ELSEVIER

Biochimica et Biophysica Acta 1194 (1994) 123–130

BBA Biochimica et Biophysica Acta

The effect of phospholipase A_2 on chloride transport by pancreatic secretory granules

Kenneth W. Gasser *, Jaclyn R. Holda

Department of Biological Sciences, Northern Illinois University, DeKalb, IL 60115-2861, USA

Received 22 March 1994

Abstract

Secretory granules from the rat pancreas contain electrolyte transport pathways that may contribute to exocrine fluid production. The Cl^- selective transport pathway was measured indirectly in isolated granules by ionophore-induced lysis after suspension in isotonic KCl. This Cl^- transport was shown to respond to alterations in the granule membrane lipid environment. Exogenously added phospholipase A_2 (PLA_2) caused an increase of up to 193% in the Cl^- specific transport by the isolated granules. In addition, the products of PLA_2 hydrolysis, lysophospholipids and unesterified fatty acids, directly increased the rate of Cl^- transport when incubated with granules in vitro. Lysophospholipids (2.0 μg/ml) increased the Cl^- transport between 280–450% (depending on the lysophospholipid species). Similarly, free fatty acids (10 μM) increased the granule Cl^- transport from 25% with capric acid (10:0) to 255% with arachidonic acid (20:4). The relative extent of stimulation by fatty acids was dependent on their carbon chain length and to a lesser extent, the degree of unsaturation. The inhibition (68%) of PLA_2 promoted granule lysis by 4-acetamido-4′-isothiocyanatostilbene 2,2′-disulfonic acid (0.5 mM) also suggests that the effect is specific for the granule Cl^- channel. Thus, the data show that zymogen granule Cl^- transport is influenced by membrane lipids and supports a role for PLA_2 in controlling electrolyte transport during stimulus-secretion coupling.

Key words: Exocrine secretion; Exocytosis; Chloride transport; Lysophospholipid; Fatty acid; Zymogen granule

1. Introduction

Exocrine secretion involves the insertion of cytoplasmic secretory granules into the plasma membrane with the subsequent release of the macromolecular product into the extracellular environment. This process requires the coordinated promotion of both membrane fusion and electrolyte transport. Two transport proteins found in the membranes of pancreatic secretory granules are; an ATP-sensitive K^+ conductance [1–3] and a Cl^- conductance inhibitable by 4-acetamido-4′-isothiocyanatostilbene 2,2′-disulfonic acid (SITS) [4–6]. The exocytotic fusion of granule membrane with the apical plasma membrane results in the insertion of these electrolyte transport proteins into the luminal facing surface of the acinar cell. These channels may promote local fluid production which solubilizes and flushes the macromolecular granule product into the acinar lumen. Alternatively, they may contribute to the efficiency of exocytotic membrane fusion by promoting vesicle swelling, and therefore widen and stabilize the fusion pore. To guard against premature granule swelling (and conceivably lysis), the activation of these transport pathways must be timed to coincide with the fusion of the granule and apical plasma membranes.

The intracellular signaling mechanisms that control stimulus-secretion coupling include the second messengers cAMP and calcium, as well as the accompanying activation of phospholipases and kinases. These signaling systems likely control vesicle movement to the plasma membrane and their subsequent fusion [7], as well as the granule electrolyte transport. For example, there is evidence to suggest that the granule Cl^- transport can be regulated by cAMP-dependent protein kinase [8], nucleotides [9], and changes in membrane fluidity [10]. In addition, PLA_2 may be an important effector enzyme in the coordination of membrane fusion and electrolyte transport during stimulus-secretion coupling. Activation of PLA_2 coincides with exocytotic

* Corresponding author. Fax: +1 (815) 7530461.

SSDI 0005-2736(94)00130-H

secretion in a variety of tissues including adrenal chromaffin cells [11,12], pancreas [13], rat synaptosomes [14,15], the sperm acrosome reaction [16]; and it is also associated with purified pancreatic secretory granule membranes [17]. Products of PLA_2 hydrolysis of membrane phospholipids cause a net increase in the fluidity of the lipid bilayer [18], which may influence granule membrane Cl^- permeability [10]. Therefore, the alteration of the granule membrane lipid environment by PLA_2 may contribute to the regulation of Cl^- transport in the exocrine secretory system.

The hydrolysis of membrane phospholipids by PLA_2 can result in the release of a variety of fatty acids, depending on the phospholipid. This fatty acid is typically unsaturated, with arachidonic acid being the most extensively studied because of its metabolism to other biologically active eicosanoids. However, free fatty acids have also been implicated in having a direct effect on membrane proteins [19,20], including ion channels [21–23]. This study investigates the effect of fatty acids and lysophospholipids on secretory granule Cl^- transport, based on the possibility that secretory granule membrane phospholipids are utilized as a substrate by PLA_2.

