Cholesterol facilitates the native mechanism of Ca\textsuperscript{2+}-triggered membrane fusion

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Summary

The process of regulated exocytosis is defined by the Ca\textsuperscript{2+}-triggered fusion of two apposed membranes, enabling the release of vesicular contents. This fusion step involves a number of energetically complex steps and requires both protein and lipid membrane components. The role of cholesterol has been investigated using isolated release-ready native cortical secretory vesicles to analyze the Ca\textsuperscript{2+}-triggered fusion step of exocytosis. Cholesterol is a major component of vesicle membranes and we show here that selective removal from membranes, selective sequestering within membranes, or enzymatic modification causes a significant inhibition of the extent, Ca\textsuperscript{2+} sensitivity and kinetics of fusion. Depending upon the amount incorporated, addition of exogenous cholesterol to cholesterol-depleted membranes consistently recovers the extent, but not the Ca\textsuperscript{2+} sensitivity or kinetics of fusion. Membrane components of comparable negative curvature selectively recover the ability to fuse, but are unable to recover the kinetics and Ca\textsuperscript{2+} sensitivity of vesicle fusion. This indicates at least two specific positive roles for cholesterol in the process of membrane fusion: as a local membrane organizer contributing to the efficiency of fusion, and, by virtue of its intrinsic negative curvature, as a specific molecule working in concert with protein factors to facilitate the minimal molecular machinery for fast Ca\textsuperscript{2+}-triggered fusion.

Key words: Sterol, Negative curvature, Vitamin E, Secretory vesicles, Exocytosis, Polyene antibiotics

Introduction

Exocytosis is an essential cellular process with numerous distinct stages. In many systems, the defining step of regulated exocytosis is the Ca\textsuperscript{2+}-triggered fusion of two apposed membranes. The mechanism of Ca\textsuperscript{2+}-triggered membrane fusion has been described by both proximity and proteinaceous fusion pore models. Both models seek to account for a mechanism that can overcome the substantial energy barriers obviating the close apposition of two bilayers and the subsequent molecular reorganization required for the merger and coalescence of these membranes (Rand and Parsegian, 1989). Protein pore models initially considered connexin-like channels linking the two fusing bilayers (Lindau and Almers, 1995). Recent proteomic, genetic, and molecular biological approaches have identified potential protein pore components that may include syntaxin (Han et al., 2004), and vacuolar ATPase $V_0$ subunits (Peters et al., 2001). Proximity models consider mechanisms that bring two bilayers into close apposition, causing bilayer reorganization and rapid formation of a lipidic fusion pore (Rand and Parsegian, 1989; Kuzmin et al., 2001; Kozlovsky et al., 2002). The stalk-pore hypothesis is a mathematical and physical description of membrane fusion based initially on data from model membrane studies (Kozlov and Markin, 1983; Siegel, 1993; Chernomordik et al., 1995a). The hypothesis accounts for the minimized energies associated with the rapid and progressive formation of transient lipid intermediates in terms that include membrane curvature (Chernomordik and Zimmerberg, 1995), or the ability of a hydrated lipid assembly to form non-bilayer structures (Gruner, 1985). By convention, positive curvature is the tendency to form convex, micelle-like structures, whereas negative curvature implies formation of concave surfaces at the lipid-water interface (Luzzati and Husson, 1962).

Cholesterol is a major component of native biological membranes. In both model and native membranes cholesterol associates to form discrete, functional microdomains (rafts) that serve as sites for specific protein-lipid interactions (Lucero and Robbins, 2004). Several proteins implicated in the exocytotic process have been shown to associate with cholesterol-rich microdomains (Lang et al., 2001): these domains have been suggested to be the sites of membrane fusion, although a more recent study suggests them to be negative regulators of the exocytotic process (Salaun et al., 2005), consistent with other membrane components functioning downstream in the actual triggered membrane fusion event (Coorssen et al., 1998; Peters and Mayer, 1998; Peters et al., 1999; Peters et al., 2001; Coorssen et al., 2003). This is at least potentially consistent with either the protein pore or proximity models for fusion. Additionally, cholesterol also serves as a source of negative curvature within the bilayer membrane (Coorssen and Rand, 1990; Chen and Rand, 1997) that can lower energy barriers to promote the formation of lipidic fusion intermediates, or support the formation and expansion of proteinaceous pores.
Here we selectively describe a role for lipidic membrane components, specifically cholesterol, in the process of triggered fusion using well-established, stage-specific preparations of cortical vesicles (CVs) isolated from sea urchin eggs. Cortex preparations, consisting of primed, release ready CVs fully docked to the plasma membrane (PM), undergo rapid exocytotic fusion in response to an increase in \([\text{Ca}^{2+}]_{\text{free}}\) (Vacquier, 1975; Baker and Whitaker, 1978; Vogel et al., 1991; Shafi et al., 1994; Vogel et al., 1996; Tahara et al., 1998; Coorssen et al., 1998; Zimmerberg et al., 2000; Blank et al., 2001; Coorssen et al., 2003). As this fully docked state can restrict access to critical components at the fusion site (Coorssen et al., 1998; Whalley et al., 2004) and as the PM can often act as a sink for reagents, experiments were also carried out using the established homotypic CV-CV fusion system (Vogel et al., 1991; Coorssen et al., 1998; Szule et al., 2003). Homotypic fusion has been documented at the cortex and proceeds rapidly through the same molecular mechanism as CV-PM fusion (Chandler, 1984; Coorssen et al., 1998; Zimmerberg et al., 2000; Coorssen et al., 2003). Cholesterol was removed from membranes using methyl-\(\beta\)-cyclodextrin (m\(\beta\)cd), an agent known to alter membrane cholesterol levels through direct binding of cholesterol into a hydrophobic pocket (Kilsdonk et al., 1995; Christian et al., 1997). Delivery of cholesterol to depleted membranes was accomplished using cholesterol-loaded m\(\beta\)cd, saturated cholesterol solutions (Alivisatos et al., 1977), and cholesterol-loaded 2-hydroxypropyl-\(\beta\)-cyclodextrin (hp\(\beta\)cd), a related cyclodextrin with a relatively lower affinity for membrane cholesterol. Studies were also carried out with polyene antibiotics, a class of molecules known to bind and effectively sequester sterols in the membrane (Weissmann and Sessa, 1967; Norman et al., 1972a; Norman et al., 1972b; Patterson et al., 1979). Cholesterol oxidase was used to metabolically ‘remove’ cholesterol from the membranes and effectively disrupt functional microdomains (Xu and London, 2000; Samsonov et al., 2001) The results of these experiments support the role of cholesterol as a pre-fusion organizer (Lang et al., 2001; Salaun et al., 2005), but also indicate that cholesterol functions more directly in the native molecular mechanism of bilayer merger.

Materials and Methods

Materials

M\(\beta\)cd, hp\(\beta\)cd, \(\alpha\)-cyclodextrin (\(\alpha\)cd), \(\beta\)-cyclodextrin (\(\beta\)cd), cholesterol oxide, amphoterin B, pimarinic, and \(\alpha\)-tocopherol (\(\alpha\)-toc) were from Sigma (St Louis, MO). Filipin III was from Calbiochem (La Jolla, CA). Cholesterol, 12:0 lysophosphatidylcholine (LPC), dioleoylphosphatidylethanolamine (DOPE) and lipid standards for HPTLC were from Avanti Polar Lipids (Alabaster, AL). All other chemicals were of at least analytical grade.

