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DIMETHYLNITROSAMINE INHIBITS THE GLUCAGON-STIMULATED ADENYLATE CYCLASE ACTIVITY OF RAT LIVER PLASMA MEMBRANES AND DECREASES PLASMA MEMBRANE FLUIDITY

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The effect of the hepatocarcinogen dimethylnitrosamine on rat liver plasma membrane adenylate cyclase activity and lipid fluidity was assessed. Glucagon-stimulated adenylate cyclase activity exhibited a complex response to increasing concentrations of dimethylnitrosamine, whereas fluoride-stimulated adenylate cyclase activity was progressively inhibited. Maximal inhibitory effects were observed at a concentration of 15 mM in both cases. The activity of detergent-solubilized adenylate cyclase was unaffected by dimethylnitrosamine. ESR analysis using a fatty acid spin probe showed that dimethylnitrosamine produced a marked, dose-dependent reduction in the fluidity of the plasma membrane with a maximal effect occurring at 20 mM. Dimethylnitrosamine also elevated the temperature at which the lipid phase separation occurred in rat liver plasma membranes, from 28°C to 31°C. The non-carcinogenic but structurally similar compound, dimethylamine hydrochloride neither inhibited adenylate cyclase nor decreased plasma membrane fluidity. It is suggested that the decrease in membrane fluidity, induced by dimethylnitrosamine, is responsible for the observed inhibition of adenylate cyclase and that dimethylnitrosamine, via its effects on membrane fluidity, could influence plasma membrane function and cellular regulation.

Introduction

Changes in plasma membrane fluidity [1–3], membrane lipid composition [4–6] and membrane functioning [4,6] have all been associated with malignant transformation. Furthermore, the activity of many integral membrane proteins has been shown to be modulated by alterations in the physical properties of their membrane environment (see Refs. 7–10).

Adenylate cyclase is a key regulatory enzyme which mediates the actions of a large variety of hormones and neurotransmitters by elevating intracellular cyclic AMP concentrations (see, for example, Refs. 11 and 12). It has been suggested that alterations in cyclic AMP concentrations and, in particular, the functioning of adenylate cyclase might exert effects on cell growth and development (see, for example Refs. 13 and 14). Indeed, transformed cells have been demonstrated to have lower cyclic AMP concentrations than the corresponding untransformed cells [15,16]. In liver [17–19] and other tissues [20–23] the activity of the integral, plasma membrane enzyme, adenylate cyclase is highly sensitive to alterations in membrane fluidity. We demonstrate here that the liver carcinogen, dimethylnitrosamine decreases the fluidity of the liver plasma membrane, as detected

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using a fatty acid spin label, and inhibits the activity of adenylate cyclase.

Materials and Methods

The I(12,3) spin label (*N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-oxosteric acid) was obtained from Syva Co., Palo Alto, CA, U.S.A. Creatine kinase, phosphocreatine, ATP, cyclic AMP, and triethanolamine hydrochloride were from Boerhinger (U.K.) Ltd. Dimethylnitrosamine was obtained from Eastman/Kodak, Rochester, NY, U.S.A. Di[^{14}C]methylnitrosamine was synthesised from di[^{14}C]methylamine hydrochloride obtained from Amersham International, Amersham, U.K. All other chemicals were of AnalaR quality from BDH Chemicals, Poole, Dorset, U.K.

Liver plasma membranes were isolated from male Sprague-Dawley rats weighing 200–300 g as described previously [24]. Fluoride- and glucagon-stimulated adenylate cyclase (EC 4.6.1.1) were assayed over linear time-courses at constant pH as previously described [24], except that membranes were preincubated with either dimethylnitrosamine or dimethylamine hydrochloride for 15 min at 4°C prior to assay. Fluoride-preactivated adenylate cyclase was solubilized with Lubrol PX as described by Swislocki et al. [25]. Dimethylnitrosamine was shown not to affect the method of determination of cyclic AMP. The determination of breakpoints in Arrhenius plots was performed using a least-squares fitting procedure as described previously by us [26]. Protein was determined using a modified microbiuret method [26].

Electron spin resonance measurements were made using a Varian E-9 X band spectrometer equipped with variable temperature accessory (Varian E-257). Measurements were made at a 2.0 G modulation amplitude, 0.30 S time constant, 4 min time scan, 10 mW microwave power on a scan range of 100 G. Sample spectra were compared with a standard manganese sample in a microwave cavity operating in the HO14 mode. The spectrometer was used in conjunction with a Nicolet 1020A signal averager and the inner and outer hyperfine splittings, $2T_{\perp}$ and $2T_{\parallel}$ were measured in terms of channels on the averager, calibrated by comparison with the separation of the manganese marker peaks. The order parameter S of rat liver plasma membranes was calculated as previously detailed [7]. The value of S reflects the membrane fluidity. or the fluidity of the membrane incorporated spin probe. S may assume a value between 0 and 1, representing a free fluid or immobilized environment, respectively.

