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Regulation of Chloride Transport in Parotid Secretory Granules by Membrane Fluidity†

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Received November 16, 1989; Revised Manuscript Received May 3, 1990

ABSTRACT: Zymogen granule membranes contain Cl^- conductance and Cl/anion exchange activities that become important for primary fluid production after fusion with the apical plasma membrane of the acinar cell. We have used steady-state fluorescence anisotropy of diphenylhexatriene derivatives and measurements of Cl^- transport in isolated secretory granules to determine the contribution of membrane fluidity to the regulation of transport across the granule membrane. Secretory granules from several unstimulated glands (rat pancreas and parotid, rabbit gastric glands) were shown to have low membrane fluidity compared to plasma membranes. In addition, Cl^- transport activity in different granule preparations showed a strong correlation to the membrane fluidity when measured with 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH), but not with 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl)-phenyl]propionic acid (PA-DPH). These data suggest that TMA-DPH preferentially partitions into a specific lipid environment associated with, or which exerts an influence on, the Cl^- transport proteins and that increases in the fluidity of this environment are associated with higher transport rates. Data from other types of plasma membranes indicate that TMA-DPH partitions much more than PA-DPH into the cytoplasmic leaflet, suggesting that this part of the granule membrane is involved in the observed fluidity changes. Furthermore, increasing the bulk membrane fluidity with the local anesthetics benzyl alcohol and *n*-alkanols increased the Cl^- transport rates up to 10-fold. This increase was apparently through specific transporters as anion selectivity was maintained in spite of the higher absolute rates. Temperature also influenced membrane fluidity and transport in a highly correlative manner. These observations are consistent with membrane fluidity acting as a major modulating factor for Cl^- transport activity in secretory granules and with exocrine acinar cells selectively regulating the fluidity of this environment.

Apart from serving as a simple boundary, biological membranes are involved in many complex physiological functions, including interactions with the integral proteins which reside within the lipid bilayer. These membrane-associated proteins typically contain large hydrophobic sequences which span or anchor in the lipid bilayer. The lipids serve as a solvent for the hydrophobic segment of the proteins and as such can have a profound effect on their properties. Studies on receptors, enzymes, and transport proteins (Abney & Owicki, 1985; Carruthers & Melchior, 1988; Farias, 1987) have shown optimal activity to require specific lipids, which serve as cofactors or stabilize the proteins through hydrophobic or electrostatic interactions and hydrogen bonding. In addition to these specific biochemical requirements, such bulk phase bilayer properties as fluidity, thickness, and surface potential may also influence the physiological activity of many integral proteins (Shinitzky, 1984; Deuticke & Haest, 1987; Watts & DePont, 1985).

Since the presentation by Singer and Nicolson (1972) of the fluid mosaic model of biological membranes, considerable attention has been directed toward the regulation and importance of membrane fluidity. Many organisms (e.g., bacteria, fish, hibernating mammals) have the ability to change the lipid composition and fluidity of membranes in response to such gross environmental conditions as temperature and thereby maintain a requisite fluidity for cellular functions (Cossins & Sinensky, 1984). However, evidence is lacking for specifically controlled fluidity changes serving as part of a regulatory system for membrane-bound enzymes or transport proteins in mammalian systems. In vitro alterations in membrane lipid composition and fluidity, or imposed in vivo changes brought about by diet, have had varied effects on the functioning of integral proteins (Shinitzky, 1984; Deuticke & Haest, 1987; Dudeja et al., 1987). Although membrane

† This research was supported by grants from the National Institutes of Health (DK-39658) and the Cystic Fibrosis Foundation (Z0298).
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0006-2960/90/0429-7282$02.50/0

transport has been shown to be sensitive to changes in bilayer composition and properties, neither the nature of the effect nor the physiological significance of membrane fluidity manipulations can be generalized.

Exocrine secretory systems are a useful physiological model to study the effect of membrane fluidity on a specific function. The secretory granules contain electrolyte transport pathways whose activities in isolated preparations can vary considerably. This variability among preparations is probably caused by varying degrees of activation of transporters in vivo. The regulatory mechanisms responsible for this activation, however, are not fully understood. Since a previous report suggested that isolated secretory granules from the toad bladder possess a lower membrane fluidity than the luminal plasma membrane (Verkman & Masur, 1988), we have investigated the relationship between granule membrane fluidity and Cl^- transport.

