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# CHOLINERGIC STIMULANTS AND EXCESS POTASSIUM ION INCREASE THE FLUIDITY OF PLASMA MEMBRANES ISOLATED FROM ADRENAL CHROMAFFIN CELLS

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## Summary

Chromaffin cell membranes from the bovine adrenal medulla were labelled with the hydrophobic fluorescent probe 1,6-diphenyl-1,3,5-hexatriene, and the fluorescence polarization ($P$) of the membrane suspensions was measured as a function of temperature. The $P$ versus $t$ profiles, between 20 and 37°C, showed two linear regions separated by a break in the vicinity of 30°C, reflecting a change in the phase behaviour of the constituent lipids. Decreases in $P$ values at higher temperatures indicated progressive fluidization of the lipid bilayer. Previous incubation with either acetylcholine (0.5 mM) or nicotine (50 $\mu$M) produced further fluidization, the extent of which depended on the presence of added $Ca^{2+}$ (2.2 mM). Thus, the flow activation energy, $\Delta E$, between approx. 30 and 37°C was 9.1 kcal/mol for acetylcholine and 8.8 kcal/mol for acetylcholine plus $Ca^{2+}$, as compared to 7.9 kcal/mol in the absence of acetylcholine and $Ca^{2+}$. In the presence of nicotine, $\Delta E$ was 11.4 kcal/mol when $Ca^{2+}$ was absent and 9.5 kcal/mol when it was present. The cholinergic blocker, hexamethonium (0.5 mM), abolished the acetylcholine- or nicotine-induced changes. 65 mM $K^+$ produced a similar fluidization, which was reversed by addition of $Ca^{2+}$. An additive effect was observed when the membranes were incubated with both nicotine and $K^+$, with $\Delta E$ = 16.6 kcal/mol in the presence of $Ca^{2+}$. These results indicate a receptor-mediated modulation of the lipid distribution between rigid and fluid regions in the membrane, which could be of importance for stimulated catecholamine secretion in the intact cell.

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Abbreviation: DPH, 1,6-diphenyl-1,3,5-hexatriene.

## Introduction

Secretion of catecholamines, in response to stimulation of the adrenal chromaffin cell by acetylcholine, is generally believed to occur by exocytosis [1,2]. This process depends on the presence of extracellular $Ca^{2+}$, and involves the fusion of the chromaffin cell membrane to that of the catecholamine storage organelle, the chromaffin granule, with subsequent extrusion of the granule contents into the extracellular space. The molecular details of the fusion event and the reason for the $Ca^{2+}$ requirement are as yet not well understood. Among others, one would like to know whether $Ca^{2+}$ acts at the level of the plasma membrane or at some intracellular site. Very recently, the preparation of intact, functional chromaffin cells, isolated in large yields from the bovine adrenal medulla, has been reported [3—5]. These cells show a catecholamine secretory response to acetylcholine, as well as $K^+$ stimulation in the presence of $Ca^{2+}$ in mmolar concentrations [3,5]. The preparation of the cell membrane and characterization of its acetylcholine receptor have also been recently achieved [6,7]. These cell membranes are a very convenient system to focus on, if the stimulatory and exocytotic events are to be studied in more detail.

Work with synthetic phospholipid bilayer vesicles has shown that these relatively simple systems exhibit increases in cation permeability, as well as cation specific ability to fuse with each other, depending on the properties of the constituent lipids. Fluidization of the lipid bilayer enhances $Na^+$ permeability [8] and fusion [9—11], and both processes proceed fastest at the temperature of the gel to liquid-crystalline phase transition. The formation of an unstable boundary between rigid and fluid domains is believed to favour the fusion event. Furthermore, $Ca^{2+}$ has been found to cause isothermal phase transitions and lateral phase separations into regions of different fluidity in vesicles consisting of lipid mixtures [10,12,13].

It is, therefore, of interest to investigate lipid fluidity in relation to the functional state of the chromaffin cell membrane. This can conveniently be studied with the aid of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH), which inserts into the core of the bilayer by virtue of its hydrophobicity [14,15]. The rotational mobility of the probe, detected by fluorescence depolarization, gives a measure of the degree of order of the fatty acid chains located in its vicinity. This degree of order will depend on whether the lipids are in a solid gel or a fluid state. In the present work we have applied the DPH technique to obtain information on the state of the lipids, as influenced by cholinergic stimulants, excess $K^+$, added $Ca^{2+}$ and the cholinergic blocker hexamethonium. We describe fluidity increases occurring in response to membrane incubation with the various agonists and discuss possible interpretations.

