Anesthetic inhibition of firefly luciferase, a protein model for general anesthesia, does not exhibit pressure reversal

G. W. J. Moss, W. R. Lieb, and N. P. Franks
Biophysics Section, The Blackett Laboratory, Imperial College of Science, Technology and Medicine, Prince Consort Road, London SW7 2BZ, United Kingdom

ABSTRACT The surprising observation that pressures of the order of 150 atmospheres can restore consciousness to an anesthetized animal has long been central to theories of the molecular mechanisms underlying general anesthesia. We have constructed a high-pressure gas chamber to test for "pressure reversal" of the best available protein model of general anesthetic target sites: the pure enzyme firefly luciferase, which accounts extremely well for animal potencies (over a 100,000-fold range). We found no significant pressure reversal for a variety of anesthetics of differing size and polarity. It thus appears that either firefly luciferase is not an adequate model for general anesthetic target sites or that pressure and anesthetics act at different molecular sites in the central nervous system.

INTRODUCTION

The discovery (Johnson and Flagler, 1950, 1951) of the pressure reversal of general anesthesia has long been thought to provide an important clue as to how general anesthetics act (Lever et al., 1971; Halsey and Wardley-Smith, 1975; Franks and Lieb, 1982; Dluzewski et al., 1983; Miller, 1985). Indeed, many workers have argued that pressure and anesthetics act antagonistically at the molecular level and therefore, because pressure can only act (at constant temperature) by reducing volume, anesthetics must be exerting their effects by increasing volume. Such arguments led to the critical volume hypothesis (Miller et al., 1973), which postulates that a critical expansion of some crucial target in the central nervous system results in the state of general anesthesia, which can then be reversed by pressure compressing the target back to its original functional state. More complex variants of the critical volume hypothesis, involving multiple targets, have also been proposed (Halsey et al., 1978). Another hypothesis (Franks and Lieb, 1982), which also assumes that pressure and anesthetics act at the same molecular sites, explains the animal data equally well: the volume changes which occur when general anesthetics bind to their targets are irrelevant to the actual mechanism of anesthesia but are nonetheless sufficient to account for pressure reversal, because increasing pressure favors dissociation of the anesthetic molecule from its targets. An alternative to these ideas is that anesthetics and pressure act at different molecular targets and that pressure reversal is a fortuitous combination of a generalized stimulation by pressure overcoming the depression brought about by anesthetics (Winter et al., 1976; Tarasiuk et al., 1991). Support for this view comes from the fact that although anesthetic potencies are quite similar for a wide range of animals, the pressures needed to reverse anesthesia vary considerably from animal to animal (Franks and Lieb, 1982) and, in some animals, anesthesia cannot be reversed by pressure at all (Simon et al., 1983; Smith et al., 1984).

The problem in testing such ideas is that the molecular target sites underlying general anesthesia have yet to be identified. In recent years (Richards, 1980; Franks and Lieb, 1982; Dluzewski et al., 1983; Miller, 1985; Franks and Lieb, 1987), attention has been focussed on two extreme alternative types of target sites: lipids and proteins. The traditional view has been that anesthetics act by dissolving in lipid regions of nerve membranes and so perturbing their structure or dynamic properties (e.g., fluidity) that the normal functioning of membrane proteins (such as ion channels) is compromised. Although criticized (Franks and Lieb, 1978; Richards et al., 1978; Franks and Lieb, 1982; Franks and Lieb, 1987) on quantitative grounds (effects on lipid bilayers are generally extremely small at surgically relevant anesthetic concentrations), lipid models have the attraction of accounting for pressure reversal, at least qualitatively, because a number of anesthetic-induced perturbations of lipid bilayers can be reversed by high pressures (Franks and Lieb, 1982; Dluzewski et al., 1983; Miller, 1985). The pressures required, however, are generally much lower than those needed to reverse the effects of general anesthetics in animals (Boggs et al., 1976;

Dr. Moss' present address is Department of Pharmacology, Sterling Hall of Medicine, Yale University School of Medicine, New Haven, CT 06510.

Mountcastle et al., 1978; Finch and Kiesow, 1979; Kamaya et al., 1979; Mastrangelo et al., 1979).