Materials and Methods

Granule and Membrane Isolation. Secretory granules were isolated from the parotid glands or the pancreas of Sprague-Dawley rats (150–200 g), or from the stomach of New Zealand white rabbits, by a methodology which has been previously described in detail (Gasser et al., 1988b; Sharma et al., 1989). Briefly, the method employs cell disruption by nitrogen cavitation in a low ionic strength buffer followed by separation using Percoll density gradient centrifugation. The granules were harvested as a high-density band (1.11–1.13 g/mL). Granule membranes were prepared from purified granules by osmotic lysis with distilled water and collection of the membranes by centrifugation at 50000*g* for 1 h.

The Percoll gradient also yielded a low-density fraction (1.03–1.06 g/mL), rich in plasma membranes. The plasma membranes from the parotids were further enriched by differential centrifugation after a 1:20 dilution of the low-density fraction in a solution consisting of 250 mM sucrose, 40 mM MOPS,[1] titrated to pH 7.0 with NaOH, 0.1 mM EGTA, and 0.1 mM $MgSO_4$. A 2000*g* pellet was discarded, and a subsequent 30000*g* pellet was resuspended in the same buffer and used as a crude plasma membrane fraction. A single animal was used for most granule preparations. High-purity brush border membranes from nonexocrine tissues such as rat small intestine and kidney proximal tubule were isolated as described by Hopfer et al. (1983).

Evaluation of Membrane Permeability. The anion (primarily Cl^-) permeability of isolated parotid secretory granules was determined by the method of ionophore-dependent granule lysis in a defined salt solution. The technique has been described in detail previously (DeLisle & Hopfer, 1986; Gasser et al., 1988a). Briefly, the method involved imposing an ionophore-dependent cation permeability such that osmotic influx and lysis were limited by the endogenous rate of anion (Cl^-) transport by the granule membrane. Lysis was followed by the change in OD_{540} of the granule suspension. The suspension solution consisted of 150 mM KCl, 20 mM HEPES titrated to pH 7.0 with KOH, 0.1 mM EGTA, and 0.1 mM $MgSO_4$. Valinomycin (10 μg/mL), an electrogenic K^+-selective ionophore, produced a maximal K^+ conductance in all experimental conditions and initiated granule lysis commensurate with the endogenous rate of Cl^- conductance; similarly, maximal concentrations of nigericin (5 μg/mL), an electroneutral K^+/H^+ exchanger, initiated lysis whose rate was limited by the endogenous rate of Cl^-/anion exchange.

Membrane Fluidity. Membrane fluidity is a general term for the degree of motional freedom within a bilayer or membrane. Fluorescent probes that insert into the membrane are frequently used to measure fluidity by steady-state fluorescence anisotropy. However, probe anisotropy can reflect the rotational rate of a fluorescence probe, the lipid order or range of probe motion, or the probe lifetime. Steady-state fluorescence anisotropy is unable to clearly differentiate between these various contributions, and therefore it is generally considered inappropriate to assign quantitative units for parameters such as microviscosity or lipid order except in ideal model conditions. However, many reports have shown it to be an effective and sensitive tool for comparative measurements of general membrane fluidity (motional freedom) (Shinitzky, 1984; Illsley et al., 1987; Sheridan & Block, 1988) and will be used in this capacity. Steady-state anisotropy values (r) vary inversely with fluidity and were determined by using the probes TMA-DPH and PA-DPH. Fluorescence polarization measurements were performed with a Perkin-Elmer LS-5B luminescence spectrometer equipped with a polarizer, and a four-cell thermostated compartment. Cuvette temperature was maintained with a Haake circulating bath and was monitored directly with a Fluke thermistor. Excitation wavelength was 362 nm (5-nm slit width) while emission was measured at 430 nm (10-nm slit width) for both probes. The results are expressed as fluorescent anisotropy (r), and $r = (I_0 - I_{90})/(I_0 + 2I_{90})$, where I_0 and I_{90} were the relative fluorescence intensities measured with the emission polarizer parallel (0°) and perpendicular (90°) to the excitation beam. No correction for system asymmetry was necessary since the correction factor equalled 1.00. This correction factor is given by $[(I_0)_H(I_{90})_V]/[(I_0)_V(I_{90})_H]$, whereby the subscripts H and V indicate horizontally and vertically polarized light, respectively.

Measurements were made on isolated plasma membranes (75 μg of protein), secretory granule membranes, or intact granules (both, about 10 μg of membrane protein). These samples were added to 3 mL of a buffer containing 150 mM KCl, 20 mM HEPES, pH 7.0, 0.1 mM $MgSO_4$, and 0.1 mM EGTA. The fluorescence fluidity probes were added from 1 mM stock solutions in DMSO with thorough mixing to give a final concentration of 0.5 μM. Membrane labeling with TMA-DPH and PA-DPH was complete within 2–3 min (Trotter & Storch, 1989). Both compounds exhibit strong fluorescence enhancement upon binding to lipids. The background light scatter was less than 5% of the recorded fluorescence signal in all situations. Furthermore, triplicate readings routinely exhibited a precision of greater than 1%. In experiments concerned with the effects of different alcohols on fluidity or transport, additions from 1000-fold concentrated stock solutions were made with mixing to the granule suspension. Granules were preincubated with any of the tested alcohols (concentrations given under Results) for approximately 5 min at 37 °C prior to starting fluorescence or transport measurements. The figures are representative experiments taken from at least four repetitions on different granule preparations. Unless otherwise indicated, data are presented as mean ± standard error of the mean. The significance of differences was tested by the pair Student's t test.