## Materials and Methods

*Membrane preparation.* Plasma membranes from bovine adrenal glands were prepared by the method of Wilson and Kirshner [6], with minor modifications. After separation of the cortex, the medulla was cut into small pieces and homogenized with 0.3 M sucrose/10 mM Tris-HCl buffer, pH 7.4, in a glass-to-glass

homogenizer, using 8—10 strokes/min for 2 min. Centrifugation (800 × *g*, 10 min, Sorvall SS 34 rotor) yielded a pellet which was rehomogenized in the same buffer and centrifuged at 600 × *g* for 10 min. The pooled supernatants from these two centrifugations were spun at 26 000 × *g* for 20 min and the pellet was resuspended in buffer, layered over 1.4 M sucrose/10 mM Tris-HCl, pH 7.4, and centrifuged at 100 000 × *g* for 90 min, using the SW-27 rotor (Beckman). The material from the interface was diluted 1 : 3 with 10 mM Tris-HCl, pH 7.4, layered over an 11% (v/v) Urografin (Schering AG, Berlin) solution in 0.3 M sucrose/10 mM Tris-HCl, pH 7.4, and spun down at 100 000 × *g* for 2 h. The interface was diluted 1 : 2 in 0.3 M sucrose/10 mM Tris-HCl, centrifuged at 100 000 × *g* for 1 h, the resulting pellet resuspended in the same buffer, layered over a discontinuous gradient of 7% and 10% Urografin and spun at 100 000 × *g* for 12 h. The interface between the 7% and 10% Urografin layers was diluted 1 : 2 in 0.3 M sucrose/10 mM Tris-HCl and subjected to a final centrifugation at 100 000 × *g* for 1 h to yield the plasma membranes. These were washed and resuspended with Brook's solution, pH 7.4, containing 135 mM NaCl, 10.7 mM KCl, 10 mM Glc and 5.7 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes).

*Protein assay.* Protein concentration was measured by the method of Hartree [16] after previous solubilization of membranes in 0.1 N NaOH.

*Enzyme assays.* Acetylcholinesterase (acetylcholine hydroxylase, EC 3.1.1.7) was assayed by the method of Ellman et al. [17], dopamine-β-hydroxylase (3,4-dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase (c-hydroxylating), EC 1.14.17.1) by the method of Kirshner et al. [18], and monoamine oxidase (amine:oxygen oxidoreductase, EC 1.4.3.4) by the method of Wurtman and Axelrod [19]. The plasma membranes showed a very high specific activity of acetylcholinesterase (0.426 ± 0.036 μM thiocholine formed/min per mg protein), low dopamine-β-hydroxylase activity (20.7 ± 6.3 nM octopamine formed/min per mg protein) and either no detectable or very low monoamine oxidase activity (9.9 ± 2.6 nM *p*-hydroxybenzaldehyde formed/min per mg protein).

*Membrane fluorescent labelling.* The plasma membranes were labelled according to the procedure described by Shinitzky and Inbar [15]. The membranes (100 μg protein) were incubated with DPH (2 μM) in Brook's solution and the labelled membranes were used immediately for the fluorescent measurements without removal of excess label. Cholinergic reagents and/or KCl (replacing an equivalent amount of NaCl), with or without $CaCl_2$, were added directly to the fluorescence cuvette as concentrated solutions and incubated at room temperature for 10 min. When hexamethonium bromide was used the labelled membrane suspensions were preincubated for 10 min with this reagent. Where needed acetylcholinesterase activity was blocked by adding 5 μg prostigmin ((*m*-hydroxyphenyl)trimethylammoniummethylsulfate dimethyl carbamate) with a preincubation lasting for 5 min.

