ISOLATED HUMAN BLOOD PLATELETS DISCRIMINATE BETWEEN ANAESTHETIC AND NON-ANAESTHETIC GASES AT HIGH PRESSURES†

D. J. L. McIVER, N. D. FIELDS AND R. B. PHILP

SUMMARY

We have compared the effects of the anaesthetic gases nitrogen and argon on adenosine diphosphate (ADP)-induced human blood platelet aggregation with the effects of the non-anaesthetic gas helium. All three gases showed dose-dependent inhibition of platelet aggregation. For nitrogen and argon there was a linear relationship between gas pressure and inhibition of aggregation over the range 15–68 atmospheres absolute (atm abs), whereas helium had a threshold for inhibition of approximately 34 atm abs. The inhibition by all gases was reversible after slow decompression. At pressures greater than 55 atm abs, nitrogen produced less inhibition than helium, indicating anaesthetic-pressure antagonism. Whereas pressure alone and the anaesthetic gases inhibited aggregation, the platelet shape change elicited by ADP was resistant to both nitrogen and helium, indicating that ADP binding and the early events in platelet activation were relatively unaffected by these conditions.

KEY WORDS


There are two important unsolved questions on the molecular and cellular mechanisms of general anaesthesia: what is the molecular nature of the general anaesthetic receptor(s) [1] and how does hydrostatic pressure reverse anaesthesia [2]?

The first question is structural: are the receptors lipids, lipid–protein interfaces or proteins [3]? If the receptors are proteins, do anaesthetics act by a global perturbation of protein structure [4] or do they act as relatively selective antagonists of the binding of endogenous ligands [5]? The evidence for and against each of these views has been discussed in detail [6, 7]. The available information favours the proposal that general anaesthesia is produced by competitive antagonism of the binding of some, as yet unidentified, central nervous system ligand(s).

The second question is thermodynamic: how can increased pressure (the only action of which is to reduce volume) reverse so many of the effects of anaesthetics on living organisms?

The most economical answer to both of these questions is the “critical volume hypothesis” [8]: anaesthetics expand and pressure compresses a single molecular site which is responsible for both anaesthesia and pressure reversal. This single site approach conveniently lends itself to mathematical and experimental modelling, but several lines of evidence indicate that the original critical volume theory provides an oversimplified representation of general anaesthesia in whole animals and that a more complex multi-site hypothesis is necessary to account for such factors as anaesthetic additivity and pressure reversal [9, 10].

Progress with answering these questions in molecular and cellular terms requires the identification of pharmacologically relevant anaesthetic binding sites in living excitable cells, demon-
strating that these binding sites fulfill the currently accepted criteria of anaesthetic action [1], and eventually isolating and purifying the binding molecules. Given the complexity of the nervous system and the current ignorance of how and where anaesthetics act on it, this remains a daunting task.

We have therefore chosen a relatively simple excitable cell—the human blood platelet—in which to study the interactions of anaesthetics and pressure. These readily available and relatively homogeneous cells are similar biochemically to central nervous system monoaminergic neurones [11]. Platelets exhibit specific ligand-induced behaviours (such as shape change and aggregation) which may be quantified readily in vitro by photometric methods [12].

Several workers have studied the effects of anaesthetics on platelet function [13-15]. Recently, it has been shown that hyperbaric helium inhibits platelet aggregation [16]. In the present studies we have examined the pressure–response relations for human platelets stimulated to aggregate by adenosine diphosphate (ADP) in the presence of the anaesthetic gases nitrogen or argon, or the non-anaesthetic gas helium. Preliminary accounts of some of this work have been presented earlier [17, 18].

METHODS

Hyperbaric apparatus

A non-magnetic stainless steel pressure chamber constructed in the Department of Physiology, State University of New York at Buffalo, and similar in design to previous descriptions [19], was used to apply gas pressure to the platelet sample. The internal dimensions of the chamber are approximately 8 cm x 6.5 cm; the 4-cm thick wall is fitted with penetrations to allow drug injection, optically flat methyl methacrylate windows rated to a pressure of 250 atmospheres absolute (atm abs), electrical connections and pressurization and depressurization ports.

The gases were industrial grade nitrogen, argon, helium, carbon dioxide and oxygen (CanOx Limited, Mississauga, Ont.) supplied in standard industrial cylinders at 2200 lbf in$^{-2}$ (150 atm abs) and delivered to the chamber at the required pressure through standard industrial regulators, calibrated by the manufacturer.

