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Evidence that the Ability to Respond to a Calcium Stimulus in Exocytosis is Determined by the Secretory Granule Membrane: Comparison of Exocytosis of Injected Bovine Chromaffin Granule Membranes and Endogenous Cortical Granules in *Xenopus laevis* Oocytes

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SUMMARY

1. To understand better the mechanisms which govern the sensitivity of secretory vesicles to a calcium stimulus, we compared the abilities of injected chromaffin granule membranes and of endogenous cortical granules to undergo exocytosis in *Xenopus laevis* oocytes and eggs in response to cytosolic Ca^{2+}. Exocytosis of chromaffin granule membranes was detected by the appearance of dopamine-β-hydroxylase of the chromaffin granule membrane in the oocyte or egg plasma membrane. Cortical granule exocytosis was detected by release of cortical granule lectin, a soluble constituent of cortical granules, from individual cells.

2. Injected chromaffin granule membranes undergo exocytosis equally well in frog oocytes and eggs in response to a rise in cytosolic Ca^{2+} induced by incubation with ionomycin.

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3. Elevated Ca^{2+} triggered cortical granule exocytosis in eggs but not in oocytes.

4. Injected chromaffin granule membranes do not contribute factors to the oocyte that allow calcium-dependent exocytosis of the endogenous cortical granules.

5. Protein kinase C activation by phorbol esters stimulates cortical granule exocytosis in both *Xenopus laevis* oocytes and *X. laevis* eggs (Bement, W. M., and Capco, D. G., *J. Cell Biol.* 108, 885–892, 1989). Activation of protein kinase C by phorbol ester also stimulated chromaffin granule membrane exocytosis in oocytes, indicating that although cortical granules and chromaffin granule membranes differ in calcium responsiveness, PKC activation is an effective secretory stimulus for both.

6. These results suggest that structural or biochemical characteristics of the chromaffin granule membrane result in its ability to respond to a Ca^{2+} stimulus. In the oocytes, cortical granule components necessary for Ca^{2+}-dependent exocytosis may be missing, nonfunctional, or unable to couple to the Ca^{2+} stimulus and downstream events.

INTRODUCTION

In regulated exocytosis mature secretory vesicles await a stimulus that triggers the events leading to fusion with the plasma membrane. A rise in cytosolic calcium is a common stimulus but is not the only initiator of regulated exocytosis. There is considerable heterogeneity among secretory cell types regarding the primary stimulus for exocytosis and also in the subset of biochemical events which can regulate responsiveness to the primary stimulus. In rat parotid cells and guinea pig pancreatic acinar cells, elevation of cAMP is sufficient to cause secretion (Spearman and Butcher, 1989; Williams and Yule, 1993). Elevations in cytosolic Ca^{2+} also stimulate secretion in these cells. In rat basophilic leukemia cells activation of protein kinase C is necessary together with a rise in Ca^{2+} (Ozawa *et al.*, 1993). In many cells protein kinase C activation enhances Ca^{2+}-dependent secretion (Nishizuka, 1986). In bovine adrenal chromaffin cells, protein kinase C activation enhances but is not necessary for calcium-dependent exocytosis (TerBush and Holz, 1990).

The Ca^{2+} concentrations which stimulate secretion vary among cells. For example, in the giant synapse of the squid, greater than 100 μM Ca^{2+} probably stimulates exocytosis (Augustine *et al.*, 1987), whereas secretion stimulated by IP_3[3]-induced Ca^{2+} release from intracellular stores in pancreatic acinar cells is triggered by Ca^{2+} concentrations at least two orders of magnitude lower (Williams *et al.*, 1989). In bovine adrenal chromaffin cells, submicromolar Ca^{2+} is

[3] *Abbreviations used*: ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); BSA, bovine serum albumin; cAMP, cyclic AMPs; DBH, dopamine-β-hydroxylase; CGL, cortical granule lectin; CGM, Chromaffin granule membranes; FITC, fluorescein isothiocyanate: IP_3, D-myoinositol 1,4,5-trisphosphate; MBSH, modified Barth's saline with Hepes; PMSF, phenylmethylsulfonyl fluoride; DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assay; PMA, ; 4αPDD,

sufficient to trigger a small amount of exocytosis, while a Ca^{2+} concentration of 100–300 μM is necessary to stimulate maximal initial rates of secretion (Bittner and Holz, 1992).

We have recently reconstituted the exocytotic function of a mammalian secretory vesicle, chromaffin granules from bovine adrenal medullae, in *Xenopus laevis* oocytes (Scheuner *et al.*, 1992). Both intact granules and granule membranes injected into the oocytes are able to undergo exocytosis upon a rise in cytosolic Ca^{2+}. Exocytosis was demonstrated by the strong correlation between the appearance of a lumenal membrane marker of the chromaffin granule, dopamine-β-hydroxylase, on the surface of the oocyte and the release of catecholamine. An intracellular calcium concentration of approximately 18 μM free calcium (buffered by injected Ca^{2+}/EGTA) supported chromaffin granule membrane exocytosis. This calcium concentration is comparable to that which stimulates exocytosis of chromaffin granules in the chromaffin cell (Dunn and Holz, 1983; Bittner and Holz, 1992). The work did not examine whether the endogenous secretory granules of the oocyte (cortical granules) undergo exocytosis in response to Ca^{2+} stimuli that trigger chromaffin granule membrane exocytosis.