*Fluorescence measurement.* The measurements of the degree of fluorescence polarization (*P*) and the fluorescent intensities (*I*) were done with the Elscint Microviscosimeter MV-1 (Elscint, Haifa, Israel), described by Inbar and Ben-Bassat [20]. This instrument measures simultaneously the intensities of the parallel ($I_\parallel$) and perpendicular ($I_\perp$) polarized light beams and displays *P* defined

by

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}.$$

$P$ values were measured at 1° intervals. They were found to be independent of the direction of the temperature change (heating or cooling) and of protein concentration. Before each measurement the suspensions were mixed by an internal magnetic stirrer. 5—10 min were necessary to reach a stable temperature and $P$ value. The microviscosity, $\overline{\eta}$, and the flow activation energy, $\Delta E$, were calculated following Shinitzky et al. [14,15,21], who showed that the Perrin equation for the rotational depolarization of a non-spherical fluorophore written in the form

$$\left(\frac{r_0}{r} - 1\right)^{-1} = \frac{\overline{\eta}}{C(r) \cdot T \cdot \tau}$$

can be used to yield $\overline{\eta}$, which is the mean of the effective viscosities opposing the rotations of the probe molecule around its main axes. In this expression, $r_0$ and $r$ are the limiting and the measured fluorescence anisotropies, respectively, $T$ is the absolute temperature, $\tau$ is the excited state lifetime and $C(r)$ is a molecular shape-dependent parameter. $r$ is related to $P$ by the expression $r = 2P/3 - P$. The product $C(r)T\tau$ is approximately constant with temperature and its value is 2.4 P for DPH [22]. This value and the value $r_0 = 0.362$ [14] were used to derive $\overline{\eta}$. The assumption that the rotations of the fluorophore are isotropic, does not apply for DPH [23—25], and hence $\overline{\eta}$ loses its strict meaning, but it can nevertheless be used as a comparative index for the changes occurring in the rotational mobility of the probe molecule under various experimental conditions. The same applies to the flow activation energy, given by the equation

$$\overline{\eta} = Ae^{\Delta E/RT}$$

from which $\Delta E$ can be derived by plotting $\ln \overline{\eta}$ vs. $1/T$.

*Osmotic Experiments.* Changes in osmolarity of the suspension medium will cause volume changes of closed vesicles and these can be monitored by the light scattering of the membrane suspensions (see, e.g. Ref. 26). Scattering was determined by measuring the absorbance at 500 nm of membrane suspensions in Brook's solution after successive additions of either distilled water or 1.4 M sucrose solution.

## Results

The temperature dependence of the DPH fluorescence polarization has been found useful in characterizing membranes of different biological origin, as well as in different functional or developmental states [14,15,20,27,28]. The shape of the $P$ vs. temperature dependence can also give an indication of a lipid phase transition by a change in slope ('break') within a certain temperature range [29]. In the following we present $P$ values recorded in the temperature range from 20 to 37°C which demonstrate the changes induced in the membrane

lipids by cholinergic stimulants causing catecholamine secretion in the isolated chromaffin cell [3].

The influence of acetylcholine and nicotine, in the absence of added $Ca^{2+}$, is shown in Fig. 1. The plots display two linear ranges, separated by a more or less sharply defined break, at which the slope $\Delta P/\Delta t$ changes. We define as 'pretransition range' the temperature interval between 20° C and the temperature of the break, and as 'posttransition range' that above the break and up to 37° C. Both acetylcholine and nicotine cause sharp decreases in the $P$ values, signifying increases in the fluidity of the membrane lipids. While the differences in slope are significant in both ranges, they are especially large in the posttransition range. In this range, nicotine has a more pronounced effect than acetylcholine, and also shifts the break to lower temperatures. The cholinergic agonists are not unique in producing the changes just described. Fig. 2 shows that excess KCl (65 mM) elicits similar effects. In contrast to the agonists, $K^+$ is believed to stimulate secretion from cells by an overall depolarization of the membrane [30], and thus could possibly have given a different response. However, the slopes and the shift of the break are similar to those in Fig. 1. Furthermore, Fig. 2 shows that addition of $Ca^{2+}$ reverses to a significant extent the changes induced by $K^+$. A similar, but less pronounced, influence of $Ca^{2+}$ on the slopes is observed also for acetylcholine and nicotine (not shown). Hexamethonium bromide is a cholinergic blocker of high specificity and has been found to inhibit acetylcholine-, as well as nicotine-induced catecholamine secretion from intact chromaffin cells [3]. It was, therefore, of interest to examine

