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α-ADRENERGICALLY MEDIATED CHANGES IN MEMBRANE LIPID FLUIDITY AND Ca^{2+} BINDING IN ISOLATED RAT LIVER PLASMA MEMBRANES

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Noradrenaline (0.1–5 μM, in the presence of 5 μM propranolol to block β-receptors), ATP (100 μM) and angiotensin II (0.1 μM), which are thought to increase cytosolic Ca^{2+} concentration by mobilizing Ca^{2+} from internal stores, increased the lipid fluidity as measured by diphenylhexatriene fluorescence polarization in plasma membranes isolated from rat liver. The effect of noradrenaline was dose-dependent and blocked by the α-antagonists phenoxybenzamine (50 μM) and phentolamine (1 μM). The response to a maximal dose of noradrenaline (5 μM) and that to ATP (100 μM) were not cumulative, suggesting that both agents use a common mechanism to alter the membrane lipid fluidity. In contrast, the addition of noradrenaline (5 μM) along with the foreign amphiphile Na^{+}-oleate (1–30 μM) resulted in an increase in membrane lipid fluidity which was equivalent to the sum of individual responses to the two agents. In the absence of Mg^{2+}, reducing free Ca^{2+} concentration by adding EGTA increased membrane lipid fluidity and abolished the effect of noradrenaline, suggesting that Ca^{2+} is involved in the mechanism by which the hormone exerts its effect on plasma membranes. Noradrenaline (5 μM) and angiotensin II (0.1 μM) also promoted a small release of $^{45}Ca^{2+}$ (16 pmol/mg membrane proteins) from prelabelled plasma membranes. The effect of noradrenaline was suppressed by the α-antagonist phentolamine (5 μM). It is proposed that noradrenaline, via α-adrenergic receptors and other Ca^{2+}-mobilizing hormones, increases membrane lipid fluidity by displacing a small pool of Ca^{2+} bound to phospholipids, removing thus the mechanical constraints brought about by this ion.

Introduction

It has been shown that the binding of certain hormones or agonists (insulin, cholinergic agonists, etc.) to their respective receptors may induce changes in the fluidity of membrane lipids as measured by fluorescence polarization or total fluorescence [1–4]. These hormone-mediated effects have been observed in preparation of isolated plasma membranes and immediately following the hormone addition, indicating that the change in membrane lipid fluidity (increase or decrease, depending on the hormones) is intimately coupled to receptor activation and is a part of the first steps which lead to the cell response. In the present work we have investigated whether noradrenaline was also able to alter the order of membrane lipids, as determined by fluorescence polarization of a diphenylhexatriene lipophilic probe in plasma membranes isolated from rat liver, a target tissue for this hormone [5]. We have attempted to relate

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Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

the effect to the Ca^{2+} movements generated by noradrenaline. This has been done, first because Ca^{2+} is known to alter the fluidity of membrane phospholipids [6], and second because noradrenaline via α-adrenergic receptors releases Ca^{2+} from intracellular organelles to exert its effects in liver [7–9]. Results have shown that noradrenaline does increase the fluidity of isolated plasma membranes. The effect is dependent on the presence of Ca^{2+} and is associated with a small release of Ca^{2+} from high-affinity binding sites. Other Ca^{2+}-mobilizing hormones cause similar responses. It is suggested that the Ca^{2+} release is responsible for the increase in the membrane lipid fluidity initiated by the hormones.

Methods

Isolated plasma membranes

Plasma membranes were isolated from fed female Wistar rats (200–250 g) essentially as described by Song et al. [10]. Briefly, animals were killed by cervical dislocation and the liver was removed and rinsed in ice-cold buffer containing 1 mM $NaHCO_3$ and 0.5 mM $CaCl_2$, pH 7.4. All the subsequent steps were performed at 0–4°C. The tissue was then minced and homogenized in 40 vol. (v/w) with 30 strokes of a loose-fitting Dounce homogeneizer. The resulting homogenate was stirred for 3 min, filtered twice through two layers of cheese cloth, then centrifuged at $100 \times g$ for 10 min. The supernatant was kept and the pellet resuspended in fresh buffer and centrifuged at $100 \times g$ for a second 10-min period. The combined supernatants were then centrifuged twice at $1500 \times g$ for 10 min and the pellet was diluted by adding 5.5-times its volume of a sucrose solution (70.74%; $d = 1.26$) to give a final density of 1.22. This suspension was placed in the bottom of cellulose tubes, then successively overlaid with 10 ml of sucrose solution ($d = 1.18$ and 1.16, respectively. The tubes were centrifuged at $66\,000 \times g$ for 2 h and the membranes recovered from the 1.16 : 1.18 interface were resuspended and centrifuged twice at $66\,000 \times g$ for 30 min. The first resuspension medium contained 30 mM Tris-HCl/1 mM EGTA, pH 7.4, and the second, 30 mM Tris-HCl, pH 7.4. The final pellet was resuspended in 30 mM Tris buffer. Aliquots of 1 mg protein were stored in liquid nitrogen. The protein concentration was determined in duplicate by the method of Lowry et al. [11] with crystalline bovine serum albumin as standard. 5′-Nucleotidase activity in the membrane fraction was between 15- and 25-times higher than in the initial homogenate.