2. Materials and methods

2.1. Secretory granule isolation

Secretory granules were isolated from the pancreas of Sprague-Dawley rats (150–200 g) by a methodology that has been previously described in detail [3]. Briefly, rats were killed by cervical dislocation while under ether anesthesia. The pancreas was removed and placed in an ice-cold, low ionic strength homogenization buffer. This buffer consisted of: 250 mM sucrose, 40 mM 3-(*N*-morpholino)propanesulfonic acid (Mops; titrated to pH 7.0 with NaOH), 0.1 mM $MgSO_4$, 0.1 mM EGTA (free calcium 0.1 μM), 1.0 mg/ml fatty-acid free bovine serum albumin (BSA), and 0.1 mM phenylmethylsulfonyl fluoride, added immediately before use. The pancreas was minced to a paste and homogenized with a glass-Teflon tissue grinder. This homogenate was disrupted further by nitrogen cavitation at 750 psi, a process that broke the majority of the cells and released the secretory granules intact. The homogenate was supplemented with Percoll stock (100%) to give a final concentration of 45% by volume, while maintaining the original homogenization buffer constituents and concentrations. A density gradient was then formed by centrifugation at $20000 \times g$ for 20 min in a Sorvall SV-288 vertical rotor. The granules formed a distinct band in the high density portion of the gradient (1.11–1.13 g/ml). Zymogen granules were always prepared fresh and stored concentrated at 4°C.

2.2. Evaluation of granule membrane chloride permeability

The anion permeability of isolated secretory granules was determined indirectly by the method of ionophore-dependent granule lysis in a defined salt solution. The technique has been described previously in detail [4,5], and measures permeabilities based on solute driven osmotic influx of fluid and the observed kinetics of the subsequent swelling and lysis of the organelle. The granules were suspended in a solution consisting of: 150 mM KCl, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes; titrated to pH 7.0 with KOH), 0.2 mM EGTA, and 0.2 mM $MgSO_4$. The Cl^- transport was identified by increasing the K^+ permeability of the membrane with the ionophore, valinomycin. As electroneutrality must be maintained for net solute accumulation and granule swelling, the endogenous Cl^- permeability of the granule membrane becomes rate-limiting. Therefore, the granules accumulate fluid and lyse at a rate dependent upon the endogenous Cl^- transport pathways in the zymogen granule membrane. Valinomycin was considered to induce a maximal K^+ permeability at 10 μg/ml based on dose-response experiments showing no further increase in granule lysis rate when valinomycin exceeded 5–8 μg/ml. Granule lysis was followed by the change in optical density (OD) of the granule suspension at 540 nm. This OD is proportionate to the number of secretory granules in suspension, with intact granules accounting for at least 90% of the optical signal. The lysis rate was quantified as the time required for the OD of the granule suspension to decrease by 50% (half-life).

PLA_2, from *Naja mocambique mocambique* (1500 units per mg protein), lysophosphatidylcholine (prepared from egg phosphatidylcholine), lysophosphatidylethanolamine (oleoyl, 18:1,[*cis*]-9), lysophosphatidylserine (monoacyl-*sn*-glycero-3-phospho-L-serine), and fatty acids were purchased from Sigma. Lysophospholipids are reported as g/ml due to uncertainty in the *sn*-1 fatty acid of lysophosphatidylcholine. Stock concentrations of lysophospholipids and fatty acids were solubilized in 100% ethanol; however, the ethanol concentration never exceeded 0.5% when in the granule suspension. This level of ethanol has been previously shown not to influence the acute granule transport behavior [5]. Furthermore, the final concentration of BSA in the test solutions was approx. 1.7 μg/ml, an amount that did not seem to significantly alter lipid availability. Granules that displayed half-time rates for lysis of less than 40 min prior to the addition of valinomycin were considered to be unstable and were excluded from further analysis.

Experimentally, the granules were incubated for 5 min at 37°C (in the presence or absence of PLA_2,

lysophospholipids, or fatty acids) prior to the addition of valinomycin. This period allowed for the hydrophobic lipids to partition from the aqueous suspension into the native granule membrane. Control experiments were always performed to quantify the granule lysis rate in the absence of valinomycin. This control value served as the baseline for comparison after addition of valinomycin.

All experiments were conducted at 37°C with a Beckman DU-64 spectrophotometer equipped with a constant temperature chamber, 6-unit sample changer, computerized data capture system, and software for kinetic analysis. Data are reported as means ± S.E. Figs. 1–4 illustrate a single experiment but are representative of at least five other experiments that displayed similar results.

3. Results

Secretory granules from the rat pancreas are osmotically stable in a solution of 150 mM KCl (pH 7.0) at 37°C when isolated as described above [1,3]. The mean half-life for granule lysis for the preparations used in the following experiments was approx. 74 min in the absence of an ionophore (valinomycin). However, when the untreated granules are suspended in KCl and permeabilized to K^+ with valinomycin, they displayed a mean half-life of approx. 31 min, an increase of over 2-fold compared to the ionophore-free control rate. This increase in lysis rate is taken to be proportional to the endogenous granule membrane Cl^- conductance, due to the electrogenic nature of valinomycin and the need to maintain electroneutrality for intragranular solute and therefore fluid accumulation. By this technique, the measured anion permeability is selective, displaying a sequence of $SCN^- > I^- > Br^- > Cl^- > F^- > SO_4^{2-} \gg$ gluconate, consistent with other reported Cl^- channels [24]. Valinomycin addition in the absence of a permeant anion did not increase the granule lysis rate over the control values. For example, when valinomycin is added to granules suspended in K^+-gluconate, the half-time for lysis was 66 ± 7 min, compared to 71 ± 8 min in the absence of valinomycin.