Preparations and fusion assays

Cortical vesicles were isolated from purple sea urchins (Strongylocentrotus purpuratus, Westwind, BC, Canada) as previously described (Coorssen et al., 1998). Animals were stored on site at \(-7^\circ\)C. All experiments were carried out in baseline intracellular medium (BIM, 210 mM potassium glutamate, 500 mM glycine, 10 mM NaCl, 10 mM Pipes, 50 \(\mu\)M Ca\(\text{Cl}_2\), 1 mM Mg\(\text{Cl}_2\), 1 mM EGTA pH 6.7) (Coorssen et al., 2002) supplemented with 2.5 mM ATP and protease inhibitors, unless otherwise stated. Standard end-point and kinetic fusion assays were carried out as previously described (Coorssen et al., 1998; Coorssen et al., 2003; Szule et al., 2003), with some modifications. CV-PM preparation (cell surface complexes, CSCs) endpoint and kinetic fusion assays were carried out on free-floating CSCs, with suspensions maintained by gentle shaking steps. During kinetic measurements CSCs were kept suspended by 2 second shaking steps (Wallac Victor II microplate reader) between the measurement of each well. Each condition was tested in sets of four replicates per experiment, and each experiment was repeated as indicated (\(n\)); data are reported as mean ± s.e.m. Final free \([\text{Ca}^{2+}]_{\text{free}}\) concentrations (\([\text{Ca}^{2+}]_{\text{free}}\)) were measured with a \(\text{Ca}^{2+}\) sensitive electrode (World Precision Instruments, Sarasota, FL) for each condition in every experiment, as previously described (Coorssen et al., 1998; Coorssen et al., 2003). \(\text{Ca}^{2+}\) activity curves were fit using the sigmoidal cumulative log-normal model (Blank et al., 1998); control conditions were fit with a two-parameter model (by definition reaching 100% fusion), whereas experimental conditions were fit with a 3-parameter model (TableCurve 2D) to determine the upper plateau extent, \([\text{Ca}^{2+}]_{\text{free}}\) sensitivity and sigmoidal-slope parameters of fusion. In the kinetic assays, a rapid phase of fusion was apparent during the period of \([\text{Ca}^{2+}]_{\text{free}}\) injection (450 msconds) corresponding to a rate of CV fusion reported here as the initial fusion rate (% fusion/second). Lysis was confirmed by light microscopy, and measured as a change in optical density after incubation with m\(\beta\)cd. Two-sample, two-tailed t-tests were used to determine differences (\(P<0.05\)) of fusion parameters between the experimental conditions and parallel, internal controls.

CSC and CV treatments

Saturated cholesterol was prepared as previously described (Alivisatos et al., 1977). Polyene antibiotics were delivered from dimethylsulphoxide (DMSO) stock solutions to a final concentration of <1% DMSO, while \(\alpha\)-tocopherol (\(\alpha\)-toc) and dioleoylphosphatidylethanolamine (DOPE) were delivered using hexadecane to a final solvent concentration of 0.05%; parallel solvent controls were carried out in every experiment but never significantly affected fusion. Stock solutions of m\(\beta\)cd, hp\(\beta\)cd, \(\alpha\)cd, and \(\beta\)cd were prepared by dissolving in BIM working buffer and added to CV suspensions at the indicated concentrations. M\(\beta\)cd- and hp\(\beta\)cd-cholesterol were prepared as previously described (Racchi et al., 1997; Sheets et al., 1999; Hao et al., 2002); briefly, cholesterol dissolved in chloroform:methanol (2:1 v/v) was dried under a stream of nitrogen and trace solvent was removed under vacuum for 2 hours. An appropriate volume of 100 mM m\(\beta\)cd or hp\(\beta\)cd was added to the dried film at a standardized molar ratio (~8:1 for m\(\beta\)cd:cholesterol and 10:1 for hp\(\beta\)cd:cholesterol) and vortexed to suspend the film. Suspensions were bath sonicated for 20 minutes, then incubated overnight at 37°C with shaking (250 rpm). Finally, suspensions were cooled to room temperature and filtered through 0.2 \(\mu\)m filters (Filter Scientific, Hampton, NH) to clarify solutions. CSC and CV suspensions (OD 1.00±0.05) were treated with m\(\beta\)cd for 30 minutes (25°C), followed by centrifugation to isolate the preparations. Resulting supernatant samples were cleared of all membrane fragments by ultra-centrifugation (100,000 g for 3 hours), and stored at –80°C until analyzed. Isolated CSCs and CVs were then suspended in BIM (OD 0.39±0.02); an aliquot was used for fusion assays, and the remainder stored at –80°C prior to lipid analysis. Cholesterol oxidase treatments were carried out at 30°C in BIM, pH 7.0, for 30 minutes. For treatments with reversible inhibitors (filipin, amP, PIM, and LPC) CVs were either treated at an optical density of 1.00±0.05 and then diluted after incubations (for filipin, amP and PIM) or treated at a final dilution of 0.40±0.02 (for LPC). Fusion assays were then carried out immediately. In all cases fusion assay results were normalized against those of controls handled in parallel.

Molecular analyses

Quantification of cholesterol in CV membrane fractions after m\(\beta\)cd
treatments was carried out using the Amplex Red Cholesterol Assay kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions.

CV membrane lipids were extracted according to Bligh and Dyer (Bligh and Dyer, 1959) with some modifications. Lipids were extracted by the sequential addition of methanol, and then chloroform, to an intermediate ratio of 0.8:2:1 (H2O:CH3OH:CHCl3, v/v/v) and subsequently brought to a final ratio of 1.8:2:2 through sequential addition of aqueous solution (1 M NaCl, 0.1 M HCl in water) and CHCl3. The resulting organic phase was recovered, dried under vacuum and stored under nitrogen at –30°C. Dried lipid films were resuspended in CHCl3:CH3OH (2:1 v/v) for high performance thin layer chromatography (HPTLC) analysis. HPTLC was carried out according to Weerheim et al. (Weerheim et al., 2002) with modifications. Extracted lipids dissolved in CHCl3:CH3OH were loaded onto silica gel 60 HPTLC plates (CAMAG Linomat IV; Wilmington, NC) pre-washed with CH3OH:ethyl acetate (6:4) and loaded onto silica gel 60 HPTLC plates (CAMAG Linomat IV; Wilmington, NC) pre-washed with CH3OH:ethyl acetate (6:4) and activated at 110°C for 30 minutes. Using the automated, sequential separation steps enabled by the CAMAG AMD 2 multi-development unit, lipids were resolved to 50 mm using CHCl3:ethyl acetate:acetone:isopropanol:ethanol:methanol:water:acetic acid (30:6:6:6:16:28:6:2, v/v), then to 78 mm with dichloromethane:ethyl acetate:acetone (80:16:4, v/v/v), and finally to 90 mm with hexane:ethyl acetate in three steps (85:15, 92:8, and 100:0 v/v, sequentially). Separated lipids were visualized with Nile Red (Fowler et al., 1987) and imaged (Ex 540 nm/Em 620 nm) with the PROXPRESS multi-wavelength fluorescent imager (Perkin Elmer, Boston, MA). For phospholipid quantification on HPTLC plates, the integrated Nile Red fluorescent signal for each species of interest was compared to a parallel dilution series of standard phospholipids. Extraction efficiency was estimated by collecting and lyophilizing the aqueous phase remaining after lipid extraction, and analyzing these lipid samples in parallel with the organic phase.

Results
Effects of cholesterol depletion on exocytotic fusion

Native, docked CV-PM preparations (cell surface complexes, CSCs) exposed to increasing [Ca2+]free undergo exocytosis in vitro yielding a classic sigmoidal Ca2+ activity curve (Vacquier, 1975; Baker and Whitaker, 1978; Vogel et al., 1991; Vogel et al., 1996; Tahara et al., 1998; Coorssen et al., 1998; Blank et al., 1998; Zimmerberg et al., 2000; Coorssen et al., 2003) with an EC50 of 45.8±8.0 µM [Ca2+]free (Fig. 1A, black circles; n=5) after an hour or more incubation in vitro. The initial rate of fusion was 77.5±14.0%/second (n=2) in response to 189±19 µM [Ca2+]free, consistent with previous reports (Blank et al., 2001). Treating CSCs with mβcd resulted in dose-dependent inhibition of the extent of triggered fusion even at high concentrations of [Ca2+]free (e.g. 1 mM), and a progressive rightward shift in Ca2+ sensitivity of up to 695 ±M [Ca2+]free (Fig. 1A, orange squares). The kinetics of fusion showed a comparable dose-dependent inhibition following treatment with mβcd (Fig. 1B); the initial rate decreased to 8.9±0.9%/second following treatment with 6 mM mβcd (n=2). Furthermore, at high doses of mβcd (>10 mM) the fusion of intact vesicles was effectively abolished. Inhibition was not a result of CV lysis or the undocking of CVs from the PM during treatment (Fig. 2), as cortices treated with mβcd were morphologically identical to parallel, untreated cortices, except that intact, unfused vesicles remained even after a Ca2+ challenge (Fig. 2A). Likewise, significant inhibition of the extent of fusion occurred at concentrations of mβcd that did not induce marked lysis (Fig. 2B).