Cortical granules in oocytes are much less able than those in eggs to undergo Ca^{2+}-activated exocytosis (Iwao, 1982; Charbonneau and Grey, 1984; Elinson, 1986; Busa *et al.*, 1985; Busa and Nuccitelli, 1985; Grey *et al.*, 1974; Kubota *et al.*, 1987; Bement, 1992). Our finding that oocytes are capable of supporting chromaffin granule membrane exocytosis prompted us to investigate the role of the secretory vesicle in determining the Ca^{2+} sensitivity of exocytosis. Exocytosis of chromaffin granule membranes and of endogenous cortical granules in oocytes and eggs was compared under identical conditions. An important aspect of these studies was our ability to measure both cortical granule and chromaffin granule membrane exocytosis in the same cells. The results suggest that the secretory vesicle membrane plays a major role in the determining the Ca^{2+} dependence of exocytosis.

MATERIALS AND METHODS

Isolation of Chromaffin Granules and Purification of Chromaffin Granule Membranes. Highly purified chormaffin granule membranes were prepared from bovine adrenal medullae (Scheuner *et al.*, 1992). Medullae were homogenized in 0.3 *M* sucrose. The supernatant from an 800 g centrifugation was recentrifuged at 27,000*g* to generate a large granule fraction that was layered onto a sucrose shelf of 1.7 *M* and centrifuged at 145,000*g* for 60 min. After centrifugation, the chromaffin granule pellet was lysed in 10 m*M* Hepes (pH 7.2), 1 m*M* EDTA, and 1 m*M* PMSF. The suspension was frozen, thawed, pelleted, resuspended in lysis buffer, and again pelleted. This final pellet was resuspended in 10 m*M* Hepes, pH 7.2, aliquoted, and stored at −70°C. For microinjection, chromaffin granule membranes were thawed, diluted to 2.8 mg protein/ml, and lightly sonicated.

Manipulation of Oocytes. Oocytes were obtained from adult female

Xenopus laevis purchased from Xenipus I, Ann Arbor, Michigan. Ovarian tissue was removed surgically. Oocytes were pulled from the tissue in MBSH (modified Barth's saline with Hepes) supplemented with 2.5 m*M* pyruvate and 50 μg/ml gentamycin sulfate (Wittaker Bioproducts). Oocytes were defolliculated with Type IV collagenase (Sigma Co., St Louis, MO), rinsed with MBSH, and incubated overnight at 19°C in L-15 Medium (Wittaker Bioproducts) diluted to 200 m0sm, plus 50 μg/ml gentamycin sulfate. Oocytes and eggs were transferred to gentamycin-free MBSH prior to use.

Oocytes or eggs were pressure-injected with suspensions of chromaffin granule membranes or buffers (Scheuner *et al.*, 1992). The oocytes were quickly washed with MBSH after injection and, within 2 min, bathed in 300 μl (unless otherwise indicated) of the indicated incubation solution at room temperature. Oocytes and eggs were cooled on ice to stop exocytosis. As a control for the immunocytochemistry, injections were performed with buffer (0.05 μl) without chromaffin granule membranes. Buffer-injected cells were subjected to equivalent procedures postinjection.

Xenopus laevis eggs were prepared from oocytes defolliculated as above, treated *in vitro* with 10 μg/ml progesterone (Sigma Co., St. Louis, MO), and incubated overnight in L-15 medium. Eggs were injected and washed in O–Ca^{2+} MBSH. We have observed that the oocytes or eggs of some frogs (approximately 40%) do not support chromaffin granule exocytosis. We are not able to predict competency based on the visual appearance of the oocytes, although we select only healthy-appearing oocytes prepared according to the above procedures.

Surface Dopamine-β-Hydroxylase Immunocytochemistry (Detection of Chromaffin Granule Membrane Exocytosis). Immunocytochemistry was performed as previously described except that the fixation step was done after the antibody incubations and washings (Scheuner *et al.*, 1992). Oocytes or eggs were incubated with rabbit anti-dopamine-β-hydroxylase (DBH) antibody, washed, and then incubated with FITC-labeled goat, anti-rabbit antibody. Oocytes or eggs were then fixed in 4% paraformaldehyde, washed, and placed in *p*-phenylenediamine mounting medium for viewing. No significant immunofluorescence appeared on the surface of cells injected without chromaffin granule membranes. A positive response is defined as easily detectable, punctate or patchy fluorescence covering 10% or more of the surface area. Many cells displayed dopamine-β-hydroxylase immunofluorescence covering up to 50% of the total surface area.