Changes in the intracellular calcium concentration of pancreatic acinar cells is thought to regulate PLA_2 and induce its association with the secretory granule membrane [17,25]. The enzyme will utilize the membrane phospholipids as substrate, yielding an unesterified fatty acid and a lysophospholipid. The subsequent transient alteration of the membrane composition could serve to regulate granule electrolyte transport. This type of lipid second messenger regulation was tested with exogenous additions of PLA_2 (*Naja mocambique mocambique*). The enzyme was incubated with secretory granules and the Cl^- dependent lysis rate quantified before and after the addition of valinomycin. Consistent with the calcium requirement of *Naja mocambique* PLA_2, the enzyme (0.5 ng/ml; $7.5 \cdot 10^{-4}$ units/ml) produced only a nominal effect on the Cl^- transport (less than 5% increase from the control) when incubated with zymogen granules in the absence of calcium. However, when 0.25 mM calcium was added to the granule suspension solution, the rate of Cl^- specific lysis increased by 139% in comparison to the untreated control (granules plus valinomycin without PLA_2) (Fig. 1). In the absence of valinomycin, the half-time for granule lysis was 46 ± 5 min at this concentration of PLA_2, illustrating that the treatment did not make the granules nonspecifically lytic. Calcium in the absence of PLA_2 caused an increase in Cl^- transport of 30% ± 9 over the control rate. However, quinacrine (0.1 mM), an inhibitor of PLA_2 [26], did not block the calcium promoted increase in Cl^- transport. This failure by quinacrine suggests that the added calcium did not activate an endogenous PLA_2 at the zymogen granule membrane. In the presence of 0.25 mM added calcium, *Naja mocambique* PLA_2 displayed a dose-dependent increase in effectiveness, promoting a 61% increase in Cl^- specific granule lysis at 50 pg/ml ($7.5 \cdot 10^{-5}$ units/ml) and a 193% increase at 1.0

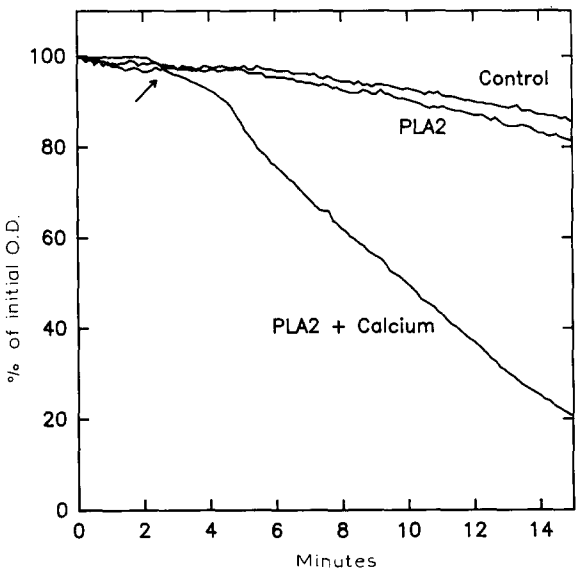

Fig. 1. The effect of exogenously applied phospholipase A_2 on zymogen granule Cl^- transport in vitro. PLA_2 (0.5 ng/ml) with and without calcium (0.25 mM) was added to a suspension of zymogen granules and preincubated for 5 min The suspension solution consisted of 150 mM KCl, 20 mM Hepes (pH 7.0), 0.2 mM EGTA, and 0.2 mM $MgSO_4$ at 37°C. Cl^- transport was measured as the rate of Cl^- dependent granule lysis after increasing the K^+ membrane permeability with the ionophore valinomycin (10 μg/ml), at the arrow. Control represents the Cl^- dependent lysis is the absence of PLA_2 treatment; PLA_2 represents Cl^- dependent lysis of the same granule population after treatment with PLA_2 in the absence of calcium; and PLA_2 + Calcium represents the Cl^- dependent lysis after treatment with PLA_2 and calcium.

Table 1
Dose dependent change in granule Cl^- transport in response to increasing concentrations in PLA_2

	% Change in Cl^- transport
PLA_2 (g/ml)	
$5 \cdot 10^{-11}$	61.5 ± 14.5
$2 \cdot 10^{-10}$	119.7 ± 31.6
$5 \cdot 10^{-10}$	140.4 ± 34.0
$1 \cdot 10^{-9}$	193.3 ± 39.9

Cl^- transport was measured as the rate of Cl^- dependent granule lysis after addition of the ionophore valinomycin (10 μg/ml). The data are expressed as the % change in Cl^- dependent lysis compared to an untreated control from the same granule population. Granules were suspended in 150 mM KCl, 20 mM Hepes (pH 7.0), 0.2 mM EGTA, and 0.2 mM $MgSO_4$ at 37°C. All treatments represent the mean ± S.E. of experiments from at least five different granule preparations. PLA_2 + 0.25 mM Ca^{2+} were added to the granule suspension and incubated 5 min at 37°C before addition of the ionophore.

ng/ml ($1.5 \cdot 10^{-3}$ units/ml), following a 5 min preincubation at 37°C (Table 1). When the stilbene SITS (0.5 mM) was added to the cytoplasmic surface of the granule membrane, 68% ± 11 of the PLA_2 (0.5 ng/ml) promoted Cl^- specific lysis was inhibited. This responsiveness to SITS is further evidence that the osmotic lysis is due to solute accumulation associated with a granule Cl^- conductance. At concentrations above 1.0 ng/ml, PLA_2 began to induce significant lysis of the zymogen granules (half-life less than 40 min) in the absence of valinomycin and was therefore excluded from further consideration.

