of anesthesia . There is no evidence, however, that anesthetics at clinically relevant concentrations can interact directly with lipid - free preparations of receptors or signal - transducing proteins. Calmodulin is a key regulator of metabolism in neurons and muscle cells . This study describes the effects of halothane on the binding of the neuropeptide vasoactive intestinal peptide (VIP) by calmodulin . It was found that : (1) Saturable complexation of (tyr^{10}-^{125} I)VIP by calmodulin was absolutely Ca^{2+}-dependent, requiring mM metal concentrations for optimal binding. (2) Halothane produced a dose - dependent, biphasic inhibition of VIP - calmodulin complexation . Relatively low concentrations of halothane (0.15-0.27 mM) inhibited the complexation strongly up to 67% inhibition. (3) The inhibitory concentrations of halothane did not influence binding of VIP by monoclonal antibody .

It could be concluded that calmodulin may be the target molecule for halothane and that the mechanism of anesthesia by halothane involves direct interaction with cellular signal-transducing proteins .

INTRODUCTION

The hypothesis that volatile anesthetics act directly on or bind specifically to membrane proteins remains controversial [1] .

Historically, three principal methods have been used to probe for sites and mechanisms of action of drugs : analysis of structure-activity relationships, functional studies, and radioligaand binding assays [2] .

Structure-activity studies of anesthetics, as characterized by the Meyer - Overton relationship[3] have broadly defined the molecular target to be hydrophobic, but they have not provided evidence distinguishing between a direct lipid or protein site of action. Functional studies, on the other hand, seem to point to anesthetic effect at multiple protein sites [4] . However, because the function of many proteins depends on membrane lipid, determination of the direct anesthetic site remains ambiguous[5] .

Radioactive ligand binding techniques, which are powerful methods for identifying and characterizing pharmacological sites of action have not been used to study the inhalational anesthetics, primarily because of their low affinity, rapid kinetics, high vapour pressure and absence of chemical antagonists[1] .

In the present study, the effect of halothane at clinically relevant concentrations has been studied on the binding of the neuropeptide VIP by calmodulin as well as its effect on the binding of VIP by monoclonal antibody.

MATERIALS AND METHODS

Peptides : Synthetic VIP was purchased from Bachem . Iodination of synthetic VIP and purification and identification of (Tyr^{10} - ^{125} I) VIP were as described by Paul et al[6]. The radiolabeled peptide was stored (-80° C) at a concentration of approximately 40nM in 0.1 N acetic acid supplemented with 0.25% bovine serum albumin and was used for assays for up to 8 weeks from the date of preparation .

Lipid free porcine brain calmodulin was purified by reversed - phase HPLC [7] .

Binding and Cross - linking : ^{125}I VIP was permitted to bind calmodulin in an atmosphere of nitrogen or halothane and 22-26 Kd_a complexes covalently-crosslinked with an NH_2- directed bifunctional reagent were separated by sodium dodecyl sulphate (SDS) - gel electrophoresis on phast gradient (8-25 %; pharmacia LKB Biotechnology Inc. followed by autoradio-graphy and measurement of relative optical density (arbitrary units) in the 22-26 Kd_a bands[7]. Halothane concentrations were determined by gas chromato-graphy[8] .

Radio-labeled VIP binding by a monoclonal antibody (C 23. 5) was estimated by precipitation of immune complexes with polyethylene glycol [9] .

Data analysis : All assays were performed in duplicate and values obtained from six separate experiments were used to calculate means and standard errors using student test [10] .

RESULTS

^{125}I VIP-calmodulin complexation in atmosphere of 0.5 % halothane gave rise to significantly $P < 0.05$ biphasic inhibition of binding up to 53.8% in calcium concentration of 100 μM in the final assay (Fig . "1", table "1"). Halothane 1-2% inhibited ^{125}I VIP-calmodulin binding by 56% & 67% respectively, in calcium concentration near saturation in the final assays fig.(2), fig. (3) and table (1).