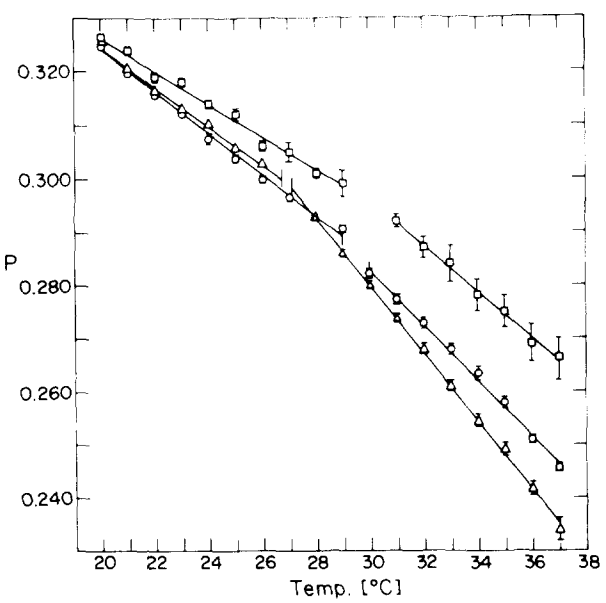


**Fig. 1. Temperature dependence of the fluorescence polarization of plasma membrane suspensions (100 μg protein/2 ml), as influenced by cholinergic agonists, in the absence of $Ca^{2+}$. The data are mean values ± S.D. (vertical bars) of several independent experiments (the number is given in Table I), to which lines were fitted by least squares. The empty intervals correspond to the region of the 'break'. □, plasma membrane; ○, plasma membrane + 0.5 mM acetylcholine; △, plasma membrane + 50 μM nicotine.**

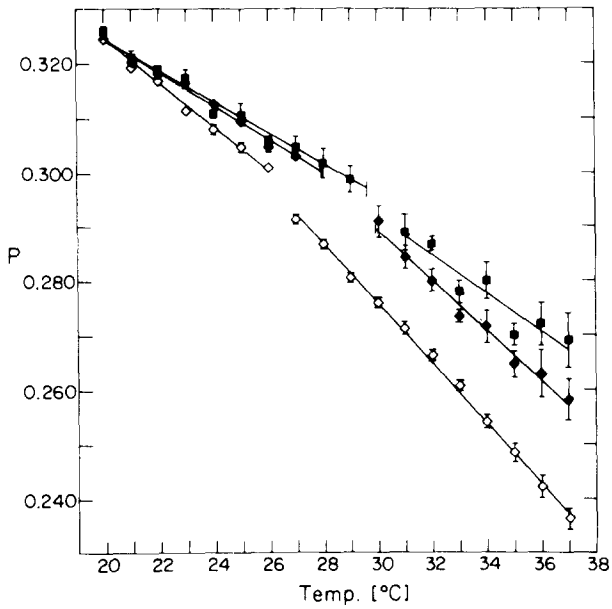


Fig. 2. Temperature dependence of the fluorescence polarization of plasma membranes, as influenced by excess $K^+$. For details, see legend to Fig. 1. ■, plasma membranes + 2.2 mM $Ca^{2+}$; ◆, plasma membranes + 2.2 mM $Ca^{2+}$ + 65 mM $K^+$; ◇, plasma membranes + 65 mM $K^+$.

whether it would modify the membrane effects of the cholinergic stimulants. Fig. 3 shows that it inhibits nicotine-induced membrane fluidization. The acetylcholine effect is also reversed by this blocker (not shown). On the other hand, hexamethonium bromide has no effect whatsoever on the $K^+$-induced increases in slope and shift in transition temperature.

As already mentioned, the fluidity changes due to the agonists on the one hand, and to the excess $K^+$ on the other, are similar, although there may be differences in basic mechanism of membrane depolarization. In order to test whether the fluorescence polarization decreases recorded in each of these cases were limiting changes corresponding to the maximum effect attainable, the two kinds of stimulants were applied simultaneously. Fig. 4 shows that the changes in slopes and $P$ values are larger than those observed for each stimulant by itself, in fact even larger than the sum of the two. Hexamethonium bromide reverses this trend, as expected, to some degree.

Table I summarizes the experimental results, as expressed by the various parameters that characterize membrane fluidity. In addition to the actually measured slopes $\Delta P/\Delta t$, the table lists the flow activation energies, $\Delta E$, in order to facilitate comparison with other membrane systems. $\overline{\eta}$ values at two selected temperatures appear for similar reasons.