PLA_2 hydrolysis of membrane phospholipids results in the production of a lysophospholipid and a free fatty acid from the *sn*-2 position. Lysophosphatidyl-choline, -ethanolamine, and -serine were directly tested to determine their contribution to the PLA_2 induced increase in Cl^- transport. Fig. 2 illustrates the change in the granule lysis rate (limited by the endogenous membrane Cl^- conductance) in response to incubation with 0.6 μg/ml lysophospholipids. The lysis rate in the presence of lysophospholipids was always greater than 40 min prior to the addition of valinomycin, although the Cl^- specific lysis rate increased significantly after addition of valinomycin. In general, the ability of the lysophospholipids to promote Cl^- transport (granule lysis) followed the order lysophosphatidyl-choline > -ethanolamine > -serine (Table 2). Lysolipid concentrations lower than 0.2 μg/ml were without significant effect on the Cl^- specific granule lysis rate. The maximal measurable response was achieved with 2.0 μg/ml lysophospholipid, causing an increase of over 400% with lysophosphatidylcholine and ethanolamine and 280% with lysophosphatidylserine, compared to the untreated control rate (granules plus valinomycin without lysolipid treatment) (Table 2). Once again, 0.5 mM SITS inhibited a significant portion of the treatment

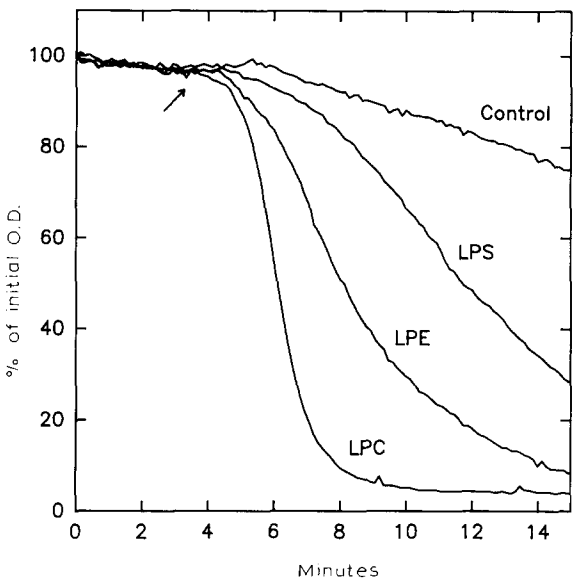

Fig. 2. The effect of lysophospholipids on zymogen granule Cl^- transport in vitro. Cl^- transport was measured as the rate of Cl^- dependent granule lysis (decrease in OD) after addition of the ionophore valinomycin (10 μg/ml), at the arrow. The granules were suspended in a solution consisting of 150 mM KCl, 20 mM Hepes (pH 7.0), 0.2 mM EGTA, and 0.2 mM $MgSO_4$ at 37°C. 2.0 μg/ml of lysophosphatidyl-choline (LPC), -ethanolamine (LPE), or -serine (LPS) were preincubated with granules for 5 min prior to initiation of transport with valinomycin. Control represents the rate of Cl^- dependent granule lysis in the absence of lysophospholipid pretreatment.

enhanced granule lysis (60% ± 10 inhibition of the 0.6 μg/ml lysophosphatidylcholine promoted rate increase). Lysolipid concentrations in excess of 2.0 μg/ml began to cause significant granules lysis in the absence of valinomycin and were excluded from further consideration.

The other product of PLA_2 hydrolysis is typically an

Table 2
Dose dependent change in granule Cl^- transport in response to increasing concentrations of lysophospholipids

	% Change in Cl^- transport concentration (μg/ml)		
	0.2	0.6	2.0
Lysophosphatidylserine	54.7 ± 18.5	84.8 ± 31.4	279.1 ± 37.6
Lysophosphatidyl-ethanolamine	38.3 ± 10.7	121.5 ± 19.7	451.6 ± 44.8
Lysophosphatidylcholine	107.4 ± 38.7	208.5 ± 91.0	419.7 ± 88.9

Cl^- transport was measured as the rate of Cl^- dependent granule lysis after addition of the ionophore valinomycin (10 μg/ml). The data are expressed as the % change in Cl^- dependent lysis compared to an untreated control from the same granule population. Granules were suspended in 150 mM KCl, 20 mM Hepes (pH 7.0), 0.2 mM EGTA, and 0.2 mM $MgSO_4$ at 37°C. All treatments represent the mean ± S.E. of experiments from at least five different granule preparations. Lysophospholipids, at the above concentrations, were incubated with granules for 5 min prior to the addition of valinomycin.

