Application of a Membrane Fusion Assay for Rapid Drug Screening

Steven S. Vogel,[1,4] Sven Beushausen,[2] and David S. Lester[3]

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Purpose. The purpose of this study is to develop an *in vitro* assay for screening drug and their effects on membrane fusion and lysis of intracellular organelles.

Methods. A 96-well microtiter-dish turbidimetric assay using membrane components of the eggs of sea urchins, a marine invertebrate, was applied to monitor granule fusion and/or lysis.

Results. Of 18 drugs screened, 16 had no effect. One antineoplastic drug, tamoxifen, disrupted intracellular membranes in a calcium independent manner. Taxol, another antineoplastic drug, specifically inhibited calcium triggered exocytosis.

Conclusions. This assay is inexpensive, simple, rapid, and does not require the sacrifice of animal life. It has the potential to identify drugs that are membrane active, as well as those which specifically perturb events involved in the secretion process.

KEY WORDS: drug screening; secretion; tamoxifen; taxol; sea urchin.

INTRODUCTION

In vitro drug screen assays utilize cultured cells. The toxicity is generally determined by monitoring such factors as dye uptake or cell " leakage". Cell leakage is a final stage of toxicity and is an indication of severe membrane disruption (1). These sorts of assays are relatively expensive and require large scale preparations of cultured cells. In this study, we use a simple, well-defined membrane system composed of plasma membrane fragments with attached intracellular organelles, secretory vesicles. In this *in vitro* system, secretory granules fuse with the plasma membrane in response to elevated concentrations of calcium. As a result, the turbidity of the solution decreases. This response can be monitored in a microtiter dish spectrophotometer as a decrease in absorbance at 405 nm. This feature of the assay has two potential applications for screening drugs. First, as sea urchin egg exocytotic granules are large (~1 μm diameter) the ability of drugs to lyse them can be easily followed by light scattering or direct microscopic examination. Thus, we have a simplified assay of screening drugs for potentially toxic effects via membrane lysis. In addition, this assay can be used to identify drugs for their ability to specifically perturb events involved in secretion, such as granule docking, triggered granule fusion, and granule content release. This is justified as the sea urchin calcium-triggered exocytosis is an established fusion process which has similar properties to those seen in other fusion systems, such as chromaffin and mast cell degranulation, viral infection, and rat liver microsome fusion (2). We have developed this assay such that rapid large scale screening can be done *in vitro* in 96-well microtiter dishes. The assay, as for previous assays (3-6) monitors the turbidity of a solution containing egg plasma membrane fragments and attached exocytotic granules from sea urchin.

MATERIALS AND METHODS

Preparation of cell surface complex: Sea urchin egg cell surface complexes (CSCs) were prepared from *Strongylocentrotus purpuratus* (Marinus, Long Beach, California). Eggs were collected in artificial sea water by intracoelomic injection of 0.5 M KCl. They were dejellied by several passes through 90 μm Nitex mesh and washed three times in ASW, and three times in PKME (50 mM PIPES, 450 mM KCl, 10 mM $MgCl_2$, 5 mM EGTA, 1 mM benzamidine, pH 6.7). Eggs were resuspended in PKMEB (50 mM PIPES, 450 mM KCl, 10 mM $MgCl_2$, 5 mM EGTA, 1 mM BAPTA, 1 mM benzamidine, pH 6.7) at a ratio of 1:10 (vol:vol) All manipulations were done at 4°C. In order to disrupt the eggs, they were homogenized with 6-10 strokes of a teflon-glass dounce homogenizer. The homogenate was centrifuged for 1 min at 200 × g in a table top centrifuge. The white pellet containing CSCs was collected. The supernatant was aspirated and the loose white pellet resuspended in HENPK buffer (3.3 mM HEDTA, 3.3 mM EGTA, 3.4 mM NTA, 50 mM PIPES, 450 mM KCl, 1 mM benzamidine, pH 6.7) by gentle vortexing to form a turbid white solution. When homogenization was inadequate a light yellow pellet with a thin white crust on top was observed. This is indicative of intact eggs mixed with CSCs. This type of pellet was resuspended in PKMEB and re-homogenized and centrifuged. At the proper concentration, a 200 μl suspension of CSCs should have an absorbance of between 1 and 2 optical density units at 405 nm. CSCs were stored on ice until ready for use. All transfers of CSCs were done with 1000 μl disposable plastic pipet tips whose opening had been enlarged by cutting to avoid shearing the CSCs. Failure to do this often resulted in a shift in the calcium dependence of exocytosis.