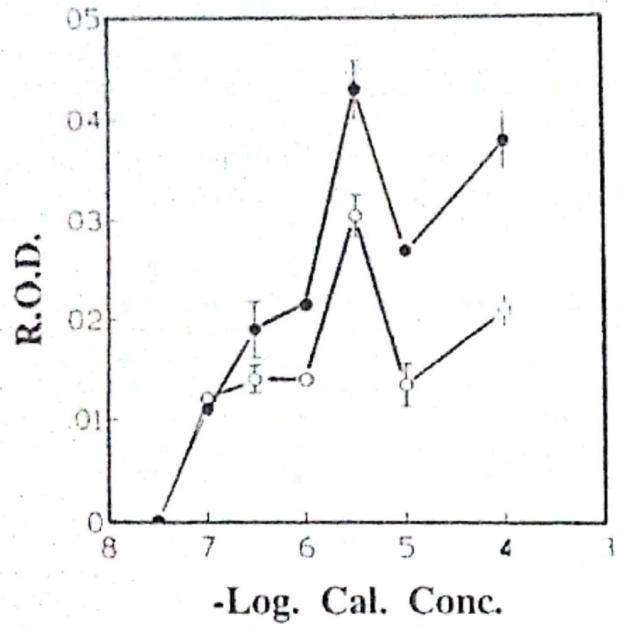

Fig. (1) : VIP-CAM. Binding in Nitrogen vs Halothane 0.5%.
● Binding in Nitrogen
○ Binding in Halothane 0.5%.
The points plotted represent the mean + SEM of six separate experiments ($P < 0.05$).

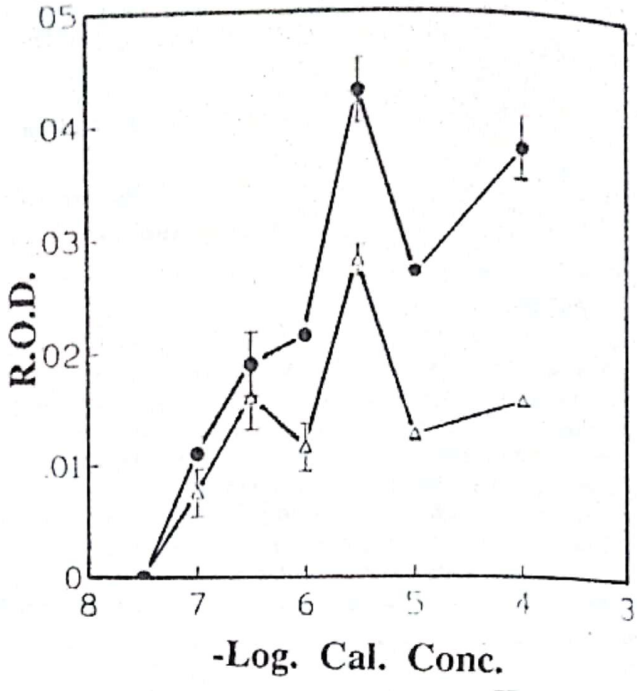

Fig. (2): VIP-CAM. Binding in Nitrogen vs Halothane 1.0%.
● Binding in Nitrogen
□ Binding in Halothane 1.0%.
The points plotted represent the mean + SEM of six separate experiments ($P < 0.05$).