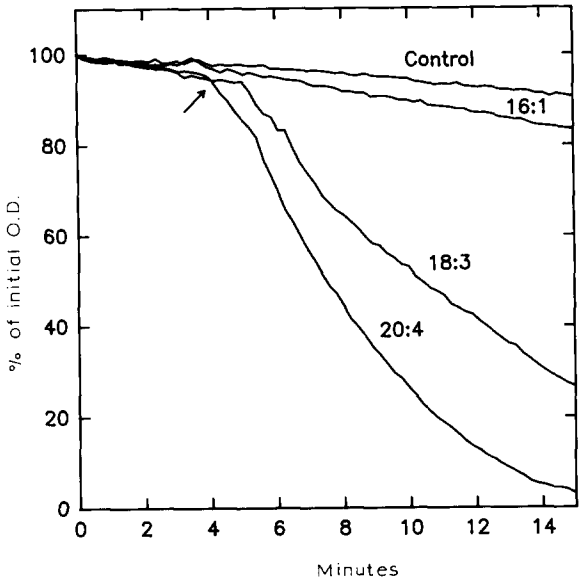

Fig. 3. The effect of unsaturated fatty acids on zymogen granule Cl^- transport in vitro. Cl^- transport was measured as the rate of Cl^- dependent granule lysis (decrease in OD) after addition of the ionophore valinomycin (10 μg/ml), at the arrow. The granules were suspended in a solution consisting of 150 mM KCl, 20 mM Hepes (pH 7.0), 0.2 mM EGTA, and 0.2 mM $MgSO_4$ at 37°C. 10 μM of palmitoleic (16:1), linolenic (18:3), or arachidonic acid (20:4) were preincubated with granules for 5 min prior to initiation of transport with valinomycin. Control represents the rate of Cl^- dependent granule lysis in the absence of fatty acid pretreatment.

unesterified unsaturated fatty acid liberated from the *sn*-2 position of the phospholipid. Fig. 3 shows that free unsaturated fatty acids (10 μM) can have a dramatic effect on the Cl^- transport after a 5 min preincubation with the isolated zymogen granules. Significant changes in the transport rate could be seen with exogenously applied fatty acids as low as 0.3 μM. The increase in transport ranged from a modest 13% increase induced by palmitoleic acid (16:1) to 40% or more after the addition of arachidonic, (20:4), linolenic (18:3), linoleic (18:2), and oleic acid (18:1) (Table 3). In general, the maximal fatty acid concentration that elicited a Cl^- specific increase in lysis rate was 10 μM. This concentration caused increases of 86% with palmitoleic (16:1), 225% with linolenic (18:3), 235% with linoleic (18:2), and 255% with arachidonic acid (20:4). Oleic acid was an exception, producing a maximal effect at 3.0 μM, above which it began to cause significant lysis in the absence of ionophore. Indeed, the 18 carbon fatty acids were the most potent activators of Cl^- transport at concentrations between 0.3 and 3.0 μM. At 3.0 μM, the ordered effectiveness for the fatty acids tested was linoleic (18:2) ~ oleic (18:1) > linolenic (18:3) ~ arachidonic (20:4) > palmitoleic (16:1) (Table 3).

Saturated fatty acids, although not a primary product of intracellular PLA_2 hydrolysis of phospholipids, also have the ability to increase the Cl^- specific rate of

Table 3
Dose dependent change in granule Cl^- transport in response to increasing concentrations of unsaturated fatty acids

	% Change in Cl^- transport concentration (μM)		
	0.3	3.0	10.0
Palmitoleic (16:1)	13.0 ± 11.0	52.4 ± 20.2	86.5 ± 27.0
Oleic (18:1)	44.4 ± 13.7	189.5 ± 35.9	
Linoleic (18:2)	55.1 ± 19.3	209.2 ± 30.7	235.1 ± 30.1
Linolenic (18:3)	42.3 ± 1.6	98.0 ± 18.9	225.2 ± 51.6
Arachidonic (20:4)	43.0 ± 16.0	100.1 ± 12.3	255.4 ± 89.7

Cl^- transport was measured as the rate of Cl^- dependent granule lysis after addition of the ionophore valinomycin (10 μg/ml). The data are expressed as the % change in Cl^- dependent lysis compared to an untreated control from the same granule population. Granules were suspended in 150 mM KCl, 20 mM Hepes (pH 7.0), 0.2 mM EGTA, and 0.2 mM $MgSO_4$ at 37°C. All treatments represent the mean ± S.E. of experiments from at least five different granule preparations. Lysophospholipids, at the above concentrations, were incubated with granules for 5 min prior to the addition of valinomycin.

granule lysis. Fig. 4 shows the response to 10 μM saturated fatty acids with the effectiveness being correlated to the carbon chain length up to 18 carbons. Dose-response data shows that lignoceric acid (24:0) was less effective than stearic acid (18:0) at all concentrations (Table 4). At a concentration of 10 μM, capric acid (10:0) caused a 26% increase in the Cl^- depend-

