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The Selective Effects of Charged Local Anaesthetics on the Glucagon- and Fluoride-Stimulated Adenylate Cyclase Activity of Rat-Liver Plasma Membranes

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The cationic local anaesthetics carbocaine and nupercaine were found to increase the fluoride-stimulated adenylate cyclase up to a maximum level; above this maximum level further increases in drug concentration inhibited the enzyme. At concentrations where this activity was stimulated, a fatty acid spin label detected an increase in bilayer fluidity, which, it is suggested, is responsible for the activation of the enzyme. A solubilized enzyme was unaffected by the drugs, a finding consistent with this proposal.

These cationic drugs began to inhibit the glucagon-stimulated activity at concentrations where they activated the fluoride-stimulated activity. It is suggested that this is due to their effect on the coupling interaction between the receptor and catalytic unit.

The anionic drugs, phenobarbital, pentobarbital, and salicylic acid, all inhibited the fluoride-stimulated enzyme. This may be due in part to a direct effect on the protein and in part to the interaction of the drugs with the bilayer. The drugs had small inhibitory effects on the lubrol-solubilized enzyme.

The glucagon-stimulated enzyme was initially inhibited by the anionic drugs at low concentrations, then activated, and finally inhibited with increasing drug concentration. The reasons for such changes are complex, but there was no evidence from electron spin resonance studies to suggest that the elevations in activity were due to increases in bilayer fluidity.

Key words: glucagon, adenylate cyclase, anaesthetics, membrane bilayer fluidity

Local anaesthetics reversibly block conduction in nerves by preventing the influx of Na^+ during impulse propagation [1]. The precise molecular mechanism by which they achieve this effect is not understood, but the wide variation in structure and charge characteristic of local anaesthetics suggests that they work through a perturbation of the

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lipid bilayer [1]. The extent of hydrophobic interactions between the local anaesthetic and the lipid bilayer, or the ease of their incorporation into the bilayer, correlates well with anaesthetic potency [2]. The form of the perturbation that affects the protein(s) responsible for Na^+ conductance may be a change in bilayer fluidity [3, 4], a change in bilayer thickness [5], and/or a perturbation of membrane proteins [6, 7].

Charged local anaesthetics can alter cell shape [8, 9], cell spreading [10], cell movement [11], cell adhesion [10], cell fusion [11], and the binding of Ca^{2+} to cell membranes [1]. These effects are believed to be mediated through the action of the drug at the level of the surface membrane [12]. Sheetz and Singer [9] have suggested that, due to the now well-documented asymmetry of composition of plasma membranes where negatively charged lipids predominate on the cytosol side of the membrane and neutral ones on the external surface [13], drugs of opposite charge may act preferentially at one or the other half of the bilayer. Certainly in artificial membranes consisting of the negatively charged lipid phosphatidylserine, the amine local anaesthetics interact efficaciously [14], whereas the negatively charged barbiturates do not [15]. Indeed, incorporation of negative charges into bilayers of neutral phospholipids causes a very large augmentation of the effect of positively charged anaesthetics [16] in increasing bilayer fluidity.

We have demonstrated that in liver plasma membranes the glucagon receptor and the catalytic unit of adenylate cyclase are discrete units able to undergo free lateral diffusion in the bilayer [17]; however, upon addition of glucagon, the receptor and catalytic unit interact to form an activated complex spanning the bilayer membrane. In the absence of the hormone, the uncoupled catalytic unit, when stimulated directly by fluoride, is only sensitive to changes in the lipid environment of the cytosol side of the bilayer; but upon physical coupling with the receptor in the presence of glucagon, the complex responds to the lipid environment of both halves of the bilayer [18, 19]. This observation, together with our knowledge that increases in bilayer fluidity activate adenylate cyclase [20, 21] and that the bilayer is asymmetric with the negatively charged lipids predominantly at the cytosol half of the rat-liver plasma membrane [22], presents us with a functionally asymmetric system of some importance.