Platelet aggregation was monitored by a modification of the turbidometric method of Born [12] using solid-state optical components mounted in an aluminium block controlled thermostatically at 37 ± 0.5 °C. Monochromatic infrared light ($\lambda = 920$ nm) from a gallium arsenide light emitting diode (Motorola LED 55C) was used to illuminate the sample; this wavelength was chosen in order to allow the capability of simultaneous spectrophotometric or luminescence measurements in the visible light range. Platelet-rich plasma (PRP) was stirred magnetically in a siliconized glass cuvette and the axial optical transmission of the PRP was measured by a phototransistor (Motorola FPT 100). The photodetector half-angle was estimated to be 3°. After amplification, the phototransistor output voltage was recorded directly on a chart recorder, or digitized by an analogue-to-digital converter (Interactive Microwave, State College, Pennsylvania) for analysis by the programme of Huzoor, Romstedt and Manhire [20], which uses a least-squares method to compute the curve of best fit to the digitized recordings, and which calculates the statistics of the light transmission changes and derived parameters such as the rate and extent of the reactions.

Pressure itself did not affect the optical transmission measurements; similar amounts of light were transmitted by a well defined sample of polystyrene latex spheres (6-μm average diameter electron microscopy calibration beads; Polysciences Inc., Warrington, Pennsylvania) under both hyperbaric and normobaric conditions. In order to prevent heat inactivation of the PRP, compression rates were restricted to 30 atm abs min$^{-1}$, which caused a temperature increase of the sample of only 0.5 °C. Greater rates caused significant warming of the sample. Reversibility of the hyperbaric effects could be demonstrated only when even slower decompression rates were used, otherwise bubbles were formed which were large enough to alter light transmission substantially. Empirically, a decompression rate of 5 atm abs min$^{-1}$ was found to avoid this problem.

Injection of the aggregating agent (ADP; Sigma Chemical Company, St Louis, Missouri) was carried out using a commercial high pressure liquid chromatography syringe (Hamilton Co., Reno, Nevada) rated to 400 atm abs. A length of Teflon tubing connected the syringe needle to the PRP.

Blood collection

Following informed consent, blood was obtained from the antecubital vein of eight ap-
paren tally normal donors (four male, four female), none of whom had consumed non-steroidal anti-inflammatory drugs for at least 2 weeks previously. Whole blood was drawn into a 10-ml plastic syringe and diluted 1:9 with 3.8% (w/v) trisodium citrate as the anticoagulant.

Preparation of plasma samples
Platelet-rich plasma (PRP) was obtained by centrifuging the whole blood at 100 £ for 20 min. Platelet-poor plasma (PPP) was obtained by centrifuging the blood at 3000 $g$ for 5 min. PRP platelet counts were carried out using unstained cell samples fixed with 2% glutaraldehyde (Polysciences). The cells were counted in a Neubauer type haemocytometer using differential interference contrast (Nomarski type) and a 100 x strain-free plan apochromat objective fitted to a Nikon Optiphot microscope. The distribution of aggregate sizes (see results) in PRP samples fixed at pressure with glutaraldehyde was determined by a similar procedure. The PRP platelet count was adjusted to 300000 £l$^{-1}$ where necessary by dilution with PPP. Four hundred-microlitre aliquots of PRP were placed in aggregometer cuvettes, and stored at room temperature under an atmosphere of 5% carbon dioxide [21]. The PRP was allowed to equilibrate with the mixture for at least 30 min.

Aggregation procedure
Cuvettes containing PRP and PPP were used to standardize the light transmittance for each experiment, the difference in transmittance between PRP and PPP being set to 80% of the full scale recording. Cuvettes containing 400 £l of temperature-equilibrated PRP were placed in aggregometer cuvettes, and stored at room temperature under an atmosphere of 5% carbon dioxide in air [21]. The PRP was allowed to equilibrate with the mixture for at least 30 min.

RESULTS
Figures 1–5 show that helium, nitrogen and argon inhibit ADP-induced platelet aggregation in the pressure range 15–68 atm abs, but there are significant differences in the details of the gas–platelet interactions.

Figure 1 shows the measurements of light transmission during a single experiment in which
samples of PRP were exposed to ADP at 1 atm abs (control) or 34 atm abs of helium or nitrogen. The latter pressure was chosen as an example because it represents the upper limit of hydrostatic (helium) pressure which may be applied without inhibiting the maximal extent of platelet aggregation, and because it illustrates the clear difference in effects of nitrogen (which is narcotic at this pressure) and helium (which is non-narcotic at this pressure). In this particular experiment, the dose of ADP which produced a biphasic wave of aggregation was $6 \times 10^{-8}$ mol litre$^{-1}$. The transient decrease in light transmission associated with the initial disk-to-spiny-sphere shape change was seen under all three conditions, as were the first and second waves of aggregation. The time to onset of shape change and of the first wave of aggregation did not differ significantly between the three conditions ($P < 0.05$). At this pressure of helium a small but statistically significant ($P < 0.01$) difference in the rate of second phase aggregation was just becoming evident—a trend which was more marked at higher pressures (see below). The fluctuation in the axial light scattering was also less in the sample exposed to helium at this pressure, which indicates that the aggregate size was smaller. This conclusion was confirmed by direct microscopic observation of the aggregate size distribution of PRP samples fixed under hyperbaric conditions by injection of glutaraldehyde (final concentration 2%) into the aggregation cuvette 5 min after the addition of ADP, and continuing stirring for 5 min before decompression (results not shown). However, by 5 min after stimulus addition (the maximum time recorded), the sample compressed by helium 34 atm abs achieved an increase in light transmission which did not differ significantly from the control response at 1 atm abs. This trend was also observed in the pooled results of six
experiments. In contrast, the same pressure (34 atm abs) of nitrogen produced a reduction in the rate and extent ($P < 0.01$ in both cases) of aggregation.