The inhibition of fusion extent, Ca2+ sensitivity and kinetics correlated with depletion of cholesterol from CSCs (Fig. 1C). As the Ca2+ activity curves (Fig. 1A) were all translationally invariant, the results are consistent with a reduced number of

Fig. 1. The dose-dependent effects of mβcd on CV-PM fusion. (A) Ca2+ activity curves (n=3) following treatments with concentrations of mβcd as indicated. (B) Kinetics of fusion of CV-PM preparations treated as in A, triggered with 189±19 µM [Ca2+]free (n=2). (C) Total cholesterol in CV-PM preparations after treatment with the indicated concentration of mβcd, expressed relative to untreated preparations. *Significant difference from control (P<0.05) and other treatments (P<0.005), **, ***significant difference from control and other treatments (P<0.001).
active fusion machines, rather than with an altered fusion mechanism (Blank et al., 1998). To ensure full access even to the sites of triggered fusion, and to enable more direct molecular quantification of treatment effects, experiments were also carried out with the well-established isolated CV system, thereby avoiding effects arising from the large molecular background imposed by the PM.

Direct effects of cholesterol depletion on vesicle fusion

Well established homotypic CV-CV fusion assays yield characteristic sigmoidal \( \text{Ca}^{2+} \) activity curves (Vogel et al., 1991; Vogel et al., 1996; Coorssen et al., 1998; Blank et al., 1998; Szule et al., 2003) with an EC50 of 36.1±7.0 \( \mu \text{M} \) \( \text{Ca}^{2+} \) free following an hour or more incubation in vitro (Fig. 3A, black circles; \( n=4 \)); these are translationally invariant to the CV-PM curves shown in Fig. 1A (Vogel et al., 1996; Coorssen et al., 1998; Blank et al., 1998; Coorssen et al., 2003). The initial rate of fusion was 57.8±4.4%/second (\( n=3 \)) in response to 71±21 \( \mu \text{M} \) \( \text{Ca}^{2+} \) free. Treating isolated, free-floating CVs with increasing concentrations of \( \text{m} \)\text{βcd} prior to fusion assays resulted in a progressive, dose-dependent rightward shift in \( \text{Ca}^{2+} \) sensitivity (to 240±4 \( \mu \text{M} \) \( \text{Ca}^{2+} \) free) and a parallel loss in the extent of fusion (Fig. 3A, orange squares).

Fig. 2. Effects of \( \text{m} \)\text{βcd} on CV docking. (A) Treatment with 0.5 mM \( \text{m} \)\text{βcd} does not alter CV density at the cortex, but affects fusion of CVs after perfusion of 113±15 \( \mu \text{M} \) \( \text{Ca}^{2+} \) free; overlay shows unfused vesicles in white, fused vesicles in green. (B) Lysis of CV-PM preparations and (C) suspensions of isolated CVs after treatment with the indicated concentrations of \( \text{m} \)\text{βcd} (\( n=3 \)).

Fig. 3. The dose-dependent effects of \( \text{m} \)\text{βcd} on CV-CV fusion. (A) \( \text{Ca}^{2+} \) activity curves (\( n=4 \)) following treatments with various concentrations of \( \text{m} \)\text{βcd} as indicated. (B) Kinetics of CV-CV fusion treated as in A, triggered with 71±21 \( \mu \text{M} \) \( \text{Ca}^{2+} \) free (\( n=3 \)). (C) Total CV cholesterol after treatment with the indicated concentration of \( \text{m} \)\text{βcd}, expressed relative to untreated CVs. *Significant difference from control (\( P<0.05 \)); **, ***, ****significant difference from control and all other conditions (\( P<0.001 \)).
Kinetics of fusion were also inhibited in a dose-dependent manner (Fig. 3B); the initial rate of fusion decreased to 4.6±1.9%/second (n=3) following treatment with 4 mM mβcd. Inhibition of CV-CV fusion was more sensitive to mβcd than was CSC fusion, probably due to higher mβcd concentrations relative to CV resulting from the absence of PM cholesterol. Lysis of isolated CVs was also more sensitive to mβcd concentration, however, at lower concentrations (≤2 mM), lysis did not exceed 10% (Fig. 2C). It was also noted that the time course of inhibition by mβcd, which was maximal within ~30 minutes, was independent of lysis, which generally occurred within 1 minute (data not shown). The dose-dependent inhibition of the extent of fusion was mathematically correlated to the number of intact, active fusion sites (n) per vesicle as previously described (Vogel et al., 1996; Coorssen et al., 1998; Blank et al., 1998) and extrapolated to estimate the number of fusion machines per native vesicle (n_max; Fig. 4 open symbols). The resulting estimates, n_max=6.5±2.7 (n=4) for CSCs, and n_max=5.7±1.2 (n=5) for CVs, are consistent with previously reported values (Coorssen et al., 1998; Coorssen et al., 2003). The ATP dependence of mβcd inhibition was examined by excluding ATP from buffers both during treatment and during the fusion assay. As previously established, the Ca²⁺-triggered fusion steps occur independently of ATP (Vacquier, 1975; Baker and Whitaker, 1978; Vogel et al., 1991; Coorssen et al., 1998; Zimmerberg et al., 2000), and there was also no effect of ATP on the inhibitory activity of mβcd (data not shown). Furthermore, the inhibition by mβcd was not reversed by simply removing mβcd from the buffers. Washing CVs (up to four wash steps) following mβcd treatment had no effect on the extent of inhibition, and there was no residual mβcd detected by HPTLC (data not shown). Thus, the inhibition of the extent of fusion, together with marked declines in Ca²⁺ sensitivity and the rate of fusion, were related to the proportion of cholesterol removed from the CV membranes (Fig. 3C) rather than to an effect of residual mβcd bound to the membrane.

HPTLC analyses revealed that the mβcd treatments only removed limited amounts of other lipidic components from CV membranes relative to cholesterol (Fig. 5D). The specificity of the observed relationship between removal of cholesterol and inhibition of fusion was confirmed by using other cyclodextrins that are less selective for cholesterol (Kilsdonk et al., 1995). The α- and β-cyclodextrins (αcd and βcd) as well as hpβcd had no significant effect on the extent, Ca²⁺ sensitivity or kinetics of triggered fusion at comparable doses to mβcd (Fig. 5A,B); however, at higher doses (5 mM), βcd had a slight (but not statistically significant) inhibitory effect on Ca²⁺ sensitivity (Fig. 5A, yellow diamonds), while αcd had inhibitory effects on fusion kinetics (Fig. 5E, green triangles). In response to 87±3 μM [Ca²⁺]free, the initial rate of fusion decreased from 60.4±1.2%/second to 27.1±1.2%/second following treatment with 5 mM βcd (n=2). Very high (≥20 mM) doses of hpβcd also inhibited the Ca²⁺ sensitivity, but not the extent of fusion (Fig. 5B, purple inverted triangles). Relative to the effects of an identical dose of mβcd (2 mM), there was no substantial removal of CV membrane cholesterol by any of these other cyclodextrins (Fig. 5C,D), although at higher doses each of the cyclodextrins tested removed significant amounts of cholesterol (P<0.001, Fig. 5C). Thus, overall, the dose-dependent effects of mβcd treatments on triggered fusion (as well as those of the other cyclodextrins at higher doses) correlated directly with the removal of CV cholesterol. If this is indeed a cause-effect relationship, the ability of CVs to fuse should be rescued by the addition of exogenous cholesterol to cholesterol-depleted CVs.