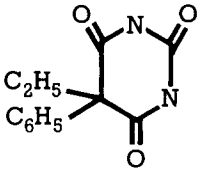
METHODS

Rat-liver plasma membranes were prepared as described by Houslay et al [23]. Fluoride- and glucagon-stimulated adenylate cyclase (EC 4.6.1.1.) were assayed at 30°C, using final concentrations of 15 mM NaF and 10^{-6} M glucagon, respectively, as previously reported [23]. The local anaesthetics did not interfere with the method of assay of cyclic AMP. Lubrol 12A9-solubilized enzyme was prepared as reported by Swislocki et al [24]; detergent-solubilized preparations are characteristically not stimulated by glucagon. Stock solutions of nupercaine and carbocaine were prepared at 100 mM. Phenobarbital and pentobarbital were made up as 40 mM solutions and the pH adjusted to 8.5 prior to dilution into the assay. Salicylic acid was made up as a 20 mM solution (pH 7.6) and then diluted into the assay (see Fig. 1 for structures of local anaesthetics). For assays of adenylate cyclase activity, the protein concentration was held at 0.4 mg/ml; the final assay pH with all constituents was 7.6. To test for reversal, membranes treated with these drugs in the assay mixture were mixed with 1 ml of 1 mM $KHCO_3$, pH 7.2, at 4°C and left for 10 min prior to centrifugation at 14,000g for 6 min. This procedure was usually repeated twice before resuspending the membranes for assay.

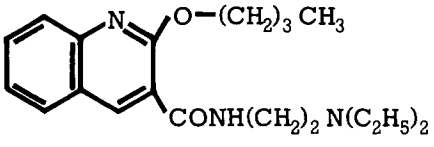
Methods for electron spin resonance (ESR) studies were essentially as described earlier in some detail [25]. No differences in order parameters or in the lipid-phase separations of these preparations occurred upon freeze-drying [21]. Furthermore, the fatty-acyl-chain profile obtained upon glc analysis of the methyl esters was unaffected by this procedure, as was the activity of adenylate cyclase [17]. Seventy μl samples of previously freeze-dried liver plasma membranes (4.3 or 1.4 mg protein/ml) were added to the N-oxyl-4′, 4′-dimethyl-oxazolidine derivative of 5-ketostearic acid, 1(12,3), that had initially been deposited on the side of a tube by evaporation from aqueous ethanol to yield probe ratios of 9 and 11 μg 1(12,3)/mg protein, respectively. Spectra were recorded on a Varian E-104A Century series ESR spectrometer at a constant temperature of 30°C [25].

The hyperfine splittings of labelled liver membranes were determined from "unexpanded spectra with magnified wings," as described by Gordon et al [25], to give an outer hyperfine splitting, $2T_{\parallel}$ and an inner hyperfine splitting $2T_{\perp}$. The inner hyperfine splitting, $2T_{\perp}$, could be accurately determined with membranes at 4.3 mg protein/ml,

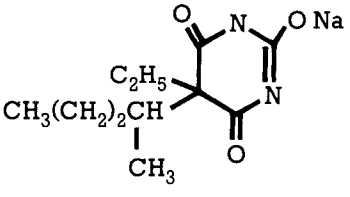
PHENOBARBITAL
(5-ethyl-5-phenylbarbituric acid)



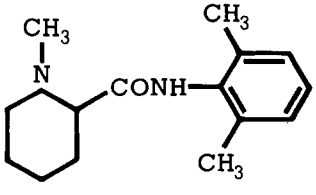
NUPERCAINE or DIBUCAINE
(2-butoxy-N-(2 diethyl-
aminoethyl cinchoninamide HCl)



PENTOBARBITAL [Na 5-ethyl-5-
(1-methylbutyl)-barbiturate]



CARBOCAINE or MEPIVACAINE
(1-methyl-2′,6′-pipecoloxylidide)



SALICYLIC ACID
(o-hydroxybenzoic acid)

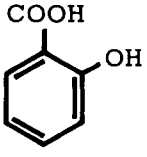


Fig. 1. Structures of local anaesthetics used in this study.

but at 1 mg/ml evaluation was less precise due to the overlap of "liquid-lines" [26]. For this reason, an order parameter $S(T_{\parallel})$ was used to assess fluidity as described previously [27]. Changes in $S(T_{\parallel})$ primarily reflect alterations in probe flexibility in rat-liver plasma membranes, with increases in $S(T_{\parallel})$ denoting decreases in the mobility of the probe. Experimentally-determined, low probe concentrations were used following the criteria set by Sauerheber et al [26].