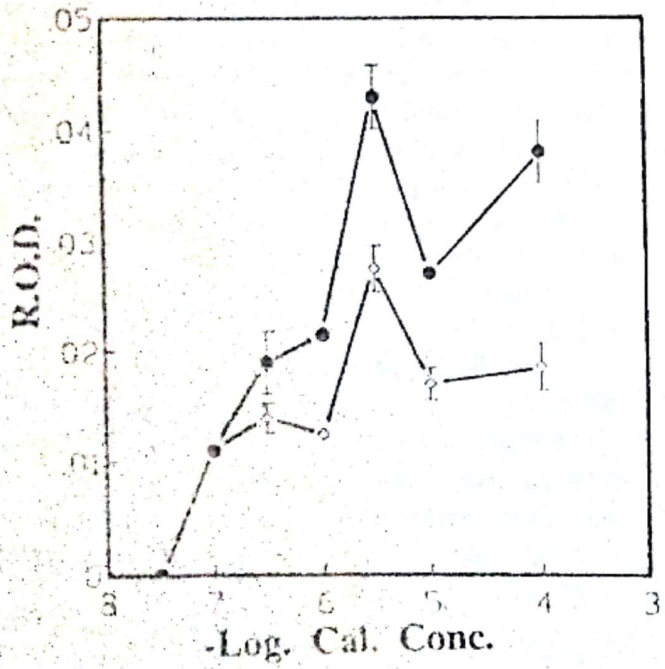

Fig (3): VIP-CAM. Binding in Nitrogen vs Halothane 2.0%.
● Binding in Nitrogen
Δ Binding in Halothane 2.0%.
The points plotted represent the mean + SEM of six separate experiments ($P < 0.05$).
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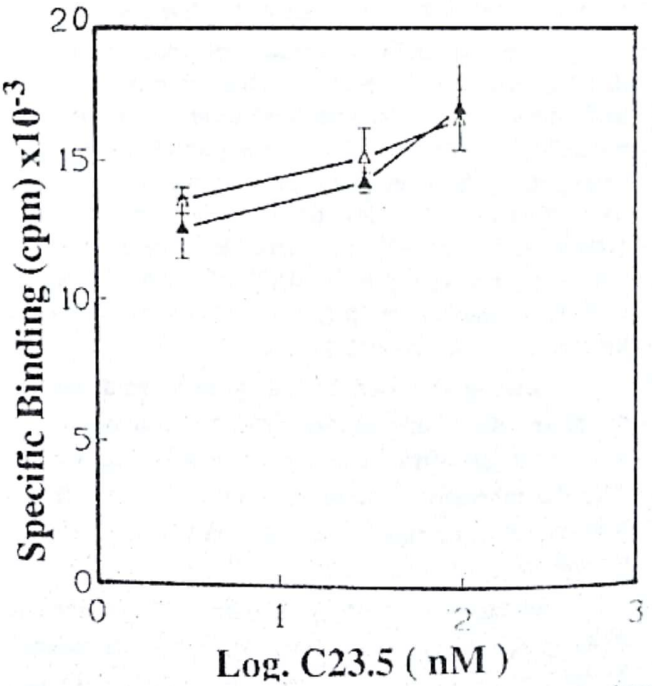

Fig. (4) : Monoclonal (C23.5) antibody - VIP Binding in nitrogen vs Halothane 0.5%.
Δ Binding in Nitrogen
Δ Binding in Halothane 0.5%.
The points plotted represent the mean ± SEM of six separate experiments ($P > 0.05$).

Zagazig J. Pharm. Sci. December. 1996
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The inhibitory concentrations of halothane did not influence binding of VIP by a monoclonal antibody (C23.5) fig (4) and fig. (5). This indicated that calmodulin was the target molecule and not VIP for the effect of different concentrations of halothane .

The concentrations of halothane in the reagent of the binding assays were determined by gas chromatography table (2) . The concentrations obtained were clinically relevant [11] .

Table (1) ^{125}1 VIP-calmodulin complexation inhibition in different halothane and0 calcium concentrations (Mean + S.E)

Halothane % / % Inhibition of binding+ SEM	Calcium Concenrations		
	1 μM	10 μM	100 μM
0.5	40.9 ± 2.3	57.1 ± 3.1	53.8 ± 1.7
1.0	40.8 ± 1.9	42.8 ± 2.1	56.4 ± 2.9
2.0	50.3 ± 2.4	55.5 ± 3.2	67.2 ± 2.3

All values are significantly different from control $P < 0.05$.

Table (2) Halothane concentration as determined by gas chromatography . (Mean+ S.E) n = 6

Halothane %	Halothane concentration
0.5	0.154 ± 0.08
1.0	0.271 ± 0.11
2.0	0.395 ± 0.14

The values obtained are the mean + SEM of six separate experiments .

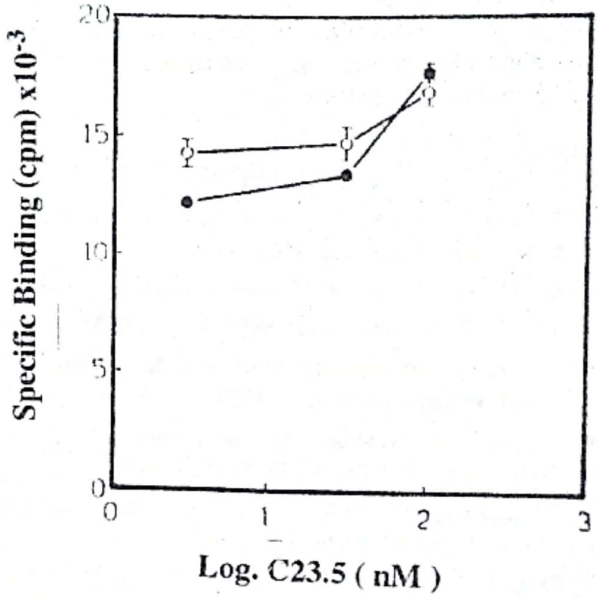

Fig. (5) : Monoclonal ($C_{23.5}$) antibody - VIP Binding in nitrogen vs Halothane 1.0% .
● Binding in Nitrogen
○ Binding in Halothane 1.0%.
The points plotted represent the mean + SEM of six separate experiments ($P > 0.05$) .