The absorbance, at 500 nm, of membrane suspensions in Brook's solution decreases by 45% when the tonicity is increased by addition of sucrose/Tris solution to a final concentration of 0.7 M sucrose. The membranes do not absorb at this wavelength so that the absorbance arises purely from light scattering. Since for particles in the range of sizes of the chromaffin cell membrane suspensions, as shown by electron micrographs [6], there exists a direct

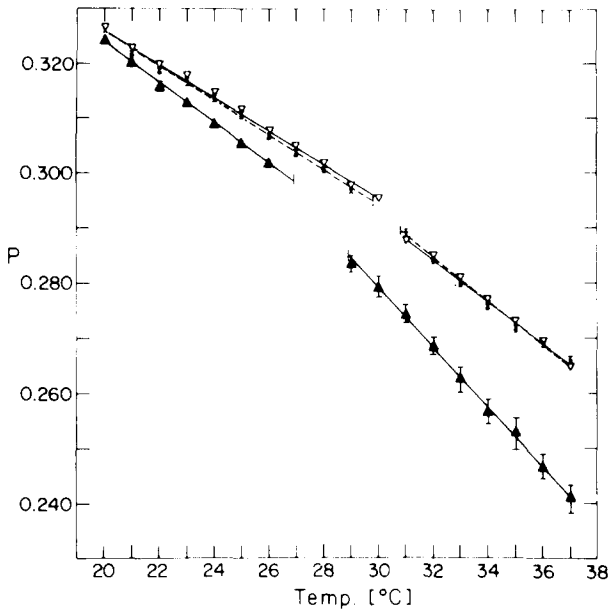


Fig. 3. Temperature dependence of the fluorescence polarization of plasma membranes, as influenced by hexamethonium, in the presence of $Ca^{2+}$. For details, see legend to Fig. 1. ▽———▽, plasma membranes + 2.2 mM $Ca^{2+}$ + 0.5 mM hexamethonium; X- - - - -X, plasma membranes + 2.2 mM $Ca^{2+}$ + 0.5 mM hexamethonium + 50 μM nicotine; ▲, plasma membranes + 2.2 mM $Ca^{2+}$ + 50 μM nicotine.

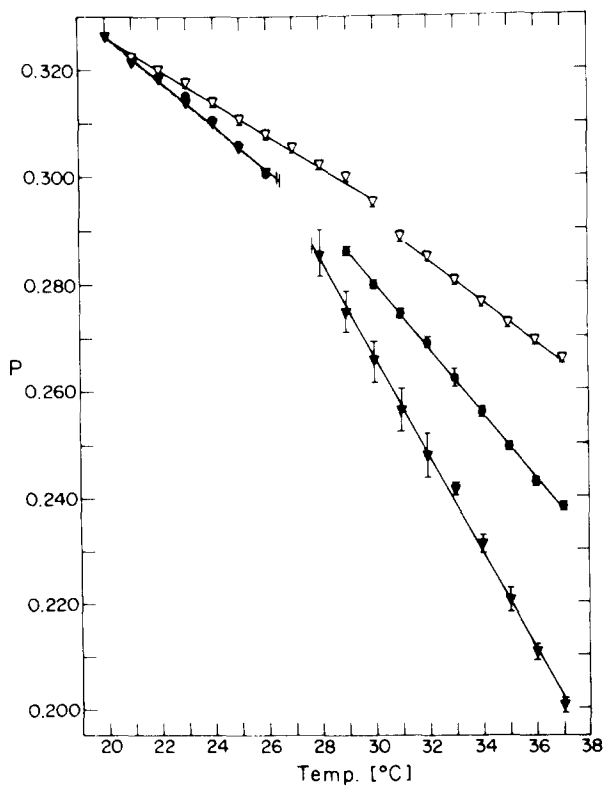


Fig. 4. Temperature dependence of the fluorescence polarization of plasma membranes, as influenced by excess $K^+$ and nicotine, in the presence of $Ca^{2+}$. For details, see legend to Fig. 1. ▽, plasma membranes + 2.2 mM $Ca^{2+}$ + 0.5 mM hexamethonium; ■, plasma membranes + 2.2 mM $Ca^{2+}$ + 0.5 mM hexamethonium + 65 mM $K^+$ + 50 μM nicotine; ▼, plasma membranes + 2.2 mM $Ca^{2+}$ + 65 mM $K^+$ + 50 μM nicotine.

