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On the Relevance of "Clinically Relevant Concentrations" of Inhaled Anesthetics in In Vitro *Experiments*

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THE use of the phrase, "clinically relevant anesthetic concentrations" has become so enshrined in *in vitro* research on anesthetic action that its importance has assumed a prominent and rarely questioned status. To review, the yardstick by which most studies of anesthetic action are measured is *sensitivity,* or whether significant effects on *in vitro* systems can be measured at concentrations of general anesthetics used in people. Our contemporary inhalational anesthetics, halothane, isoflurane, and sevoflurane, produce aqueous concentrations of approximately 0.3 mM at 1 minimum alveolar concentration (MAC) and at 37°C.[1] Thus, the logic goes, for an *in vitro* preparation or some other model system, to be in any way clinically relevant, it too must be affected, preferably by 50% of some maximal response at the same concentration. There are several implicit, but rarely discussed, assumptions underlying this logic, which we will consider in the following paragraphs.

Most *in vitro* responses to a drug or ligand are nonlinear and, when plotted against the log of concentration, fit a sigmoid relation (Hill or logistic equation) reasonably well. Such relations are characterized as being "saturable," meaning that progressively smaller increases in the target response occur with increases in ligand concentration beyond a specific point. If this *in vitro* response directly controls some behavior of an organism, one might also expect that behavior to reach a maximum, or "ceiling," as the administered dose of the drug is increased. But is anesthesia saturable? It depends on how we define a state that we know very little about. The popular operational definition is the absence of a motor response to a noxious stimulus in an unparalyzed animal. This is known also as the MAC response (*m*inimum *a*lveolar *c*oncentration of inhaled anesthetic to prevent this response in 50% of a population).[2] This is a categoric (binomial in this case) definition, and any such definition of necessity results in a saturable response. However, does an arbitrary behavioral definition require that the underlying central nervous system (CNS) events be saturable? Although the MAC response is a relatively clear behavioral endpoint, in terms of CNS function it is considerably less clear. We know that all noxious stimuli are not equal and that the dose–response curve for a simple skin incision, for example, is different from that describing intubation of the trachea.[3] That different CNS functions have different sensitivities is also shown by the existence of the MAC-awake,[4] and MAC-bar[5] values. Further, no discrete electroencephalographic changes have been identified as indicating the achievement of MAC concentrations.[6] Brain electrical activity becomes progressively depressed until approximately 3 MAC (approaching 1 mM aqueous concentration) for many of our agents[7] until cardiovascular depression becomes critical. If cardiovascular and respiratory components are removed, as in, for example, cell culture, reversible influences of these compounds can be measured well above 3 MAC.[8]

Such progressive actions of anesthetics speak against finding a saturable CNS action that underlies binomial responses exemplified by MAC, and are more likely explained by progressive, simultaneous actions at many targets of comparable sensitivity. Alternatively, progressive CNS dysfunction could result from a more layered

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"sequential" action on progressively less-sensitive targets. If the latter is true, it may be reasonable to use sensitivity to identify targets underlying each response (although see discussion of K_d/EC_{50} relation below), but only if one proposes that important single targets are responsible for each specific endpoint. However, if the progressive simultaneous action model holds, then the response of the whole organism is an integrated, fluctuating, nonlinear result of effects at many sites, and it makes little sense to use sensitivity to select among them. Because we really do not know the underlying pharmacologic basis for any of the actions of inhaled anesthetics, distinction between the simultaneous or sequential models is not yet possible, and, therefore, using sensitivity to clinically relevant anesthetic concentrations as a way of relating *in vitro* responses to CNS function must be viewed with considerable caution.

Other than EC_{50} or MAC values, dose–response relations contain additional useful information. For example, the steepness, or slope, is often used to gauge the degree of cooperativity of events that contribute to the observed response. In a well-known example of this, oxygen binding to hemoglobin shows a Hill slope of about three, reflecting the cooperative interaction between the binding of successive oxygen molecules. Binding interactions involving a single ligand, or multiple ligands binding to a single protein at noninteracting sites, typically show Hill slopes of around one. It is useful to keep in mind that about a 100-fold increase in ligand concentration is necessary to "saturate" a dose–response relation with a Hill slope of one. In contrast, the Hill slope for the MAC response is extremely steep—approximately 20.[1] Some would argue that this is due solely to the categoric (quantal) nature of the measurement (an infinite slope in an individual). Statistically, the slope of concentration–percent relations only quantitates pharmacodynamic variation in a population, which may or may not reflect cooperativity or coupling in underlying molecular targets. It is clear, however, that the remarkably steep slopes of inhaled anesthetic concentration–percent population curves are shared by few other drugs. The most plausible explanation for such highly conserved sensitivity to general anesthetics is that there are multiple contributing systems, each of which might be influenced to only a small degree by the anesthetic. Figure 1 shows such an effect. In the probable circumstance that many separate *in vitro* responses contribute to a behavioral effect (indicated by the response of one in fig. 1), and only a small contribution of each is required to produce this effect, then the apparent concentration–effect curve steepens and shifts considerably to the left—closely resembling the concentration–percent curves for MAC. Further, multiple contributing targets provide for redundancy in individuals with molecular heterogeneity. In other words, in people with genetic or acquired differences in this or that signaling system, actions of a drug at many targets will reduce the effect of these differences on the ultimate observed response. Thus, multiple sites of action could explain the remarkably well-conserved anesthetic sensitivity across the animal kingdom, even among response-selected organisms,[9–12] or in those with sensitive targets that were removed or altered genetically.[12–14] The possibility of multiple important targets is consistent with the integrated mechanism mentioned before, and therefore adds further uncertainty about the logic of using the sensitivity of *in vitro* systems as a criterion for their relevance.