We note (unpublished experiments) that the polarity-corrected order parameter, S, decreased with increasing concentrations of the cationic anaesthetics, implying that the decreases in $S(T_{\parallel})$ do indeed monitor increases in bilayer fluidity. The definitions of these order-parameter expressions [27], used to evaluate the flexibility of the liver plasma membrane-incorporated fatty-acid spin labels, are:

$$S(T_{\parallel}) = \tfrac{1}{2}\left[\frac{3(T_{\parallel} - T_{xx})}{(T_{zz} - T_{xx})} - 1\right] \quad (1)$$

and

$$S(T_{\perp}) = \tfrac{1}{2}\left[\frac{3[(T_{zz} + T_{xx}) - 2T_{\perp}]}{(T_{zz} - T_{xx})} - 1\right] \quad (2)$$

$$S = \frac{(T_{\parallel} - T_{\perp})(a_N)}{(T_{zz} - T_{xx})(a'_N)} \quad (3)$$

where T_{xx} and T_{zz} are the hyperfine splitting elements of the static interaction tensor ($\underset{\sim}{T}$) parallel to the static Hamiltonian ($\underline{H}$), principal nuclear hyperfine axes x and z, respectively. The x axis is parallel to the N-O bond direction, and the axis is parallel to the nitrogen $2p\pi$ orbital. Values for the elements of $\underset{\sim}{T}$ used in this study were taken as before [27]. The symbols a'_N and a_N represent the isotropic hyperfine coupling constants for the probe in the membrane and crystal states, respectively [ie, $a'_N = 1/3\,(T_{\parallel} + 2T_{\perp})$ and $a_N = 1/3\,(T_{zz} + 2T_{xx})$].

Syva Co., Palo Alto, California, provided the 1 (12,3). Creatine kinase, ATP, cyclic AMP, creatine phosphate, and triethanolamine HCl were from Boerhinger (UK) Ltd., East Sussex, UK. Sucrose was of AR grade from either Calbiochem, La Jolla, California or from BDH Chemicals, Dorset, UK. Glucagon was a kind gift from Dr. W. W. Bromer, Lilly Research Laboratories, Indianapolis. Lubrol 12A9 was a kind gift from ICI pharmaceuticals, Cheshire, UK. Phenobarbital, pentobarbital, and salicylic acid were from Sigma. Nupercaine and carbocaine were kind gifts from Ciba-Geigy Ltd., Macclesfield, UK. All other chemicals were of AR grade from DBH Chemicals, Dorset, UK.

RESULTS

Glucagon and fluoride routinely stimulated the adenylate cyclase activity of our preparation 30- and 7-fold, respectively, as previously reported [20, 23].

Cationic Drugs

The effects of the amine local anaesthetics nupercaine and carbocaine (Fig. 1) on adenylate cyclase activity were investigated. As shown in Figure 2A and B, increasing concentrations of these compounds led to a point where the fluoride-stimulated activity

was enhanced. This activation reached a peak at about 40% above the initial activity of the enzyme; further increases in drug concentration strongly inhibited the activity. When these experiments were repeated on an enzyme preparation that had been solubilized with the nonionic detergent, Lubrol 12A9, the fluoride-stimulated activity exhibited no response to these concentrations of drugs. In contrast to the effect of these agents on the fluoride-stimulated activity, the glucagon-stimulated activity began to be inhibited by the drugs (Fig. 3A, B) at concentrations where the fluoride-stimulated activity began to increase. Further addition of these compounds led to a progressive inhibition of the glucagon-stimulated activity. It is of particular interest that the above perturbations on adenylate cyclase activity were seen with nupercaine at a 10-fold lower concentration

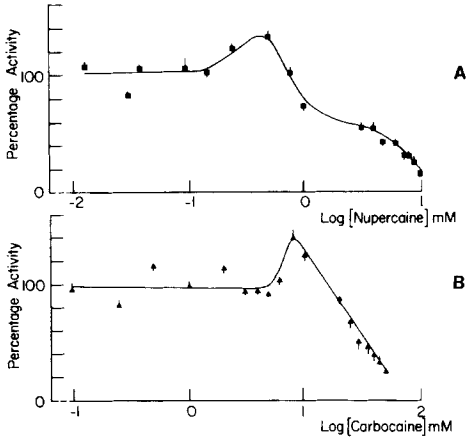


Fig. 2. The action of cationic drugs on the fluoride-stimulated adenylate cyclase activity of rat-liver plasma membranes. A) Nupercaine. B) Carbocaine. Experiments were performed on three different plasma membrane preparations. Errors are given as ± SEM on six determinations of activity.