The method of preparation of rat liver plasma membranes for ESR studies was as described earlier using an experimentally determined 'low probe' concentration [7] of 9 μg of probe per mg of protein. All samples were preincubated at 4°C for 15 min after the addition of dimethylnitrosamine or dimethylamine hydrochloride before analysis.

In some experiments plasma membranes samples were washed (three times) to remove dimethylnitrosamine by the addition of 10 vol. of 1 mM $KHCO_3$ (pH 7.4) at 4°C followed by centrifugation for 5 min at $14000 \times g$.

Dimethylnitrosamine might affect liver plasma membrane fluidity by partitioning into sites within the bilayer of the liver plasma membrane. If the following assumptions hold, (i) there are a finite number of dimethylnitrosamine sites; (ii) dimethylnitrosamine binding is reversible; (iii) ΔS, the percentage change in fluidity induced by dimethylnitrosamine is proportional to the density of membrane sites occupied by dimethylnitrosamine; and (iv) one class of membrane sites is present, then by a similar reasoning to that expounded by us in some detail elsewhere [27], for the interaction of Ca^{2+} with lipids of this membrane, a plot of $\Delta S_{max}/\Delta S$ vs. 1/[dimethylnitrosamine] should yield a straight line. The slope of such a straight line will reflect the association constant (K_a) for the dimethylnitrosamine binding sites in the membrane.

Results

Increasing concentrations of dimethylnitrosamine progressively inhibited the fluoride-stimulated adenylate cyclase activity of liver plasma membranes (Fig. 1a). A maximum inhibitory effect of about 40% occurred at around 10 mM dimethylnitrosamine. However, the non-carcinogenic analogue of dimethylnitrosamine, dimethylamine failed to inhibit this activity; instead at a concentration around 5 mM it elicited a small

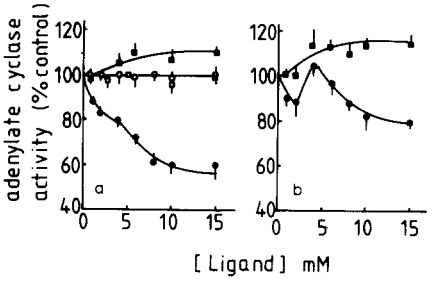

Fig. 1. The response of adenylate cyclase to dimethylnitrosamine and dimethylamine. (a) Adenylate cyclase activity was stimulated by 15 mM NaF and the response to dimethylnitrosamine (●, ○) and dimethylamine (■, □) observed using either liver plasma membranes (●, ■) or a solubilized (○, □) enzyme preparation. (b) Adenylate cyclase was stimulated by 1 μM glucagon and the response to dimethylnitrosamine (●) and dimethylamine (■) noted. All assays were performed at 30 °C taking initial rates of cyclic AMP production from linear time-courses. Each point represents the mean (±S.D.) of six determiantions using three different membrane preparations.

increase, of around 10%, in the fluoride-stimulated activity (Fig. 1a). Indeed this may correspond to an inflection occurring at around this concentration in the inhibitory response of fluoride-stimulated adenylate cyclase to dimethylnitrosamine (Fig. 1a).

In contrast to these results, if the fluoride-stimulated adenylate cyclase activity was solubilized using the non-ionic detergent Lubrol PX, then neither dimethylnitrosamine nor dimethylamine exerted effects on adenylate cyclase activity over this concentration range (Fig. 1a).

Increasing concentrations of dimethylnitrosamine exerted a complex effect on the glucagon-stimulated adenylate cyclase activity of rat liver plasma membranes (Fig. 1b). An initial inhibitory phase was followed by a small activation and then a progressive inhibition upon raising the dimethylnitrosamine concentration further. The maximum inhibitory effect was elicited at a concentration of around 10 mM dimethylnitrosamine. In contrast, the non-carcinogenic analogue, dimethylamine elicited a small activation of the activity (Fig. 1b). This occurred at around 2–5 mM dimethylamine and was comparable, in both this regard and in magnitude, to the small activation phase observed with dimethylnitrosamine. A functional glucagon-stimulated adenylate cyclase activity cannot be solubilized [25] for a study of drug effects in a membrane-free environment.