Results

We had previously shown that Cl^- conductance and Cl/anion exchange activity is low in granules isolated from the

[1] Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PA-DPH, 3-[*p*-(6-phenyl-1,3,5-hexatrienyl)phenyl]propionic acid; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate.

Table I: TMA-DPH Steady-State Fluorescence Anisotropy as a Measure of Membrane Fluidity from Plasma and Secretory Granule Membranes

membrane	anisotropy (r)[a]
plasma membranes	
parotid	0.225 ± 0.003
kidney	0.202 ± 0.011
intestine	0.223 ± 0.003
secretory granule membranes	
pepsinogen	0.287 ± 0.003
pancreas	0.264 ± 0.010
parotid	0.275 ± 0.004
limiting hindered anisotropy[b]	
r_∞	0.120
anisotropy without rotation	
r_0	0.390

[a] Results are expressed as mean ± standard error of the mean of at least four separate preparations. [b] The minimal steady-state anisotropy in 1,2-dimyristoylglycero-3-phosphocholine vesicles (r_∞) at 55 °C and the maximal anisotropy (r_0) measured with the probe dissolved in −65 °C propylene glycol [taken from Prendergast et al. (1981)].

pancreas of animals pretreated with antagonists for secretion, while those isolated after intracardial injection of the secretagogues, cholecystokinin or secretin, had very high levels (Gasser et al., 1988a). In a variety of exocrine systems (e.g., pancreas, parotid, chief cells), granules isolated from untreated animals show variable Cl^- transport activity, presumably due to a variable degree of spontaneous activation of transport pathways in vivo. To determine the contribution of the physical state of lipids to this spontaneous variation in chloride transport in different granule preparations, the membrane fluidity was assessed by steady-state fluorescence anisotropy measurements with DPH analogues. Table I summarizes the anisotropy results with TMA-DPH obtained from secretory granules from three different types of unstimulated glands (rat parotid and pancreas, and rabbit gastric pepsinogen granules) as well as plasma membranes from three sources (rat parotid acinar cells as well as brush border membranes from rat small intestine and rabbit kidney proximal tubule). A paired comparison of TMA-DPH and PA-DPH anisotropy between intact granules and granule membranes derived from the granules showed no significant difference. Therefore, most measurements involving granules were made on membranes and not intact organelles. The secretory granules exhibited a much higher fluorescence anisotropy than the plasma membranes, reflecting a significantly lower granule bilayer fluidity. This relationship was consistent for all groups with the secretory granules exhibiting mean anisotropies (r) ranging from 0.264 to 0.287 compared to the plasma membranes range of 0.202–0.225.

When the TMA-DPH anisotropy of each granule preparation was plotted against its Cl^- conductance (measured as the half-time for lysis after the addition of valinomycin), a strong correlation was evident (Figure 1). This relationship suggests that the TMA-DPH-measured environment is associated with the regulation of Cl^- transport in the membrane. Interestingly, when PA-DPH anisotropy was plotted versus Cl^- conductance, no correlation was apparent (Figure 2). These results suggest that the two probes do not measure fluidity in the same environment and that Cl^- transport proteins occupy an environment more efficiently measured by TMA-DPH. This interpretation is supported by data showing that the Cl^- conductance is substantially increased by TMA-DPH (11.7 ± 3.4 h^{-1}) over controls (2.8 ± 0.9 h^{-1}; $p < 0.05$), but not by PA-DPH (1.6 ± 0.3 h^{-1}; not significantly different from controls) or the uncharged parent molecule DPH (3.0 ± 0.8 h^{-1}; not significantly different from controls). Although

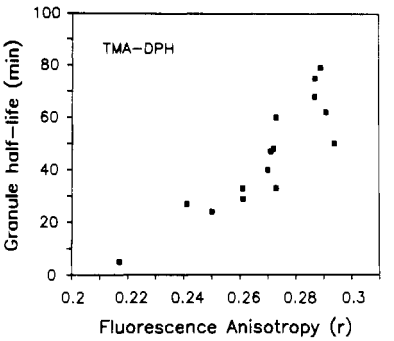

FIGURE 1: Relationship between TMA-DPH steady-state fluorescence anisotropy of granule membranes and Cl^- conductance in intact granules from different preparations. The Cl^- conductance was determined by valinomycin-induced granule lysis in isotonic KCl, pH 7.0, and is reported as the half-time for lysis. TMA-DPH anisotropy was measured on membranes prepared from the isolated granules, except for two of the experiments with intact granules.