Cholesterol acts in the fusion mechanism

Bypassing the access difficulties at fully docked sites, we found a direct effect of cholesterol on triggered bilayer merger, resulting in a selective rescue of fusion. Delivery of cholesterol using mβcd as a carrier molecule did not fully recover the extent of fusion, and resulted in no rescue of Ca²⁺ sensitivity or kinetics (Fig. 6A, green triangles). Additionally, exposing untreated (native) CV to cholesterol-loaded mβcd (mβcd-cholesterol) caused a significant rightward shift in Ca²⁺ sensitivity and a decrease in the extent of fusion (data not shown), similar to that seen with unloaded mβcd (Fig. 6A, blue circles). Taking these results to indicate nonspecific effects of mβcd, particularly with cumulative exposures, we sought alternate methods of delivering cholesterol to the CV membrane. Cholesterol delivery using saturated solutions was only able to partially recover the extent, but not the Ca²⁺ sensitivity or kinetics of fusion (Fig. 6A,D orange triangles). Exogenous cholesterol delivered with hpβcd caused full recovery of the extent, Ca²⁺ sensitivity and kinetics of fusion (Fig. 6B-D, red triangles, Table 1). Molecular analysis revealed the specificity of both mβcd inhibition and the recovery of fusion (Fig. 6E, Table 1). Untreated, native CVs had 70.6±3.3 amol (4.25±0.20 ×10⁷ molecules) of cholesterol per CV (n=9), and this correlates with a cholesterol:phospholipid ratio of 0.60±0.034. After treatment with 2 mM mβcd, cholesterol levels drop to 49.5±2.0 amol per CV (n=4), with a cholesterol:phospholipid ratio of 0.41±0.021 (Table 1). The effective delivery of cholesterol to cholesterol-depleted CVs resulted in the full recovery of total cholesterol per CV (and thus the native cholesterol:phospholipid ratio) and correlated with the complete rescue of fusion extent, Ca²⁺ sensitivity and kinetics (Fig. 6B-D, Table 1). For each method of cholesterol delivery, rescue of the ability to fuse (e.g. extent) correlated in a dose-dependent manner with the total cholesterol content; however, at lower concentrations (≤2 mM), lysis did not exceed 10%.
amount of recovered membrane cholesterol (Fig. 6D), yet the Ca$^{2+}$ sensitivity and kinetics of fusion were only effectively recovered when the total CV membrane cholesterol concentration was recovered to native levels. Untreated, native CVs supplemented with cholesterol had a slight, but statistically insignificant left-shift in Ca$^{2+}$ sensitivity, and a slight corresponding increase in fusion kinetics without affecting the extent of fusion (Fig. 7A,B). Taken together, these results suggest different specific roles for cholesterol in both the pre-fusion and triggered steps of native bilayer merger. First, to ensure the efficiency of fusion (e.g. Ca$^{2+}$ sensitivity and rate of fusion), cholesterol probably acts via microdomains to optimize the interactions of other critical upstream factors (Lang et al., 2001; Chamberlain et al., 2001; Ohara-Imaizumi et al., 2004); roles for related sterols in priming and docking have also been proposed (Kato and Wickner, 2001; Fratti et al., 2004). Now, by selective rescue, a second more central role for cholesterol as a direct component of the native fusion mechanism is revealed.

**Testing for specific roles of cholesterol**

In order to further characterize the effects of altering cholesterol levels in the membrane, and to test the hypothesis of two select roles for cholesterol in the fusion pathway, we sought additional methods of altering the effective cholesterol concentration in the membrane. As oxidized cholesterol (the ketone, cholesten-3-one) is known to disrupt rafts (Xu and London, 2000; Samsonov et al., 2001), we enzymatically manipulated cholesterol levels in the CV membrane using cholesterol oxidase (Fig. 8A). The resulting inhibition of fusion extent was dose dependent, correlating with total cholesterol levels. Only following treatments with very high enzyme concentrations (1 U/ml cholesterol oxidase) that caused a loss of 52.3±0.5% of the total CV cholesterol, was Ca$^{2+}$ sensitivity also significantly shifted to the right (Fig. 8B, green triangles). Thus, effects on the efficiency of fusion were only seen when cholesterol was depleted to levels comparable to those seen after treatments with mβcd (Fig. 6E, Table 1).

![Graphs and images from Fig. 5](https://example.com/fig5.png)

Fig. 5. Effects of cyclodextrins on CV-CV fusion. CVs treated with 0 mM (closed circles) or with (A) concentrations of α-cd (n=2), or β-cd as indicated (n=2). Dashed lines in A represent the 99% confidence intervals of the fusion plot for untreated CVs. (C) Total CV cholesterol assayed after treatment with the indicated cyclodextrins. Results are expressed as percentage of untreated control CVs. *, ** significant difference from control and all other conditions (P<0.001). (D) HPTLC analysis of supernatant extracts following incubation with 0 mM or 2 mM of the indicated cyclodextrin. Lipid standards include cholesteryl esters (CE), triacylglycerol (TAG), free fatty acids (FFA), diacylglycerol (DAG), cholesterol (Chol), monoacylglycerol (MAG), ceramide (Cer), cardiolipin (Card), diacylphosphatidylethanolamine (PE), diacylphosphatidylinositol (PI), lysophosphatidylethanolamine (LPE), diacylphosphatidylserine (PS), diacylphosphatidylcholine (PC), lysophosphatidylserine (LPS), sphingomyelin (Sph), and lysophosphatidylcholine (LPC). The prominent band in the mβcd lane is mβcd. (E) Kinetics of CV-CV fusion following treatments as in A, in response to 87±3 μM [Ca$^{2+}$]$_{free}$ (n=2).
Role of cholesterol in membrane fusion

We also capitalized on the selective cholesterol binding of polyene antibiotics. Of the antibiotics tested, filipin and amphotericin B (ampB), with the highest relative affinities for cholesterol (Norman et al., 1972a; Norman et al., 1972b; Patterson et al., 1979), produced similar, dose-dependent inhibitions of fusion extent (Fig. 9A,B). Although also causing dose-dependent inhibition, the lower affinity antibiotic pimaricin (PIM) (Patterson et al., 1979) was a less potent inhibitor of the extent of fusion (Fig. 9C). The more potent antibiotics, filipin and ampB, also had significant inhibitory effects on the kinetics of Ca\(^{2+}\)-triggered fusion (Fig. 9D). In response to 108±17 μM [Ca\(^{2+}\)]\(_{\text{free}}\), the initial rate of fusion decreased from 79.4±1.1%/second to 34.4±1.6%/second and 5.7±0.1%/second with 76 μM filipin and 50 μM ampB, respectively (n=2/3). PIM did not significantly inhibit kinetics, even at a dose of 100 μM (Fig. 9D). These inhibitory effects were selective, in that all the polyene antibiotics tested inhibited the extent of fusion, had no effect on Ca\(^{2+}\)-sensitivity and affected kinetics only at higher doses, if at all.

### Table 1. Molecular analyses of cholesterol-depleted and recovered CV

| Sample                        | Fusion EC50 (μM) | Fusion extent (%) | Fusion kinetics (%/second)\(^1\) | Cholesterol/CV (amol) | CH:PL\(^2\) |
|-------------------------------|-----------------|------------------|---------------------------------|-----------------------|------------|
| Control                       | 28.4±2.8        | 100.0**          | 92.4±6.1                       | 70.6±3.3              | 0.602±0.034|
| Cholesterol depleted\(^3\)    | 50.0±6.6*       | 78.6±4.5*        | 43.5±4.1*                      | 49.5±2.0*             | 0.411±0.021*|
| Cholesterol recovered\(^4\)   | 28.3±8.6        | 95.2±7.7         | 85.9±9.7                       | 74.8±4.9              | 0.637±0.045|

*Significant difference from control (P<0.05).  
\(^1\)Initial rate in response to 96±11 μM [Ca\(^{2+}\)]\(_{\text{free}}\).  
\(^2\)Molar cholesterol:phospholipid ratio.  
\(^3\)Treated with 2 mM mβcd for 30 minutes at 25°C.  
\(^4\)Treated with 2 mM hpβcd-cholesterol for 30 minutes at 25°C after treatment with 2 mM mβcd.  
**100% fusion by definition.