Calcium buffers: A collection of 1X HENPK buffers with 2X concentrations of calcium were prepared by adding different amounts of $CaCl_2$ to a 2X HENPK stock solution. The pH of these buffers were adjusted to 6.7 by the addition of KOH, and the concentration of the salts, chelators and pipes adjusted to 1X HENPK by addition of double distilled, deionized H_2O. Free calcium concentrations were measured for these buffers after they had been mixed 1:1 (vol/vol) with 1X HENPK using a calcium electrode (World Precision Instruments, Inc., Sarasota, Florida) or with the fluorescent calcium indicator rhod-2 (Molecular Probes, Eugene, Oregon) using the function (free calcium) = K_d × (F-Fmin)/

[1] Laboratory of Theoretical and Physical Biology, National Institute of Child Health and Human Development, National Institutes of Health, Bldg. 10, Rm. 10D-09, Bethesda, Maryland 20892.

[2] Laboratory of Neurobiology, National institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892.

[3] Division of Research and Testing, Center for Drug Evaluation and Research, Food and Drug Administration, Laurel, Maryland 20708

[4] To whom correspondence should be addressed.

Abbreviations: EGTA, ethylene glycol bis(b-aminoethyl ether) N,N,N′,N′-tetraacetic acid; HEDTA,N-hydroxyethylene-diaminetriacetic acid; NTA, nitrilotriacetic acid; CSC, cell surface complex; TMB, 3,3′,5,5′-tetramethyl benzidine.

(Fmax-F) and an apparent K_d value of 4.0 μM in this buffer (7).

Chemicals: Tamoxifen was from Aldrich. Taxol and vinblastine were purchased from Calbiochem. Cytarabine was from Upjohn. Fluoxetine was prepared from capsules of Prozac (Eli Lilly and Co.). Fenfluramine was obtained from Research Biologicals Inc. Cyclosporine B and suramin were gifts from Drs. A. Aszalos and S. McPherson (FDA, Laurel, Maryland), respectively. All other drugs were obtained from Sigma Chemical Co. Drugs were added to the CSC suspensions providing a final concentration of 1% DMSO. This was found to have no effect on calcium dose-response controls. Ellipticine and cytarabine were solubilized in double distilled H_2O.

Assays for exocytosis: CSCs in 1X HENPK buffer were incubated at room temperature for 15 min. in the presence of DMSO (1% final concentration) as a control, or with specific drugs in 1% DMSO. CSCs (100 μl) or drug treated CSCs (100 μl) were transferred into 96-well microtiter dish wells and 100 μl of various 1X HENPK/2X calcium buffers were added. After a 10 min incubation at room temperature to allow the fusion reactions to reach completion, the turbidity of the individual points were measured at 405 nm in a microtiter dish spectrophotomer (Molecular Devices, Menlo Park, California). To measure the amount of soluble protein secreted from the CSC preparation in response to calcium, CSCs were pelleted by spinning the micro-titer dish in a table top centrifuge for 10 min at 10 x g. The supernatant (20 μl) was assayed for protein from each microtiter-dish well using the BCA protein assay (Pierce, Rockford, Illinois) using dilutions of bovine serum albumin in HENPK buffer to generate a standard curve. To assay ovoperoxidase activity released during cortical granule exocytosis, we added 50 μl of TMB/H_2O_2 solution (Pierce, Rockford, Illinois) in HENPK to each well and incubated at room temperature for 15 min. CSCs were pelleted by centrifugation in a table top centrifuge for 10 min at 10 x g, and 100 μl of supernatant was transferred to a new microtiter dish for spectroscopic analysis of TMB absorbance in a microtiter dish spectrophotometer using a 405 nm filter.

RESULTS

A cell surface complex, composed primarily of large fragments of sea urchin egg plasma membrane with docked cortical granules (Figure 1A), can be prepared by differential centrifugation of a homogenate made from unfertilized sea urchin eggs (8). When the free calcium concentration is raised above a threshold value (approximately 10 μM calcium), cortical granules begin to fuse with the plasma membrane (Figure 1 B). At high concentrations of calcium, virtually all of the membrane docked cortical granules fuse with the plasma membrane, liberating their proteinaceous cores, leaving large sheets of plasma membrane (Figure 1C).