In native membranes we and others (for review, see Refs. 17 and 28) have demonstrated that Arrhenius plots of glucagon-stimulated adenylate cyclase exhibit a well-defined break at around 28 °C (here 27.8 ± 0.5 °C). This is caused by a lipid phase separation which has been proposed to occur in the external half of the bilayer of liver plasma membranes [17,28]. In the presence of dimethylnitrosamine (8 mM) this break was shifted (Fig. 2) to a higher temperature, 31.0 ± 0.5 °C with activation energies of 33.2 ± 4.6 $kJ \cdot mol^{-1}$ and 90.3 ± 8.3 $kJ \cdot mol^{-1}$ found at temperatures above and below the break, respectively (errors are S.D., $n = 4$). This compares with activation energies of 29.2 ± 3.3 and 83.3 ± 9.1 $kJ \cdot mol^{-1}$ for native membranes (All errors are S.D., $n = 4$). In contrast Arrhenius plots of fluoride-stimulated adenylate cyclase activity were linear. It has been proposed that this is because under such conditions the catalytic unit of adenylate cyclase and its guanine nucleotide regulatory unit are not associated with the glucagon receptor and only experience the lipid environment of the inner half of the bilayer where no lipid phase separation occurs over the temperature range examined [17,28,29]. In the presence of dimethylnitrosamine (8 mM) Arrhenius

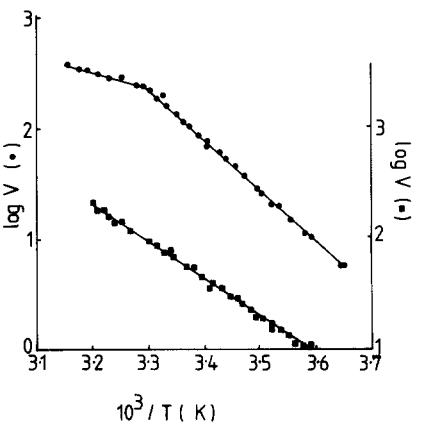

Fig. 2. Arrhenius plots of glucagon- and fluoride-stimulated adenylate cyclase activity. Arrhenius plots of the glucagon- (●) and fluoride- (■) stimulated adenylate cyclase activities assayed in the presence of 8 mM dimethylnitrosamine are shown. Activity is expressed as μunits/mg protein. A typical plot is shown.

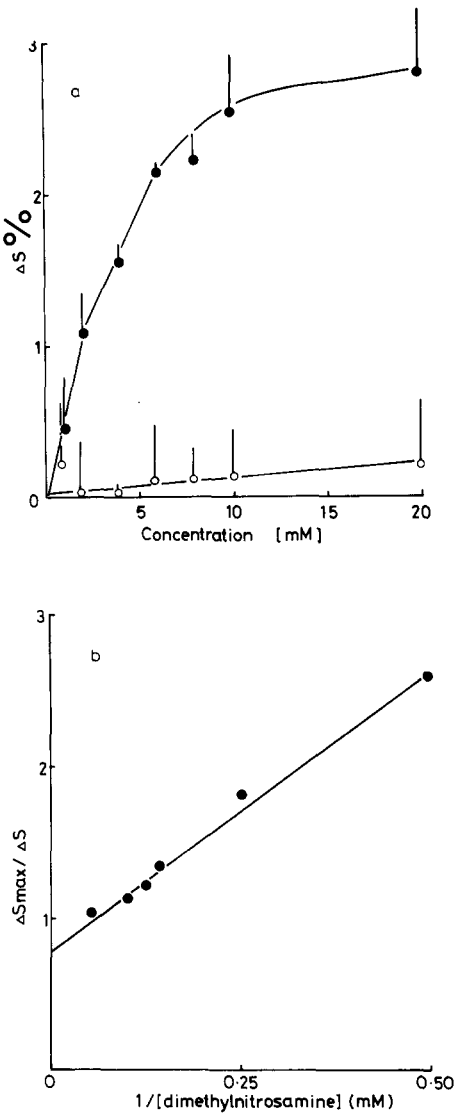

Fig. 3. The effect of dimethylnitrosamine on plasma membrane fluidity. (a) The percentage change in the polarity corrected order parameter S for I(12,3)-labelled liver plasma membranes, at 30 °C, in response to dimethylnitrosamine (●) and dimethylamine (○) are shown. Each point represents the mean (± S.D.) obtained using three different membrane preparations. Under control conditions the value of S obtained is 0.621. (b) As defined in the methods and more fully in Ref. 27 an association constant for those dimethylnitrosamine sites on the plasma membrane which decrease membrane fluidity can be calculated. Here $\Delta S_{max}/\Delta S$ is plotted against 1/[dimethylnitrosamine]. ΔS_{max} is the maximal percentage change in S at saturating dimethylnitrosamine concentrations. Data are from Fig. 3a. Curve (parabolic) fitting analysis [35] of these data yielded a K_a of 5.1 ± 1.0 (S.D.) for these data.