The alternative hypothesis is that general anesthetics act simply by binding directly to particularly sensitive proteins and modifying their function (Franks and Lieb, 1978; Richards et al., 1978; Richards, 1980; LaBella, 1981; Franks and Lieb, 1982). The strongest support for this view comes from recent observations (Franks and Lieb, 1984) that the lipid-free protein firefly luciferase can be competitively inhibited by a diverse range of general anesthetics at concentrations which are almost identical to those which induce general anesthesia in animals, over a 100,000-fold range of concentrations. Moreover, the abrupt cutoffs in anesthetic potency which are observed with long-chain compounds in animals are mimicked by similar effects on the firefly enzyme (Franks and Lieb, 1985; Moss et al., 1991).

Thus firefly luciferase is probably the best available protein model of general anesthesia. If anesthetics and pressure do indeed act by direct antagonism at the same protein target sites, one would expect the anesthetic inhibition of firefly luciferase to be overcome by high pressure. We decided to test this prediction. To do this, however, it was first necessary to design and construct a rapid-mixing system suitable for assaying the luciferase enzyme at high pressures. The system had to be able to perform the multiple assays required for kinetic analysis in a reasonable amount of time, because the luciferase enzyme inactivates slowly and spontaneously with time at room temperature. Furthermore, it was designed to operate using helium as the pressurizing agent. This was because much of the available data (including all of the data for mammals) has been obtained using helium rather than hydrostatic pressure, and the physiological effects of helium and hydrostatic pressure are not always equivalent (Macdonald and Wann, 1978; Dodson et al., 1985).

MATERIALS AND METHODS

Purification of luciferase enzyme

Details of the procedures used to obtain pure (~99%) preparations of the firefly (*Photinus pyralis*) luciferase enzyme have been published elsewhere (Branchini et al., 1980; Franks and Lieb, 1986). The highly purified enzyme was stored as a stock solution in 0.4 M ammonium sulphate, 1 mM EDTA, pH 7.8 at 4°C. D-luciferin, desiccated firefly lanterns and ATP (grade I) were purchased from Sigma Chemical Company (St. Louis, MO). *N*-Glycylglycine and $MgSO_4$ were obtained from BDH, Dagenham. All reagents used were of the highest purity available from the above suppliers and were used without further purification.

High-pressure rapid-mixing chamber

The high-pressure chamber we constructed is shown schematically in Fig. 1 *A*. The control reaction was initiated by rapidly injecting a small amount of solution containing ATP and the enzyme from a glass syringe into a reaction vial containing a solution of the substrate firefly luciferin. Before the injection, the vial could be continually stirred, so as to allow equilibration of the solution with the helium pressurizing gas. The chamber was fabricated from a single piece of type 303/S21 stainless steel, and it was rated for a maximum working pressure of 300 atm. The luciferase reaction was initiated by operating a high-torque 90° rotary solenoid which turned a lead screw (double-threaded, 2-mm pitch) and advanced a polytetrafluoroethylene (PTFE) block attached to the plunger of a 5-ml glass syringe. This syringe held a buffered solution containing the luciferase enzyme, oxygen, ATP and, when required, anesthetic. The injected solution (160 μl) was delivered into a small glass reaction vial which contained 500 μl of a buffered solution of magnesium sulfate and the substrate luciferin. All solutions were buffered at pH 7.8 using 25 mM *N*-glycylglycine. Light from the reaction passed through a sapphire window (6.4 mm thick) before being focussed by a lens and directed to a sensitive photomultiplier via a silvered Mylar mirror (to protect the photomultiplier tube in the advent of a failure of the sapphire window). The glass reaction vial was one of twenty, mounted on a rotary carousel driven by a stepper motor. Each vial was located in a gear wheel which rotated the vial about its own axis several times for each circuit of the carousel. Stirring bars in each vial allowed rapid equilibration of the solutions in the vial with the high-pressure helium gas in the chamber. A system of microswitches ensured accurate positioning of each vial under the injection syringe. The design was such that none of the moving parts in the chamber required lubrication. The chamber pressure was monitored continuously using a piezoelectric sensor, and the temperature was recorded using a thermocouple mounted in a dummy sample vial (not shown in the figure) containing 500 μl of water. The signal from the photomultiplier was passed through a current-voltage converter and stored on a digital storage oscilloscope and chart recorder. Luciferase activity was taken as the peak light intensity, i.e., the maximum rate of photon emission (Franks and Lieb, 1984; Moss et al., 1991).