Cortical Granule Lectin Release from Individual Oocytes and Eggs. Cortical granule lectin release was measured from oocytes or eggs via ELISA. When lectin release was measured from EGTA (10 m*M* EGTA, 10 m*M* Hepes, pH 7.2) or Ca^{2+}/EGTA (9.93 m*M* $CaCl_2$, 10 m*M* EGTA, 10 m*M* Hepes, pH 7.2, 10μ*M* free calcium)-injected cells, the cells were injected with 0.05 μl of the solutions while resting in a pool of 60 μl of MBSH. Where indicated, chromaffin granule membranes were suspended in these solutions for coinjection into the oocytes. The medium bathing the cells was recovered after the incubation period and lectin release was measured directly. Under these conditions, more than 7 ng of cortical granule lectin release is readily detectable. The total lectin content of the

cell is approximately 395 ng (Monk and Hedrick, 1986); thus, secretion of 1.8% or more of the total cortical granule lectin could be detected. Because of the large lectin content in samples obtained from stimulated eggs, these samples were diluted 5 to 10-fold for assay.

When cortical granule exocytosis was measured from oocytes or eggs incubated in the presence or absence of 10 μM ionomycin (Table II), the incubation solution was diluted 8- to 10-fold to minimize the inhibitory effect of ionomycin on the assay. The lectin content was compared to standard lectin samples containing an equivalent amount of ionomycin. Secretion of 25 ng (6% of the total lectin) is the minimum amount of lectin accurately detected under these conditions.

Wells in a 96-well plate were washed twice with 85% ethanol. Samples (38–48 μl) were added to wells containing concentrated coating buffer. The final composition was 1 M Na_2/CO_3, 1 M $NaHCO_3$, pH 9.6 (60 μl). Lectin adsorption was accomplished by overnight incubation at 4°C. Wells were washed thrice with wash buffer (10 mM Tris base, 150 mM NaCl, 0.05% Tween 20, pH 7.5) and blocked by incubation in wash buffer with 2.5 mg/ml BSA for 3.5 hr at room temperature. Goat anti-cortical granule lectin antibody (1:1000 dilution in wash buffer) was incubated with the wells overnight at 4°C or 1 hr at room temperature. Wells were washed three times with wash buffer and incubated for 1 hr at room temperature with 1:1000 horseradish peroxidase-conjugated secondary antibody (Sigma Co., St. Louis, MO). The wells were washed and the peroxidase reaction was developed using a modified ABTS method (Childs and Bardsley, 1975; Shindler *et al.*, 1976; Makinen and Tenovuo, 1982; Engvall, 1980). The substrate buffer contained 50 mM citric acid pH 4.0, 0.23 mM H_2O_2, 0.54 mM ABTS. The absorbance was measured at 404 nm.

RESULTS

Xenopus laevis Eggs Support Chromaffin Granule Membrane Exocytosis

In a previous study we found that chromaffin granule membranes were able to undergo calcium-activated exocytosis in approximately 40% of the injected oocytes. The endogenous cortical granules in eggs are much better able than those in oocytes to undergo calcium activated exocytosis (Iwao, 1982; Charbonneau and Grey, 1984; Elinson, 1986; Busa *et al.*, 1985; Busa and Nuccitelli, 1985; Grey *et al.*, 1974; Kubota *et al.*, 1987; Bement, 1992). If this difference is caused by differences in cytosolic or other factors not associated with the secretory vesicle, then the ability of injected chromaffin granule membranes to undergo exocytosis may be greater in eggs than in oocytes. To investigate this possibility, eggs were microinjected with chromaffin granule membranes and then incubated in standard MBSH in the absence or presence of ionomycin (10 μM). Ionomycin caused the surface appearance of DBH in 10 of 30 eggs (33%) (Table I). Incubation of eggs in the absence of ionomycin caused the surface appearance of DBH in only 1 of 36 eggs (3%). Figure 1B demonstrates a typical response to

Table I. Ionomycin Induces Chromaffin Granule Membrane Exocytosis in *Xenopus laevis* Eggs[a]

	Number of eggs		% expressing surface DBH
	expressing surface DBH	Total injected	
0 μM ionomycin (1% DMSO)	1	36	3
10 μM ionomycin	10	30	33

[a] Eggs were injected in 0-Ca^{2+} MBSH buffer with chromaffin granule membranes (0.14 μg protein) and incubated for 20 min in the absence or presence of ionomycin in standard MBSH. The eggs were then processed for surface dopamine-β-hydroxylase (DBH) immunocytochemistry and evaluated individually for DBH immunofluorescence. The positive DBH responses reflect strong immunofluorescence on >10% of the cell surface. Data from two experiments were combined.

ionomycin. A speckled and sometimes aggregated DBH-specific immunofluorescence was apparent over approximately 40% of the surface area of the egg. In comparison, a representative egg incubated in the absence of ionomycin displayed virtiually no surface immunofluorescence (Fig. 1A).

These results are comparable to those obtained previously in frog oocytes and indicate that there is little difference between the capacity of eggs and oocytes to support calcium-activated exocytosis of injected chromaffin granule membranes.