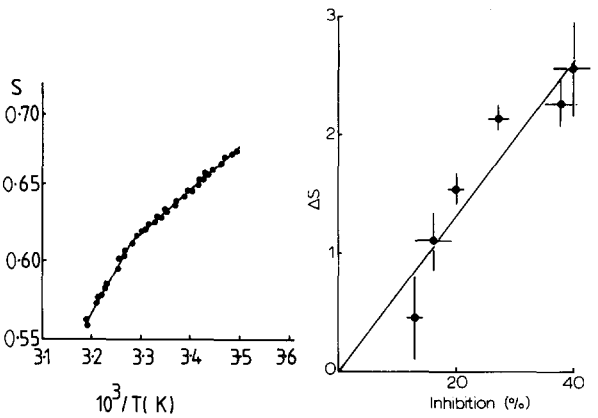

Fig. 4. Effect of dimethylnitrosamine on the temperature dependence of the order parameter S. The value of the order parameter S for I(12,3)-labelled plasma membranes is plotted as a function of the reciprocal of the absolute temperature (see Refs. 7 and 27). Membranes were labelled with an experimentally determined low probe concentration (see Methods). Control membranes identified the high temperature onset of the lipid phase separation occurring at 28 °C as defined by us and others previously [17,28]. Here data are shown for membranes exposed to 8 mM dimethylnitrosamine.

Fig. 5. The correlation between the inhibition of fluoride-stimulated adenylate cyclase activity and the decrease in membrane fluidity induced by dimethylnitrosamine. ΔS, the percentage change in the order parameter S with respect to control values, measured in the absence of dimethylnitrosamine, is plotted against the percentage inhibition of fluoride-stimulated adenylate cyclase induced by increasing dimethylnitrosamine concentrations (from Figs. 1 and 3a). Each point is the mean of six determinations of both ΔS and also fluoride-stimulated enzymic activity on at least three plasma membrane preparations. Error base represent ± 1 S.D.

plots of fluoride-stimulated adenylate cyclase remained linear yielding an activation energy of 74.4 ± 4.3 kJ · mol^{-1} compared with an activation energy of 63.4 ± 3.6 kJ · mol^{-1} observed in its absence (All errors are S.D., $n = 4$).

Upon ESR analysis of liver plasma membranes labelled with the fatty acid spin probe I(12,3) we observed that concomitant with the addition of increasing concentrations of dimethylnitrosamine there was a parallel increase in the value of the polarity corrected order parameter S (Fig. 3a). This suggests that dimethylnitrosamine decreased the fluidity of liver plasma membranes. In contrast, dimethylamine HCl failed to exert an effect on S, or the inner and outer hyperfine splittings.

Arrhenius-type plots of the polarity-corrected order parameter S for I(12,3)-labelled plasma membranes exhibit a well-defined discontinuity at 28°C due to a lipid phase separation occurring in these membranes (Fig. 4). This has been detected previously using ESR and a variety of other physical techniques (for review, see Refs. 7, 17 and 28). Here we note (Fig. 4) that in the presence of dimethylitrosamine (8 mM) the high temperature onset of this lipid phase separation was increased to 31.7 ± 0.3°C (S.D., $n = 3$).

The decrease in plasma membrane fluidity effected by dimethylnitrosamine was found to be fully reversed by a simple washing procedure. This involved dilution with 1 mM $KHCO_3$ buffer (pH 7.2) followed by centrifugation at $15\,000 \times g$ for 10 min at 4°C; such a procedure was repeated three times. This was consistent with our observations that plasma membranes incubated with di[^{14}C]methylnitrosamine (0.3 μCi/mg of protein) for up to 4 h at 30°C showed no retention of radioactivity after washing as above.

A plot of $\Delta S_{max}/\Delta S$ vs. 1/[dimethylnitrosamine] yielded a straight line (correlation coefficient $r = 0.9954$) indicating that dimethylnitrosamine decreases bilayer fluidity by partitioning reversibly into a single set of sites in the membrane (Fig. 3b). From this data a value of 5.1 ± 1.0 mM can be obtained for the apparent association constant (K_a) for binding of dimethylnitrosamine to these membrane sites (S.D., $n = 3$).