RESULTS AND DISCUSSION

We first determined the effects of high pressures of helium on the activity of the luciferase enzyme in the absence of anesthetics. Fig. 1 *B* shows the control response at ambient pressure (1 atm helium), and Fig. 1 *C* shows the response at elevated (150 atm) pressure. We found that the peak in the light output did not change significantly over the range of helium pressures between 1 to 150 atm. Furthermore, double-reciprocal plots obtained by measuring reaction velocities at different luciferin concentrations (see inset to Fig. 2) showed that the K_m for luciferin was independent of pressure (see Fig. 2). Thus, there were no significant changes with pressure in either the substrate-binding reaction or the subsequent catalytic steps. The absence of any significant change in control luciferase activity with pressure rules out one a priori possible mechanism of pressure reversal. Pressure might simply increase enzyme activity

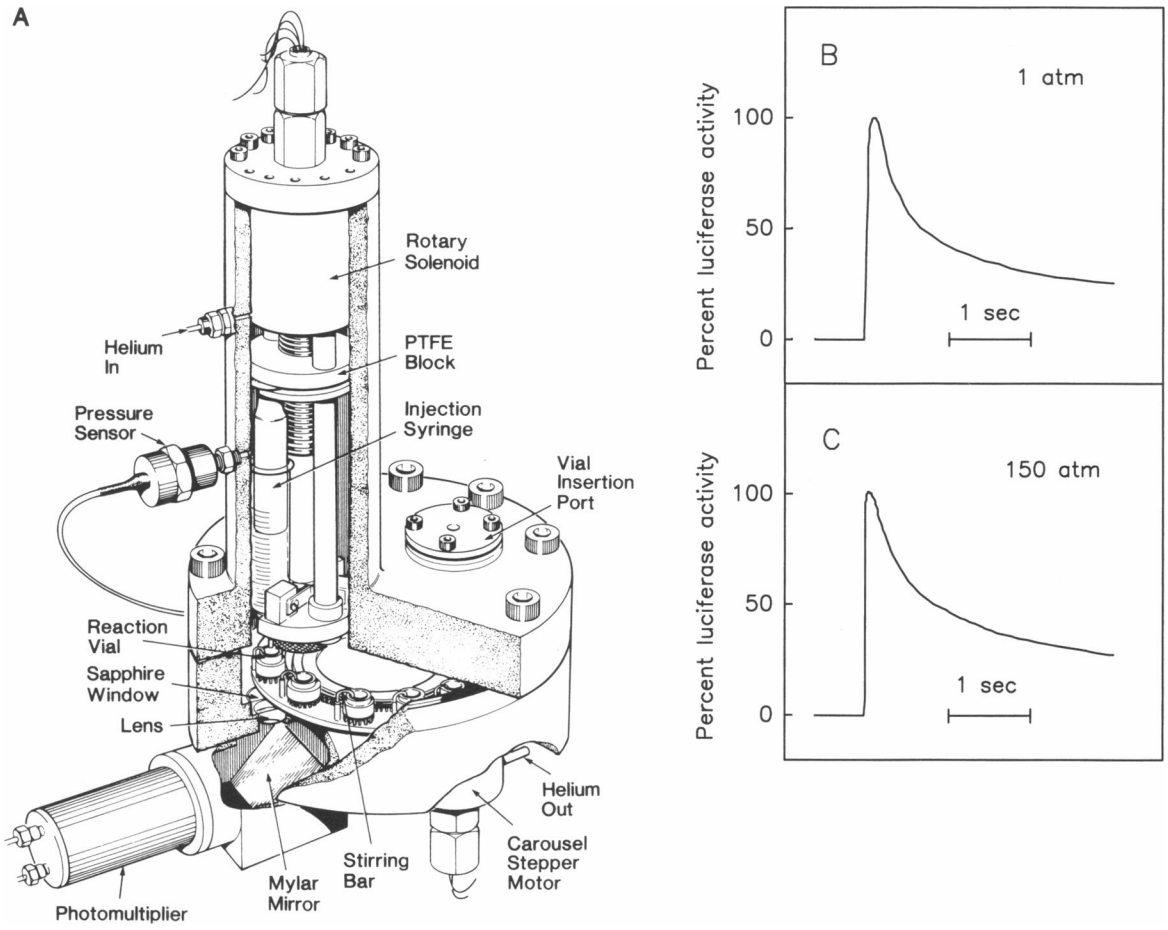

FIGURE 1 (*A*) A schematic drawing of the high-pressure chamber used for the luciferase experiments. See text for details. (*B*) A typical record from the luciferase reaction at 1 atm helium. (*C*) A typical record from the luciferase reaction at 150 atm helium. In both cases, activity is expressed as a percentage of the control activity at 1 atm helium. The final reagent concentrations were 10 nM luciferase enzyme, 2 mM ATP, 6.7 mM magnesium sulfate and 10 μM luciferin in 25 mM *N*-glycylglycine buffer at pH 7.8. The pressure was applied using helium gas (BOC, 99.99% pure) at a rate of ~ 10 atm min^{-1}. Usually 10 min were allowed to achieve equilibration, at which time the sample temperature was generally within 1°C of the ambient temperature, which was typically 20°C.

to compensate for the anesthetic inhibition. In the case of the luciferase enzyme, however, the overall volume of activation for the reaction is evidently too small for this to be a significant effect.