We also capitalized on the selective cholesterol binding of polyene antibiotics. Of the antibiotics tested, filipin and amphotericin B (ampB), with the highest relative affinities for cholesterol (Norman et al., 1972a; Norman et al., 1972b; Patterson et al., 1979), produced similar, dose-dependent inhibitions of fusion extent (Fig. 9A,B). Although also causing dose-dependent inhibition, the lower affinity antibiotic pimaricin (PIM) (Patterson et al., 1979) was a less potent inhibitor of the extent of fusion (Fig. 9C). The more potent antibiotics, filipin and ampB, also had significant inhibitory effects on the kinetics of Ca\(^{2+}\)-triggered fusion (Fig. 9D). In response to 108±17 μM [Ca\(^{2+}\)]\(_{\text{free}}\), the initial rate of fusion decreased from 79.4±1.1%/second to 34.4±1.6%/second and 5.7±0.1%/second with 76 μM filipin and 50 μM ampB, respectively (n=2/3). PIM did not significantly inhibit kinetics, even at a dose of 100 μM (Fig. 9D). These inhibitory effects were selective, in that all the polyene antibiotics tested inhibited the extent of fusion, had no effect on Ca\(^{2+}\)-sensitivity and affected kinetics only at higher doses, if at all.

Fig. 6. Addition of exogenous cholesterol to cholesterol-depleted CVs. Homotypic CV-CV fusion assays of CVs treated with 0 mM or 2 mM mβcd, and recovered by delivering cholesterol using (A) 2 mM mβcd as a vehicle (n=3), saturated cholesterol solutions (n=4), and (B) with 2 mM hpβcd as a vehicle as indicated (n=4). Vertical dashed lines in A and B indicate the EC50 of each curve. (C) Kinetics of CV-CV fusion triggered with 67±14 μM [Ca\(^{2+}\)]\(_{\text{free}}\) (n=4), after treatments as in A and B. (D) Total CV cholesterol assayed before and after 2 mM mβcd treatment, and with subsequent recovery with the indicated delivery methods. *Significant difference from control and all other conditions (P<0.005); **, ***significant differences from control and all other conditions (P<0.05).
Cholesterol as a critical negative curvature membrane component
Since cholesterol acts in the membrane as a molecule supporting negative curvature, and also through interactions with membrane proteins and other membrane constituents, we investigated the effects of alternative curvature analogues in cholesterol-depleted membranes. In a direct physical role, cholesterol could act focally at the fusion site to promote/support the formation of highly curved fusion intermediates. If this hypothesis concerning the negative curvature of cholesterol facilitating the native triggered fusion mechanism is correct, then introduction of other negatively curved molecules should rescue the ability to fuse whereas the introduction of positive curvature components would be expected to further inhibit fusion in cholesterol-depleted CV. Lysophosphatidylcholine (LPC) is a native membrane component of high positive curvature that has been previously shown to cause potent, dose-dependent, fully reversible inhibition of Ca\(^{2+}\)-triggered membrane fusion (Chernomordik et al., 1993; Vogel et al., 1993; Gunther-Ausborn et al., 1995; Chernomordik et al., 1995a; Chernomordik et al., 1995b). Addition of a low dose of LPC caused reversible inhibition of the extent of fusion, without significant effect on Ca\(^{2+}\) sensitivity (Fig. 10A, yellow circles); the same dose of LPC added to cholesterol-depleted CV further inhibited the extent of fusion (Fig. 10A, red squares). Inhibition by LPC was fully reversed by adding 1 mM hp\(\beta\)cd (Fig. 10B, purple triangles). Hp\(\beta\)cd was found to act as a molecular sink, apparently binding excess LPC in solution, and thus shifting the intercalation equilibrium of LPC with the membrane. The addition of 1 mM hp\(\beta\)cd to m\(\beta\)cd- and LPC-treated CVs again reversed the inhibition, back to that originally observed following m\(\beta\)cd treatment alone (Fig. 10B, green squares). Addition of exogenous

Fig. 7. The effects of supplementing native, untreated CVs with cholesterol using hp\(\beta\)cd. (A) Ca\(^{2+}\) activity curves of CVs treated with 0 mM or 5 mM hp\(\beta\)cd-cholesterol (n=3). Inset: total CV cholesterol of samples: 1, untreated CVs; 2, treated with 5 mM hp\(\beta\)cd-cholesterol. Results are presented as percentage of control cholesterol; *significant differences from control (P<0.001). (B) Kinetics of CV-CV fusion in response to 96±11 \(\mu\)M [Ca\(^{2+}\)]\(_{free}\) (n=3) as in A.

Fig. 8. The effects of cholesterol oxidase activity on CV-CV fusion. (A) Ca\(^{2+}\) activity curves of CVs treated with 0 U/ml (n=3), 0.01 U/ml (n=3), 0.1 U/ml (n=3), or 1.0 U/ml cholesterol oxidase (n=3). Vertical dashed lines indicate the respective EC50 values. (B) Total cholesterol of CVs treated as indicated. Results are expressed as percent of untreated CVs; *, **significant difference from control and all other samples (P<0.005).
cholesterol to mβcd-treated, LPC-inhibited CVs resulted in the full recovery of both the extent and Ca\textsuperscript{2+} sensitivity of fusion (Fig. 10B, orange diamonds).

Two other membrane components of high intrinsic negative curvature, α-tocopherol (α-toc) and dioleoylphosphatidylethanolamine (DOPE) (Rand et al., 1990; Epand et al., 1996; Leikin et al., 1996; Chen and Rand, 1997; Bradford et al., 2003), were tested for their ability to substitute for cholesterol in selectively rescuing fusion ability. Incorporation of α-toc or DOPE into cholesterol-depleted CVs produced a selective recovery of fusion extent, without rescuing either Ca\textsuperscript{2+} sensitivity or kinetics (Fig. 11A,B); the resulting Ca\textsuperscript{2+} activity curves remained translationally invariant relative to parallel controls. In response to 111±14 μM [Ca\textsuperscript{2+}]\textsubscript{free}, the initial rate of fusion was 68.1±5.5%/second before treatment and 9.0±2.5%/second after 2 mM mβcd treatment; following recovery with α-toc and DOPE the initial fusion rates were 14.9±2.2%/second and 18.2±1.7%/second, respectively (n=3). Initial fusion rates following delivery of DOPE and α-toc to the CV membrane were not significantly different from that following treatment with 2 mM mβcd. Membrane incorporation of both α-toc and DOPE was verified by HPTLC (data not shown). Since both α-toc and DOPE are native membrane components of high negative curvature, but without the ability to promote the formation of rafts comparable to those containing cholesterol, their selective rescue of the extent of fusion (but not Ca\textsuperscript{2+} sensitivity or kinetics) is consistent with the idea that negative curvature agents promote or support transient fusion intermediates, whereas the full, physiological Ca\textsuperscript{2+} sensitivity of fusion involves the interaction of additional factors through cholesterol-rich microdomains.

**Discussion**

Using a well-established model system specific for the Ca\textsuperscript{2+}-triggered fusion steps of regulated exocytosis, which is also highly amenable to direct molecular manipulations and to rigorous molecular analyses, we have shown for the first time that a native lipid membrane component, cholesterol, facilitates the minimal molecular machinery of native membrane merger for fast, Ca\textsuperscript{2+}-triggered fusion. The role of cholesterol in the membrane is twofold. First, acting as a membrane organizer, cholesterol aids in spatially orienting critical molecular components at the fusion site (via microdomains) to ensure the efficiency of triggered membrane merger. Second, the results are consistent with a direct link between curvature stress and the native fusion pathway. As an abundant native membrane component of intrinsic negative curvature, cholesterol lowers energy barriers to the formation of transient fusion intermediates, promoting rapid fusion pore formation and/or expansion.
CV functional characteristics and membrane composition

CVs are reasonably well characterized, both functionally and physically. Here, we confirm the robust consistency of previously established curve shape parameters and the translational invariance of the classic sigmoidal Ca$^{2+}$ activity curves for both CV-PM and CV-CV fusion, and the rapid kinetics of fusion (Vogel et al., 1991; Vogel et al., 1996; Coorssen et al., 1998; Blank et al., 1998; Blank et al., 2001; Coorssen et al., 2003; Szule et al., 2003). The slight rightward shift in Ca$^{2+}$ sensitivity (EC50) reported here, relative to previous work, is the result of the 3-4°C lower holding temperature we now use to better maintain the gravid adult urchins in captivity.