Figure 2 shows the effect of increasing the nitrogen or helium pressure to 68 atm abs, the maximum pressure investigated in the present study. The trends were reversed when compared with figure 1: helium was more inhibitory than nitrogen. The second phase of aggregation was virtually abolished by helium at this pressure. The difference between nitrogen and helium is significant at the $P < 0.05$ level.

Figure 3 shows that the effects of helium 68 atm abs pressure were reversible. When compression and decompression were carried out as described in the methods section, indistinguishable responses to ADP at 1 atm abs were obtained before and after exposure of the PRP to hyperbaric conditions. The effects of nitrogen were similarly reversible (results not shown).

Figure 4 shows the pooled results of 5-min light transmission measurements obtained from six separate experiments in which PRP samples were exposed to various pressures of nitrogen or helium. In addition, a comparison of the more potent anaesthetic gas argon was made at 34 atm abs. All conditions (with the exception of helium 34 atm abs) were significantly different ($P < 0.05$ or less) from the control responses at 1 atm abs. The trends shown in figure 3 are confirmed in this larger sample. In addition, argon inhibited aggregation significantly more than nitrogen at the same pressure ($P < 0.05$).

In figure 5, the data for the effects of helium or nitrogen in the range 15–68 atm abs are plotted as gas dose (pressure)–response curves, with responses normalized to the control response at 1 atm abs as 100%.

**DISCUSSION**

The present observations may be summarized as follows: First, anaesthetic gases and pressure alone show dose-dependent and reversible inhibition of platelet aggregation, and in the limited sample studied here there is a correlation with anaesthetic potency: the more potent anaesthetic gas argon has greater inhibitory effects than the...
weaker gas nitrogen. The latter point requires more detailed examination with a wider range of soluble (i.v.) and gaseous anaesthetics (e.g. nitrous oxide, fluorinated hydrocarbons). Second, an anaesthetic-pressure antagonism is evident: significantly less inhibition of platelet aggregation is seen with nitrogen than with helium at the greater pressures (68 atm abs). Finally, there is a good numerical correlation between the threshold pressure we observed for helium inhibition of platelet aggregation (34 atm abs) and the onset of signs of the high pressure neurological syndrome in mice (approximately 30 atm abs, dependent on the rate of compression [23]).

The thermodynamic effect of pressure on any physical or chemical process is to reduce volume, the conjugate variable of pressure. Consider, for example, a pressure sensitive process such as a thermotropic lipid phase transition (a popular target for theories of anaesthesia, if not necessarily for anaesthetics themselves). Pressure sensitivity dictates that there must be molar volume differences between the initial and final equilibrium states ($\Delta V$) or between a transition state activated complex and the equilibrium state ($\Delta V^*$. When pressure is applied to a system which can undergo a non-isovolumetric transition, the state of lower molar volume is preferred, which is the standard prediction of the Clapeyron–Clausius equation

$$dT/dP = T \cdot \Delta V/\Delta H$$

where $T$ = the transition temperature; $P$ = pressure; $\Delta V$ and $\Delta H$ = discontinuities in molar volume and enthalpy associated with the phase transition.

The molecular effect of pressure on most substances is to hinder molecular motion—that is, most reactions are “frozen” by increased pressures, and temperature and pressure act usually in opposite directions. Water at physiological temperatures is a notable exception to this rule; its unusually bulky structure [25] leads to the familiar observation of ice melting under pressure. It has been recognized for some time that protein–solvent interactions are sensitive to pressure [26], as are several ligand–binding events which involve molar volume changes [27]. More recently, it has been shown that the temperatures of lipid phase transitions are increased by increased pressures [28].

Which of these processes, alone or in combination (the solvent water, proteins, solutes or lipids) may be involved in the actions of anaesthetics and pressure on the molecular and cellular events involved in stimulus–response coupling in blood platelets?