Lipid extraction and vesicle preparation

Vesicles were prepared from lipid extracted from isolated rat liver plasma membranes in chloroform/methanol (2 : 1, v/v). The resulting extract was evaporated under reduced pressure, then sonicated under N_2 at 4°C for 10 min in a medium containing 50 mM Tris-HCl/5 mM $MgCl_2$/100 μM EGTA/5 μM propranolol, pH 7.4, using a probe sonicator (Branson). The sonicate was centrifuged ($40\,000 \times g$, 45 min, 4°C) to separate unilamellar vesicles (supernatant) from undispersed lipids and multilamellar liposomes (pellet).

Steady-state fluorescence polarization

The method used was that of Shinitzky and Barenholz [12] with some modifications. Liver plasma membranes and liposomes were labelled with the fluorescent probe diphenylhexatriene. The probe was stored in tetrahydrofuran at a concentration of 2 mM and, immediately before use, it was diluted 2000-fold by injection into 50 mM Tris-HCl (pH 7.4), 5 μM propranolol and when indicated, 5 mM $MgCl_2$ and/or EGTA, $CaCl_2$. The solution was sonicated for 5 min with a Branson probe sonicator. 2-ml aliquots of the sonicated solution were mixed with 5–10 μl of membranes (final concentration 30–40 μg membrane protein/ml) or 100 μl of liposomes (final concentration 50 μg phospholipid/ml). The mixtures were incubated for 10 min at 37°C to incorporate diphenylhexatriene. When required, antagonists, phentolamine (1 μM) and phenoxybenzamine (50 μM), were added at the beginning of the 10-min pre-incubation period.

Fluorescence polarization was measured at 37°C on a Jobin and Yvon JY 3C spectrofluorimeter equipped with excitation and emission polarizers. Excitation wavelength was 360 nm and emission wavelength 430 nm. The recording system gave digital reading, as well as a chart tracing, of I_{vv}, I_{vh}, I_{hh} and I_{hv}, values corresponding to the emission intensities (I) recorded in the vertical (v) and

horizontal (h) positions of the polarizers. Fluorescence polarization was calculated as:

$$P = \frac{I_{vv} - I_{vh}(I_{hh}/I_{hv})}{I_{vv} - I_{vh}(I_{hh}/I_{hv})}$$

Recording of the fluorescence intensities in the four positions was performed from the 30th s following the addition (10–30 μl) of the drugs, or the $CaCl_2$ solutions (or H_2O for controls) up to the 90th s and under agitation with a small magnet. Temperature was monitored with a circulating water-bath. Background scatter obtained from suspensions of membrane alone and probe alone, which amounted to less than 5%, was not subtracted as corrections. Measurements were made at least in triplicate. Absorption spectra of all the agents used in this study were recorded to examine possible interference with diphenylhexatriene. None of them showed absorbance at 360 nm.