We then went on to determine the effects of pressure on the enzyme in the presence of a variety of general anesthetic agents to test the possibility that the binding equilibrium between the anesthetics and enzyme is altered at high pressure, due to a change in the volume of the system upon binding. For these experiments, we introduced anesthetics into the ATP injection syringe so that any pressure reversal that might be observed could not be due to a simple redistribution of anesthetics between the aqueous solution and the gas phase in the chamber. This was important because we have previously shown (Franks and Lieb, 1982) that much of the pressure reversal observed with gases for mice (Miller et al., 1978) can be accounted for simply in terms of the expected changes in solubility from the gas phase. Pressure-reversal data (Halsey et al., 1978; Miller and Wilson, 1978) for intravenous agents, however, are not so easily explained because unreasonably large binding volumes would be required to account for pressure reversal in terms of displacement from the target sites.

Our initial results with methoxyflurane, however, showed that the anesthetic-inhibited luciferase reaction was not significantly affected when pressure was applied, indicating that any pressure effects on anesthetic binding must be small (see Fig. 3). Because anesthetics inhibit the luciferase enzyme by competing for the

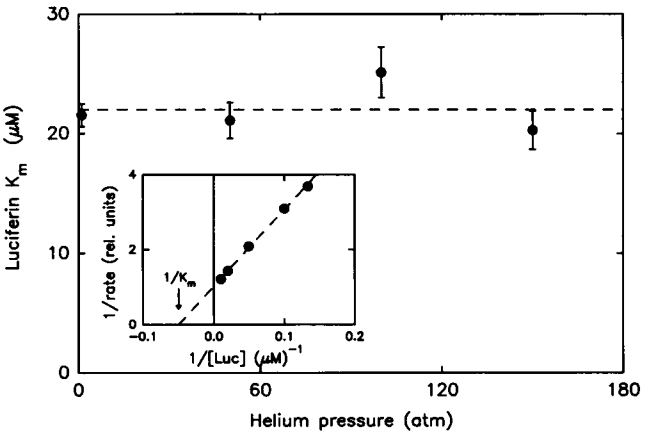

FIGURE 2 The K_m for luciferin does not change significantly with pressure. At each pressure, the K_m was determined using double-reciprocal plots (*inset*) over a range of luciferin concentrations. The error bars represent standard errors. (*Inset*) A typical double-reciprocal plot (at 1 atm). Each point represents the mean of at least three enzyme assays, and the standard errors are smaller than the size of the symbols. The double-reciprocal line was fitted by the method of weighted least squares, with the errors in K_m determined as described in detail elsewhere (Curry et al., 1990).

substrate luciferin, any changes in anesthetic binding constants would be most accurately quantified by measuring changes in the apparent K_m for luciferin (rather than in the anesthetic/enzyme dissociation constant K_i, because a determination of K_i inevitably includes the random error in the Michaelis constant K_m.) We therefore measured the apparent luciferin K_m as a function of helium pressure in the presence of anesthetics.