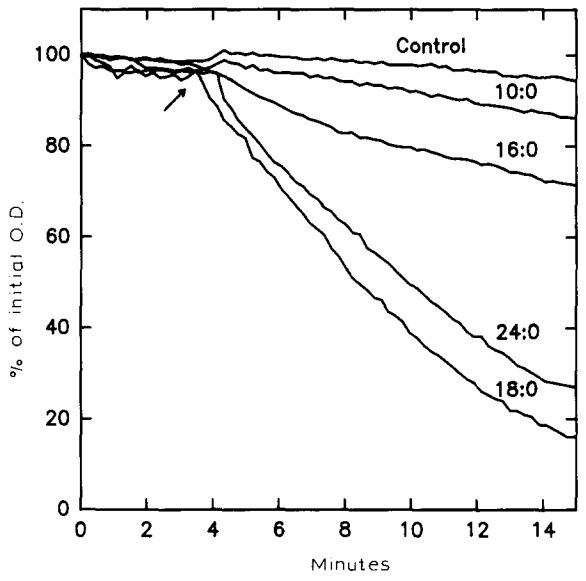

Fig. 4. The effect of saturated fatty acids on zymogen granule Cl^- transport in vitro. Cl^- transport was measured as the rate of Cl^- dependent granule lysis (decrease in OD) after addition of valinomycin (10 μg/ml), at the arrow. The granules were suspended in a solution consisting of 150 mM KCl, 20 mM Hepes (pH 7.0), 0.2 mM EGTA, and 0.2 mM $MgSO_4$ at 37°C. 10μM of capric (10:0);palmitic (16:0), stearic (18:0), or lignoceric acid (24:0) were preincubated with granules for 5 min prior to initiation of transport with valinomycin. Control represents the rate of Cl^- dependent granule lysis without fatty acid pretreatment.

Table 4
Dose dependent change in granule Cl^- transport in response to increasing concentrations of saturated fatty acids

	% Change in Cl^- transport concentration (μM)		
	0.3	3.0	10.0
Capric (10:0)	3.2± 8.0	25.2± 7.0	26.1± 5.0
Palmitic (16:0)	2.4±11.8	46.3±22.3	40.1±16.0
Stearic (18:0)	21.9± 5.9	95.0±28.0	130.7±43.4
Lignoceric (24:0)	16.7± 9.3	31.2± 7.0	93.4±35.0

Cl^- transport was measured as the rate of Cl^- dependent granule lysis after addition of the ionophore valinomycin (10 μg/ml). The data are expressed as the % change in Cl^- dependent lysis compared to an untreated control from the same granule population. Granules were suspended in 150 mM KCl, 20 mM Hepes (pH 7.0), 0.2 mM EGTA, and 0.2 mM $MgSO_4$ at 37°C. All treatments represent the mean±S.E. of experiments from at least five different granule preparations. Lysophospholipids, at the above concentrations, were incubated with granules for 5 min prior to the addition of valinomycin.

ent granule lysis, palmitic (16:0) a 40% increase, stearic (18:0) a 130% increase, and lignoceric (24:0) a 93% increase in the putative Cl^- transport.

The degree of unsaturation is typically related to the ability of a fatty acid to increase membrane fluidity. However, in these experiments, the number of double bonds was not a steady predictor of fatty acid potency in stimulating Cl^- transport, although all unsaturated fatty acids were more potent than saturated fatty acids of equal chain length. For example, a comparison of 18 carbon fatty acids with 0–3 double bonds shows a pattern of linoleic (18:2) ~ oleic (18:1) > linolenic (18:3) > stearic (18:0). The overall ability of fatty acids (saturated and unsaturated) to increase granule Cl^- transport displayed a pattern of linoleic (18:2) ~ oleic (18:1) > linolenic (18:3) ~ arachidonic (20:4) > stearic (18:0) > palmitoleic (16:1) > lignoceric (24:0) > palmitic (16:0) > capric (10:0).

Based on the indirect nature of the Cl^- transport measurements, it could be argued that PLA_2 or lipid treatments increase the apparent Cl^- transport rate by making the secretory granules more vulnerable to osmotic lysis. However, when the lysis rate of treated granules was measured in response to an acute 150 mOsm reduction of the solution osmolarity with distilled H_2O, no significant difference could be detected compared to untreated granules. The half-time for osmotic lysis was 32 ± 9 min for untreated granules ($n = 8$); whereas treatment of zymogen granules with PLA_2 (0.5 ng/ml), arachidonic acid (3.0 μM), or lysophosphatidylcholine (0.6 μg/ml) yielded half-lives of 28 ± 8 ($n = 4$), 31 ± 6 ($n = 3$), and 30 ± 7 ($n = 3$), respectively, after an osmotic challenge. This suggests that the results cannot be explained as a simple increase in the membrane leakiness or susceptibility to lysis.

4. Discussion

The carbon chain length of fatty acids (saturated and unsaturated) was identified as an important characteristic in their capacity to promote granule Cl^- transport. The ability of saturated fatty acids to stimulate Cl^- transport increased with the length of the carbon chain up to stearic acid (18:0) and decreased with the 24 carbon lignoceric acid. This relationship between chain length and physiological response has been noted previously [27–30] and has been suggested to reflect an influence on membrane thickness or fluidity. Our results show 18 carbon fatty acids, along with the 20 carbon arachidonic acid, to be the most effective modulators of Cl^- permeability in the concentration range of 0.3 to 10 μM.

Although fatty acid unsaturation was a significant factor as well (ie., all unsaturated fatty acids were more effective than saturated fatty acids of equal chain length), a correlation between the degree of unsaturation and transport activation was less well defined. For example, when comparing 18 carbon fatty acids, the potency followed a pattern of linoleic (18:2) ~ oleic (18:1) > linolenic (18:3) > stearic (18:0). Chain length remains a critical feature for unsaturated fatty acids as shown by the comparison of oleic (18:1) and palmitoleic acid (16:1). Although both fatty acids contain a double bond at carbon number 9, oleic acid promotes Cl^- transport to a much greater extent than palmitoleic, 189% vs. 52% at 3.0 μM.

