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A COMPARISON OF THE EFFECT OF EXPERIMENTAL GENERAL ANAESTHETICS ON NERVE IMPULSE BLOCKADE AND ON A PROTEINACEOUS TARGET SITE

Jaime Requena, Oscar Morán and Pedro Malavé,

Centro de Biociencias, Instituto Internacional de Estudios Avanzados (IDEA)
Apartado 17606 Parque Central, Caracas 1015A. VENEZUELA

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While it has been reported that general anaesthetics inhibit the enzyme luciferase and thus reduce the light output of the reaction with luciferin, we find that in squid giant axons injected with luciferin and luciferase, treatment with experimental general anaesthetics at concentrations sufficient to block axonal conduction leads to an increase in the light production by the reaction. This potentiation of the protein activity is best observed when luciferin concentration is above the apparent association constant. Our findings raise doubts regarding the suitability of luciferase as a model for the target region of general anesthetic action.

The inhibitory interaction of a variety of experimental general anaesthetics, such as n-alkanes and n-alkanols, with the water soluble enzyme luciferase has been advanced as strong support for the hypothesis that general anaesthesia can be understood in terms of interactions of the anaesthetic agent with a proteinaceous site (1-3). Although certain aspects of some of the evidence presented (2-3) has been questioned (4), it is nevertheless accepted that under the conditions employed in the in vitro test there is, following exposure to the anaesthetic agent, an inhibition in the light produced by the enzyme system. Controversy arises, mainly, from a comparison of the effect of chemical compounds under such widely disparate conditions as relating in vitro tests with in vivo experiments.

An important part of the protein site thesis (2-3) is that the light emission is inhibited by the experimental anaesthetics through a simple competition with luciferin for a hydrophobic pocket within the enzyme. While the occurrence of hydrophobic pockets in water soluble proteins are scarcely controversial, the existence of similar pockets in membrane proteins is intrinsically less likely owing to their partially hydrophobic environment. With this kind of considerations in mind, it was though of interest to observe the light emitted by the luciferase in an in vivo preparation and compare,

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simultaneously, the ability of some anaesthetics to block the nerve impulse generation. This sort of experiment could help to determine the validity of the model implicit in the comparison of anaesthetic potency data in in vivo and in vitro preparations.

An obvious choice for the general anaesthetics in vivo target site is the squid giant axon. This preparation has been shown to be the best understood excitable tissue. Moreover, at so-called clinical concentrations, the experimental anaesthetics affect the functioning of the ionic channels responsible for the generation and propagation of its action potentials (5).

MATERIALS AND METHODS

Giant axons from the tropical squid Loligo plei were carefully dissected out of the mantle and cleaned of connective tissue. They were mounted in a closed chamber designed for a rapid flow of artificial seawater (ca. 1 ml/min). This set up permitted solution changes to be accomplished in 15 s without the appearance of any artifact (6). The experimental arrangement, also allowed the simultaneous measurements of the transmembrane potential and the glow emitted by the luminescent systems. The microelectrode used for the potential measurement was coupled to a specially designed microliter syringe which permitted the axial injection of the enzyme and substrate in the axoplasm (7). The light produced by the chemiluminescent reaction was collected and carried, by an optical guide, to a photon counting apparatus. Under these conditions, the light output was very stable. It should be noticed that the initial glow (L1) depended upon the level reached by the enzyme system in the axoplasm, which varied somewhat from axon to axon. The composition of the artificial sea water (ASW) used to bath the axon was in mM:440 NaCl, 10 KCl, 10 $CaCl_2$, 50 $MgCl_2$, 10 Tris buffer pH 7.8 (2-Amino 2(hydroxymethyl) 1,3-propandiol) and 0.1 EDTA (Ethylenediamine tetraacetic acid). Luciferase pure and lyophilized from Photinus pyralis, was obtained from SIGMA Chemical (type VI, St. Louis, MO.) it was dissolved in 1 M K-MOPS (3-[N-Morpholino] propanesulfonic acid), buffered at pH 7.6 and at a concentration of 11.4 units/ml. Luciferin (synthetic, lyophilized, also from SIGMA) was dissolved in the same buffer at 20 mM concentration. 0.15 microliter of an equal mixture of luciferin and luciferase was injected into the axon, over a length of 12 mm. The axoplasmic concentrations of the reactants for the light reaction were normally: Luciferase 0.3 units/ml and luciferin 0.5 mM. The physiological concentration of electrolytes in the axons is (in mM):27 Na, 410 K, 4.2 Mg, 0.07 Ca, pH=7.3 (13). All experiment carried out at 18 $\pm$ 0.2° C. Superfusing solution were fed through Teflon lines to avoid loss of experimental general anaesthetics.