Limitations of the fluorescence polarization method

The steady-state fluorescence polarization has been interpreted in terms of 'microviscosity' i.e., the rate of rotational diffusion of the probe within the membrane [12]. This has been done by applying a classical hydrodynamic expression of the Perrin type to the fluorescence anisotropy (r_s). However, this derivation assumes that the environment of the probe is isotropic, as in a reference mineral oil. Recent time-resolved fluorescence anisotropy measurements in pure lipid membrane and in cell membranes have shown the fluorescence anisotropy not to decrease to zero — which would be the case in an isotropic medium — but to a finite value, r_∞ (for a review, see Jahnig [13]). This indicates that the probe does not assume all possible orientations with equal probability after a prolonged time period, and reflects the structural order in membrane. Then the steady-state fluorescence anisotropy, r_s, is resolved into a kinetic part, r_f which is related to the rotational relaxation time of the probe, itself proportional to the 'microviscosity', and into a static part, r_∞, neglected in the 'microviscosity' concept. The contribution of each term depends on the absolute value of r_s: in very fluid phase (very low r_s) the Perrin equation must hold because $r_\infty = 0$ and $r_s = r_f$, but in more ordered phase ($r_s > 0.2$) the kinetic contribution is very low, and $r_s \approx r_\infty$. In the range of $0.13 < r_s < 0.28$ (which is the case for most natural membranes) r_∞ can be deduced from r_s by the following equation [14]:

$$r_\infty = \tfrac{4}{3} r_s - 0.10$$

In rat liver plasma membranes we have calculated that the structural contribution represents 85% of the total anisotropy, leaving only 15% for the kinetic contribution. Thus the measured steady-state fluorescence polarization of diphenylhexatriene in our membrane preparations is mainly the reflection of the degree to which the fluorophore rotations are restricted by the molecular packing (order) of the lipids (and/or the proteins) rather than its rotational rate [14,15]. In the following we have used only the fluorescence polarization value, P, as defined in the above paragraph, as an indication of the lipid structural order parameter of the membrane or, as suggested by Van Blitterswijk et al. [14], the reciprocal of lipid fluidity.

$^{45}Ca^{2+}$-binding to isolated rat liver plasma membranes

Isolated plasma membranes were incubated in media containing 1 $\mu Ci \cdot ml^{-1}$ of the tracer for a period of 20 min. 30 s after hormone addition, the membrane suspensions were filtered through Wathman GF/C filters and rinsed with the incubating solution. The filters were then dissolved in 10 ml Triton/toluene scintillation mixture (30 : 70 v/v) and, after stabilization, counted in a liquid spectrophotometer (Intertechnique).

Total Ca^{2+} – and Mg^{2+}

The total Ca^{2+} and Mg^{2+} contents of plasma membranes and that contaminating solutions were determined by absorption spectrophotometry.

Chemicals

The following chemicals were purchased from Sigma: (−)-noradrenaline, (±)-propranolol, *N*-adenosine triphosphate, angiotensin II, sodium oleate. Diphenylhexatriene was from Aldrich. Radioactive Ca^{2+} was purchased from INR (Belgium) and CEA (France). Phentolamine (regitine) was a gift from Ciba-Geigy (France) and pheno-

xybenzamine (dibenziline) was a gift from Smith, Kline & French (France).

Other reagents and chemicals used throughout this investigation were of analytical-reagent grade.

Results

The effect of noradrenaline on the membrane fluidity

The fluorescence polarization factor P of the probe diphenylhexatriene incorporated in liver plasma membranes was measured at 37°C in a medium containing 50 mM Tris-HCl/5 mM $MgCl_2$/100 μM EGTA/1 μM diphenylhexatriene/5 μM propranolol. The average value of P was 0.267 ± 0.002 ($n = 64$). Individual P values ranged from 0.210 to 0.290, depending on plasma membrane preparations. This presumably resulted from slight contamination of the preparation with intracellular membranes (probably endoplasmic reticulum).

Fig. 1 shows that noradrenaline (in the presence of 5 μM propranolol to block β-receptors) caused a reduction in P in diphenylhexatriene-labelled plasma membranes. As described in the Methods section, a decrease in P indicates a reduction in the molecular packing of membrane lipids surrounding the probe or an increase in membrane lipid fluidity. The maximum reduction in P was low (around 5%) and was observed with 5 μM noradrenaline with an EC_{50} value of 0.1 μM. Propranolol itself (5 μM) had no apparent effect on P. Fig. 1 also shows that the effect of noradrenaline could be blocked by pre-incubating the membranes with the α-adrenoreceptor antagonists phentolamine (1 μM) or phenoxybenzamine (50 μM). They, like propranolol, did not alter P. These findings suggest that noradrenaline-induced change in membrane fluidity was mediated by the activation of α-adrenergic receptors.

Further confirmation that the effect was receptor-mediated and did not result from a nonspecific interaction of the hormones with the membrane lipids or with the fluorescent probe was obtained by the following experiments. Lipids were extracted from isolated plasma membranes by chloroform/methanol treatment, then sonicated to obtain unilamellar vesicles devoid of membrane proteins. Table I shows that, in contrast with native membranes, no effect of noradrenaline on fluorescence polarization of diphenylhexatriene-labelled vesicles could be detected.