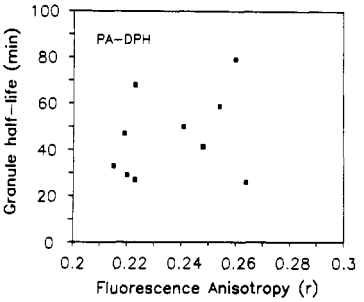

FIGURE 2: Relationship between PA-DPH steady-state fluorescence anisotropy of granule membranes and Cl^- conductance of intact granules from different preparations. The Cl^- conductance was determined by valinomycin-induced granule lysis in isotonic KCl, pH 7.0, and is reported as the half-time for lysis. PA-DPA anisotropy was measured on membranes prepared from the isolated granules, except for two experiments with intact granules.

the basis for the probe-associated transport enhancement is not known, it indicates by another mechanism the selective interaction between the TMA-DPH probe and the Cl^- transporter(s).

To test the hypothesis that membrane fluidity can influence Cl^- transport, the effects of agents known to alter membrane fluidity were examined. Benzyl alcohol, a water-soluble, local anesthetic, has been shown to increase the fluidity of model phospholipid bilayers as well as biological membranes (Gordon et al., 1980). Indeed, benzyl alcohol increased the fluidity of parotid secretory granule membranes in a concentration-dependent manner. This increase was expressed in a decrease in anisotropy of both TMA-DPH and PA-DPH with 10, 20, 30, and 40 mM benzyl alcohol causing a 2%, 5%, 8%, and 10% decrease, respectively. Other alkanols which can serve as local anesthetics were also effective modulators of membrane fluidity. For example, octanol was a more potent fluidizing agent than benzyl alcohol, causing a 5% decrease at 1 mM and a 10% decrease in anisotropy at a concentration of approximately 3–4 mM as compared to 20 and 40 mM, respectively, for the latter aromatic alcohol.

In support of our hypothesis, the fluidizing alcohols had a dramatic effect on the Cl^- transport rates of parotid secretory granule membranes. Benzyl alcohol caused a concentration-dependent increase of up to 10-fold in valinomycin-induced granule lysis without promoting lysis in salt solutions in the absence of ionophores (Figure 3A). This increase was independent of the type of ionophore or counterion present, suggesting an increase in specific Cl^- conductance. For example, qualitatively similar increases were obtained with benzyl

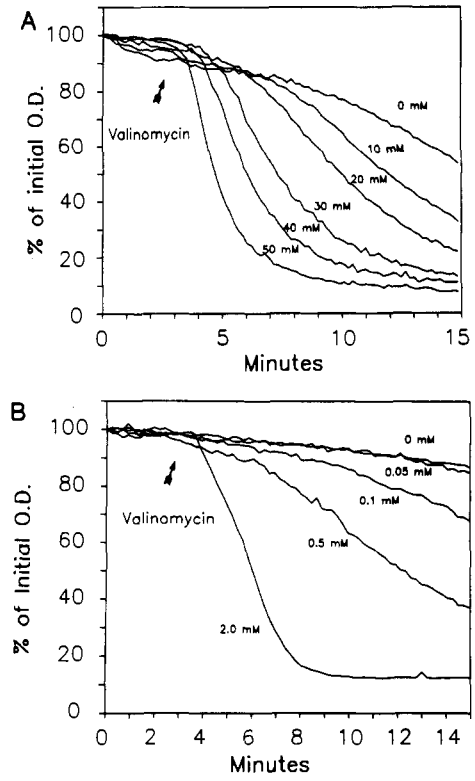

FIGURE 3: Effect of pretreatment with various concentrations of the fluidizing agents benzyl alcohol (A) or octanol (B) on granule membrane Cl^- conductance. Conductance was measured as the valinomycin-induced granule lysis rate when suspended in isotonic KCl, 20 mM HEPES, pH 7.0, 1.0 mM EGTA, and 0.1 mM $MgSO_4$ at 37 °C. Valinomycin (10 μg/mL) was added at the arrow to the granule suspension, creating a maximal K^+ conductance and a condition whereby the rate-limiting step for granule lysis is the endogenous Cl^- conductance.