Sea urchin CSCs have a high optical absorbance, primarily due to light scattering off the dense proteinaceous contents of the cortical granules (9). When granules fuse with the egg plasma membrane these contents are released and dispersed resulting in a reduction in the measured absorbance. In Figure 2, comparisons are made of the calcium dose response curves for light scattering and the release of a specific granule component, ovoperoxidase, or the release of the granule protein contents. In all three cases we see no exocytotic activity below 1 μM calcium, and a sharp transition in the light scattering signal, the release of ovoperoxi-

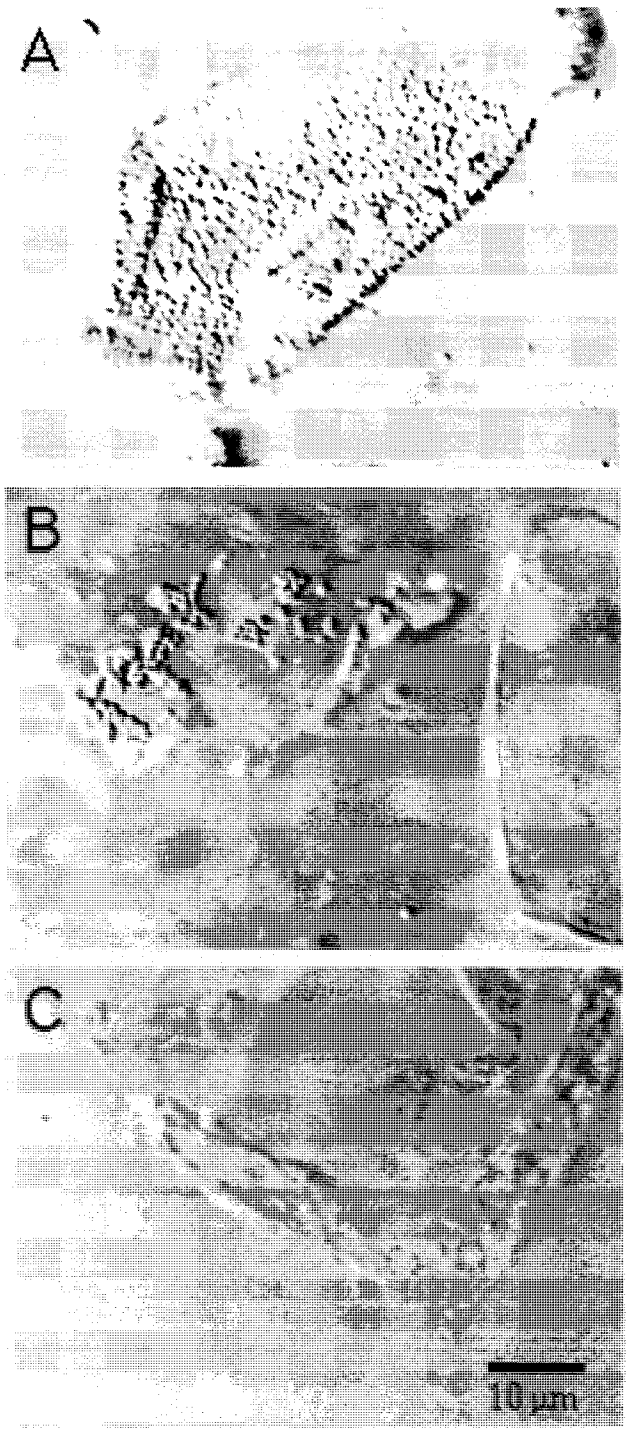

Fig. 1. Differential interference contrast microscopy was used to image the sea urchin egg Cell Surface Complex (CSCs) in calcium free buffer (A), buffer containing 23.1 μM calcium (B), and buffer containing high calcium (C). Note that most of the granules in this preparation are associated with the egg plasma membrane sheets (A), and the disappearance of these 1 μm granules with increased calcium concentration.

