Bovine Chromaffin Granule Membranes Undergo Ca^{2+}-regulated Exocytosis in Frog Oocytes

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Abstract. We have devised a new method that permits the investigation of exogenous secretory vesicle function using frog oocytes and bovine chromaffin granules, the secretory vesicles from adrenal chromaffin cells. Highly purified chromaffin granule membranes were injected into *Xenopus laevis* oocytes. Exocytosis was detected by the appearance of dopamine-β-hydroxylase of the chromaffin granule membrane in the oocyte plasma membrane. The appearance of dopamine-β-hydroxylase on the oocyte surface was strongly Ca^{2+}-dependent and was stimulated by coinjection of the chromaffin granule membranes with $InsP_3$ or Ca^{2+}/EGTA buffer (18 μM free Ca^{2+}) or by incubation of the injected oocytes in medium containing the Ca^{2+} ionophore ionomycin. Similar experiments were performed with a subcellular fraction from cultured chromaffin cells enriched with [^{3}H]norepinephrine-containing chromaffin granules. Because the release of [^{3}H]norepinephrine was strongly correlated with the appearance of dopamine-β-hydroxylase on the oocyte surface, it is likely that intact chromaffin granules and chromaffin granule membranes undergo exocytosis in the oocyte. Thus, the secretory vesicle membrane without normal vesicle contents is competent to undergo the sequence of events leading to exocytosis. Furthermore, the interchangeability of mammalian and amphibian components suggests substantial biochemical conservation of the regulated exocytotic pathway during the evolutionary progression from amphibians to mammals.

A variety of techniques have been developed to study the physiological and biochemical basis for regulated exocytosis from cells and neurons. The use of permeabilized cells (2, 6, 15, 24) and patch clamp techniques (19) has permitted direct control of the intracellular milieu and has greatly facilitated the analysis of factors which regulate exocytosis. However, it has not been possible to manipulate the secretory vesicle apart from the cell. In the present study we have injected bovine chromaffin granules, the secretory vesicles from adrenal chromaffin cells, into frog oocytes. We demonstrate that the injected chromaffin granules undergo Ca^{2+}-triggered exocytosis. This approach may allow the identification of the components of the secretory vesicle necessary for regulated secretion.

Frog oocytes and eggs are secretory cells. Endogenous cortical granules in frog eggs undergo exocytosis when the cytoplasmic Ca^{2+} concentration is elevated by fertilization (3), incubation with Ca^{2+} ionophore (4, 5), injection with Ins^1-(1,4,5)P_3 (4), mechanical stimulation (5, 8, 17), or expression and activation of exogenous plasma membrane receptors (14). Manipulations to increase cytoplasmic Ca^{2+} are less able to cause cortical granule exocytosis in frog oocytes (immature eggs) (5, 7, 12). However, direct microinjection of Ca^{2+} (9) or high concentrations of A23187 (12) can induce cortical granule exocytosis.

Exocytosis of chromaffin granules was monitored by two techniques. Dopamine-β-hydroxylase (DBH)[1] is both soluble within the chromaffin granule and bound as an integral membrane protein in the chromaffin granule membrane (27). Soluble DBH is released into the medium upon exocytosis (23). The antigenic sites of membrane-bound DBH are intragranular and become exposed on the chromaffin cell surface upon exocytosis (21, 22). If injected chromaffin granule membranes undergo exocytosis in the frog oocyte, then membrane-bound DBH should be exposed on the oocyte surface. Purified chromaffin granule membranes or intact granules were injected into *Xenopus laevis* oocytes and the oocytes stimulated with manipulations which increase cytoplasmic Ca^{2+}. The appearance of DBH on the surface of the oocyte was measured with an immunocytochemical technique. The release of [^{3}H]norepinephrine from oocytes injected with [^{3}H]norepinephrine-containing intact granules was also used as a measure of exocytosis.

Materials and Methods

Isolation of a P_2 Fraction Containing Intact Chromaffin Granules and Purification of Chromaffin Granule Membranes

Fresh bovine adrenal medullae were homogenized in 0.3 M sucrose, 10 mM

1. *Abbreviation used in this paper*: DBH, dopamine-β-hydroxylase.