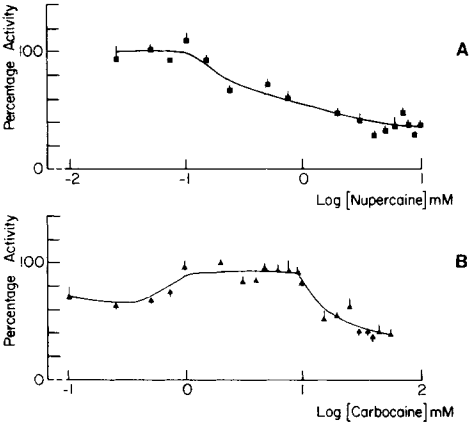


Fig. 3. The action of cationic drugs on the glucagon-stimulated adenylate cyclase activity of rat-liver plasma membranes. A) Nupercaine. B) Carbocaine. Experiments were performed on three different plasma membrane preparations. Errors are given as ± 1 SEM on six determinations of activity.

than with carbocaine. All of the effects described were fully reversible upon washing to remove the anaesthetic. None of the drugs caused time-dependent changes in activity as time courses were linear over 10 min.

Nupercaine and carbocaine were examined for their ability to affect the fluidity of liver plasma membranes over the concentrations tested. As demonstrated in Figure 4A and B, both of these drugs initiate increases in the membrane fluidity [decrease $S(T_{\parallel})$] at concentrations that correspond to those augmenting the fluoride-stimulated adenylate cyclase and inhibiting the glucagon-stimulated activity. However, the concentration at which nupercaine began to fluidize liver membranes was 10-fold less than the corresponding concentration for carbocaine. The changes in fluidity induced by carbocaine were similar at either membrane concentration (4.3 or 1.4 mg protein/ml); as shown in Figure 4, nupercaine was examined only at 1.4 mg protein/ml.

Anionic Drugs

Three anionic drugs, phenobarbital, pentobarbital, and salicylic acid (Fig. 1), were examined for their effects on the adenylate cyclase activity of rat-liver plasma membranes. Increase in concentration of all of these drugs effected a progressive inhibition of the fluoride-stimulated activity (Fig. 5A, B, C). With the lubrol-solubilized preparation, the maximum amount of inhibition of activity observed at the highest drug concentrations used was 30 ± 10% for pentobarbital (20 mM), 20 ± 10% for phenobarbital (20 mM), and 10 ± 10% for salicylic acid (10 mM), compared with 90 ± 2% (pentobarbital),

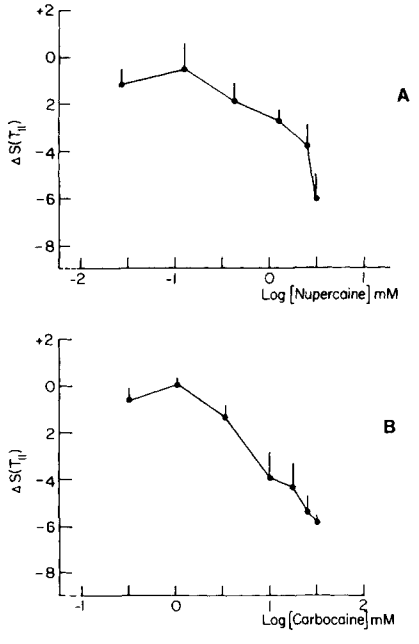


Fig. 4. The effect of cationic drugs on the fluidity of rat-liver plasma membranes. These plots show the percentage change in the order parameter $S(T_{\parallel})$, $\Delta S(T_{\parallel})$, for 1(12,3)-labelled rat-liver plasma membranes as a function of drug concentration; $S(T_{\parallel})$ is defined as in Gordon and Sauerheber [27]. Errors are given as ± 1 SEM for four determinations using three different membrane preparations. A) Nupercaine. B) Carbocaine. Experiments were carried out with nupercaine at 1.4 mg protein/ml and with carbocaine at either 1.4 or 4.3 mg protein/ml.