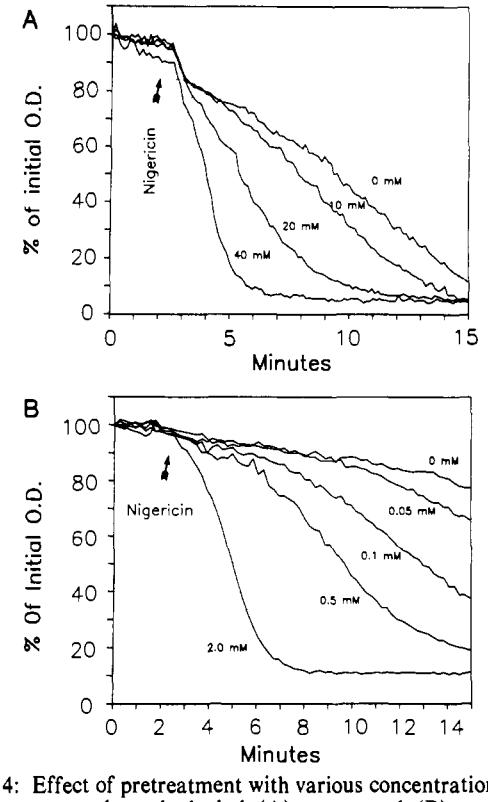

FIGURE 4: Effect of pretreatment with various concentrations of the fluidizing agents benzyl alcohol (A) or octanol (B) on granule membrane Cl^-/anion exchange. Anion exchange was measured as the nigericin-induced granule lysis rate when suspended in isotonic KCl, 20 mM HEPES, pH 7.0, 1.0 mM EGTA, and 0.1 mM $MgSO_4$ at 37 °C. Nigericin (5 μg/mL) was added at the arrow to the granule suspension, creating a maximal K^+/H^+ exchange and a condition whereby the rate-limiting step for granule lysis is the endogenous Cl^-/anion exchange.

alcohol when nonactin or gramicidin replaced valinomycin as the electrogenic ionophore and NaCl or LiCl replaced KCl in the suspension solution (data not shown).

In keeping with its measured effect on fluidity, octanol had a proportional effect on the parotid granule Cl^- conductance, with 3 mM octanol causing a 6-fold increase in rate (1.5 ± 0.8 to 9.3 ± 2.3 h^{-1}; inverse of the half-time for valinomycin-induced lysis in isotonic KCl at 37 °C) (Figure 3B). The ability of members of a homologous series of alkanols, of which octanol is a member, to promote Cl^- transport in parotid secretory granules was also measured. The concentrations of alkanols required to increase the control Cl^- conductance by 2-fold were approximately 50 mM propanol, 40 mM butanol, 10 mM pentanol, 0.2 mM octanol, and 0.05 mM decyl alcohol. Their efficacy as a transport stimulant was directly related to the chain length of the alkanol and the degree to which they fluidized the granule membrane. The sequence was also similar to their reported potency as local anesthetics or their ability to block sodium channels in nerve membrane vesicles (Rodriguez et al., 1988).

Several types of data argue that the elevated Cl^- conductance in the presence of fluidizing agents represents greater channel activity and not the creation of nonspecific leaks in the membrane: (1) The anion conductance pathway of the secretory granule membrane had previously been shown to have an anion selectivity sequence of $SCN^- > I^- > Br^- > Cl^- > F^- > SO_4 \gg$ gluconate (Gasser & Hopfer, 1989), similar to that reported for other Cl^- channels (Halm et al., 1988). This sequence was not altered by the addition of benzyl alcohol, even though the rates were 5-fold faster. (2) In the absence of valinomycin, the alcohols did not influence the lysis rate. (3) Increasing lipid fluidity did not render the granules more susceptible to osmotic lysis. For example, granules suspended in a 300 mOsm solution exhibited a half-life of 4.20 ± 1.17 h which decreased to 0.83 ± 0.21 h after dilution into medium of 150 mOsm; addition of 10, 20, or 40 mM benzyl alcohol did not significantly alter the lysis rates in the 150 mOsm medium, yielding half-times of 0.75 ± 0.32, 0.92 ± 0.51, and 0.68 ± 0.43 h, respectively.

Since the Cl^- transport is measured by an ionophore-dependent mechanism, it could also be argued that the observed increase in rate is due to an effect of fluidity on the added ionophore and not the endogenous Cl^- transport protein. However, the same concentration of valinomycin that was used in the experiments with Cl^- induced a much higher lysis rate when the granules were suspended in isotonic KSCN (i.e., the salt of a membrane permeant anion), and this rate was not significantly affected by benzyl alcohol: 115 ± 43 h^{-1} vs 142 ± 53 h^{-1} (n = 5). Thus, valinomycin-induced K^+ transport was not rate limiting for salt influx into the granules and lysis in the transport experiments with Cl^-, regardless of the membrane fluidity. Therefore, a change in Cl^- conductance is necessary to account for the observed changes in lysis rates. Furthermore, the effects of alcohols on the granule lysis rates were similar with gramicidin which forms a cation channel and whose activity is not greatly influenced by membrane fluidity (Krasne et al., 1971).