The physiological significance of these results is based on the proposed use of the granule phospholipid bilayer as a substrate by PLA_2. The transient activation of this enzyme during stimulus-secretion coupling would in turn cause the transient accumulation of PLA_2 reaction products, lysophospholipids and unesterified fatty acids, in the membrane. Eventually, these intermediates can be reacylated in the phospholipid metabolic cycle by acyltransferases, returning the membrane to its former status and composition. Clearly, a major physiological function of PLA_2 is the liberation of arachidonic acid, which serves as a precursor for a large number of biologically active eicosanoids. However, there is increasing evidence that some PLA_2 mediated events are independent of further arachidonic acid metabolism. In these cases a physiological response is thought to be elicited directly by the transient membrane accumulation of PLA_2 products.

Several solute transport systems have been shown to be directly influenced by free fatty acids or lysophospholipids. Calcium accumulation in pancreatic islets was increased by lysophospholipids [31] and glucose

transport by red blood cells was inhibited by nanomolar concentrations of non-metabolizable lysophospholipids [32]. K^+ channels may also be regulated by PLA_2 products. The ATP-sensitive K^+ channel was inhibited by lysophospholipids in the pancreas [3] and in cardiac myocytes [23]; whereas outwardly-rectifying (ATP-insensitive) K^+ channels were directly activated by arachidonic acid and other unsaturated fatty acids in cardiac [33] and smooth muscle [22].

The mechanism by which these products influence solute transport could involve a direct effect of the fatty acid or lysophospholipid on the integral membrane protein, or be mediated through bulk changes in membrane structure and fluidity. Previous evidence has shown that the lipid bilayer fluidity of exocrine secretory vesicles is variable and that the Cl^- transport rate across those granule membranes was directly correlated to the fluidity [10]. If membrane fluidity or lipid composition is a regulatory event in the control of exocytotic secretion, there must also be a mechanism to alter the membrane on a time scale consistent with exocytosis. Physiologically, this could be accomplished by PLA_2, since it liberates products that have been shown to increase membrane fluidity or influence solute transport systems [3,18,32]. Furthermore, stimulus-secretion coupling in pancreatic acinar cells includes the activation of PLA_2 [13]. There are other routes for the liberation of unesterified fatty acids, such as phospholipase C production of diacylglycerol and the subsequent deacylation by glycerol lipases; however, PLA_2 has been specifically identified at the cytoplasmic membrane surface of activated secretory granules [17]. PLA_2 likely partitions to the membrane in response to an increase in cytoplasmic calcium. Since the secretory granules in this study were isolated from unstimulated cells in a low Ca^{2+} concentration, they probably lacked endogenous PLA_2.

Our results support a mechanism whereby PLA_2 promotes a transient modification of the secretory granule membrane in vivo as part of stimulus-secretion coupling. The subsequent increase in the membrane concentration of unesterified fatty acids and lysophospholipids could in turn promote granule Cl^- transport. Indeed, the fatty acid levels required (3–10 μM) to promote a significant Cl^- transport response by the granules in vitro are within the physiological range observed after activation of some secretory cells [13,34,35]. Furthermore, the levels of lysophospholipids employed in this study were estimated to range from approx. 0.8–9.0 mol% of the total granule phospholipids, if all the lysolipid partitioned to the granule membrane. Due to the transient nature of lysolipid accumulation, it is difficult to put this value in physiological context; although previous reports suggest that this level is in the physiological range [34,36]. The activation of granule Cl^- transport by PLA_2 may then help to drive primary fluid production after exocytotic fusion, or alternately, have a more fundamental role in exocytosis, contributing to vesicle swelling and the efficiency of fusion pore formation or stabilization [6,37,38].

A physiological model consistent with the available information would suggest that resting or unstimulated acinar cells contain secretory granules with a low capacity for electrolyte transport. Net salt transport in this state would be tonically blocked by ATP inhibition of the granule K^+ channel [2,3]. Furthermore, the Cl^- channel would be inhibited by the phosphorylation state [8] and a rigid vesicle membrane [10]. Stimulus-secretion coupling includes an increase in cytoplasmic cAMP and Ca^{2+} leading to the activation of protein kinase A and PLA_2. These enzymes may convert the granule into an active or primed state by protein kinase A phosphorylation of the Cl^- channel and PLA_2 alteration of the granule membrane with the transient accumulation of lysophospholipids and unesterified fatty acids. There is evidence to suggest that these primed granules have a greatly enhanced fusion capacity with the apical membrane [38,39]. Net salt transport by the granule would require an additional signal to override the ATP-inhibition of the K^+ channel, possibly involving granule contact with the apical membrane. Importantly, the model allows those activated granules that do not make contact and fuse with the apical membrane to be recycled back to the resting state after termination of the stimulus signal. This could be accomplished by the dephosphorylation of the Cl^- channel and reacylation of PLA_2 products, which would in turn restore the original membrane composition and electrolyte transport capacity.