70 ± 5% (phenobarbital), and 97 ± 2% (salicylic acid) for the membrane-bound enzyme. In marked contrast to these effects, the drugs appeared to inhibit the glucagon-stimulated adenylate cyclase activity at low concentrations, then markedly activate it up to a peak, whereupon further increases in drug concentration progressively inhibited the activity (Fig. 6A, B, C).

All of these drug effects were fully reversible upon washing the membranes to remove the drug. None of these changes in activity were time-dependent, as rates were linear over the 10 min time course of the assay.

Over the concentration range tested, it is clear that salicylic acid had no effect on the fluidity of rat-liver plasma membranes (Fig. 7B). In experiments with phenobarbital, we found that at 4.3 protein/ml of this drug had no effect on the fluidity of the membranes, while at 1.4 mg protein/ml it actually decreased the fluidity as indicated by an increase in $S(T_{\parallel})$ (Fig. 7A). Unfortunately, we were not able to use lower protein concentrations than 1.4 mg protein/ml for ESR studies because that necessitated an increase in probe-membrane ratio, which significantly perturbs the spectra obtained (see [25, 26] for discussion).

Increases in $S(T_{\parallel})$ with phenobarbital could be due either to rigidization or a relatively large increase in the polarity of the probe. This uncertainty cannot be resolved by examining the effects on S, since the increase in $S(T_{\parallel})$ occurs only at low mg protein/ml where the "fluid" component substantially interferes with the $2T_{\perp}$ measurement. However, we consider it more likely that the increase in $S(T_{\parallel})$ with increasing phenobarbital is due to a rigidity change.

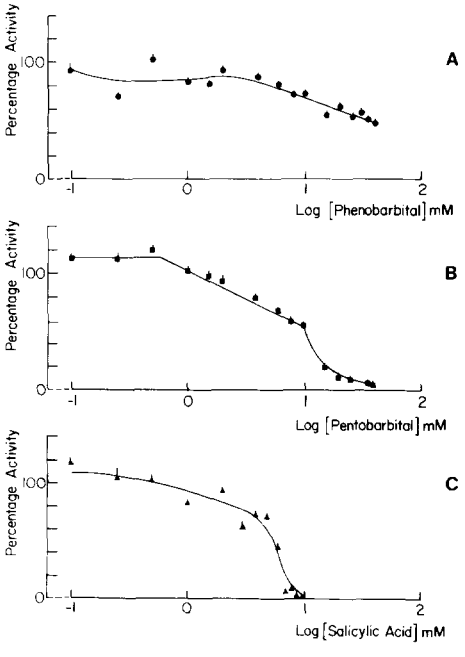


Fig. 5. The action of anionic drugs on the fluoride-stimulated adenylate cyclase activity of rat-liver plasma membranes. A) Phenobarbital. B) Pentobarbital. C) Salicylic acid. Experiments were performed on three different membrane preparations. Errors are given as ± 1 SEM on six determinations of activity.

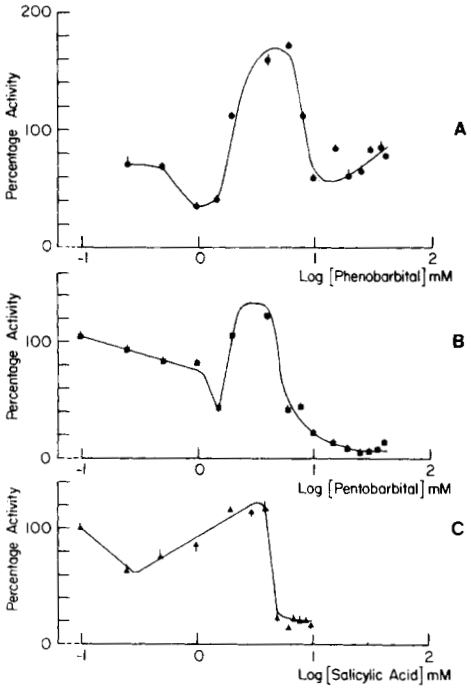


Fig. 6. The action of anionic drugs on the glucagon-stimulated adenylate cyclase activity of rat-liver plasma membranes. A) Phenobarbital. B) Pentobarbital. C) Salicylic acid. Experiments were performed on three different membrane preparations. Errors are given as ± 1 SEM on six determinations of activity.