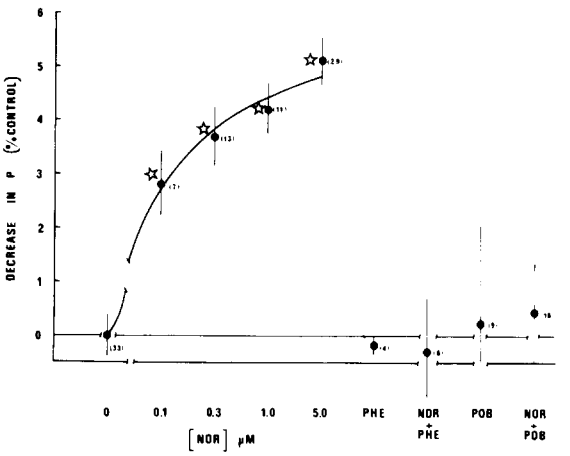

Fig. 1. The effect of noradrenaline on the fluorescence polarization of diphenylhexatriene-labelled isolated plasma membranes. Rat liver plasma membranes were incubated in a medium containing 50 mM Tris-HCl (pH 7.4)/5 mM $MgCl_2$/100 μM EGTA/5 μM propranolol/1 μM diphenylhexatriene. Fluorescence polarization (P) was measured 30 s after the addition of either noradrenaline or distilled water (see Methods). Decrease in P has been expressed as a percentage of the control values for each experiment ± S.E. of the number of values indicated in brackets. The control value was 0.265 ± 0.0026 ($N = 33$). * Significantly different from controls ($P \leq 0.05$). When used, phentolamine (PHE, 1 μM) and phenoxybenzamine (POB, 50 μM) were added at the beginning of the preincubation period.

Effect of ATP and angiotensin II on the membrane fluidity

It has been suggested that externally applied ATP (through P_2 purinergic receptors), in common with α-adrenergic agonists, mobilize internal Ca^{2+} to extert its action in mammalian liver [16,17]. To test whether ATP also alters the membrane lipid order, its effect on P was examined in the same preparations of isolated rat liver plasma membranes. It was found that ATP (100 μM, a maximal dose in rat liver, see Ref. 17) caused a decrease in P ($5.9 \pm 1.0\%$, $n = 10$) which was not significantly different from that initiated by 5 μM noradrenaline (5.1 ± 0.6; $n = 11$). Angiotensin II (0.1 μM), which is thought to use the same mechanism as α-agonists and ATP, also provoked a decrease in P (about 6%, results not shown).

TABLE I

LACK OF EFFECT OF NORADRENALINE (5 μM) ON THE FLUORESCENCE POLARIZATION OF DIPHENYLHEXATRIENE-LABELLED LIPOSOMES

These were prepared by extracting lipids from isolated rat liver plasma membranes. The effect of noradrenaline on plasma membrane is given for comparison. The same incubating medium and experimental protocol was used as described for Fig. 1. *P* values have been given as means ± S.E. of the number of observations indicated in brackets.

	Controls	Noradrenaline	Noradrenaline + phentolamine
Isolated plasma membranes	0.250 ± 0.004 (16)	0.232 ± 0.005 [a] (10)	0.247 ± 0.003 (11)
Liposomes	0.214 ± 0.004 (4)	0.213 ± 0.003 (4)	–

[a] Significantly different ($P \leq 0.05$) when compared to paired controls.

The response to a maximal dose of noradrenaline (5 μM) and that to 100 μM ATP were not cumulative (4.1 ± 0.7, $n = 10$). The similarity of the magnitude of responses and the lack of additivity suggested that, after occupation of their respective receptors, noradrenaline and ATP might use a common mechanism to alter the movements of membranes lipids.

In contrast, as illustrated in Table II, the decrease in *P* induced by noradrenaline was cumulative with that produced by sodium oleate. Sodium oleate is an amphiphile molecule which is known to disorder membranes by intercalating between fatty acyl chains of phospholipids [18]. Table II shows that in the range of concentrations tested (1–30 μM) the effect of sodium oleate was dose-dependent, as already reported in plasma membranes prepared from other tissues [18,19]. It may be seen that the addition of submaximal (1 μM) or maximal (5 μM) doses of noradrenaline along with amphiphile resulted in a decrease in *P* which was equivalent to the sum of the individual responses to the two agents, an indication that the hormone altered the dynamic properties of membrane lipids by a mechanism different from that used by the amphiphile.