Acknowledgements

This study was supported in part by NIH BRSG S07 RR07176 and the Northern Illinois University Graduate School. We thank Robert Thumme for technical assistance.

References

[1] Gasser, K.W., DiDomenico, J. and Hopfer, U. (1988) Am. J. Physiol. 255, C705–C711.
[2] Thevenod, F., Chathadi, K., Jiang, B. and Hopfer, U. (1992) J. Membr. Biol. 129, 253–266.
[3] Gasser, K.W. and Holda, J.R. (1993) Am. J. Physiol. 264:G137–G142.
[4] DeLisle, R.C. and Hopfer, U. (1986) Am. J. Physiol. 250, G489–G496.
[5] Gasser, K.W., DiDomenico, J. and Hopfer, U. (1988) Am. J. Physiol. 254, G93–G99.
[6] Fuller, C.M., Eckhardt, L. and Schulz, I. (1989) Pfluegers Arch. 413, 385–394.

[7] Almers, W. (1990) Annu. Rev. Physiol. 52, 607–624.
[8] Fuller, C.M., Deetjen, H., Piiper, A. and Schulz, I. (1989) Pfluegers Arch. 415, 29–36.
[9] Thevenod, F., Gasser, K.W. and Hopfer, U. (1990) Biochem. J. 272, 119–126.
[10] Gasser, K.W., Goldsmith, A. and Hopfer, U. (1990) Biochemistry. 29, 7282–7288.
[11] Frye, R.A. and Holz, R.W. (1984) J. Neurochem. 43, 146–150.
[12] Frye, R.A. and Holz, R.W. (1985) J. Neurochem. 44, 265–273.
[13] Chaudhry, A., Laychock, S.G. and Rubin, R.P. (1987) J. Biol. Chem. 262, 17426–17431.
[14] Yoshihara, Y. and Watanabe, Y. (1990) Biochem. Biophys. Res. Commun. 170, 484–490.
[15] Nishikawa, T., Tomori, Y., Yamashita, S. and Shimizu, S.-I. (1989) J. Pharm. Pharmacol. 41, 450–458.
[16] Imai, A., Ida, K., Matsunami, K., Matsuda, T. and Tamkaya, T. (1990) Comp. Biochem. Physiol. 95B, 635–639.
[17] Rubin, R.P., Thompson, R.H. and Laychock, S.G. (1990) Biochim. Biophys. Acta 1045, 245–251.
[18] Vance, D.E. and Vance, J. (1991) Biochemistry of Lipids, Lipoproteins and Membranes, p. 596, Elsevier, New York.
[19] Giaume, V., Randriamampita, C. and Trautman, A. (1989) Pfluegers Arch. 413, 273–279.
[20] Murakami, M., Takada, K. and Muranishi, S. (1992) Biochim. Biophys. Acta 1117, 83–89.
[21] Rustenbeck, I. and Lenzen, S. (1989) Biochim. Biophys. Acta 982, 147–155.
[22] Ordway, R.W., Walsh, Jr., J.V. and Singer, J.J. (1989) Science 244, 1176–1179.
[23] Kim, D. and Duff, R.A. (1990) Circ. Res. 67, 1040–1046.
[24] Halm, D.R., Rechkemmer, G., Schoumacher, R.A. and Frizzel, R.A. (1988) Comp. Biochem. Physiol. 90, 597–601.
[25] Rubin, R.P., Withiam-Leitch, M. and Laychock, S.G. (1991) Biochem. Biophys. Res. Commun. 177, 22–26.
[26] Lapetina, E.G., Billah, M. and Cuatrecasas, P. (1981) J. Biol. Chem. 256, 5037–5040.
[27] Cevc, G. (1991) Biochemistry 30, 7186–7193.
[28] In't Veld, G., Driessen, A.J.M. and Konings, W.N. (1992) Biochim. Biophys. Acta 1108, 31–39.
[29] In't Veld, G., Driessen, A.J.M., Op den Kamp, J. and Konings, W.N. (1991) Biochim. Biophys. Acta 1065, 203–212.
[30] Gruber, H.J. and Low, P.S. (1988) Biochim. Biophys. Acta 944, 414–424.
[31] Rustenbeck, I. and Lenzen, S. (1992) Cell Calcium 13, 193–202.
[32] Melchior, L.D., Carruthers, A., Makriyannis, A., Duclos, Jr., R.I. and Abdel-Mageed, O.H. (1990) Biochim. Biophys. Acta 1028, 1–8.
[33] Kim, D. and Clapham, D.E. (1989) Science 244, 1174–1176.
[34] Metz, S.A. and Dunlop, M. (1990) Biochem. Biophys. Res. Commun. 167, 61–66.
[35] Wolf, B.A., Turk, J., Sherman, W. and McDaniel, M.L. (1986) J. Biol. Chem. 261, 3501–3511.
[36] LeBel, D. and Beattie, M. (1984) Biochim. Biophys. Acta 769, 611–621.
[37] Helm, C.A., Israelachvii, J.N. and McGuiggan, P.M. (1992) Biochemistry 31, 1794–1805.
[38] Gasser, K.W., Stahl, A.M. and Holda, J.R. (1993) FASEB J. 7, A577.
[39] Karli, U.O., Schafer, T. and Burger, M.M. (1990) Proc. Natl. Acad. Sci. USA 87, 5912–5915.