Conditions Which Support Chromaffin Granule Membrane Exocytosis in *Xenopus laevis* Oocytes Do Not Elicit the Cortical Granule Reaction

To investigate the ability of endogenous cortical granules to undergo exocytosis under the conditions of our experiments, we measured the release from individual oocytes of a soluble protein stored within cortical granules, cortical granule lectin. The lectin is a specific marker for cortical granule exocytosis (Nishihara *et al.*, 1986; Monk and Hendrick, 1986). The ELISA we established could accurately measure secretion of lectin from individual cells stimulated with ionomycin with a sensitivity of greater than approximately 6% of the total cortical granule lectin in an oocyte or egg (see Materials and Methods). Ionomycin stimulated the cortical granule reaction in eggs but not from oocytes (Table II). Every egg secreted cortical granule lectin. This is the first reported measurement of calcium-regulated cortical lectin secretion from individual cells. The amount of calcium-triggered lectin release from individual eggs is consistent with previous measurements of lectin release from groups of 100 eggs (Monk and Hedrick, 1986). None of the oocytes released a significant amount of cortical granule lectin in response to ionomycin. Therefore, it is unlikely that the oocytes supporting chromaffin granule membrane exocytosis represent a subpopulation of cells that are also capable of supporting exocytosis of their endogenous secretory vesicles. The calcium stimulus provided by ionomycin was sufficient in eggs but not in oocytes to activate cortical granule exocytosis.

It was possible that injected chromaffin granule membranes supplied the oocyte with components which not only permitted the injected membranes to undergo exocytosis but also conferred to the endogenous cortical granules the

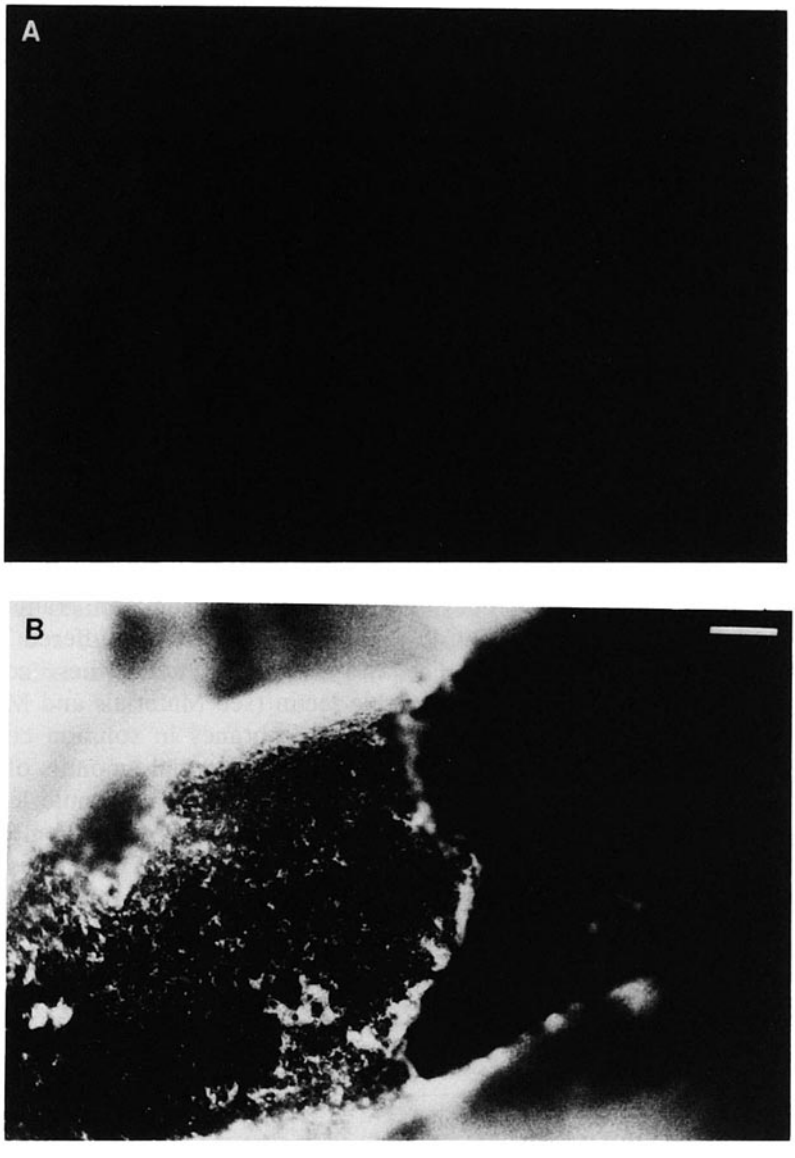

Fig. 1. Ionomycin induces chromaffin granule membrane exocytosis in *Xenopus laevis* eggs. Eggs were injected in Ca^{2+}-free MBSH buffer and then incubated for 20 min in the absence or presence of 10 μM ionomycin. The eggs were then processed for surface dopamine-β-hydroxylase immunocytochemistry. (A) Egg injected with membranes and incubated for 20 min in buffer alone. DBH was not expressed on the surface of the egg. (B) Egg injected with membranes and incubated in ionomycin. DBH was expressed over 40% of the egg surface. Both micrographs depicts regions of the animal hemisphere of the egg. The scale bar represents 50 μm and applies to both A and B.