DISCUSSION

The binding assay approach to the characterization of halothane or other inhalational anesthetic binding sites has been neglected because of the low affinity of any apparent binding and the high volatility of the agents[12]. This approach has also been unattaractive because of difficulties in conceiving of a common and specific functional site that could accommodate the large variety of structures capable of producing anesthesia[13]. Nevertheless, recent studies have suggested that the halocarbon anesthetics could produce their effect by a direct interaction with protein[14]. as opposed to an indirect & nonspecific effect on membrane lipid . The calcium-calmodulin system is a key signal transducing pathway that regulates the activity of several membrane-bound and sytosolic enzymes, including adenylcyclase, cyclic nucleotide phosphodiesterase, Ca^{2+}-ATP ase, protein kinase, and myosin light chain kinase[15] .

Calmodulin has also been implicated in the release of neuropeptides by exocytosis[16] . In the present study Ca^{2+}-dependent inhibition of VIP-calmodulin complexation by halothane and its inability to inhibit VIP - antibody binding suggest that calmodulin is the target molecule for halothane . The inhibitory effect was observed at halothane concentrations (0.15 mM & 0.27 mM, equivalent to 0.5 & 1.0 vol .%, respectively) in the range producing anesthesia *in vivo*, providing support for the hypothesis that halothane interaction with calmodulin is clinically relevant. The mechanism of the interaction is likely to be complex, involving quaternary complexation of the anesthetic, calmodulin, Ca^{2+} and calmodulin binding proteins . Sush an interaction is expected to modify the conformational structure of calmodulin and its ability to bind calcium & subsequently to bind VIP . This interpretation is in agreement with that previously obtained[11] . Who stated that anesthetics induce coformational changes in the substrates of enzymes and impaired their functions . The results of this study may explain Also the initial activation of prtein kinase C (PKC) by halothane and the following attenuation of PKC - dependent effects seen by Hemming and Adamo,[17] . Such an effect which may be due to halothane modification of Ca^{2+} - calmodulin binding . Although Ca^{2+} is necessary for

potent inhibitor of PKCA[18,19]. These observations support the possibility that the mechanism of anesthesia by halothane involves direct interaction with cellular signal transducing proteins.

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تثبيط الهالوثان لارتباط الكالموديولين مع البروتينات العصبية

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من الفروض وجود علاقة ارتباط بين التحدر العمومى الغازى والبروتينات العصبية كمستقبلات تتم من خلالها عملية التحدير الكلى ولكن لا توجد دلائل واضحة على ذلك لأسباب عدة أهمها عدم وجود أدوية مضادة تمنع التحدر من عمله بالاضافة الى سرعة حركية هذه المواد والضغط العالى للازم لاثبات ذلك .

وفى هذه الدراسة تم عمل نموذج لاثبات ذلك من خلال عملية ارتباط الكالموديولين مع البروتين المعوى الوعائى النشط فى وجود الهالوثان وفى عدم وجوده . ثم اجراء تجارب لاثبات أيا منهما الذى تأثر بالهالوثان وذلك من خلال ارتباط مادة السيروتين المعوى الوعائى النشط مع الأحساس المضادة الخاصة به فى وجود الهالوثان من عدمه . وقد أثبتت هذه الدراسة أن ارتباط الكالموديولين مع البروتين المعوى الوعائى النشط يضعف ويقل بنسبة تصل الى ٦٠ ٪ فى وجود الهالوثان بينما عملية ارتباط البروتين المعوى الوعائى النشط مع الأحساس المضادة الخاصة به لا تتأثر بالهالوثان من عدمه وهذا ما يؤكد تأثر حزيء الكالموديولين وليس حزيء البروتين المعوى الوعائى النشط بالهالوثان .

وعليه فان حزيء الكالموديولين قد يكون هو المستقبل لعمل الهالوثان وتؤدى عملية الارتباط هذه الى سلسلة من الاتفاعلات الكيميائية داخل الخلية وهذا ما يستدعى تتبع هذه المسارات فى أبحاث قادمة للوقوف على الميكانيكية الحركية لعمل المخدرات العمومية