TABLE I

EFFECT OF STIMULANTS, $Ca^{2+}$ AND HEXAMETHONIUM ON $\Delta P/\Delta t$, MICROVISCOSITY, $\bar{\eta}$, AND FLOW ACTIVATION ENERGY, $\Delta E$, OF THE LIPID BILAYER IN CHROMAFFIN CELL MEMBRANES

Membranes (100 $\mu$g protein) were incubated in Brook's solution, pH 7.4, with the following additions: $Ca^{2+}$, 2.2 mM; acetylcholine (ACh), 0.5 mM; nicotine, 50 $\mu$M; hexamethonium, 0.5 mM. Data in parentheses are the number of experiments, carried out usually on as many different membrane preparations. $\Delta P/\Delta t \pm$ S.E.; $\bar{\eta} \pm$ S.E.; $t_{tr} \pm$ S.D. The coefficient of determination ($r^2$) was higher than 0.98 in all measurements.

| Incubation conditions | Pretransition range | | | $t_{tr}$ (°C) | Posttransition range | | |
|---|---|---|---|---|---|---|---|
| | $\Delta P/\Delta t$ $\times 10^3$ ($^\circ C^{-1}$) | $\bar{\eta}_{25}$°C (P) | $\Delta E$ (kcal/mol) | | $\Delta P/\Delta t$ $\times 10^3$ ($^\circ C^{-1}$) | $\bar{\eta}_{37}$°C (P) | $\Delta E$ (kcal/mol) |
| — (6) | −3.1 ± 0.2 | 4.17 ± 0.01 | 5.4 | 30.0 ± 1.0 | −4.3 ± 0.2 | 2.74 ± 0.02 | 7.9 |
| $Ca^{2+}$ (4) | −2.9 ± 0.2 | 4.15 ± 0.01 | 5.0 | 30.3 ± 0.7 | −3.8 ± 0.2 | 2.77 ± 0.02 | 6.1 |
| $Ca^{2+}$ + hexamethonium (4) | −3.1 ± 0.1 | 4.17 ± 0.01 | 5.4 | 30.5 ± 0.5 | −3.8 ± 0.1 | 2.72 ± 0.01 | 6.7 |
| ACh (4) | −3.9 ± 0.1 | 3.92 ± 0.01 | 6.7 | 29.5 ± 0.5 | −5.2 ± 0.1 | 2.31 ± 0.01 | 9.1 |
| ACh + $Ca^{2+}$ (5) | −3.1 ± 0.3 | 4.06 ± 0.01 | 5.4 | 29.1 ± 0.9 | −4.9 ± 0.2 | 2.53 ± 0.01 | 8.8 |
| ACh + $Ca^{2+}$ + hexamethonium (4) | −3.2 ± 0.1 | 4.10 ± 0.01 | 5.5 | 30.4 ± 0.7 | −3.7 ± 0.1 | 2.71 ± 0.01 | 6.5 |
| Nicotine (3) | −3.6 ± 0.1 | 3.99 ± 0.01 | 6.3 | 26.9 ± 0.2 | −6.4 ± 0.2 | 2.09 ± 0.02 | 11.4 |
| Nicotine + $Ca^{2+}$ (6) | −3.7 ± 0.1 | 3.96 ± 0.01 | 6.4 | 27.9 ± 1.0 | −5.5 ± 0.3 | 2.20 ± 0.01 | 9.5 |
| Nicotine + $Ca^{2+}$ + hexamethonium (4) | −3.2 ± 0.1 | 4.14 ± 0.01 | 5.6 | 30.3 ± 0.5 | −4.0 ± 0.1 | 2.71 ± 0.01 | 7.2 |
| $K^+$ (3) | −4.0 ± 0.1 | 3.92 ± 0.01 | 6.8 | 26.5 ± 0.5 | −5.5 ± 0.1 | 2.13 ± 0.01 | 9.5 |
| $K^+$ + $Ca^{2+}$ (4) | −3.0 ± 0.2 | 4.11 ± 0.01 | 5.3 | 28.9 ± 1.0 | −4.6 ± 0.4 | 2.53 ± 0.02 | 8.2 |
| $K^+$ + $Ca^{2+}$ + hexamethonium (5) | −2.9 ± 0.1 | 4.10 ± 0.01 | 5.1 | 28.5 ± 0.5 | −4.4 ± 0.1 | 2.57 ± 0.01 | 7.8 |
| $K^+$ + nicotine + $Ca^{2+}$ (5) | −4.2 ± 0.2 | 3.96 ± 0.01 | 7.2 | 27.1 ± 0.6 | −9.2 ± 0.2 | 1.57 ± 0.01 | 16.6 |
| $K^+$ + nicotine + $Ca^{2+}$ + hexamethonium (5) | −4.2 ± 0.1 | 3.96 ± 0.01 | 7.3 | 27.7 ± 1.3 | −6.0 ± 0.1 | 2.14 ± 0.01 | 10.7 |