Table II. Ionomycin Induces Cortical Granule Lectin Release from Eggs but Not from Oocytes[a]

	Total ng CGL release/cell ± SE
Oocytes	
−Ionomycin	ND ($n = 16$)
+Ionomycin (10 μM)	27 ± 5 ($n = 16$)
Eggs	
−Ionomycin	ND ($n = 21$)
+Ionomycin (10 μM)	291 ± 26 ($n = 21$)

[a] Oocytes or eggs were incubated individually in 50–60 μl of MBSH in the absence or presence of 10 μM ionomycin for 20 min. Cortical granule lectin (CGL) release from each cell was determined. Ionomycin stimulated cortical granule secretion from 100% of the eggs and from none of the oocytes. *n*, number of cells; ND, not detected.

ability to undergo exocytosis. This was investigated by measuring cortical granule lectin release from individual oocytes injected with chromaffin granule membranes and either EGTA or a Ca^{2+}/EGTA solution that buffered Ca^{2+} at approximately 18 μM (Table III). The assay sensitivity under these conditions was 1.8% secretion of total cortical granule lectin (see Materials and Methods). Every egg injected with chromaffin granule membranes in solution containing 18 μM free Ca^{2+} (buffered with EGTA) secreted substantial amounts of cortical granule lectin (286 ± 63 ng; $n = 9$ eggs) as expected. Cortical granule lectin was not released from any of the oocytes injected with chromaffin granule membranes together with either EGTA or Ca^{2+}/EGTA (18 μM free Ca^{2+}). Despite the absence of cortical granule lectin release, some of the oocytes were capable of

Table III. Chromaffin Granule Membrane Exocytosis Occurs Independently of Cortical Granule Exocytosis in *Xenopus laevis* Oocytes[a]

	Positive DBH immunocytochemistry	CGL release (ng) ± SE
Injected with CGM/10 mM EGTA	0/9	1.2 ± 0.5
Injected with CGM/Ca^{2+}/EGTA buffered to 18 μM free calcium	3/7	2.0 ± 0.6

[a] Oocytes were injected with chromaffin granule membranes (CGM; 0.14 μg protein) in 10 mM EGTA or in 10 mM EGTA with calcium buffered to 18 μM free calcium. Cortical granule lectin (CGL) release was measured from inidividual oocytes 15 min after injection. These same cells were then processed for dopamine-β-hydroxylase immunocytochemistry and evaluated for surface immunofluorescence. The positive DBH responses reflect strong immunofluorescence on >10% of the cell surface. Activation of eggs by injection of the Ca^{2+}/EGTA buffer resulted in cortical granule lectin release from 100% of the eggs (286 ± 63 ng of CGL per egg; $n = 9$).

chromaffin granule membrane exocytosis (three of seven oocytes). Thus the calcium stimulus triggered chromaffin granule membrane exocytosis in oocytes in which cortical granule exocytosis did not occur.

The experiments indicate that the *Xenopus laevis* oocyte and egg are equally competent to support chromaffin granule membrane exocytosis despite differences in the ability of endogenous cortical granules to undergo exocytosis.

Phorbol Ester-Treated Oocytes Support Chromaffin Granule Membrane Exocytosis

Cortical granules in oocytes are not absolutely blocked from undergoing exocytosis. Activation of protein kinase C by phorbol ester is sufficient to cause full cortical granule exocytosis (Bement and Capco, 1989). We investigated whether activation of oocyte protein kinase C would be sufficient to elicit chromaffin granule membrane exocytosis in injected oocytes. PMA ($0.6\,\mu M$) caused strong dopamine-β-hydroxylase responses in 10 of 31 cells (Table IV and Fig. 2). This concentration of PMA is optimal in stimulating cortical granule exocytosis (Bement and Capco, 1989). Inactive phorbol ester did not stimulate chromaffin granule membrane exocytosis. Thus, despite their differing abilities to respond to a calcium stimulus, cortical granules and chromaffin granule membranes are alike in their responsiveness to protein kinase C activation in the oocyte.

DISCUSSION

The focus of the present study was to investigate the cellular components which determine Ca^{2+} sensitivity in regulated exocytosis. In a previous study we demonstrated that both chromaffin granule membranes and intact chromaffin granules injected into *Xenopus laevis* oocytes undergo Ca^{2+}-dependent

Table IV. Active Phorbol Ester Induces Chromaffin Granule Exocytosis in *Xenopus laevis* Oocytes[a]

	Number of Oocytes		% expressing surface DBH
	Expressing surface DBH	Total injected	
Buffer	1	14	7
$0.6\,\mu M$ 4αPDD	0	16	0
$0.6\,\mu M$ PMA	10	31	32

[a] Oocytes were preincubated for 13 min in MBSH buffer alone or buffer containing 4αPDD or PMA. The oocytes were then injected in MBSH buffer with chromaffin granule membranes ($0.14\,\mu g$ protein). Treatment with buffer, 4αPDD, or PMA was continued for an additional 15 min. The oocytes were then processed for surface DBH immunocytochemistry. The positive responses reported reflect strong immunofluorescence on >10% of the cell surface. No immunofluorescence was displayed on cells injected with buffer without chromaffin granule membranes and treated in an equivalent manner. The data are from two combined experiments.