RESULTS AND DISCUSSION

Figure 1 displays a composite record of a typical experiment in a squid axon. The left panel shows the classical decrement in amplitude of the elicited action potential as induced by the progressive increase in concentration of 1-pentanol. Recovery from the alcohol treatment, as shown in the last part of the record by the return of the action potential amplitude to its initial value, was almost completefew minutes after the test solution was replaced by ASW. As reported elsewhere (5-8), at concentrations above 15 mM

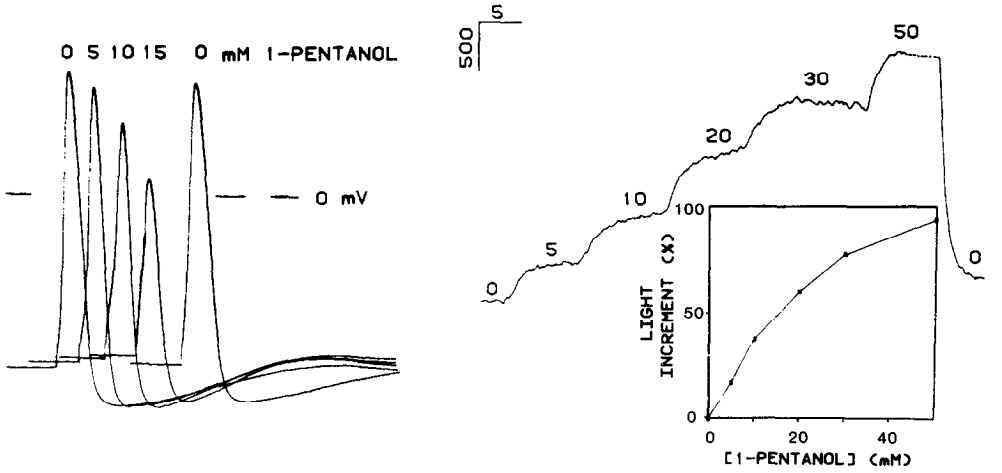

Figure 1.- The effect of 1-pentanol on the generation of action potential and on the luminescence of the luciferin + ATP + O_2 reaction catalyzed by luciferase on the same giant axon. Left panel shows, superimposed, the effect on the amplitude of the intracellular recorded action potential of 0, 5, 10, 15 and 0 mM of 1-pentanol in the artificial seawater (ASW). The time marks in the zero voltage base line correspond to 1 ms. The initial resting potential was -60 mV. Individual traces, taken 3 min after exposure to the test conditions, were displaced along the abscissa for the sake of clarity. Right panel shows the time course for the luminescence of the enzyme system luciferin luciferase when exposed to 0, 5, 10, 20, 30, 50 and 0 mM of 1-pentanol in the ASW. Initial glow was about 1900 counts/s. Calibration bars in the figure correspond to 500 counts/s and 5 min. Insert shows the fractional increment in light emitted (as percent), as a function of the 1-pentanol concentration in the sea water (in mM). Axon diameter was 400 micron.

1-pentanol in the artificial seawater bathing the axon, the action potential amplitude declined to zero. The right panel shows, for the same axon, the time course for the luminescence of the luciferase reaction observed during the periods of exposure to 1-pentanol referred left . With each increment in alcohol concentration there was a proportional enhancement of the light emitted such that, and as shown in the insert, with 50 mM of the alcohol in the extracellular fluid there is almost a doubling in the luminescence. At 15 mM 1-pentanol the light emitted by the enzyme system is about 50 % higher than that in the absence of the alcohol. For this particular experiment with 1-pentanol, the effect on the luciferase activation could be describe by an unimolecular absorption reaction with an apparent constant of about 45 mM. The observed effect upon the enzyme reaction are also reversible as shown at the end of the record where the light level returns to baseline with the removal of the 1-pentanol from the ASW bathing the axon.