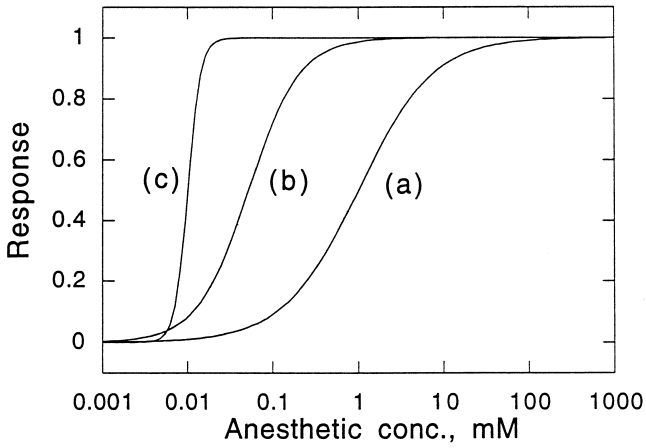

Fig. 1. Theoretical concentration–response curves for a general anesthetic. The y-axis represents the response, with 1 being "anesthesia." Curve a is a standard one-site model with K_d = 1 mM, EC_{50} = 1 mM, and a Hill number of 1.0. Curve b is for 10 different sites (all with a K_d = 1 mM) with equal additive effects. The EC_{50} for this curve is 50 μM, and the Hill number is now approximately 2. Curve c is for 50 different sites (all with a K_d = 1 mM), again with equal additive effects. The EC_{50} for curve c is now only 10 μM and the Hill number almost 6. Plots were generated using $y = y_{max} \cdot [drug]^n/(EC_{50}^n + [drug]n)$, where n is the Hill number and EC_{50} is the concentration at half-maximal effect.

On moving from the organism to the organ and then to the cellular level, signaling features, such as nonlinearity, threshold effects, amplification, feedback, and others, all tend to produce ambiguity as to the magnitude of an *in vitro* response necessary to have an effect at the next higher organizational level. This is especially true in the CNS, in which only vague qualitative ideas of regional contributions to higher brain function are now surfacing. Further, although highly receptor-specific drugs

have allowed us to gauge coupling characteristics between the receptor and behavior in some cases (such as the α_2 agonists),[15] compounds that interact with many targets could have larger ultimate effects with smaller individual actions. A familiar analogy is temperature. In general, a 10°C change in temperature only alters enzyme activity by a factor of two to three (the Q_{10}), whereas a comparable change in either direction in the intact organism has profound effects. Thus, extrapolating back to anesthetics, the requirement for 50% responses (activation or inhibition) at "clinical concentrations" becomes difficult to support if one acknowledges the possibility of a similarly diffuse mechanism. The likelihood of a diffuse mechanism for the inhaled anesthetics becomes difficult to refute when one acknowledges the large number of models and *in vitro* systems in which function is altered by a pharmacologically narrow concentration range of drug (0.2–2.0 mM).