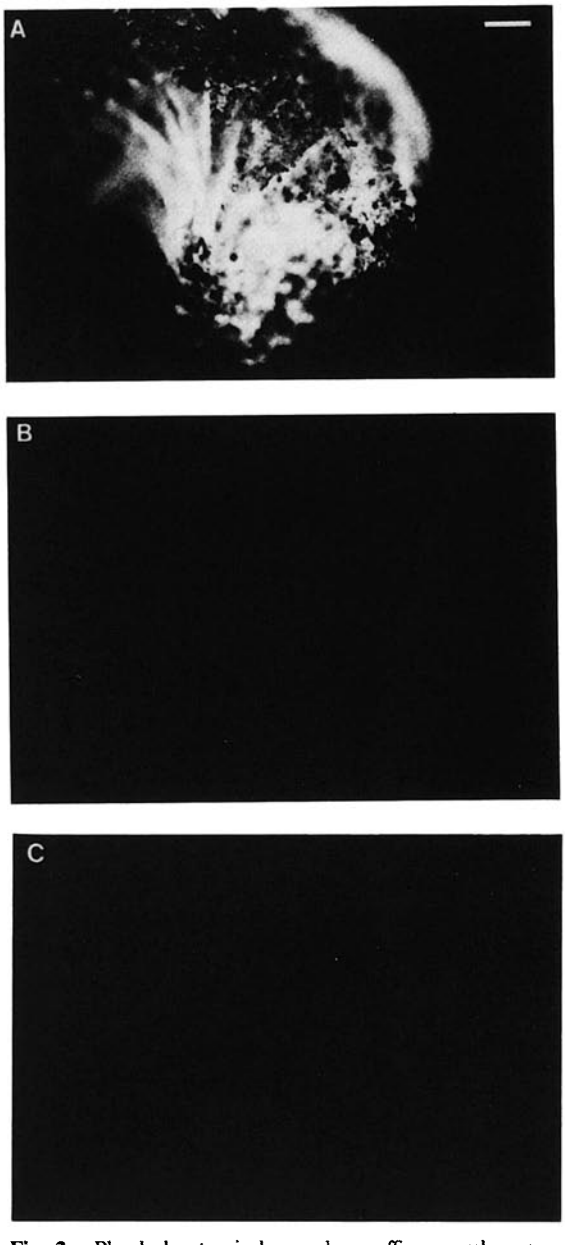

Fig. 2. Phorbol ester induces chromaffin granule membrane exocytosis in *Xenopus laevis* oocytes. Oocytes were preincubated for 13 min in MBSH buffer alone or buffer containing 4-αPDD or PMA. The oocytes were incubated in standard MBSH buffer during injection with chromaffin granule membranes. Ooctyes were then incubated in the continuing absence or presence of 4-αPDD or PMA for 15 min and processed for surface dopamine-β-hydroxylase immunocytochemistry. (A) Oocyte stimulated with PMA. DBH was expressed over approximately 50% of the surface. (B) Oocyte incubated with 4-αPDD. There was no response. (C) Oocyte incubated without phorbol ester. There was no response. The scale bar represents 100 μm and applies to A–C.

exocytosis with the oocytes plasma membrane. In this study we compared the abilities of injected chromaffin granule membranes and of endogenous cortical granules to undergo exocytosis in *Xenopus laevis* oocytes and eggs in response to a rise in cytosolic Ca^{2+}.

The following observations suggest that the secretory vesicle membrane determines the ability of the membrane to undergo Ca^{2+}-dependent exocytosis.

(1) Injected chromaffin granule membranes, but not endogenous cortical granules, are able to undergo exocytosis in frog oocytes. However, oocyte cortical granules are competent to undergo exocytosis without a Ca^{2+} signal upon activation of PKC. Thus, the oocyte has the capacity to support Ca^{2+}-regulated exocytosis, but it must be presented with a vesicle that can respond to a Ca^{2+} signal.

(2) Injected chromaffin granule membranes undergo exocytosis no better in eggs than in oocytes despite the much greater ability of endogenous cortical granules to undergo exocytosis in eggs than in oocytes. Thus, cellular changes which confer increased Ca^{2+} sensitivity to the endogenous granules upon maturation of the oocytes to the egg do not enhance chromaffin granule membrane exocytosis.