relationship between the intensity of scattered light and particle size [26], these results indicate a reduction in radius, and hence shrinking of the particles. Addition of distilled water in order to reduce the tonicity by approx. 30% leads to the opposite effect, indicating particle swelling. These size variations signify that an appreciable fraction, and possibly all, of the membranes are osmotically responsive, and hence form closed vesicles.

## Discussion

The main feature of the data reported in the present paper is the significant increase in membrane fluidity, when the membranes are incubated with cholinergic agonists, at concentrations similar to those that induce optimal catecholamine secretion in the intact cells [3]. The response is blocked by the cholinergic antagonist hexamethonium bromide, which also blocks catecholamine secretion [3]. Fluidity is found to increase also upon incubation with excess KCl. Hexamethonium bromide is inactive in this case, suggesting a different mechanism for the fluidity change elicited by excess KCl. The fluidity increase is largest in the posttransition range (Table I), i.e. those temperatures that are more relevant in the physiological sense. The break is not significantly shifted by acetylcholine and only to a very limited degree ($\Delta t_{tr} \approx 2$—$3^{\circ}$C) by nicotine and by $K^+$. It is not sensitive to the presence of added $Ca^{2+}$, except in the case of $K^+$ ($\Delta t_{tr} \approx 1^{\circ}$C). $Ca^{2+}$, however, largely reverses the decreases in $P$ values induced by the stimulants (Table I).

At the outset, two basically different interpretations may be assumed for the observed effects. One is that receptor-lipid interactions may be modified by the binding of the agonists, and DPH molecules located in the lipid regions near the receptor are able to detect these modifications. A modification in the mobility of a spin-labeled long-chain acylcholine has been found in membrane fragments of *Electrophorus electricus* upon incubation with cholinergic ligands [31], but no parallel measurements of lipid phase fluidity were made. De Robertis has envisaged the cholinergic receptor as a proteolipid incorporating the ion channels as an integral part [32]. The acetylcholine receptor from the electric organ of *Torpedo marmorata* is an integral membrane protein [33], surrounded by an immobilized boundary layer of lipids [34]. If the same applies also to the acetylcholine receptor on chromaffin cells, then the observed fluidity changes may arise from rearrangements in the protein-lipid complex induced by the agonists. It is also conceivable that conformationally induced opening or closing of the ion channels involved in the membrane depolarization of the intact cell could be linked to the degree of order of their lipid environment. The other possibility which, in contrast to the first one, would accomodate also the excess $K^+$ effect, is that secretagogue-induced membrane depolarization corresponding to altered ionic environment and potential gradients, could exert either local or long-range effects on the organization of the lipid phase [35]. The latter alternative requires closed vesicles allowing establishment of ionic gradients across the membrane. The osmotic experiments indicate that closed vesicles constitute a sizeable fraction and possibly the whole preparation. However, until it can be proved that these vesicles do actually maintain a $K^+$ gradient, this interpretation remains conjectural and the

origin of the $K^+$ effect unexplained. Experiments directed towards this question are in progress.

Evidence for the operation of two basically different mechanisms [30] was shown in Fig. 4, where the effects of nicotine and $K^+$ were seen to be more than additive, and only partially reversed by hexamethonium bromide. Thus both mechanisms can simultaneously modify the lipid properties and, in fact, they may reinforce each other. It can be imagined that an increase in the fluidity stemming from one of these processes would facilitate the other, if the lipid domains involved are in close contact or partial overlap.