The experiments referred above were realized with a final concentration of luciferin of 500 µM. This value is 30x higher than the reported K_m (15 µM at very low ionic strength) for the enzyme system in vitro

TABLE 1

SUMMARY OF EFFECTS OF VARIOUS CHEMICALS COMPOUNDS UPON THE LIGHT EMITTED BY LUCIFERASE INJECTED INTO AXONS

AXON NUMBER	AGENT	[CONC] (Mm)	LUMINESCENCE INITIAL GLOW(L1) (photons/s)	LUMINESCENCE FINAL GLOW(L2) (photons/s)	$\frac{L_2-L_1}{L_1}$ (%)	AXON ½ BLOCK (mM)
100387A	1-PENTANOL	100.0	950	1700	78.9	15.0
		50.0	950	1600	68.4	
190287A		15.0	3680	4800	30.4	
050387A		15.0	1875	2600	38.6	
		10.0	1875	2400	28.0	
		5.0	1875	2125	13.3	
031187A*		15.0	5150	6500	26.2	
210287A	1-HEXANOL	5.5	4420	5820	31.7	3.5
230287A	1-BROMOBUTANE	0.032	3120	3820	22.4	0.014
250287A		0.032	2150	2500	16.3	
		0.016	2425	2750	13.4	
		0.011	2000	2200	10.0	
190287B	1-BROMOPENTANE	0.010	4000	4580	12.5	0.006
210287B		0.010	3680	4000	8.7	
210287C	HALOTHANE	5.0	4050	4250	5.0	2.5
210287D	BENZOCAINE	1.0	6460	6780	4.9	1.0

* Luciferin concentration about 25 μM. All other experiments at ca. 500 μM.

(2-3). In order to optimize the competitive inhibitory mechanism, experiments were carried out at 25 μM luciferin. In Table 1, a result for 1-pentanol at this level of luciferin is reported and it was roughly indistinguishable from those obtained at high concentration of luciferin.

Notwithstanding this, if the concentration of luciferin is lowered further still, to a value below the K_m, the behavior of the luminescent reaction becomes biphasic following exposure to an alkanol. In effect, there is first a inhibition of the light emitted by the enzyme succeeded by a recovery phase. This phenomenon is shown in Fig. 2. An axon was injected with enough luciferin to yield a final concentration just below 10 μM and exposed to three concentrations of 1-pentanol. It can be seen that there is an apparent fractional reduction in the steady state level of light and of the order of 10, 28 and 67 % for 10, 20 and 50 mM of the alcohol. Also, it should be noticed that concomitant with the removal of the alcohol from the artificial sea water there is a large, reversible, overshoot in the light signal.

Given the form of the light response observed at this low level of luciferin, the true extent of the inhibition produced by the experimental agent is very difficult to ascertain. In effect, the transient response of the luminescence during alcohol exposure reveals an initial phase composed of a

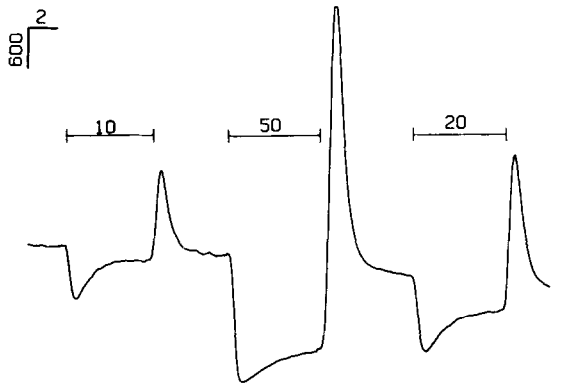

Figure 2.- The effect of 1-pentanol on the luminescence of the luciferin luciferase reaction in a giant axon injected with only 10 μM of luciferin. Figure shows the time course for the luminescence when tested with 10, 50 and 20 mM of pentanol in the artificial sea water. The axon was exposed to the alcohol concentration (in mM) shown above the horizontal thick line which covers the period of exposure. Calibration bars corresponds to 600 counts/s and 2 min. Axon diameter 375 microns. Initial resting glow corresponds to 2400 counts/s.

fast interaction of the alcohol with an inhibitory site of the enzyme, but it also shows a much slower recovery, or late potentiation phase, reminiscent of the enhancement in luminosity described above. Conversely, the large overshoot in luminescence during removal of the alcohol discloses first, a strong reduction in the extent of the inhibition caused by the alcohol, while the return to the baseline that follows reflects, in turn, the reversal of the potentiation. This late phenomenon caused by the much slower withdrawal of the alcohol from the stimulatory site.