The results of these experiments were uniformly negative. There was no significant change with pressure in the apparent K_m for luciferin (K_m^{app}) and hence

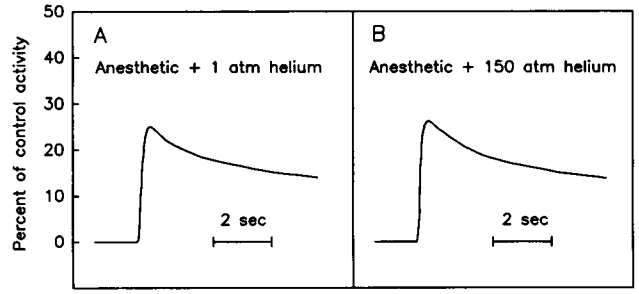

FIGURE 3 Anesthetic inhibition of the luciferase reaction is not reversed by high pressure. The data show the light output from the luciferase reaction in the presence of 0.53 mM methoxyflurane, expressed as a percentage of the activity in the absence of anesthetic and at 1 atm helium pressure. Responses at (*A*) 1 atm and (*B*) 150 atm. The final luciferin concentration was 10 μM.

(because the control K_m was constant) in the binding of the anesthetics to the enzyme. This is shown in Fig. 4 for the volatile general anesthetics methoxyflurane and chloroform as well as for the aliphatic alcohol *n*-octanol. We obtained similar negative results (not shown) with *n*-hexanol (0–1.5 mM) benzyl alcohol (0–4 mM), *n*-decanol (0–15 μM), and the local anesthetic benzocaine (0–64 μM), although with the latter two agents we did observe a small increase in potency with pressure. The small increases in the potencies of *n*-decanol and benzocaine presumably reflect small increases in the binding of these relatively large agents. Whereas this effect may be interesting, it is in the opposite direction to that needed to account for pressure reversal in terms of displacement of anesthetics from their target sites (Franks and Lieb, 1982). We also confirmed (for the case of *n*-decanol) that there was no change with pressure in the stoichiometry of anesthetic binding to the enzyme. Thus, for molecules of varying size and polarity, we observed no evidence of any significant degree of pressure reversal.

Our results are clear cut: there is no significant pressure reversal of the anesthetic inhibition of firefly luciferase. This leaves open two alternatives: either (*a*) firefly luciferase is a poor model for the target sites underlying general anesthesia, perhaps accounting for anesthetic inhibition but not for pressure reversal, or (*b*) firefly luciferase is a good model for general anesthetic target sites, but pressure reversal of general anesthesia is due to pressure acting at different molecular sites than those which are affected by anesthetics (Winter et al., 1976; Tarasiuk et al., 1991). As to the first alternative, it is of course possible that a protein will be found which can account for both anesthetic potencies and pressure reversal. Indeed, it should be remembered that the discovery of pressure reversal in animals (Johnson and Flagler, 1950, 1951) was preceded by a report (Johnson et al., 1942) of pressure reversal in luminescent bacteria, and it might be thought that the bacterial luciferases which catalyze the light-emitting reactions are good candidates. However, pressure reversal of anesthetic inhibition of bacterial luminescence does not occur for some agents (e.g., sodium barbital), is marginal for others (e.g., diethyl ether), and even in favorable cases (e.g., chloroform) is modest at pressures (150 atm) which produce reversal in animals (Johnson et al., 1942). Furthermore, while it has been reported (Strehler and Johnson, 1954) that a crude cell-free extract can exhibit qualitatively similar effects to those found with intact cells, pressure reversal has yet to be demonstrated using a purified bacterial luciferase. This is important in view of a recent finding (Curry et al., 1990) that the purified bacterial luciferase from *Vibrio harveyi* is remarkably insensitive to a number of general anesthetics, which