Dependence on divalent cations

The next experiments were performed to test the possibility that noradrenaline was able to alter

TABLE II

ADDITIVITY OF *P* RESPONSES OF DIPHENYLHEXATRIENE-LABELLED PLASMA MEMBRANES TO NORADRENALINE (1–5 μM, IN THE PRESENCE OF 5 μM PROPRANOLOL) AND TO THE AMPHIPHILE SODIUM OLEATE (1–30 μM)

In the range of concentrations tested the effect of this agent was dose-dependent. When noradrenaline and sodium oleate were applied together they were added simultaneously to the membrane suspension. The combination of two agents produced a decrease in *P* which was equivalent to the sum of their individual responses irrespective of whether submaximal and maximal doses of the hormone were used. Same incubation medium as in Fig. 1. Results have been expressed as a percentage of control values ± S.E. of the number of experiments given in brackets.

Oleate		Noradrenaline		Oleate + noradrenaline
Dose (μM)	Decrease in *P* (%)	Dose (μM)	Decrease in *P* (%)	Decrease in *P* (%)
1	4.42 ± 0.79 (4)	1	3.60 ± 0.91 (4)	7.43 ± 1.56 (3)
5	12.8 ± 1.60 (11)	5	4.62 ± 0.76 (6)	18.2 ± 1.30 (11) [a]
30	17.2 ± 0.70 (4)	5	4.69 ± 1.28 (10)	21.3 ± 1.32 (4) [a]

[a] Significantly different ($P < 0.05$) from oleate alone.

membrane lipid order by interfering with Ca^{2+} bound to membrane phospholipids. This hypothesis is based on two grounds: first, bound Ca^{2+} may control membrane lipid fluidity by reducing the mean separation of the negative polar heads of phospholipids (see Ref. 6); second, α-adrenergic agonists are known to displace Ca^{2+} from various intracellular pools in liver [8,9]. This was examined by studying the influence of Ca^{2+} on *P* and on the decrease in *P* initiated by noradrenaline. To avoid a possible competitive effect of Mg^{2+}, this cation was not added to the media. The concentration of free Ca^{2+} was estimated using the association constant for Ca-EGTA complex proposed by Bartfai [20]. Table III shows that reducing the estimated free Ca^{2+} concentration not only decreased *P* but also abolished the effect initiated by the hormone. Remarkably, the free Ca^{2+} concentration had to be decreased below 10 nM for the effect on *P* to become apparent, and this was also the concentration at which noradrenaline no longer had an effect on *P*. These results confirm that Ca^{2+} modulates lipid fluidity in isolated rat liver plasma membranes and suggest that a pool bound to high-affinity binding sites may be involved in the mechanism of action of the hormone.

It was found that in the presence of 5 mM Mg^{2+} the effect on *P* of progressively reducing the Ca^{2+} concentation from 2 mM to 0.5 nM was much less pronounced. This decrease in Ca-dependency of *P* made likely the previous suggestion that millimolar concentrations of Mg^{2+} could replace traces of Ca^{2+} in ordering membrane lipids. This observation is supported by experiments reported in Table IV. In the presence of 5 mM Mg^{2+}, the reduction in the Ca concentration neither substantially affected *P* nor influenced the response to noradrenaline (5 μM). In contrast, reducing Ca^{2+} and removing added Mg^{2+} (which left only the contaminating Mg^{2+} and that bound to plasma membrane, i.e., 2.5 μM) greatly reduced *P* and suppressed the effect of the hormone. Though the dose-dependence of the Mg^{2+} effect has not been studied in detail, enough was done to show that at least at high concentrations this ion could replace Ca^{2+} if the latter was unavailable.