In Table 1 we have summarized our experiments. For the bromoalkanes series there is a maximum fractional increase in luminescence of the order of 20 % for a saturating solution. For the normal alkanol series, the increment went as high as 80 % for the maximum range of concentration tested. At the level of concentration at which action potential amplitude reduces to half (an listed in the last column), the average luminescence increment is of the order of 30 % for the alkanols, while for the bromoalkanes it is only of the order of 10 %. This table also illustrates that the described effect is not limited to the functional derivatives of alkanes. It was also seen in compounds widely used in medical practice. Thus, there was a statistically significant 5 % increment in the light emitted by luciferase for halothane while a similar effect was observed for the local anaesthetic benzocaine, also at nerve block doses.

An enhancement of the light produced by luciferase could arise from an increment in ATP concentration resulting from interaction of the

anaesthetic agent with the metabolic machinery of the cell. Unlikely as it seems, mainly because ATP is buffered by argenine phosphate in the squid axon (9), this point was independently tested using the phosphoglyceric phosphokinase assay. In paired axons, it was found that there was not a significative variation in ATP from the control value of 1.8 mM during exposure to 25 mM 1-pentanol for 15 min.

Alternative explanations must, thus, revolve around changes in intracellular parameters. For example, although Ca_i and pH_i are known to be altered slightly during octanol exposure in axons (10-11), in the present experiments, they can not be invoked to justify an increase in light production. For the latter ion, changes should be negligible given the large amount of buffer injected into the axoplasm together with the enzyme. Nevertheless, the reported effect during alkanol exposure in unbuffered axons is to induce alkalinization of the order of 0.003 pH units (11). This level of change would not affect the light reaction given the sensitivity of luciferase to hydrogen ions (12). Regarding Ca ions, experimental general anaesthetics promote an increase of the free Ca concentration of the order of tens of nanomolar, a range not very significant, especially since they are in the axoplasm about 3-5 mM of divalent cations such as Mg (13).

A more realistic explanation for the enhancement effect could well be a conformational change in the enzyme due to interactions with elements of the axoplasm matrix. This could lead to a shift into a reaction pattern highlighted by a concentration of luciferin above the K_m. This pattern is evident, as well, at very low levels of luciferin where a biphasic response for the light signal was observed following treatment with an alcohol. This type of response has to be a consequence of the different rate of interaction of the experimental anaesthetic with two sites, one very fast and responsible of the inhibition, the other, much slower and capable of enhancing the light production. It should be mentioned that this reaction pattern was not possible to be reproduced in vitro. In effect, experiments in a rapid mixture all glass test chamber at low (15 µM) or high (500 µM) luciferin, either with luciferase at 10 nM or 100 nM (0.06 units/ml), only showed an inhibition for pentanol. In this case the reaction was initiated by the addition of ATP (2 mM final concentration) into 315 µl of 25 mM glycyl glycine buffer mixture.

It would, thus, appear that in vivo and in presence of the anaesthetic agent an hitherto dormant site, better observed when luciferin concentration is above the K_m, becomes regulatory with an excitatory character. Nevertheless, to accommodate the observation in in vitro tests with our main observation, that of a net enhancement in light production during

in vivo exposure to experimental anaesthetics, it is necessary to consider that the interaction resulting in a boost in light production has to dominate any inhibitory interaction, probably through a diminishment in competition at any inhibitory site.

Luciferase seems to be unsuitable as a model for a proteinaceous target site for general anaesthetic, mainly because is is quite unclear how simple organic substances such as alcohols interact and influence light emission. All in all, until a protein is found that responds in a plausible manner to general anaesthetics, the purely proteinaceous site hypothesis for anaesthesia will remain unattractive.

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