Unfortunately, the present studies do not yield unique answers to these questions: the “simplicity” of platelet aggregation as a model biological process is more apparent than real. Isolated platelets deploy the full biochemical repertoire of excitable cells, and aggregation is itself the ultimate result of a complex series of preceding events. The present results indicate that several of these events may be sensitive to anaesthetics, pressure, or both. This conclusion is consistent with the idea that there may be many classes of anaesthetic binding site in vivo, a long-held view that has now been given a name of its own: the “degenerate perturbation hypothesis” [3]. What do the present studies indicate for potential molecular candidates for anaesthetic or pressure sensitivity in ADP-induced platelet aggregation?

The events between ligand binding to platelet surface receptors and the formation of photometrically detectable aggregates has been summarized elsewhere [11]. Neither helium pressure nor nitrogen affects the platelet shape change to ADP in the concentrations used, and these agents do not affect the initial rate of first phase aggregation, indicating that under these conditions, ADP–receptor interactions, initial calcium mobilization, actin–myosin interactions and first phase fibrinogen receptor expression do not have a pressure- or anaesthetic-sensitive activation volume $\Delta V^*$. However, the extent of first phase aggregation is reduced by helium, and less so by nitrogen, indicating that the steady first phase aggregation reaction does have an overall positive reaction volume, $\nu$, and that anaesthetics antagonize pressure effects on the underlying molecular processes. The fact that nitrogen narcosis occurs only under hyperbaric conditions complicates the assignment of these effects to “anaesthesia” or pressure per se and is a further incentive to carrying out these types of studies with substances which are anaesthetics at atmospheric pressure, for example [29]. As they stand, the present results do not enable us to distinguish between the single site (i.e. critical volume) and multi-site models of pressure–anaesthetic interactions in isolated platelets.

Even more marked effects are seen on the second phase of aggregation—that is, on those events associated with the release of platelet
granules. At 68 atm abs this phase is inhibited completely by helium pressure and reduced significantly by nitrogen. Candidates for pressure and anaesthetic sensitivity in secondary aggregation include the granule release reaction and the post-release events involved in cell–cell adhesion (aggregation). Distinguishing between pre-release and post-release events requires direct measure of the pressure and anaesthetic sensitivity of the release reaction itself. We attempted to measure granule release by the traditional technique of luciferase determination of granule ATP secretion [30], using a fibre light guide and photon counting of luciferin–luciferase luminescence. However, these studies were complicated by the direct effects of anaesthetics and pressure on the indicator reaction; when corrections for these effects were made it was difficult to discern effects on the platelet release itself. A better approach would be to measure the amount of granule release under conditions where the experimental variables pressure and anaesthetics do not interfere with the measurement or to study pre-release events in the presence of a pharmacological inhibitor of release.

Molecular mechanisms in the release reaction which could be pressure or anaesthetic sensitive include calcium translocation (release from stores or binding to calcium-dependent effector molecules), granule–plasma membrane interactions (possibly protein-mediated) and lipid mixing or fusion between the adhering membranes. In the latter process (membrane fusion) pressure studies have recently shown a direct inhibitory effect attributed to freezing of the lipid chains [31, 32], and possible prevention of the adoption of non-bilayer phases which may be intermediates in the fusion process. Direct evidence that anaesthetics, in clinically effective doses, significantly alter intracellular calcium transport is both limited and controversial [33]. If, however, calcium translocation in platelets does turn out to be sensitive to pressure or anaesthetics, or both, modellistic considerations [34] would predict effects on calcium release rather than binding, as the former reaction is expected to have a positive reaction volume. Protein-facilitated lipid vesicle fusion has been studied in detail using the adrenal chromaffin granule protein synexin as a prototype [35], but the pressure and anaesthetic sensitivities of this reaction have not yet been determined, and an analogous reaction in platelet granule release has not yet been identified.

Other reactions which could be candidates for the inhibitory effects of pressure or anaesthetics on second phase aggregation include the expression of fibrinogen receptors (the so-called glycoprotein IIa–IIb complex [36, 37]) or the binding of fibrinogen itself. None of these possibilities has yet been explored.

These studies of pressure–anaesthetic interactions in platelets raise more questions than they answer, but they do perhaps indicate an approach to investigating the molecular and cellular mechanisms of anaesthetic actions in excitable cells which may help in answering the question “what are the molecular and cellular receptors for general anaesthetics?”

ACKNOWLEDGEMENTS

Supported by operating grants from the Heart and Stroke Foundation of Ontario, the Ontario Thoracic Society and the Medical Research Council of Canada (to D.J.L.M.) and by contract from the Defence and Civil Institute of Environmental Medicine, Downsview, Ontario (to R.B.P.).

REFERENCES