Hepes (pH 7.2), 1 mM EDTA, and 1 mM PMSF. The supernatant from a 800 $g \times 10$ min centrifugation was recentrifuged at 27,000 $g \times 10$ min to generate a large granule fraction (P_2 fraction). This was layered onto a discontinuous sucrose gradient containing 0.8, 1.3, and 1.7 M sucrose. All the sucrose solutions contained 1 mM EDTA and 5 mM Hepes, pH 7.2 (no PMSF). The tubes were centrifuged at 145,000 $g \times 60$ min. The pellet at the bottom of the tube consisted of highly purified chromaffin granules. An extensive analysis using a variety of enzymatic markers for various subcellular organelles and catecholamine for intact chromaffin granules indicated that chromaffin granules were purified five to eightfold with virtually no mitochondrial, lysosomal, or plasma membrane contamination. The chromaffin granules in the pellet were lysed in 10 mM Hepes, 0.2 mM EDTA, pH 7.2, frozen, and thawed. The chromaffin granule membranes were pelleted by centrifugation at 30,000 $g \times 20$ min and resuspended in lysis buffer. They were again centrifuged at 30,000 $g \times 20$ min and the chromaffin granule membrane pellet was resuspended (5 mg protein/ml) in 10 mM Hepes, pH 7.2 (no EDTA), aliquoted and stored at −70°C.

In some experiments (e.g., see Fig. 1 *E*) the P_2 fraction from fresh adrenal medullae was lysed in 1 mM DTT and 10 mM Hepes, pH 7.2, and the resulting membranes were washed twice by centrifugation and resuspension (final concentration 12 mg protein/ml). A P_2 fraction was also prepared without osmotic lysis from primary cultures of purified, bovine chromaffin cells (25 million cells) for injection of intact granules.

Manipulation of Oocytes

Oocytes (1.1–1.3 mm diameter) were obtained from female *Xenopus laevis*. The follicular layer was removed after a 1–2-h incubation at room temperature with 2 mg/ml Type IV collagenase (Sigma Chemical Co., St Louis, MO) in solution containing 82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.6. Oocytes were incubated overnight at 19°C in MBSH (88 mM NaCl, 1 mM KCl, 2.4 mM $NaCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 2.55 mM Na pyruvate, and 10 mM Hepes, pH 7.4) with 1 mg/ml BSA. Oocytes were injected with suspensions of chromaffin granule membranes or lysed or intact P_2 fractions (0.05 μl) using 20–25 μm tip diameter, beveled injection pipets. As a control for the immunocytochemistry, injections were performed with buffer without chromaffin granule membranes or a P_2 fraction.

Immunocytochemistry

Immediately after an experiment, oocytes were fixed on ice (4% paraformaldehyde, 0.1 M Na cacodylate, pH 7, 30 min), washed thrice with ice cold MBSH with 5 mg/ml BSA. All subsequent incubations were on ice. Oocytes were incubated with rabbit anti-DBH antibody (1:150 dilution of serum) for 0.5–16 h in MBSH with 5 mg/ml BSA. Oocytes were washed thrice for 30 min each and then incubated for 2 h with FITC-labeled goat, antirabbit antibody. Oocytes were washed thrice for 15 min each, placed in *p*-phenylenediamine mounting medium (10 mg/ml *p*-phenylenediamine into PBS, pH 9.0/glycerol, 1:9) and viewed with a Nikon Diaphot inverted fluorescent microscope at 100–400 times magnification. This same protocol was used to demonstrate the incorporation of DBH into chromaffin cell plasma membranes upon exocytosis (Scheuner, D., and R. W. Holz, unpublished observations). Fixation does not permit antibody to enter the oocyte and interact with intracellular DBH. Oocytes that were incubated with primary and secondary antibodies at 4°C before fixation showed the same pattern of surface expression of DBH as oocytes incubated with antibodies after fixation.