Partial reversal of the fluidity increases by $Ca^{2+}$ may be due to formation of ordered gel-like regions by interaction of $Ca^{2+}$ with negatively charged phospholipids [10,12,13]. Since DPH monitors $P$ values, which are an average over all labeled lipid domains [15,22], only part of which may be involved in and influenced by the stimulation process, the $Ca^{2+}$-induced increase in $P$ values may be partly or totally unrelated to the physiological event. On the other hand, bound $Ca^{2+}$ may be an integral constituent of the receptor-lipid complex. Agonist binding may free some of this $Ca^{2+}$, thereby reducing any restrictions on the lipid mobility, and this equilibrium would be sensitive to the $Ca^{2+}$ concentration in the medium. Acetylcholine-induced alterations in the binding of $Ca^{2+}$ to the acetylcholine receptor of *Torpedo californica* and *E. electricus* have in fact, been observed by Chang and Neumann [36]. A competitive interaction of $Ca^{2+}$ and acetylcholine with the receptor has been proposed to be involved in the control of the ion fluxes that are part of the nerve excitation process [37,38]. It appears reasonable to assume that $Ca^{2+}$ fulfils multiple roles in stimulus-secretion coupling and that secretagogue-induced $Ca^{2+}$ release from the membrane and a possible intracellular $Ca^{2+}$ requirement are distinct aspects of the overall event.

Comparison of $\Delta P/\Delta t$ for acetylcholine and nicotine shows a much larger effect for the latter, although the concentration used was ten times smaller than that of acetylcholine. Published data for binding of these agonists to the isolated receptor from *E. electricus* [39] indicates that acetylcholine binds more strongly than nicotine. It is possible that the receptor from chromaffin cells has the reverse order of affinity for the cholinergic agonists. Alternatively, nicotine may produce a larger perturbation in the lipid region associated with the receptor, perhaps because its aromatic moiety can interact more strongly with the lipids by virtue of hydrophobic forces.

The $\overline{\eta}_{25}$ and $\overline{\eta}_{37}$ values appearing in Table I are within the range of microviscosities found for other cell membranes [15]. It should be noted that the data published so far do not include membranes incorporating cholinergic receptors, with the exception of neuroblastoma [28], but in that case no measurements under stimulating conditions were made. On the other hand, $\Delta E$ varies very markedly in the posttransition range (Table I). The values in the presence of any one of the stimulants are much higher than those in their absence. The latter values are in the range recorded for other cell membranes, as indeed are also all the values in the pretransition range. It appears that the stimulants used reduce the order of the lipid regions in the posttransition range, which roughly corresponds to the range of physiological temperatures, to a degree not usually encountered in other cell membranes.

In conclusion, we believe that the marked increases in membrane fluidity, induced by cholinergic agonists and by excess $K^+$, may have physiological relevance, in that they may facilitate, or even be one of the requirements for stimulus-secretion coupling. Increased membrane fluidity may favour receptor interactions and channel formation by an increased mobility of the protein, somewhat in analogy to Cuatrecasas' proposal [40] for hormone-receptor interaction. Further, it may also enhance the ion fluxes, which are known to be part of stimulus-secretion via an increase in membrane permeability [8]. Finally, as already mentioned in Introduction, it may be necessary for the exocytotic event. Furthermore, the fate of $Ca^{2+}$ may be rationalized in terms of our data. Increased fluidity of the lipid regions could favour higher $Ca^{2+}$ fluxes through the membrane, in accordance with the increased permeability observed in synthetic phospholipid vesicles above their melting temperature [8]. This $Ca^{2+}$ may be needed intracellularly for the granule-membrane interaction leading to exocytosis. In addition, $Ca^{2+}$ by inducing some liquid-crystalline to solid-gel transitions during the stimulatory event could lead to segregation of rigid from fluid lipid domains, the boundaries of which would constitute centers for the initiation of fusion, again in analogy to synthetic membrane systems [8,9,11].

Membrane fluidity has been found in the present work to be enhanced by interaction with the stimulant and by elevation of the temperature. In this respect, it will be interesting to investigate the temperature dependence of catecholamine release in intact stimulated chromaffin cells. Such experiments are at present under way. It should be pointed out that the adrenal chromaffin cell, which in many respects can be considered as an analogue of the postganglionic sympathetic neuron [1], presents advantages in isolation, as well as study, over other, more inaccessible systems with similar function in the nervous system.

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