Greater membrane fluidity also increased the rate of Cl^-/anion exchange. The effect is demonstrated for benzyl alcohol in Figure 4A. As with the conductance pathway, the anion exchange pathway has a unique anion selectivity sequence, acetate > $I^- > Br^- > F^- > Cl^- \gg SO_4 \gg$ gluconate

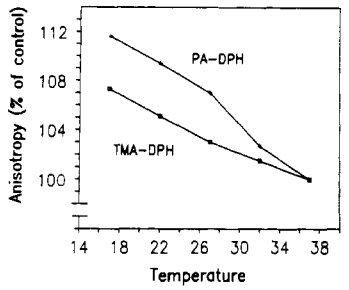

FIGURE 5: Effect of temperature on membrane steady-state fluorescence anisotropy using TMA-DPH (■) and PA-DPH (+). The data points represents the means of at least four determinations with the standard error being 2% or less.

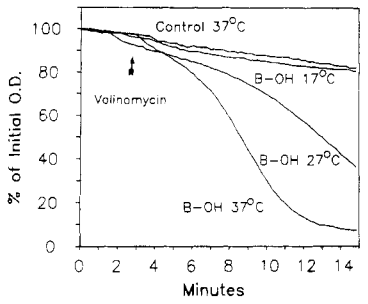

FIGURE 6: Effect of temperature on valinomycin-induced granule lysis (Cl^- conductance) in isotonic KCl, pH 7.0, after fluidization of the granule membrane with 25 mM benzyl alcohol (B-OH).

(Gasser et al., 1989), which was retained after benzyl alcohol treatment. The Cl^-/anion exchange responded similarly to octanol, with 3 mM octanol generating a 5-fold increase in rate (Figure 4B).

Temperature is known to have a profound effect on membrane fluidity, with decreasing temperature causing an increase in membrane order (decrease in fluidity) and therefore a corresponding increase in steady-state anisotropy. Figure 5 confirms this temperature-dependent change in membrane fluidity in terms of TMA-DPH and PA-DPH anisotropy. More interestingly, a decrease in temperature is also associated with decreased Cl^- transport. The temperature-dependent transport changes could therefore be used to test whether bulk fluidity changes, which includes the fluidity of specific lipid microenvironments, can account for the effects of alcohols on Cl^- transport. If membrane fluidity is the sole mediator involved, then temperature should reverse the effects of alcohols on Cl^- transport. This result was indeed observed. For example, a decrease in temperature from 37 to 17 °C completely reverses the benzyl alcohol stimulated increase in Cl^- conductance (Figure 6). Again, there is a good correlation between the measured Cl^- conductance and TMA-DPH anisotropy. For instance, the addition of 25 mM benzyl alcohol produced a decrease in TMA-DPH anisotropy of approximately 7%; conversely, a 20 °C decrease in temperature produced an increase in TMA-DPH anisotropy of approximately 7%. Figure 6 shows that these opposing effects on membrane fluidity are manifest functionally as changes in granule membrane Cl^- conductance.

The relationship between bulk fluidity changes of the entire granule membrane, brought about by alcohol and temperature, and Cl^- conductance is summarized in Figure 7A,B. It is noteworthy that there is a good correlation between the two parameters, regardless of the means employed to artificially change the membrane fluidity. The correlation is qualitatively similar to that observed between the Cl^- conductance of native granules and discrete TMA-DPH fluidity values from a

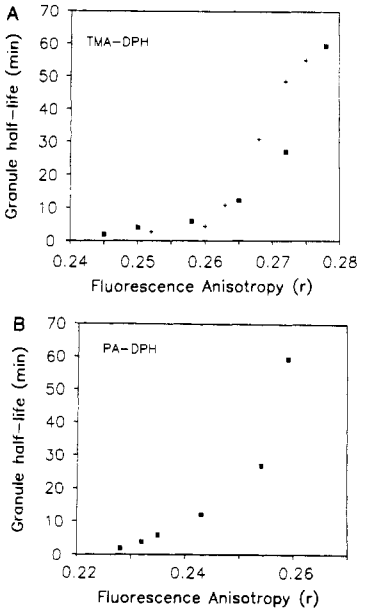

FIGURE 7: Relationship between changes in steady-state fluorescence anisotropy induced by such bulk fluidizing agents as benzyl alcohol and temperature and Cl^- conductance of parotid secretory granule membranes. The Cl^- conductance was determined by valinomycin-induced granule lysis in isotonic KCl, pH 7.0, and is reported as the half-time for lysis. (■) represents benzyl alcohol modulated granule membrane anisotropies from a single granule preparation and the associated Cl^- transport rate using different benzyl alcohol concentrations, whereas (+) represents temperature-modulated granule membrane anisotropies from the same granule preparation. (A) TMA-DPH-determined anisotropy. (B) PA-DPH-determined anisotropy.