Discussion

Glucagon-stimulated adenylate cyclase is an integral plasma membrane protein. We have previously demonstrated that increases in bilayer fluidity, achieved using either neutral [7] or charged [30–32] local anaesthetics, lead to the activation of this enzyme. On the other hand, decreases in bilayer fluidity achieved by manipulating the cholesterol content of isolated plasma membranes [19,33] lead to the inhibition of adenylate cyclase activity. All of these studies have indicated that adenylate cyclase activity is augmented by increased bilayer fluidity, presumably by relieving the constraint imposed upon the enzyme by the bilayer.

A number of lipophilic compounds that partition into membranes have been shown to alter membrane fluidity (see, for example, Ref. 10). Here, however, we observed that the carcinogen, dimethylnitrosamine reversibly decreased the fluidity of the liver plasma membrane (Fig. 3). In concert with its ability to decrease membrane fluidity it also inhibited the activity of adenylate cyclase. This inhibitory effect of dimethylnitrosamine was presumed to be exerted via the bilayer as the activity of the solubilized enzyme was unaffected by dimethylnitrosamine. Furthermore, there was a close correlation (Fig. 5) between the decrease in both adenylate cyclase activity and in bilayer fluidity achieved by dimethylnitrosamine (correlation coefficient $r = 0.9671$). This provides additional evidence to support our contention [7,18] that the activity of adenylate cyclase is highly sensitive to alterations in membrane fluidity.

In rat liver plasma membranes there is a lipid phase separation occuring at 28°C, which we have attributed to the lipids in the external half of the bilayer only (for discussion, see Refs. 17 and 28). This can be detected by esr analysis (Fig. 4) and from Arrhenius plots of glucagon-stimulated adenylate cyclase activity (Fig. 3). In both instances, dimethylnitrosamine increased the onset temperatue of this lipid phase transition to 31–32°C. This is again consistent with our observation that dimethylnitrosamine is able to decrease the fluidity of the bilayer and supports our contention that such a change in the lipid environment is able to modulate adenylate cyclase activity.

The precise mechanism by which dimethylnitrosamine decreases the fluidity of the bilayer remains to be determined. However, we have noted that by increasing the cholesterol content of liver plasma membranes, their fluidity was decreased [33] and the temperature of onset of this lipid phase separation was raised [34]. Our observations with dimethylnitrosamine are clearly in accord with this. Manipulation of cell membrane cholesterol content is, however, arduous [19,33] and thus dimethylnitrosamine provides a useful tool for rapidly and simply decreasing bilayer fluidity.

In contrast to dimethylnitrosamine, its non-carcinogenic analogue dimethylamine failed to

either decrease bilayer fluidity or to inhibit adenylate cyclase (Fig. 1). Instead, it exerted a small simulatory effect on the enzyme, which corresponded to an equally small stimulatory phase observed in the overall inhibitory response of the enyzme to dimethylnitrosamine (Fig. 1). It is unlikely that this effect was due to a direct action on the protein as the activity of the solubilized enzyme was unaffected by either compound (Fig. 1). This does tend to suggest that this small stimulation was mediated via the bilayer, although presumably not being due to any fluidity changes. There is considerable evidence to suggest that adenylate cyclase activity is influenced by specific types of phospholipids (see Ref. 17) and that perturbation of this interaction can lead to changes in enzyme activity (see Ref. 17). It is possible that both of these compounds could exert their small stimulatory effects by acting at the headgroup region of the bilayer and hence promoting an interaction with the enzyme at this level.

It is then of particular interest that the carcinogen dimethylnitrosamine can inhibit adenylate cyclase activity significantly whereas its non-carcinogenic analogue, dimethylamine, cannot. The basis for these observations would appear to be related to our novel observation that the carcinogen, dimethylnitrosamine but not the related non-carcinogen, dimethylamine, is able to decrease bilayer fluidity. It is tempting to suggest that the lowered cyclic AMP concentrations and diminished response to stimulatory hormones, that would be expected to occur in response to dimethylnitrosamine, might play a part in the mechanism of carcinogenesis. However, we [7] and others (see Refs. 8–10) have demonstrated that the activity of a variety of other plasma membrane enzymes are affected by alterations in lipid fluidity. It may be, therefore, that the decrease in lipid fluidity achieved by dimethylnitrosamine is a necessary prelude to the (tumour) promotional event itself.

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