Cholesterol is a major component of the CV membrane (Decker and Kinsey, 1983), and of many other types of secretory vesicles (Table 2). Here we report a cholesterol:phospholipid ratio of 0.602±0.034 for the CVs of *S. purpuratus*. An earlier study reported a ratio of 1.33±0.12 for *Lytechinus variegatus* (Decker and Kinsey, 1983). Such differences are not unexpected between species, particularly considering the different native environments; *L. variegatus* is found in the warmer waters off the coast of Florida, and *S. purpuratus* in the cold waters off the coast of British Columbia. It is known that organisms vary the lipid composition of cellular membranes in response to temperature (Jin et al., 1999; Rilfors and Lindblom, 2002; Sanina and Kostetsky, 2002), and as increasing total concentrations of cholesterol tend to increase order and generally decrease the fluidity of bilayer membranes, animals inhabiting warmer waters would be expected to have a higher proportion of membrane cholesterol. The importance of cholesterol in exocytosis is suggested by the substantial difference in cholesterol content between the CVs and plasma membranes. Decker and Kinsey reported CV membrane cholesterol to be fully 2.3-fold higher than that of the plasma membrane (Decker and Kinsey, 1983). Secretory

![Fig. 10. Effects of a positive curvature agent, LPC, on CV-CV fusion. Ca$^{2+}$ activity curves of untreated CVs and CVs treated with (A) 10 μM LPC, or 2 mM mβcd, or treated first with 2 mM mβcd followed by treatment with 10 μM LPC (n=3) as indicated. Vertical dashed lines indicate the EC50 of each curve. (B) Samples paralleled to A first treated with 10 μM LPC and subsequently with 1 mM hpβcd (n=3). Following sequential treatments with 2 mM mβcd and 10 μM LPC, samples were treated with 1 mM hpβcd, or 1 mM hpβcd-cholesterol (n=3). Blue line indicates the 2 mM mβcd-treated sample as in A.](image)

![Fig. 11. Addition of negative curvature lipids to cholesterol-depleted CVs. (A) Ca$^{2+}$-activity curves of untreated CVs (n=3), CVs treated with 2 mM mβcd (n=3), or CVs treated with 2 mM mβcd and then with 10 μM α-tocopherol (n=3) or 10 μM DOPE (n=3). Vertical dashed lines indicate the EC50 of each curve. (B) Fusion kinetics of CVs treated as in A, in response to 111±14 μM [Ca$^{2+}$]$_{free}$ (n=3).](image)
Role of cholesterol in membrane fusion

Table 2. Cholesterol:phospholipid ratios of different secretory vesicles

| Species and tissue                              | Type | Vesicle | PM |
|------------------------------------------------|------|---------|----|
| Bovine adrenal medulla (Blaschko et al., 1967) | CG   | 0.53±0.08 | –  |
| Bovine adrenal medulla (DaPrada M. et al., 1972)| CG   | 0.40±0.03 | –  |
| Bovine adrenal medulla (Dreyfus et al., 1977)  | CG   | 0.53±0.05 | –  |
| Bovine adrenal medulla (Zinder et al., 1978)   | CG   | 1.04     | 0.64|
| Ox pancreas (White and Hawthorne, 1970)        | ZG   | 0.56     | –  |
| Guinea Pig pancreas (Meldolesi et al., 1971)   | ZG   | 0.55±0.06 | 0.51±0.03 |
| Bovine mammary gland (Keenan et al., 1979)     | SecV | 0.34±0.01 | –  |
| Bovine splenic nerve trunk (Lagercrantz, 1971)  | DCG  | 0.50±0.08 | –  |
| Rat brain (adult) (Breckenridge et al., 1973)   | SV   | 0.58±0.09 | –  |
| Guinea Pig cerebrovascular cortex (Baker et al.,1975) | SV | 0.50±0.04 | –  |
| Guinea Pig cerebellar cortex (Nagy et al., 1976)| SV   | 0.51±0.06 | –  |
| Ray (Narcine brasiliensis) electric organ (Deutsch and Kelly, 1981) | SV | 0.50±0.043 | –  |
| Ray (Torpedo marmorata) electric organ (Baker et al., 1975) | SV | 0.42±0.04 | –  |
| Ray (Torpedo marmorata) electric organ (Nagy et al., 1976) | SV | 0.42±0.04 | –  |
| Ray (Torpedo marmorata) electric organ (Michaelson et al., 1983) | SV | 0.63 | –  |
| Urchin (Lytechinus variegatus) oocyte (Decker and Kinsey, 1983) | CV | 1.33±0.12 | 0.55±0.14 |
| Urchin (Strongylometrus purpuratus) oocyte       | CV   | 0.60±0.034 | –  |

1Type of vesicle: CG, chromaffin granule; ZG, zymogen granule; SecV, secretory vesicle; DCG, dense core granule; SV, synaptic vesicle; CV, cortical vesicle.

2Molar cholesterol:phospholipid ratio of secretory vesicles.

3Molar cholesterol:phospholipid ratio of plasma membrane.

vesicles from various tissues and across numerous species are likewise enriched in cholesterol (summarized in Table 2). Reported cholesterol:phospholipid ratios vary from 0.34 to 1.04, with most vesicle types being significantly enriched in cholesterol relative to the plasma membrane, as with CVs.

Cholesterol depletion correlates with loss of fusion capacity

Inhibition of exocytosis and the depletion of cholesterol from CV-PM preparations are strongly correlated, and independent of indirect effects of mβcd. As we have rigorously characterized the lytic properties of mβcd in our preparations, we can clearly and consistently differentiate between fusion, lysis or undocking. Inhibition of fusion by mβcd does not result from lysis, which occurs on a substantially faster time scale. Furthermore, despite the known microdomain destabilizing effects of mβcd, and the noted dispersal of SNARE proteins (Lang et al., 2001), there was no evidence of CV undocking from the PM (Fig. 2A), consistent with previous findings that inter-membrane SNARE protein interactions do not specifically define the docked state (Coorssen et al., 1998; Coorssen et al., 2003). This stably docked state of CVs in mature oocytes perhaps explains why we find no evidence for undocking, in contrast to the observed effects on insulin secretory granules following treatment of intact cells with mβcd (Ohara-Imaizumi et al., 2004); perhaps these latter vesicles were tethered but not yet fully docked. Disruption of rafts may thus interfere with the docking process but not effectively reverse docking once established. Resolution of this question will require better molecular and physical understanding of the tethered and docked states.

Like CV-PM fusion, CV-CV fusion was also significantly inhibited by mβcd, independent of its earlier lytic effects (Fig. 2C). Since lysis by mβcd is quite rapid (<1 minute) compared to the slower timescale for inhibition of the extent, Ca²⁺ sensitivity and kinetics of fusion (>10 minutes) these effects are well separated and thus unlikely to be related. Additionally, inhibition of fusion by removal of cholesterol is not a result of vesicle de-priming. As the CVs are by definition docked, primed and release ready, Ca²⁺-triggered CV-PM and CV-CV fusion are ATP independent (Vacquier, 1975; Baker and Whitaker, 1978; Vogel et al., 1991; Shafi et al., 1994; Vogel et al., 1996; Tahara et al., 1998; Coorssen et al., 1998; Zimmerberg et al., 2000; Blank et al., 2001; Coorssen et al., 2003; Whalley et al., 2004). Likewise, the effects of mβcd were also independent of ATP; exclusion of ATP from buffers both during mβcd treatments and during Ca²⁺ activity (fusion) assays had no effect on the parameters of triggered fusion, precluding effects such as de-priming and subsequent ATP-dependent re-priming upon supplementation with exogenous cholesterol. Thus, despite a suggested role for cholesterol in priming (Kato and Wickner, 2001; Fratti et al., 2004), this stage is clearly not as labile in CVs.