(3) The ability of chromaffin granule membranes to respond to a Ca^{2+} stimulus is not transferred to endogenous cortical granules of oocytes within the 20 min between injection of the chromaffin granule membranes and cooling of the oocyte. Thus, within the time frame of the experiments, Ca^{2+}- responsiveness of the chromaffin granule membranes is a nontransferable property of the chromaffin granule membrane.

We conclude that the difference in the abilities of chromaffin granule membranes and cortical granules to respond to a Ca^{2+} signal in the oocyte is likely to reflect either differences in secretory vesicle membrane components necessary for the recognition of a Ca^{2+} stimulus or differences in coupling to events downstream from a Ca^{2+} stimulus. For example, in the oocyte, cortical granule components necessary for Ca^{2+}-dependent exocytosis may be missing, nonfunctional, or unable to couple to the Ca^{2+} stimulus and downstream events (Bement and Capco, 1990). It seems unlikely that the smaller size of the chromaffin granule (0.3-μm diameter) than the cortical granule (1-μm diameter) permits the chromaffin granule or chromaffin granule membrane to interact better with the oocyte plasma membrane, since most of the cortical granules are within 200 nm of the plasma membrane (Bement and Capco, 1990). However, it is possible that the cortical granule is locked in position by the cytoskeleton so that it cannot contact the plasma membrane and respond to the Ca^{2+} stimulus. Thus, the cortical granule membrane may prevent its exocytosis by specific interactions with other cellular components.

Our analysis has not identified the biochemical components responsible for the ability of chromaffin granules to undergo calcium-dependent exocytosis in the oocyte. This study has also not determined whether the Ca^{2+} sensor is on the secretory granule membrane or resides elsewhere in the cell. However, work in other laboratories has implicated two calcium-sensitive, lipid-binding proteins in exocytosis-calpactin I (Ali *et al.*, 1989; Sarafian *et al.*, 1991) and synaptotagmin

(Brose *et al.*, 1992; Elferink *et al.*, 1993; Bommert *et al.*, 1993). Rabphilin3a, a binding protein for the *ras*-like protein Rab3a, has also been suggested to be a calcium sensor in exocytosis (Shirataki *et al.*, 1993; Kishida *et al.*, 1993; McKiernan *et al.*, 1993). Work is continuing to determine the role of these proteins in exocytosis.

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REFERENCES

Ali, S. M., Geisow, M. J., and Burgoyne, R. D. (1989). A role for calpactin in calcium-dependent exocytosis in adrenal chromaffin cells. *Nature* **340:**313–315.

Augustine, G. J., Charlton, M. P., and Smith, S. J. (1987). Calcium action in synaptic transmitter release. *Annu. Rev. Neurosci.* **10:**633–693.

Bement, W. M. (1992) Signal transduction by calcium and protein kinase C during egg activation. *J. Exp. Zool.* **263:**382–397.

Bement, W. M., and Capco, D. G. (1989). Activators of protein kinase C trigger cortical granule exocytosis, cortical contraction, and cleavage furrow formation in *Xenopus laevis* oocytes and eggs. *J. Cell Biol.* **108:**885–892.

Bement, W. M., and Capco, D. G. (1990). Transformation of the amphibian oocyte into the egg: Strudtural and biochemical events. *J. Electron Microsc. Techn.* **16:**202–234.

Bittner, M. A., and Holz, R. W. (1992). Kinetic analysis of secretion from permeabilized adrenal chromaffin cells reveals distinct components. *J. Biol. Chem.* **267:**16219–16225.

Bommert, K., Charlton, M. P., DeBello, W. M., Chin, G. J., Betz, H., and Augustine, G. J. (1993). Inhibition of neurotransmitter release by C2-domain peptides implicates synaptotagmin in exocytosis. *Nature* **363:**163–165.

Brose, N., Petrenko, A. G., Südhof, T. C., and Jahn, R. (1992). Synaptotagmin: A calcium sensor on the synaptic vesicle surface. *Science* **256:**1021–1025.

Busa, W. B., and Nuccitelli, R. (1985). An elevated free cytosolic Ca^{2+} wave follows fertilization in eggs of the frog, *Xenopus laevis*. *J. Cell Biol.* **100:**1325–1329.

Busa, W. B., Ferguson, J. E., Joseph, S. K., Williamson, J. R., and Nuccitelli, R. (1985). Activation of frog (*Xenopus laevis*) eggs by inositol trisphosphate. I. Characterization of Ca^{2+} release from intracellular stores. *J. Cell Biol.* **101:**677–682.

Charbonneau, M., and Grey, R. D. (1984). The onset of activation responsiveness during maturation coincides with the formation of the cortical endoplasmic reticulum in oocytes of *Xenopus laevis*. *Dev. Biol.* **102:**90–97.

Childs, R. E., and Bardsley, W. G. (1975). The steady-state kinetics of peroxidase with 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) as chromogen. *Biochem. J.* **145:**93–103.

Dunn, L. A., and Holz, R. W. (1983). Catecholamine secretion from digitonin-treated adrenal medullary chromaffin cells. *J. Biol. Chem.* **258:**4989–4993.