Effect of noradrenaline and angiotensin II on $^{45}Ca^{2+}$ binding to plasma membranes

A possible explanation for the above results is that the hormones act by displacing Ca^{2+} bound to plasma membranes, thus removing the mechanical constraints of Ca^{2+} on phospholipids. This hypothesis was tested by determining the effect of

TABLE III

THE EFFECT OF DECREASING THE FREE Ca^{2+} CONCENTRATION OF THE INCUBATION MEDIUM ON THE FLUORESCENCE POLARIZATION OF DIPHENYLHEXATRIENE-LABELLED RAT LIVER PLASMA MEMBRANES AND ON THE DECREASE IN *P* PRODUCED BY NORADRENALINE (5 μM)

The incubation medium was the same as in Fig. 1 except that it did not contain Mg^{2+}, and that Ca^{2+} and EGTA were modified as indicated. The estimated free-Ca^{2+} concentration was calculated from concentrations of EGTA, added Ca^{2+} and from traces of Ca^{2+} present in the media (approx. 4 μM, which includes Ca^{2+} bound to plasma membranes and the contaminating Ca^{2+} present in solutions) as indicated in Methods. The concentration of Mg^{2+} amounted to 2.5 μM. Fluorescence polarization factors (*P*) are given as means ± S.E. of four experiments.

Added Ca	EGTA	Estimated [Ca]	Control	Noradrenaline
100 μM	0	104 μM	0.280 ± 0.002	0.263 ± 0.004 [b]
0	0	4 μM	0.280 ± 0.002	0.268 ± 0.003 [b]
0	10 μM	156 nM	0.277 ± 0.003	0.264 ± 0.004 [b]
0	100 μM	10.4 nM	0.276 ± 0.004	0.262 ± 0.002 [b]
0	500 μM	2.0 nM	0.265 ± 0.005 [a]	0.266 ± 0.004
0	2 mM	0.5 nM	0.262 ± 0.006 [a]	0.267 ± 0.005

[a] Significantly different ($P \leq 0.05$) when compared to control values measured in the presence of 104 μM Ca^{2+} and in the absence of EGTA.

[b] Significantly different ($P \leq 0.005$) when compared to paired controls.

TABLE IV

THE EFFECT OF ALTERING Mg^{2+} AND Ca^{2+} CONCENTRATIONS IN THE BATHING FLUID ON *P* AND ON THE DECREASE IN *P* PROVOKED BY NORADRENALINE (5 μM)

Same incubation medium as in Fig. 1 except that EGTA varied from 0.1 to 2 mM. Contaminating Mg^{2+} was determined and the estimated free Ca^{2+} was calculated as indicated in the legend of Table III. *P* is given as the mean ± S.E. of the number of experiments indicated in brackets.

Mg^{2+}	EGTA (mM)	Estimated $[Ca^{2+}]$ (nM)	Control	Noradrenaline
5 mM	0.1	10.4	0.252 ± 0.004 (13)	0.237 ± 0.004 (7) [b]
5 mM	2	0.5	0.245 ± 0.002 (7)	0.233 ± 0.002 (7) [b]
2.5 μM	2	0.5	0.228 ± 0.008 (4) [a]	0.232 ± 0.006 (4)

[a] Significantly different ($P \leq 0.05$) when compared to control values measured in the presence of 5 mM Mg^{2+} and 0.1 mM EGTA
[b] Signifcantly different ($P \leq 0.05$) when compared to paired controls.

noradrenaline and angiotensin II on the binding of $^{45}Ca^{2+}$ to membranes. The effect of ATP was not examined because it is a Ca^{2+} ligand and because there was a possibility that it could generate some Ca uptake by contaminating endoplasmic reticulum vesicles. The membranes were incubated in Ca^{2+}-EGTA buffers containing $^{45}Ca^{2+}$, which resulted in an estimated free Ca^{2+} concentration of 10–20 nM for 20 min, a period long enough to allow membrane Ca^{2+} to exchange (the time constant of this component is 8 min (unpublished results)).

In the absence of 5 mM Mg^{2+}, $^{45}Ca^{2+}$ bound to plasma membranes incubated under these conditions amounted to 108 ± 8 pmol · mg^{-1} membrane protein ($n = 86$). Application of a maximal concentration of noradrenaline (5 μM) for 30 s triggered a small but significant release of $^{45}Ca^{2+}$ of 15.7 ± 3.3 pmol · mg^{-1} ($n = 87$). The release was difficult to detect not only because it was quantitatively small but also because, for an unknown reason, only 70% of the membrane preparations were responsive to the hormone. This effect was blocked (net $^{45}Ca^{2+}$ loss: 2.4 ± 2.1 pmol · mg^{-1}, $n = 82$) by pre-incubating membranes with the α-antagonist phentolamine (5 μM). Angiotensin II (0.1 μM) promoted a similar response (19.6 ± 7.9 pmol · mg^{-1}).