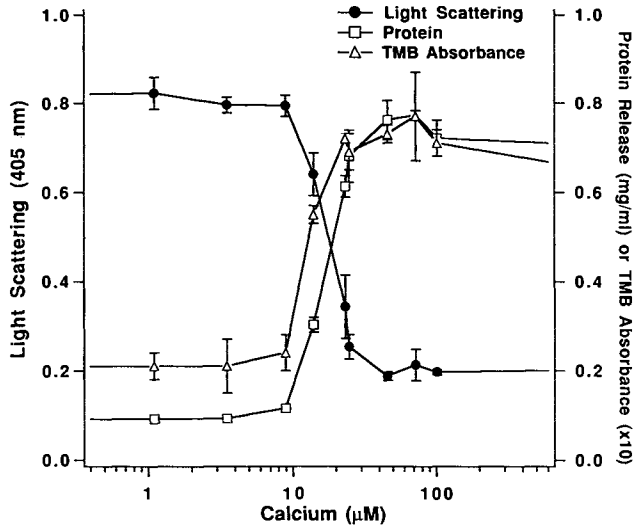

Fig. 2. Comparison of calcium dependent turbidity change (●) in CSCs assayed in a 96-well micro-titer dish spectrophotometer. Exocytotic release of ovoperoxidase activity (△), and total protein release (□) as a function of free calcium concentration. Note that all secretory responses are occurring at calcium concentrations between 3-50 μM. All points are mean ± SEM, n = 3

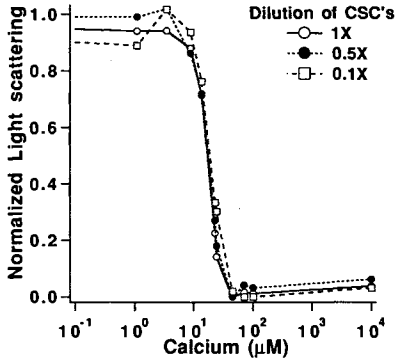

Fig. 3. Concentration dependence of the calcium dose-response of CSCs. The normalized calcium dose-response for exocytosis was determined for our standard CSCs (○) and CSCs diluted 1:1 (●) and 1:10 (□) with HENPK buffer.

dase activity into the buffer, and the release of proteins, between 10 and 30 μM calcium. There is no change in the light scattering signal of CSCs treated with known inhibitors of exocytosis in response to elevated calcium concentrations (3, 6, 10).

Sea urchin cortical granules contain high concentrations of intraluminal calcium (11). Upon fusion, intravesicular calcium may be released, which could drive our *in vitro* preparation to complete fusion. In addition, the assay may be highly sensitive to the concentration of CSCs in the microtiter dish wells. Both of these features would potentially result in shifts of the calcium-dose response. However, these behaviors are not seen. At intermediate concentrations of calcium (between 10 and 30 μM), intermediate numbers of granules fuse with the plasma membrane as determined by light scattering, protein release, ovoperoxidase activity (Figure 2), and microscopy (Figure 1B). Furthermore, when the light scattering assay is run with CSCs at various concentrations, there are identical dose response curves for calcium triggered exocytosis at CSC concentrations spanning an order of magnitude (Figure 3). All of these observations support the concept that exogenous calcium induces fusion of the granules with the plasma membrane and subsequent release of the intravesicular contents.

To demonstrate the applicability and specificity of this assay for drug screening, 18 drugs were screened for their ability to perturb calcium triggered exocytosis (Table I). Many of the drugs were tested in a dose-dependent manner at what are considered to be high concentrations for *in vitro* conditions. Most of these agents did not perturb our assay. This suggests that at the concentrations tested (far above clinical relevance) they were not membrane lytic and did not interfere with the calcium triggered fusion of docked secretory granules. By contrast, addition of tamoxifen, an antineoplastic drug whose cellular target is the estrogen receptor, to CSCs resulted in a dramatic decrease in light scattering, even in the absence of calcium (Figure 4). In calcium free buffer, addition of 100 μM tamoxifen resulted in a 35% decrease in the light scattering signal (n = 3, p = 0.0168 in paired Student t-test). This behavior, and the membrane active nature of tamoxifen (12) are both consistent with the possibility that tamoxifen is lysing membranes of sea urchin cortical granules. In contrast, taxol, an antineoplastic drug whose mechanism of action is thought to be the stabilization of microtubules, did not lyse cortical granules, but shifted the calcium dose-response curve to the right. This resulted in a requirement of higher calcium concentrations for fusion (Figure 4). A significant reduction in the amount of fusion was observed at calcium concentrations of 29 and 65 μM ($p = 0.0221$ and $p = 0.0344$ respectively, paired Student T-test). A taxol dose-response curve revealed that it was inhibitory at micromolar concentrations (Figure 5). Addition of 11.6 μM taxol resulted in a shift of the calcium concentration needed for 50% granule fusion from 21.5 to 29 μM calcium. These studies suggest that this assay has the capability of selecting drugs that affect the membrane fusion process.