Elferink, L. A., Peterson, M. R., and Scheller, R. H. (1993). A role for synaptotagmin (p65) in regulated exocytosis. *Cell* **72:**153–159.

Elinson, R. P. (1986). Fertilization in amphibians: The ancestry of the block to polyspermy. *Int. Rev. Cytol.* **101**:59–100.

Engvall, E. (1980). Enzyme immunoassay ELISA and EMIT. *Methods Enzymol.* **70**:419–438.

Grey, R. D., Wolf, D. P., and Hedrick, J. L. (1974). Formation and structure of the fertilization envelope in *Xenopus laevis*. *Dev. Biol.* **36**:44–61.

Iwao, Y. (1982). Differential emergence of cortical granule breakdown and electrophysiological responses during meiotic maturation of toad oocytes. *Dev. Growth Differ.* **24**:467–477.

Kishida, S., Shiratiki, H., Sasaki, T., Kato, M., Kaibuchi, K., and Takai, Y. (1993). *Rab3a* GTPase-activating protein-inhibiting activity of Rabphilin-3a, a putative *Rab3A* target protein. *J. Biol. Chem.* **268**:22259–22261.

Kubota, H. Y., Yoshimoto, Y., Yoneda, M., and Hiramoto, Y. (1987). Free calcium wave upon activation in Xenopus eggs. *Dev. Biol.* **119**:129–136.

Makinen, K. K., and Tenovuo, J. (1982). Observations on the use of guaiacol and 2,2′-azino-di(3-ethylbenzhtiazoline-6-sulfonic acid) as peroxidase substrates. *Anal. Biochem.* **126**:100–108.

McKiernan, C. J., Brondyk, W. H., and Macara, I. G. (1993). The Rab3A GTPase interacts with multiple factors through the same effector domain mutational analysis of cross-linking of Rab3A to a putative target protein. *J. Biol Chem.* **268**:2449–24452.

Monk, B. C., and Hedrick, J. L. (1986). The cortical reaction in *Xenopus laevis* eggs: Cortical granule lectin release as determined by radioimmunoassay. *Zool. Sci.* **3**:459–466.

Nishihara, T., Wyrick, R. E., Working, P. K., Chen, Y.-H., and Hedrick, J. L. (1986). Isolation and characterization of a lectin from the cortical granules of *Xenopus laevis* eggs. *Biochemistry* **25**:6013–6020.

Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. *Science* **233**:305–312.

Ozawa, K., Szallasi, Z., Kazanietz, M. G., Blumberg, P. M., Mischak, H., Mushinski, J. F., and Beaven, M. A. (1993). Ca^{2+}-dependent and Ca^{2+}-independent isozymes of protein kinase C mediate exocytosis in antigen-stimulated rat basophilic RBL-2H3 cells. *J. Biol. Chem.* **268**:1749–1756.

Sarafian, T., Pradel, L.-A., Henry, J.-P., Aunis, D., and Bader, M.-F. (1991). The participation of annexin II (calpactin I) in calcium-evoked exocytosis requires protein kinase C. *J. Cell Biol.* **114**:1135–1147.

Scheuner, D., Logsdon, C. D., and Holz, R. W. (1992). Bovine chromaffin granule membranes undergo Ca^{2+}-regulated exocytosis in frog oocytes. *J. Cell Biol.* **116**:359–365.

Shindler, J. S., Childs, R. E., and Bardsley, W. G. (1976). Peroxidase from human cervical mucus, the isolation and characterisation. *Eur. J. Biochem.* **65**:325–331.

Shirataki, H., Kaibuchi, K., Sakoda, T., Kishida, S., Yamaguchi, T., Wada, K., Miyazaki, M., and Takai, Y. (1993). Rabphilin-3A, a putative target protein for smg p25A/rab3A p25 small GTP-binding protein related to synaptotagmin. *Mol. Cell. Biol.* **13**:2061–2068.

Spearman, T. N., and Butcher, F. R. (1989). Cellular regulation of amylase secretion by the paratid gland. In *Handbook of Physiology, Section 6. The Gastrointestinal System* (S. G. Schultz, Ed.), American Physiological Society, Bethesda, MD, pp. 63–77.

TerBush, D. R., and Holz, R. W. (1990). Activation of protein kinase C is not required for exocytosis from bovine adrenal chromaffin cells: The effects of protein kinase C(19-31), Ca/CaM kinase II(291-317), and staurosporin. *J. Biol. Chem.* **265**:21179–21184.

Williams, J. A., and Yule, D. I. (1993). Stimulus-secretion coupling in pancreatic acinar cells. In *The Pancreas: Biology, Pathobiology and Disease*, 2nd ed (V. L. W. Go, Ed), Raven Press, New York, pp. 167–189.

Willams, J. A., Burham, D. B., and Hootman, S. R. (1989). Cellular regulation of pancreatic secretion. In *Handbook of Physiology, Section 6. The Gastrointestinal System* (S. G. Schulz, Ed.), American Physiological Society, Bethesda, MD, pp. 419–441.