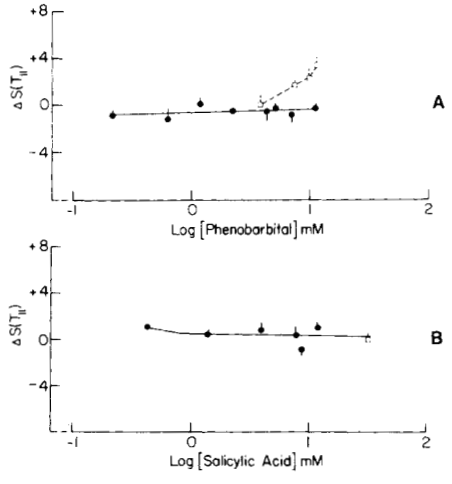


Fig. 7. The effect of anionic drugs on the fluidity of rat-liver plasma membranes. These plots show the percentage change in the order parameter $S(T_{\|})$, $\Delta S(T_{\|})$, for 1(12,3)-labelled rat-liver plasma membranes as a function of drug concentration; $S(T_{\|})$ is defined as in Gordon and Sauerheber [27]. Errors are given as ± 1 SEM for four determinations using three different membrane preparations. A) Phenobarbital. B) Salicylic acid. Solid lines and closed circles denote experiments carried out at 4.3 mg protein/ml; broken lines and open rectangles are those carried out at 1.4 mg protein/ml.

DISCUSSION

All of the anaesthetics achieved profound effects on the activity of adenylate cyclase from rat-liver plasma membranes. In instances where activation was observed, we do not believe it to be due to latent activity because extensive investigations [18, 28] have failed to provide evidence for significant permeability barriers in our membrane preparations.

The cationic anaesthetics examined in our study activated the fluoride-stimulated activity at concentrations where they achieved an increase in the fluidity of the bilayer. In previous studies conducted with liver plasma membranes, low benzyl alcohol concentrations (up to 30–40 mM) progressively activated basal and fluoride-stimulated adenylate cyclase and also increased the lipid fluidity [20, 21]. It is tempting to attribute this activation, which has also been seen with short-chain alcohols [29], to the increase in bilayer fluidity relieving a physical constraint upon the protein, leading to an increase in conformational flexibility of the protein and hence activation. Support for this hypothesis comes from the observation that nupercaine initiates increases in both the fluoride-stimulated adenylate cyclase activity and the membrane fluidity at a concentration that is an order of magnitude less than that noted for carbocaine.

The catalytic unit of adenylate cyclase is firmly associated with the cytosol half of the bilayer and only experiences changes in the lipid environment of this bilayer half, unless it is coupled to the glucagon receptor [18, 19]. Since it is in this half of the bilayer that the negatively charged lipids predominate [22], and cationic local anaesthetics efficaciously act to increase the fluidity of such lipids in model systems [14, 15], it seems reasonable to assume that a significant fraction of the increase in bilayer fluidity observed in our ESR studies occurred in the half of the bilayer that originally faced the cytosol. That these effects on activity were not seen with the solubilized enzyme and that detergents can activate the enzyme [20] support our contention that, in this instance, the drugs act at the level of the membrane and that the native enzyme is constrained by the physical properties of the bilayer lipids.