DISCUSSION

We have developed a simple *in vitro* assay to screen drugs for their ability to perturb calcium triggered exocytosis. This assay uses membrane components isolated from unfertilized sea urchin eggs. Consequently, the membrane repair mechanisms present in intact cells (13) are unlikely to be available under the conditions of this assay. Thus, we have the potential of monitoring membrane effects at the level of the plasma membrane and the secretory vesicle membrane. Because eggs can be collected from gravid animals without sacrifice, this assay has an advantage that it does not require the loss of animal life. The preparation of the CSCs takes approximately 1.5 hours to prepare and sufficient material can be collected from one animal to compare samples for 4 to 5 multiwell dishes. In this study we show that the exocytotic fusion of sea urchin egg cortical granules with the egg plasma membrane can be followed by simply monitoring the turbidity (absorbance) of a preparation of egg CSCs in a 96-well microtiter dish spectrophotometer. The legitimacy of using a microtiter dish spectrophotometer to

Table I. Screening of Various Drugs for Their Ability to Perturb Calcium Triggered Exocytosis

Drug	Therapy	Target	Concentration, μM	Effect (n)
amitryptilline	anti-depressant	5-HT uptake	320	NE (3)
camptothecin	antineoplastic	DNA	30	NE (2)
compound 48/80	histaminergic	receptor activation	160	NE[a] (2)
cyclophosphamide	antineoplastic	DNA	360	NE (3)
cyclosporine A	anti-viral	immune system	85	NE (3)
cytarabine	antineoplastic	DNA	500	NE (3)
daunorubicin	antineoplastic	DNA	370	NE (3)
dideoxycytosine	anti-viral	membrane fusion	470	NE (3)
doxorubicin	antineoplastic	DNA	500	NE (3)
ellipticine	antineoplastic	DNA	40	NE (1)
fenfluramine	anti-bulemic	5-HT storage	375	NE (2)
fluoxetine	anti-depressant	5-HT uptake	320	NE (3)
methotrexate	antineoplastic	DHFR	220	NE (3)
suramin	anti-viral	immune system	70	NE (3)
tamoxifen	antineoplastic	estrogen receptor	135	lysis (5)
tamoxifen citrate	antineoplastic	estrogen receptor	180	lysis (5)
taxol	antineoplastic	cytoskeleton	120	inhibition (5)
vinblastine	antineoplastic	cytoskeleton	110	NE (3)
3′-azido-3′-deoxythymidine	AIDS	DNA	350	NE (3)

[a] While compound 48/80 had no effect on light scattering, we did notice that treated CSCs adhered to the surface of the microtiter dish wells.

measure light scattering and thus fusion activity in CSC's has been shown by comparing it with assays which directly measured the release of cortical granule contents after exocytosis. Also, direct microscopic observation correlates the number of unfused cortical granules to the degree of exocytosis.

A decrease in turbidity of the CSC suspension indicates either the fusion of granules with the plasma membrane, or lysis of granules. While a decrease in turbidity observed at sub-threshold calcium concentrations (<1 μM) is usually indicative of granule lysis, membrane fusion can occur under special circumstances in calcium free buffers (10). Also, granules may fuse with the plasma membrane, but granule content dispersal can be inhibited resulting in no change in observable light scattering (14). The addition of drugs to sea urchin CSCs might cause the lysis of granule membranes, the inhibition of granule content release, or directly effect the secretion process triggered by calcium. This multi-well assay cannot be used to distinguish these mechanistic possibilities. Other approaches should be used for investigating these mechanisms of action (5, 9, 15, 16). The procedure described in this manuscript provides a simple and rapid means of identifying and classifying membrane-active drugs.