number of preparations (Figure 1). However, as opposed to the specific lipid environments measured in native membranes in Figure 1, bulk fluidizing agents alter the entire membrane such that fluidity changes are reflected equally in the lipid environments probed by TMA-DPH and PA-DPH. These figures show, by their qualitative similarity, that Cl^- transport responds to manipulations of bulk membrane fluidity (alcohols and temperature; Figure 7) in the same manner as a more specific endogenous mechanism (Figure 1). These results therefore support the conclusion that the fluidity of a specific lipid environment within the secretory granule membrane (manipulated by both endogenous and exogenous means) is an important regulatory component in the control of Cl^- transport.

Discussion

Electrolyte transport pathways thought to be involved in the production of primary fluid from exocrine glands are associated with secretory granule membranes (DeLisle & Hopfer, 1986; Gasser et al., 1988a,b; Sharma et al., 1989). The regulation of this transport probably occurs by a receptor-mediated pathway, as the secretagogues cholecystokinin and secretin stimulate Cl^- transport in zymogen granules from the pancreas (Gasser et al., 1988a). The intracellular regulation may involve protein kinase systems (Fuller et al., 1989a), calcium-mediated pathways (Gasser et al., 1988b; Fuller et al., 1989b), intragranular pH gradients (Gasser & Hopfer, 1990), or modulation by adenine and guanine nucleotides (Thevenod et al., 1989); however, the changes in Cl^- transport rate that can be achieved in isolated granules through the above regulatory pathways have not been sufficient to account for the variability observed with granule preparations from different animals. Membrane fluidity was investigated as a possible explanation because of its recognized influence on many membrane-me-

diated processes (Shinitzky, 1984) and the reported difference in fluidity between secretory granules from the urinary bladder and the typical plasma membrane (Verkman & Masur, 1988).

Fluidity measurements of secretory granule membranes by steady-state fluorescence anisotropy along with parallel measures of membrane electrolyte permeability provided valuable information on both physical and functional parameters in the granule membrane. TMA-DPH and PA-DPH were equally effective probes for measuring bulk fluidity changes associated with alcohol addition or changes in temperature, yielding identical percentage changes in anisotropy. However, only TMA-DPH, a cationic probe, displayed a strong correlation between discrete anisotropy values of native granule membranes and the endogenous Cl^- transport activity of different preparations. Biological membranes are not homogeneous structures, and it is concluded that TMA-DPH and PA-DPH preferentially partition into, and report fluidity from, different lipid environments within the membrane. Considerable evidence exists that the cytofacial and extracellular leaflets of plasma membrane bilayers differ in surface charge as well as the composition of phospholipids, fatty acids, and cholesterol (Sweet & Schroeder, 1988). Interestingly, Schroeder (Schroeder, 1988; Schroeder et al., 1988) presented evidence for plasma membranes of fibroblast and nerve cells that the DPH derivatives differentially partition into the two halves of the bilayer: TMA-DPH preferentially localized in the cytofacial and PA-DPH in the extracellular leaflet. Extrapolation to the secretory granules suggests that selective fluidity changes occur in the cytofacial lipid sheet of their membranes. Changes in membrane fluidity have been reported in a wide variety of tissues in response to a number of physiological stimuli [see review by Sweet and Schroeder (1988)].

The specific correlation between TMA-DPH anisotropy and Cl^- conductance suggests that a selective environment, potentially regions of the cytofacial leaflet, is physiologically important for Cl^- transport. Thus, site-specific agents, as part of a regulatory mechanism, could produce significant fluidity changes within a microenvironment without dramatically influencing the fluidity of all the lipid environments in the membrane. Indeed, preliminary data in our laboratory show that micromolar levels of some fatty acids are sufficient to increase the Cl^- transport and to decrease TMA-DPH, but not PA-DPH, anisotropy in parotid granule membranes.