In the presence of 5 mM Mg^{2+} or 150 mM KCl the $^{45}Ca^{2+}$ bound to plasma membrane amounted to 36 ± 2 ($n = 21$) and 78 ± 3 ($n = 39$) pmol · mg^{-1}, respectively. The net loss of $^{45}Ca^{2+}$ was abolished by Mg^{2+}, whereas, though reduced to 4 pmol · mg^{-1}, the $^{45}Ca^{2+}$ release was maintained in the presence of physiological concentrations of KCl. These results also suggest that Mg^{2+} could replace Ca^{2+} in the coupling mechanism of α-receptors.

Discussion

In this study we have measured the steady-state fluorescence polarization of the probe diphenylhexatriene to investigate the physical changes of membrane lipids initiated by noradrenaline, ATP and angiotensin II. Although this method is now routinely performed in many laboratories, care must be taken in its application in biological membranes. First, with regards to the location of the probe, studies have indicated that in artificial membranes constituted of pure lipids the lipophilic rod-shaped diphenylhexatriene intercalates between the acyl chains of the phospholipids whether they are in the gel or liquid crystalline state, independently of saturation of the fatty acyl chains or of the presence of cholesterol with equal affinity [21]. In artificial membranes containing proteins it has been shown that this probe also reports the dynamics of the boundary lipid regions in addition to those of the bulk lipid bilayer [15]. The proposal that in the presence of proteins diphenylhexatriene-fluorescence polarization values could also reflect interaction of the probe with hydrophobic parts of the proteins [22] has been

recently contradicted by Van Blitterswijk et al. [14]. Thus, it is likely that, in our membrane preparations, diphenylhexatriene probed the different domains of the lipid bilayer including the boundary lipid regions in contact with membrane receptors. Second, as stated in Methods, the steady-state fluorescence anisotropy of diphenylhexatriene-labelled membranes cannot be interpreted in terms of microviscosity, i.e., the rate rotational diffusion of the probe, but in terms of membrane lipid order (the reciprocal of lipid fluidity), reflecting the degree to which the fluorophore rotations are restricted to a cone formed by the molecular packing of the phospholipids [14,15]. The finding that hormones cause a decrease in the fluorescence polarization may be thus interpreted as an increase in the fluidity of the fatty acyl chains surrounding the probe [14].

The effect of noradrenaline appears to be mediated by the activation of α-adrenergic receptors. The alternative possibility that noradrenaline decreased membrane lipid fluidity by unspecifically interacting with membrane phospholipids can be ruled out on the following grounds. First, the effect of the hormone is dose-dependent, with an EC_{50} of about 0.1 μM, which is in agreement with other physiological responses of rat liver cells to this hormone [23]. Second, the effect can be prevented by the α-receptor antagonists phenoxybenzamine or phentolamine, which by themselves do not alter the fluorescence polarization of probe-labelled membranes. Third, under identical experimental conditions, the effect of noradrenaline is abolished in vesicles of lipids extracted from native plasma membranes and devoid of membrane receptors. The fact that ATP and angiotensin II, which in common with noradrenaline are believed to release Ca^{2+} from internal stores in liver [8,16], alter similarly the membrane fluidity is in agreement with this view. The present results have shown that the responses to maximal doses of noradrenaline and ATP were not cumulative, suggesting that though acting through separate receptors (α-adrenergic and presumably P_2 purinergic receptors [16,24]) the two hormones use a common mechanism to increase lipid fluidity. In this context it is interesting that the effect of the foreign amphiphile sodium oleate, which presumably alters membrane lipid order in a non-receptor-mediated way [18,19], is cumulative with that of noradrenaline. At each dose of sodium oleate tested, the membrane remained responsive to the hormone, indicating separate mechanisms of action.