Tamoxifen decreased the turbidity of CSCs in calcium free buffers. Tamoxifen is an amphipathic compound which is known to partition into membranes (17), subsequently al-

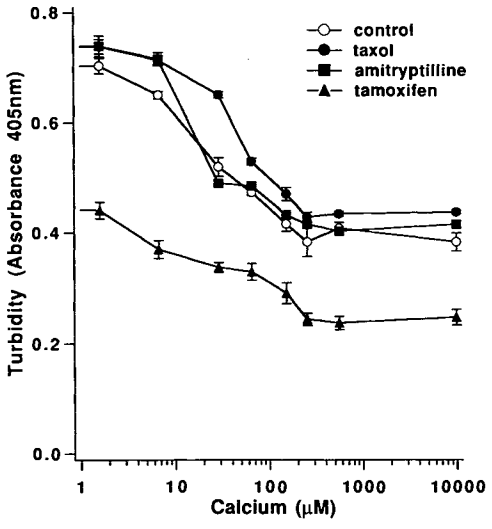

Fig. 4. Drug induced perturbation of CSC exocytotic calcium-dose response. The calcium dose-response of CSCs were determined for control CSCs treated with DMSO (1%) and CSCs treated with 100 μM taxol, amitryptilline, or tamoxifen in 1% DMSO. Each point is mean ± SEM, n=4.

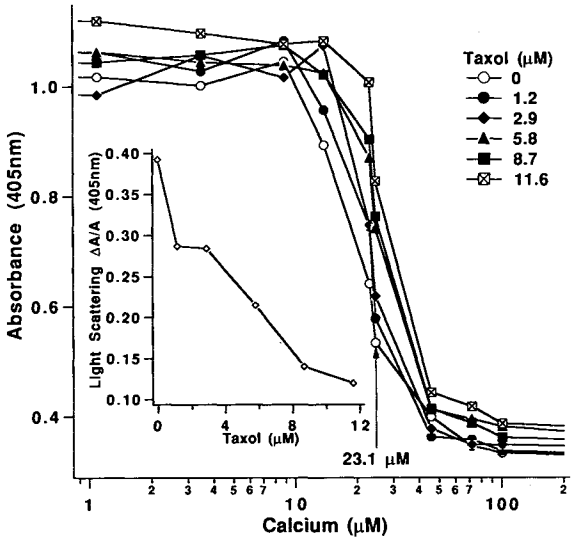

Fig. 5. Calcium dose response for cortical granule exocytosis in CSCs in the presence of different concentrations of taxol. Insert is the taxol dose response curve for data collected at 23.1μM calcium (see arrow) plotted as the change in absorbance / the initial absorbance.

tering biophysical properties of the bilayer (12). This suggests that tamoxifen can interact with membranes of intracellular organelles, a proposed intracellular receptor site (18). Thus, the assay can be used to identify membrane active drugs. Interestingly other hydrophobic drugs tested (e.g. daunarubicin) did not lyse granules, suggesting that these compounds may not perturb intracellular membranes with higher membrane curvature.

The second potential application of this assay, the identification of drugs which perturb the secretory process, was demonstrated upon treatment of the CSC preparation with taxol. Taxol inhibited calcium-dependent exocytosis, resulting in a requirement for higher calcium. Taxol is known to stabilize microtubules in sea urchin eggs (19), as it does in mammalian cells (20), so this finding is consistent with its site of action being the cytoskeleton. It also supports other studies that show that taxol can inhibit secretion from mammalian systems (21-23). It is interesting that vinblastine, a drug which destabilizes microtubules, did not perturb calcium triggered exocytosis. Consistent with this, other cytoskeletal modifying agents fail to modulate cortical granule exocytosis (24). This might reflect the important feature that in unfertilized sea urchin eggs cortical granules are already docked to the plasma membrane and fusion depends on the microtubule network being in a disassembled state (19). Alternatively, taxol inhibition of calcium triggered exocytosis might be working through a microtubule-independent mechanism. Our finding, in conjunction with reports of taxol inhibition of secretion in many different cell types raises the intriguing possibility that the antineoplastic mechanism of taxol may involve the inhibition of membrane trafficking required for rapid cell growth. Regardless of taxol's mechanism of action, we have demonstrated that this assay can be used to identify drugs which can specifically target the secretory process.

Due to the common characteristics of the fusion process (2), our assay may be used to rapidly identify drugs which perturb the mechanism of exocytosis in mammalian cells, as well as identify agents that are membrane lytic. Both plasma membrane and intracellular organelle membrane effects can be measured. This is significant as the different curvature of the intracellular membranes would have specific susceptibility to membrane active drugs (25). It can also be used to rapidly produce drug dose-response curves, and for structure-function studies (6). While it is not the aim of this assay to replace existing *in vitro* assays, it does provide a rapid screen of drugs which may contribute a new way of classifying drugs and a potential new class of drugs, i.e. site of action membrane fusion, in particular, exocytosis.

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