The Cl^- permeability of isolated granules from nonsecreting glands (i.e., from animals pretreated with antagonists of secretion) is at the low end of the normal transport variability seen in the absence of any pretreatment, while that of granules after pretreatment with secretagogues is much higher (Gasser et al., 1988a). Therefore, the strong correlation between membrane fluidity, as measured by TMA-DPH, and Cl^- conductance suggests that unstimulated granules in vivo have a very low membrane fluidity whereas activated granules with a high transport rate have an increased fluidity. This correlation is evident in Figure 1. An increase in fluidity may serve to enhance Cl^- channel activity just prior to fusion of granules with the plasma membrane. This would allow for "active" Cl^- secretion across, what after fusion functionally becomes, the luminal plasma membrane.

The physiological mechanism for causing acute changes in membrane fluidity is speculative; however, the available information suggests several feasible pathways. Lipid methylation has been shown to cause an increase in fluidity under some conditions (Rakhit, 1984). Furthermore, receptor-mediated second-messenger signals in some cells include a transient increase in methyl transferase activity (Hirata & Axelrod, 1980; Rimon et al., 1978). If this activity is sufficiently site-specific, it could induce significant changes in transporter activity. Second, Valentino et al. (1986) have presented evidence consistent with fluidity being influenced by cytoskeletal attachments, with protein anchors decreasing membrane fluidity. As secretory granules are intimately associated with the cytoskeletal network of the acinar cells, control by this route could be feasible. Another route involves the well-known rapid and transient increase in lipid metabolism associated with receptor stimulation (Rustenbeck & Lenzen, 1989; Nishizuka, 1984; Soling et al., 1989). Since, the physiological effector for transport may be site-specific and not cause changes in the bulk membrane fluidity, relatively low concentrations may be necessary. The degree of fluidity change necessary to influence protein conformation or protein equilibrium within the membrane in a manner as discussed above is not known. The fluidity changes measured in different granule preparations were significant, with the TMA-DPH anisotropy ranging from 0.215 to 0.309. As a basis of reference, the maximal steady-state TMA-DPH anisotropy measured in the "gel" state or in the anisotropy measured without rotation (r_0) was reported to be 0.39, and the minimum anisotropy measured in a system with limited hindrance (r_∞) was approximately 0.14 (Prendergast et al., 1981) (Table I). Therefore, the native granule membranes, at 37 °C, display a range in anisotropy encompassing approximately 40% of what could be thought of as the maximal TMA-DPH anisotropy range.

Aside from these physiologically possible mechanisms of fluidity modulation, in vitro alcohol-induced changes in fluidity provide further support for a direct relationship between membrane fluidity and Cl^- transport. The data demonstrate a consistent response of both the Cl^- conductance and Cl^-/anion exchange to discrete changes in fluidity, independent of the alcohol or the temperature used to effect the change. It is unlikely that these artificially promoted levels of fluidity, and therefore transport, are subject to misinterpretation due to artifactual extremes of fluidity as they remained in the physiological range. For example, 40 mM benzyl alcohol or 3 mM octanol reduce the TMA-DPH anisotropy by approximately 11–12%, well within the range of fluidities measured for the native plasma membranes and isolated granules with high Cl^- transport (Figure 1, Table I).

The physical mechanism by which membrane fluidity affects membrane-associated processes remains open to discussion; however, there are general possibilities which can be considered. Shinitzky (1984) has presented a model whereby changes in membrane fluidity cause vertical or lateral displacement of membrane proteins. Changes in fluidity are associated with alterations in the equilibrium position of a protein in the bilayer based on its net interaction between the ambient lipids, other proteins, and the aqueous environment. The supposition is that lateral displacement would open or close channels formed by the interaction of transmembrane protein segments, while vertical displacement would expose or hide catalytically important sites on the protein. Indeed, several reports support the type of physical relocation within the bilayer that would be necessary for this hypothesis (Borochov & Shinitzky, 1976; Armond & Stachelin, 1979). However, without precise information on protein structure and the nature of neighboring proteins, it is not possible to predict the direction of response for a given transporter (i.e., stimulation or inhibition) to changes in fluidity. The relative rigidity of the bilayer may also influence protein conformational changes thought to be necessary for transporting an electrolyte across the membrane. Krebs et al. (1987), using circular dichroism

and fluorescence spectroscopy, have demonstrated distinct changes in protein shape associated with calcium transport in the Ca^{2+}-ATPase. Restriction of motional freedom within the bilayer would create an energetically less favorable environment to accommodate or permit the peptide–lipid rearrangements necessary for conformational changes. Therefore, physiological control of membrane fluidity would be an effective modulator of electrolyte transport activity.

ACKNOWLEDGMENTS

We are grateful to Dr. Joyce Jentoft for generously supplying us with access to her laboratory and the Perkin-Elmer spectrometer. We also acknowledge Dr. Frank Thevenod for numerous helpful discussions.

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