Interestingly, these cationic drugs did not cause an enhancement of the glucagon-stimulated activity, but instead began to inhibit the enzyme at concentrations where activation of the fluoride-stimulated activity commenced (Figs. 2, 3). One might expect that, since the catalytic unit was activated due to an increase in the fluidity of the surrounding lipid, the coupled catalytic unit would exhibit a parallel increase in activity; this clearly was not the case. That the inhibition ensued at a similar concentration to the activation of the catalytic unit suggests that they have a common origin. Presumably, this is the interaction of the cationic drug with the bilayer leading to an increase in fluidity, an event that we have suggested is preferentially localized to the inner half of the bilayer. However, this may well affect the coupled enzyme activity in a different manner. We have proposed that the vertical positioning of the catalytic unit in the bilayer alters upon its coupling to the glucagon receptor [20]. Nuclear magnetic resonance (NMR) studies [30] indicate that the positively charged group of the cationic anaesthetic is located at the same level as the negatively charged phospholipid phosphate; thus, a change in the location of the protein in the vertical plane of the bilayer could lead it to react unfavorably with the surrounding lipid or drug. An alternative explanation centers on the demonstration that negatively charged phospholipids are intimately involved in the coupling process between the receptor and catalytic unit [31–34]. From thermodynamic considerations, it has been suggested that this interaction between the units occurs at

the cytosol-facing side of the plasma membrane [35]. Because there is good evidence from NMR studies for a strong interaction between cationic anaesthetics and negatively charged phospholipids [36], the involvement of such lipids in the coupling process may well yield an inhibitory response that would mask any activation caused by an increase in bilayer fluidity.

The anionic anaesthetics highlight the differences in response of the coupled and uncoupled activities to charged drugs. In each case, the glucagon-stimulated activity was inhibited by the drug at low concentrations, then activated as the drug concentration rose to nerve-blocking concentrations, and was finally inhibited at high levels of the drug. It is apparent that there were no significant increases in bilayer fluidity associated with the concentrations of drugs effecting the observed activations. With phenobarbital, we actually observed an increase in the rigidity of the bilayer. Such an observation has been noted for pentobarbital in model phospholipid bilayers containing less than 14 mol% cholesterol [37]. In this instance, it was suggested that the barbiturate could have an ordering effect on the bilayer in an analogous fashion to cholesterol. It is possible that the changes in activity we observed with these anionic drugs are due to changes in fluidity in areas of the bilayer in which the proteins are restricted, and the probe, by presenting us with an average picture, may lead to the masking of such effects. However, the simplest explanation for the effects of these drugs on the activities is that they interact directly with the protein itself. The inhibition of the fluoride-stimulated activity is likely to be due to similar direct effects on the protein, and indeed, our observations that these anionic drugs inhibited the solubilized enzyme suggest that they can affect the protein directly or are able to alter the surface charge and activity. If the anionic drugs act preferentially at the external surface of the membrane because the negatively charged phospholipids in rat-liver plasma membranes appear to predominate at the opposite face [22], then we might well expect to observe selective effects on the glucagon-stimulated activity.

Our studies emphasize the fact that, at anaesthetically relevant concentrations of drugs, the activity of a functioning membrane protein can be disturbed. It is likely that some of these effects are mediated at the lipid-protein interface in the bilayer and that this can be a rather specific event peculiar to a particular protein, rather than a general membrane perturbation such as lipid fluidization.

The inhibitory effects on adenylate cyclase, observed at the higher anaesthetic concentrations used in this study and with high concentrations of the neutral anaesthetic benzyl alcohol [20, 21], could be interpreted as preventing annular/boundary lipid [38, 39] from interacting with sites on the protein. Certainly, that specific headgroup phospholipids have been implicated in the functioning of adenylate cyclase [31–34] provides corroboration for this notion. Occupancy of such sites by the anaesthetic would then lead to inhibition of activity because either the anaesthetic itself was inhibitory or the displaced lipid was essential for activity. It is tempting to offer such an *Annular Lipid Displacement Model* for the phenomenon of local anaesthesia where the protein in question is the sodium channel.

As a final note we would like to suggest that some of the changes in cell activity discussed in the introduction may be due in part to changes in cyclic AMP levels effected by the action of local anaesthetics on adenylate cyclase. Certainly tetracaine has been shown to block glucagon-mediated gluconeogenesis in the liver [40], which would be in accord with our observed inhibition of glucagon-stimulated adenylate cyclase by cationic anaesthetics. Furthermore, dibucaine (nupercaine) can alter cyclic AMP levels in fat

cells [41] in a manner consistent with our studies and those of Hepp et al [42], who observed an inhibition of noradrenaline-stimulated adenylate cyclase in fat cells by tetracaine.

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