Progressing now to the most fundamental level, we consider the actual binding interaction between ligand and target, and the structural and dynamic consequences that couple binding with target function. Although incompletely understood in most proteins, coupling is rarely linear, and, therefore, anesthetic dissociation constant (K_d)—a measure of binding affinity—values are predicted to be quite different from the observed effective concentration for a 50% change in some response (EC_{50}) values. This is, of course, entirely in keeping with the behavior of many higher affinity ligands. Fentanyl, for example, has analgesic[16] or inhibition of intestinal motility[17] EC_{50} plasma values in the 0.5–5 nM free-concentration range. Conversely, measured K_d values for fentanyl binding to μ opiate receptors range from 7 nM to 400 nM,[17,18] the higher numbers (lower affinity) generally reflecting more physiologic conditions (the presence of sodium, G proteins, and guanosine 5′-triphosphate). Although perhaps expected from the amplification inherent in G-protein coupling, a wide separation of EC_{50} and K_d can also be seen in the ligand-gated ion channels. Pancuronium suppresses twitch tension by 50% at a free drug concentration of 0.3–0.4 μM,[19] whereas the K_d for the nicotinic acetylcholine receptor has been reported to be about 60 μM.[20] Although uncertain, because of the lack of examples, it seems possible that, for some ligands (those binding weakly to allosteric sites), a high level of occupancy may be necessary to produce EC_{50} effects. In other words, K_d could also be lower than EC_{50}. Finally, it is important to realize that it is not the apparent affinity (what is usually measured by binding assays) of a ligand protein interaction that dictates the effect on protein function, but rather the difference in affinity of the ligand for the different conformers (*e.g.*, active *vs.* resting) of a protein. Thus, it is possible for a ligand–protein interaction to exhibit relatively high affinity in binding assays, but have little functional consequence if there is no binding preference for the different functional conformers. Therefore, the direction and magnitude of a difference between binding affinity and the functional consequence is not predictable *a priori*, suggesting that their equivalence is a poor criterion of the functional relevance of binding sites.

Complicating issues further is the rarely considered "excluded-volume" effect. The typical ligand-binding study is performed in dilute solution, allowing the reactants to behave in a thermodynamically ideal fashion. However, *in vivo*, organelles, cytoskeletal components, soluble proteins, and other macromolecules and solutes occupy so much space that the thermodynamic activities of water, ligands, and proteins may be quite different than their actual concentrations, dramatically influencing their binding equilibria. Thus, increases in affinity of 5- to 20-fold have been reported for various protein–ligand interactions in the presence of high cosolute concentrations.[21,22] A decrease in the apparent affinity for a ligand–protein combination is also possible in the presence of a high concentration of cosolute that enhances solubility or binds weakly to the ligand. In addition to these cosolute effects on the ligand's activity, the target also may be altered by changes to its immediate environment produced by its isolation. For example, the conformation, dynamics, and hence ligand binding of many membrane proteins is controlled by the membrane potential, which is routinely altered by the isolation procedure or study conditions. Although all of these effects are important for understanding "effect-site" concentrations, they also indicate that caution must be exercised when extrapolating activity and binding constants determined in dilute *in vitro* systems in the *in vivo* setting.

Based on the forgoing discussion, it appears that rigorous adherence to clinical concentrations for *in vitro* binding studies may serve only to negate the possibility of deriving any useful parameters from the binding curves. And, as argued previously, complete concentration–effect relations are also necessary to characterize the functional response of an *in vitro* system, so that meaningful comparison (*e.g.*, of the EC_{50}) is possible. What volatile general anesthetic

concentrations should be examined in our *in vitro* experiments? We suggest that titration with anesthetic be continued until the response stops changing, so that curve fitting is possible and the appropriate parameters can be obtained with precision. Further, because we[23] and others[24,25] have noted that biphasic responses occur in some experimental systems, examination of the full concentration range is necessary to characterize the interaction to postulate and test mechanisms. Many investigators simply ignore the higher concentration (> 1 mM) effects as nonrelevant, assuming they result from different, toxic mechanisms, such as the wonderfully ambiguous "membrane" or "solvent" effects. Although different mechanisms of action at high concentrations are possible (such as lipid-mediated effects), there is little evidence to suggest that 0.1–10 mM anesthetic produces anything but a progression of the same mechanisms that cause clinical anesthesia. Again, this is entirely consistent with the behavior of more familiar, higher-affinity ligands, which produce progressively greater responses over a 100-fold concentration range. There are, of course, practical limitations to adding anesthetic until the *in vitro* response stops changing, in that most inhaled anesthetics have a maximum solubility less than two orders of magnitude higher than their MAC values, which introduces another set of complications and ambiguities that are beyond the scope of this discussion.

In summary, we simply must acknowledge what we do not know. Although clinically relevant concentrations of anesthetic are certainly relevant to the study of integrated responses in the intact animal, their relevance to *in vitro* studies must be tempered by our lack of understanding how various *in vitro* systems contribute to integrated responses. Collectively, we are beginning to construct a database of effects of anesthetics on various *in vitro* preparations, but we still uncertain how to integrate them into the anesthesia model. We share the frustration of investigators trying to approach the problem of how anesthetics work, but we believe that the strategy should not be to arbitrarily establish criteria that force a premature narrowing of our field of view. We must be thorough in our characterizations of how different anesthetics affect various systems and at what concentrations, so that when an integrated model that describes how each component contributes to the functioning of the nervous system becomes available for testing, we will be ready.

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