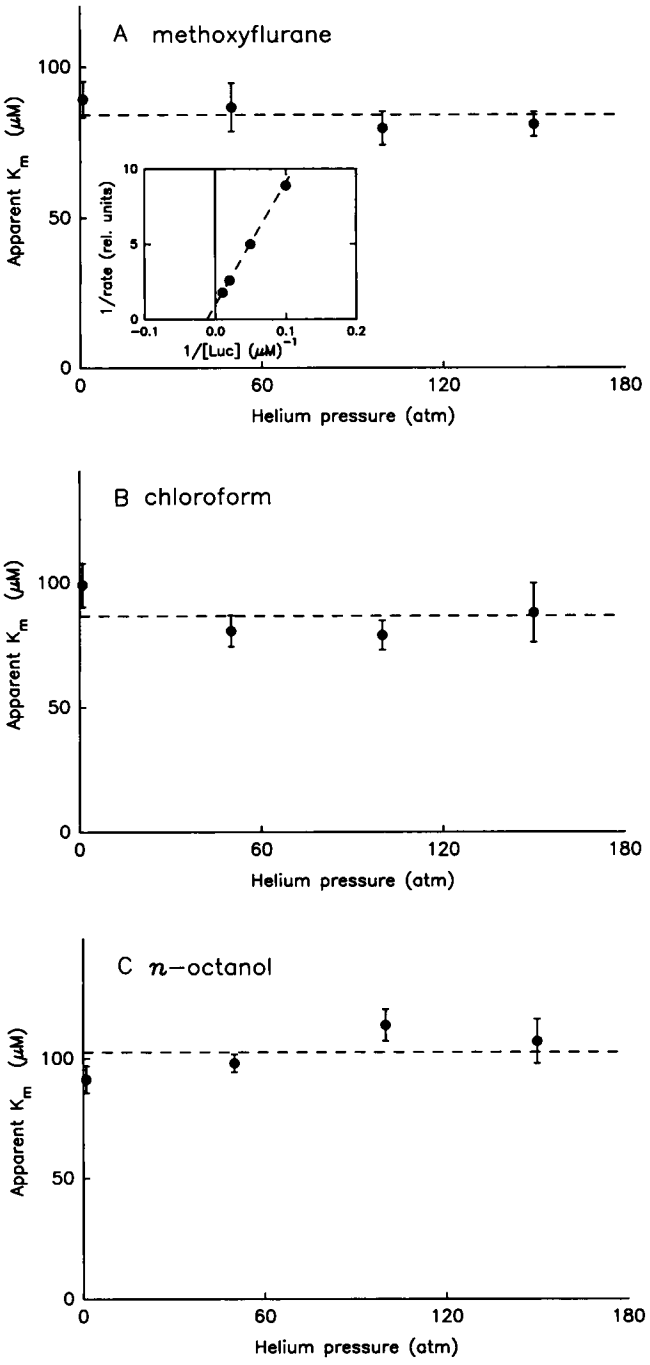

FIGURE 4 Binding of anesthetics to the luciferase enzyme does not change significantly with pressure. The data are for (*A*) methoxyflurane, (*B*) chloroform and (*C*) *n*-octanol and show the apparent K_m for luciferin (K_m^{app}) as a function of helium pressure. Each K_m^{app} point and its associated standard error was determined using a double-reciprocal plot (see *inset* to *A*), as described in the legend to Fig. 2. K_m^{app} is simply related to the dissociation constant K_i for the anesthetic (Franks and Lieb, 1984) but can be determined more accurately than K_i itself. The final anesthetic concentrations were 0.53 mM methoxyflurane, 2 mM chloroform and 0.776 mM *n*-octanol, which all reduced the enzyme activity to roughly 25% of the control.

suggests that the anesthetic and pressure sensitivities of purified enzymes may be very different to those of intact bacterial cells. Whether or not a protein will be found that is both sensitive to a wide range of anesthetics and whose inhibition is reversed by high pressure remains to be seen.

The second alternative, that anesthetics and pressure act at different sites, has always been a possibility that had to be taken seriously (for a review, see Wann and Macdonald [1988]). When complex neuronal responses are considered, there are cases where pressure does act antagonistically (Angel et al., 1980) and other cases where it does not. For example, it has very recently been shown (Tarasiuk et al., 1991) that the depressive effects of a barbiturate on the rhythmic activity of a medullary respiratory center were mimicked, rather than antagonized, by high pressures of helium. At the cellular level pressure has been shown to partially reverse the effects of anesthetics on axonal conduction (Kendig et al., 1975; Roth et al., 1976). Strikingly, however, the effects of anesthetics and pressure on synapses, generally regarded as the most likely target site for anesthetics (Pocock and Richards, 1991), have been shown to be synergistic rather than antagonistic (Kendig et al., 1975; Kendig and Cohen, 1976; Wann et al., 1980; Wann and Macdonald, 1988). Our present results with the firefly luciferase enzyme, one of the best molecular models for anesthetic target sites, adds to this body of evidence that calls into question the usual assumption that pressure and anesthetics act at the same molecular target sites.

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