One possible interpretation of the rightward shift in Ca²⁺ sensitivity following cholesterol depletion is a change in the local [Ca²⁺]free due to alteration of the local lipid composition at the fusion site. The measurement of local, near-membrane Ca²⁺ concentrations remains problematic, and as a result, all work in the field is limited to measures of bulk [Ca²⁺]free. The relationship between bulk and local [Ca²⁺]free thus remains an unknown variable, particularly in terms of routine, direct measurements. However, in these experiments, such an alteration in the local [Ca²⁺]free at fusion sites, resulting from local compositional changes of lipids, appears unlikely. Considering the very rapid transbilayer movement of cholesterol [t½ ~1 second (Leventis and Silvius, 2001; Steck et al., 2002)], its removal from the outer leaflet of the CV membrane would be expected to cause a redistribution of lipids from the inner leaflet, resulting in an increased density of charged lipids on the outer monolayer. This would only serve to increase the local [Ca²⁺]free at membrane interfaces, and potentiate fusion via the inter-bilayer binding of Ca²⁺ between the anionic lipid headgroups (Feigenson, 1989; Coorssen and Rand, 1995). Such potentiation of fusion was never seen.

Since cyclodextrins are generally able to bind numerous hydrophobic or lipophilic molecules, we must ask if we have correctly identified cholesterol as the primary target of mβcd,
and thus as the critical membrane component correlating with the observed inhibitory effects. The first indication that inhibitory effects of mβcd correlated with the selective removal of CV cholesterol came from coupled molecular-functional analyses of the effects of related cyclodextrins. At doses comparable to that of mβcd, αcd, βcd and hpβcd neither inhibited fusion, nor affected CV membrane cholesterol concentrations (Fig. 5), but did exhibit a similar complement of other lipids from the membrane (Fig. 5D). However, at higher doses each cyclodextrin produced some inhibitory effects on fusion parameters, correlating with significant removal of cholesterol from the CV membrane, comparable to that seen with mβcd. Higher doses of αcd had inhibitory effects on fusion kinetics (Fig. 5C) whereas βcd caused slight inhibitory effects on Ca²⁺ sensitivity (Fig. 5A). In both cases the extent of inhibition and cholesterol removal was approximately equivalent to that seen following the treatment of CVs with 1 mM mβcd. Thus, mβcd is ~5-fold more potent than either αcd or βcd in removing CV membrane cholesterol. As hpβcd does not have significant inhibitory effects at concentrations of<20 mM, mβcd is ~10-fold more potent than this analogue. The somewhat selective effects of the different cyclodextrins, αcd for kinetics and βcd or hpβcd for Ca²⁺ sensitivity are most probably explained by somewhat differential targeting of membrane microdomains and/or the selective solubilization of other components within these rafts (Elliott et al., 2003; Kiely et al., 2003; Aachmann et al., 2003; Bacia et al., 2004).

The dose-dependent inhibition of fusion by mβcd can most simply be described in terms of two parameters – the extent and Ca²⁺ sensitivity of fusion – as it is known that the latter is directly related to the kinetics (rate) of fusion via Ca²⁺ activation of fusion complexes (Blank et al., 2001). The effect of mβcd on membranes is likewise twofold and inter-related, causing the dose-dependent removal of cholesterol from membranes, and the subsequent disruption of cholesterol-rich microdomains, resulting in the dispersal of raft constituents. Here we show, for the first time, that the two fusion parameters are separable, and related to the two molecular effects of mβcd treatment.

**Membrane domains and the recovery of fusion efficiency**

The addition of exogenous cholesterol to cholesterol-depleted membranes results in a dose-dependent recovery of ability to fuse (e.g. the extent of fusion), but full rescue of the Ca²⁺-sensitivity and kinetics of fusion only occurs upon recovery of membrane cholesterol to native levels (Fig. 6). The separation of the two inhibited fusion parameters, ability to fuse and efficiency of fusion, allows us to speculate as to the mechanism of inhibition by mβcd. First, mβcd removes cholesterol from membranes in a dose-dependent manner. This is recovered through the addition of exogenous cholesterol to the CVs, but results in a graded recovery of only the extent of fusion (Fig. 6A). Second, mβcd is disruptive to cholesterol-rich microdomains, and causes the dispersal of raft constituents (e.g. proteins and other lipid components). In order to fully recover both the ability of CVs to fuse and the efficiency of fusion, both recovery of native cholesterol levels and reformation of appropriate cholesterol-rich microdomains appear to be necessary. The spontaneous recovery of the Ca²⁺ sensitivity and kinetics of fusion after full recovery of the native CV membrane cholesterol level is consistent with a critical total cholesterol concentration required to support formation of rafts (Parasassi et al., 1995). Additionally, model membrane studies show that increasing cholesterol concentration in lipid bilayers results in a decreased transition temperature to the liquid ordered phase (Parasassi et al., 1995; Filippov et al., 2003). Thus, at a fixed temperature, varying the cholesterol concentration in membranes identifies a critical concentration of cholesterol for the ‘spontaneous’ transition to the liquid ordered phase. Certainly the presence of other specific membrane components will also promote the local formation of domains, as will the rapid rate of transbilayer redistribution (e.g. flip-flop) that is characteristic of cholesterol in biological membranes (Leventis and Silvius, 2001; Steck et al., 2002).

**Microdomain disruption affects Ca²⁺ sensitivity of fusion**

To further understand the apparent dual effect of mβcd on Ca²⁺-triggered fusion, in that it inhibits both the extent and the Ca²⁺ sensitivity of fusion, we carried out treatments having alternate effects on membrane cholesterol. Enzymatic manipulation of CV membrane cholesterol with cholesterol oxidase resulted first in potent inhibition of the extent of fusion, followed by inhibition of the Ca²⁺ sensitivity only after more aggressive enzyme treatments (Fig. 8), comparable to the effects of mβcd (Fig. 3). The ketone end product of cholesterol oxidation, cholessten-3-one, is known to inhibit domain formation in model membranes (Xu and London, 2000) and to cause dispersal of microdomain constituents (Samsonov et al., 2001). Thus, cholesterol oxidase functionally removes cholesterol from the membrane by conversion to a dissimilar end product, while it physically eliminates functional microdomains through the disruptive effects of high local cholessten-3-one concentrations.

Contrary to the effects of both mβcd and cholesterol oxidase treatments, it has previously been shown that polyene antibiotics do not effectively disrupt rafts in a manner consistent with the effects of mβcd (Awasti-Kalia et al., 2001); the mode of action of filipin (Norman et al., 1972a; Norman et al., 1972b; Lopes et al., 2004), the best studied of the polyene antibiotics, suggests a cholesterol clustering action rather than a broadly disruptive action. Likewise, the widespread use of filipin as a fluorescent and ultrastructural probe for membrane sterol clustering also argues against effective or extensive domain disruption (Orci et al., 1981; Keller and Simons, 1998; Gagescu et al., 2000; Grebe et al., 2003; Fratti et al., 2004). In this study, we carefully selected concentrations of polyene antibiotics to approximate stoichiometric ratios with total CV cholesterol. The highest dose of filipin (76 μM) corresponds to an approximate 1:2 ratio of filipin to outer leaflet cholesterol (mol/mol); lower doses correspond to 1:4, 1:10 and 1:100 filipin:cholesterol. As estimates of the stoichiometry of cholesterol:filipin binding ratio are approximately 1.5-2:1 (Norman et al., 1972b), the treatment with 76 μM filipin correlates to approximately 50% total cholesterol binding. Comparable removal of cholesterol from CVs by 4 mM mβcd treatment results in approximately 60% inhibition of fusion extent (Fig. 3A, orange squares), consistent with the inhibition by filipin (Fig. 9A, yellow squares). Since all polyene antibiotics tested dose-dependently affected the extent of fusion, with no significant effects on the Ca²⁺ sensitivity of fusion, we interpret the latter as indicating...
that raft constituents are not effectively or generally dispersed (Awasthi-Kalia et al., 2001). Rather, it would appear that functional aggregates of critical domain components are locally retained, but inhibited. In all other examples (Figs 1, 3, 8), the treatments known to consistently disrupt cholesterol-rich microdomains correlate directly with rightward shifts in the Ca$^{2+}$ sensitivity of fusion.