The reduction of the free-Ca^{2+} concentration reduces the polarization of the probe and, at the same time, it blocks the response of the membrane to noradrenaline. This suggests that calcium ions are directly involved in the mechanism by which the hormone alters P and that they interfere at a step of the sequence which follows the hormone-receptor interaction but which precedes the effect on membrane lipids. In plasma membranes isolated from rat liver, as in those prepared from other tissues, divalent cations have been shown to reduce the separation between phospholipids, and consequently the mobility of hydrocarbon chains (see Ref. 6). So a possible explanation for these results is that noradrenaline and other Ca^{2+}-mobilizing hormones alter the membrane lipid fluidity by displacing a Ca^{2+} pool bound to membrane phospholipids, thus removing the mechanical constraints brought about by this ion. The observation that Ca^{2+}-mobilizing hormones release Ca^{2+} from isolated plasma membranes is in agreement with this view. Shlatz and Marinetti [25] had suggested that adrenaline can increase the affinity of isolated rat liver plasma membranes for Ca^{2+}. However, Yamagami and Terayama [26] were later unable to confirm this result. In both cases, however, the Ca^{2+} concentration of the media was quite high (1 and 10 μM, respectively). In membrane preparations incubated at lower free-Ca^{2+} concentrations similar to those used previously, which incidentally had the advantage of reducing substantially the nonspecific binding of Ca^{2+}, noradrenaline and angiotensin II initiated small releases of Ca^{2+}. The adrenergic stimulation was blocked by phentolamine, indicating that, as for the change in membrane lipid fluidity, the effect on Ca^{2+} binding was mediated by activation of α-receptors.

Using the dissociation constant proposed by Bartfai [20] it may be calculated that in the absence of added Mg^{2+} the concentration of free Ca^{2+} required for the hormone to be effective was only 10–20 nM. These values are not far below those estimated for free-Ca^{2+} concentrations in the cytosol of mammalian cells, which range from 10

to 200 nM [7,27]. However, in the presence of 5 mM Mg^{2+}, the effect of Ca^{2+} on the membrane fluorescence polarization and on the response to noradrenaline was abolished. Similarly, Mg^{2+} and K^+ reduced Ca^{2+} binding and Ca^{2+} release initiated by the hormone. This probably resulted from the fact that high concentrations of these cations displace Ca^{2+} from its polar head-group binding sites [28–30]. Although a complete analysis of this competitive effect has not been performed in this study, it appears that, at least at high concentrations, Mg^{2+} could replace Ca^{2+} in the coupling mechanism of the hormone if the latter was unavailable. The nature of these interactions between cations, specially at physiological ion concentration, merits further study.

The fact that Ca^{2+}-mobilizing hormones can bring about a decrease in *P* and a release of Ca^{2+} bound in isolated plasma membranes is evidence that these events are intimately coupled to receptor activation. However, there is little to indicate the exact mechanism of action of the hormones or the nature of the phospholipids involved. The observation that the effects on *P* as well as Ca^{2+} movements are very small could indicate that the hormone-receptor interaction is likely to affect a limited number of phospholipids at only a few sites adjacent to the receptor. In rat hepatocytes Ca^{2+}-mobilizing hormones promote a rapid breakdown of inositol-containing phospholipids [31]. It has been recently reported [32] that in the presence of deoxycholate the breakdown of phosphatidylinositol may also occur in isolated rat liver plasma membranes stimulated by vasopressin. In furtherance of this theory, it could be interesting to investigate whether the degradation of phosphatidylinositides could be evoked in our preparations of isolated plasma membranes.

In the context of the sequence of events initiated by noradrenaline, ATP or angiotensin II in rat liver, it may be interesting to discuss the relevance of the small displacement of Ca^{2+} bound to plasma membranes. It is thought that these hormones increase cytosolic Ca^{2+} by releasing Ca^{2+} from intracellular stores [8,9]. Murphy et al. [7] indirectly determined a variation of free-Ca^{2+} concentration from a basal level of 190 to 400 nM following receptor activation. Assuming that rat hepatocytes contain 1.8 μl cell water [16] and 30 μg plasma membrane protein per mg dry weight [33], the release of Ca^{2+} from plasma membrane should result in a net increase of about 270 nM. Strictly speaking, this amount should be large enough to account for the observed increase in cytosolic free Ca^{2+}. However, the estimation does not take into account the buffering power of intracellular proteins or organelles (Ca^{2+}-binding proteins, calmodulin, etc.) which necessarily would reduce the theoretical variation of Ca^{2+}. Moreover, the net movements of Ca^{2+} initiated in cells [9] are several orders of magnitude larger than those promoted in isolated plasma membranes. So, we are inclined to think that the small Ca^{2+} release could play a role in the mechanism by which Ca^{2+} is mobilized from other internal stores. For example, it could trigger a more important loss of Ca^{2+} from internal stores by a system of Ca^{2+}-induced Ca^{2+} release similar to that already described in cardiac and muscle cells [34].

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