Supplementation of native CV membranes with exogenous cholesterol results in a statistically insignificant leftward shift in the Ca$^{2+}$ sensitivity of fusion, and a likewise insignificant promotion of the kinetics of fusion (Fig. 7), despite an increase in CV cholesterol to $152\pm9\%$ ($n=3$). This implies that the lipid components of the CV membrane are carefully ‘tuned’ to a critical cholesterol concentration that supports the formation and maintenance of functional microdomains (Parasassi et al., 1995). The effects of supplementing cholesterol above native densities does not yield more efficient domains that further promote the Ca$^{2+}$ sensitivity or kinetics of fusion.

Cholesterol contributes negative curvature to the fusion process

As we have identified correlations between changes in the Ca$^{2+}$ sensitivity of fusion and treatments that disrupt rafts, we also sought to test for correlations between the observed changes in extent of fusion with the second proposed role of cholesterol in the CV membrane. In addition to the organizational role cholesterol plays in the formation and maintenance of microdomains, it also contributes negative curvature stress to lipid bilayers (Coorssen and Rand, 1990; Rand et al., 1990; Chen and Rand, 1997). Thus, with its intrinsic negative curvature and high focal density in certain membrane microdomains, this abundant native sterol (and perhaps related molecules) could well serve to reduce local energy constraints and thereby promote the progression of energetically favourable fusion intermediates.

The dose-dependent recovery of the extent of fusion with exogenous cholesterol delivery, without parallel rescue of the Ca$^{2+}$ sensitivity (Fig. 6A), suggests that the curvature role of cholesterol in the CV membrane is selectively associated with fusion ability. Exogenous cholesterol can also overcome the inhibitory effects associated with the incorporation of LPC (high positive curvature) in the vesicle membrane (Fig. 10A). The fully effective and selective recovery of the fusion ability of CVs (extent of fusion), following incorporation of the native ‘curvature analogues’ α-toc and DOPE (Epand et al., 1996; Bradford et al., 2003), is also fully consistent this hypothesis (Fig. 11). Since neither α-toc nor DOPE are capable of supporting the formation of functional microdomains comparable to those involving cholesterol, the contribution of α-toc and DOPE to the process of Ca$^{2+}$-triggered membrane merger is most simply interpreted in terms of a direct physical contribution of local curvature stress, promoting or supporting the formation of highly curved, transient fusion pore intermediates. Additionally, since all recovered fusion curves, including those for each cholesterol delivery method as well as the recoveries with DOPE and α-toc, are translationally invariant to Ca$^{2+}$ activity curves for untreated control CVs, recovered membrane fusion still proceeds through the same molecular mechanism (Vogel et al., 1996; Blank et al., 1998).

Changes in fusion parameters in response to inhibitory reagents has been the subject of elegant mathematical analysis and modelling (Vogel et al., 1996; Tahara et al., 1998; Blank et al., 1998; Blank et al., 2001). Such analyses have been used here to reveal characteristics of the inhibitory effects of mβCD. As the Ca$^{2+}$ activity curves of both CSCs and CVs treated with various concentrations of mβCD are translationally invariant with respect to untreated controls and each other, it is not that fusion is proceeding via an alternate pathway, but rather that native fusion machines are progressively inactivated as increasing amounts of cholesterol are lost from the membrane. Thus, we can extrapolate back from the progressive decline in the extent of fusion in order to estimate the total number of functional fusion machines ($\langle n_{\text{Max}} \rangle$) on a native vesicle (Fig. 4) (Vogel et al., 1996; Blank et al., 1998). Since these values are both internally consistent, and consistent with previously reported values of $\langle n_{\text{Max}} \rangle$ (Coorssen et al., 1998; Coorssen et al., 2003), we conclude that the inhibition of fusion by mβCD is a direct result of a decrease in the number of intact, active fusion machines per vesicle. This implies that cholesterol is itself a component of the fusion machinery, or is at the very least intimately associated with and influencing critical components. Thus, through cholesterol removal and subsequent replacement, we have effectively ‘switched’ fusion machines off and on.

Although perhaps most consistent with the stalk-pore hypothesis of fusion pore formation (Kozlov and Markin, 1983; Chernomordik and Zimmerberg, 1995; Chernomordik et al., 1995a; Chernomordik et al., 1995b; Chanturiya et al., 1997; Kozlovsky et al., 2002), alternate local effects of cholesterol including the direct facilitation of protein-based pores can not be excluded. Nonetheless, these studies clearly differentiate the dual role of cholesterol in the process of Ca$^{2+}$-triggered membrane fusion: as a prefusion organizer contributing to the efficiency of fusion (e.g. Ca$^{2+}$ sensitivity and kinetics) and as a native membrane component, critical to fast, functional fusion machinery, contributing negative curvature to facilitate the formation and/or expansion of fusion intermediates, thereby supporting the intrinsic ability of vesicles to fuse.

Cholesterol as a component of the fusion machine

The direct role of cholesterol in Ca$^{2+}$-triggered membrane fusion can be interpreted in two ways. The simplest interpretation is that cholesterol contributes negative curvature to the membrane, promoting the formation of transient lipidic fusion intermediates (Kozlov and Markin, 1983; Chernomordik and Zimmerberg, 1995; Chernomordik et al., 1995a; Chernomordik et al., 1995b; Chanturiya et al., 1997; Kozlovsky et al., 2002). Of increasing complexity, in a proteinaceous fusion pore model, cholesterol could contribute to the mixing and coalescence of apposed membranes via expansion of a proteinaceous fusion pore (Lindau and Almers, 1995; Peters and Mayer, 1998; Bayer et al., 2003). In this model, lipids with negative curvature could be seen as essential to pore invasion, expansion and subsequent mixing of two bilayers following the initial opening of a protein pore. The well characterized disruption of rafts by mβCD, in many different biological membranes, also results in the irreversible destabilisation of protein interactions and the dispersal of protein components. In secretory cells, such microdomains are critical to a range of pre-fusion states, and domain disruption has been shown to result in the dispersal of proteins that function in the exocytic pathway (Lang et al., 2001; Chamberlain et al., 2001;
triggers membrane merger neither unequivocally supports nor rules out a role for proteins in triggered fusion steps. It seems likely that the membrane components of all secretory vesicles have been highly ‘tuned’ through evolution to allow variations in the extent, Ca$^{2+}$ sensitivity and rate of fusion while preserving the same underlying molecular framework (Table 2). Evidence suggests that the energetic contributions of proteins alone may generally be insufficient to fully and effectively breach the hydration layer (Rand and Parsegian, 1989) or to effect complete bilayer merger (Coorssen et al., 2002; Coorssen et al., 2003; Szule et al., 2003). The differential optimization of the vesicle lipidic matrix to facilitate the functions of the protein components of the fusion machine, might well explain differences in the rate and Ca$^{2+}$ sensitivity of exocytotic release processes between different cell types and across species. Given the energetic complexity of the fusion process, necessitating inherent protein-lipid interactions in native membranes, we think it appropriate to consider cholesterol as a critical component of the minimal essential fusion machine. Cholesterol is responsible for the pre-fusion organization of components critical for Ca$^{2+}$ sensing and contributing to the efficiency of fusion; a possible role for annular cholesterol in regulating the functions of specific proteins (such as the affinity of the Ca$^{2+}$ sensors) must also be considered. Subsequently, cholesterol also contributes directly to the triggered fusion step, promoting fast fusion pore formation by virtue of its high focal native density and intrinsic negative curvature.

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