Release of [^{3}H]Norepinephrine

[^{3}H]Norepinephrine-labeled chromaffin granules of high specific activity were prepared from cultures of purified chromaffin cells (25 million cells/10-cm diameter dish) incubated overnight with 10 μCi/ml [^{3}H]norepinephrine (12 C_i/mmol). [^{3}H]Norepinephrine is specifically taken up into intracellular chromaffin granules (13). A subcellular fraction (P_2) enriched in chromaffin granules was prepared without osmotic lysis. This method proved to be much more efficient for loading chromaffin granules with [^{3}H]norepinephrine than incubation of purified granules with [^{3}H]norepinephrine in the presence of MgATP (20). Isolated chromaffin granules are prepared in and are stable in 340 mOsM solution; they lyse at the normal oocyte osmolality of 200 mOsM. Intact chromaffin granules were injected into oocytes preequilibrated for 1 h with 340 mOsM MBSH (MBSH with 125 mM sucrose). At 340 mOs the oocytes were somewhat shrunken and the surface depressed and folded in some areas. The morphological changes did not appear to alter the responsiveness of the oocytes. Subsequent incubations before fixation were performed with 340 mOsM MBSH. The percentage of the total radioactivity secreted during a 20-min incubation ± 10 μM ionomycin was determined.

Results

The Appearance of DBH on the Surface of Oocytes Is Stimulated by a Variety of Manipulations Which Increase Cytoplasmic Ca_2^+

Cytoplasmic Ca^{2+} was increased by (*a*) incubation of injected oocytes with the Ca^{2+} ionophore ionomycin (10 μM); (*b*) co-injection of the granule membranes with Ins-(1,4,5)-P_3 (100 μM) to release intracellular Ca^{2+} stores; and (*c*) coinjection of granule membranes with ~18 μM Ca^{2+} free buffered with 9.9 mM Ca^{2+} and 10 mM EGTA. Injection volumes were 50 nl/oocyte which corresponds to 10% of the cytoplasmic volume of the oocytes. The amount of injected chromaffin granule membranes (0.1–0.2 μg protein) was sufficient to give a cytoplasmic concentration of granule membrane in the oocyte of approximately 10% that in chromaffin cells. In oocytes injected without chromaffin granule membranes, no specific fluorescence was detected with or without manipulations which increased cytoplasmic Ca^{2+}. Examples of responses in oocytes injected with chromaffin granule membranes are shown in Fig. 1. In the absence of a Ca^{2+} stimulus there was no appreciable appearance of DBH on the oocyte surface in 90% of the oocytes injected with chromaffin granule membranes (126 out of 142 oocytes, see Fig. 1 *A* for an example). Infrequent responses in the absence of a Ca^{2+} stimulus may have resulted from damage to Ca^{2+} sequestration sites when the injection pipet penetrated the oocyte. Indeed, the frequency of responses in the absence of a controlled manipulation to increase cytoplasmic Ca^{2+} increased with large diameter injection pipets.

Approximately 50% of the oocytes (84 out of 173 oocytes) responded to a 20-min Ca^{2+} stimulus with the appearance of DBH antigen on the surface of the oocyte (measured by FITC immunocytochemistry). The fractional response was independent of the method of stimulation and was similar for injections of purified chromaffin granule membranes, intact chromaffin granules in a P_2 fraction, and lysed chromaffin granules in a P_2 fraction. Because fluorescence occurred away from the injection site, the response was not a result of locally damaged membrane. By varying the plane of focus it was determined that the fluorescence appeared on the oocyte surface and not within the oocyte. Fluorescence appeared both over the animal and vegetal poles. Specific fluorescence was not observed in the absence of primary antibody or with nonimmune primary rabbit antibody. The three methods of raising cytosolic Ca^{2+} gave qualitatively similar results. In the strongest responses DBH epitope covered 40% or more of the surface with a patchy distribution (Fig. 1, *B* and *C*). Sometimes the fluorescence outlined 2–7-μm diameter spots of non-fluorescent surface (Fig. 1 *F*). Images often suggested that the larger circular outlines represented the coalescence of smaller ones. The circumference of the circles may represent the distribution of release sites or may outline underlying structures. Oocytes occasionally responded with a patchy fluorescence distributed over a smaller area of the oocyte. In some cases oocytes responded with many bright speckles (Fig. 1 *D*), some of which were

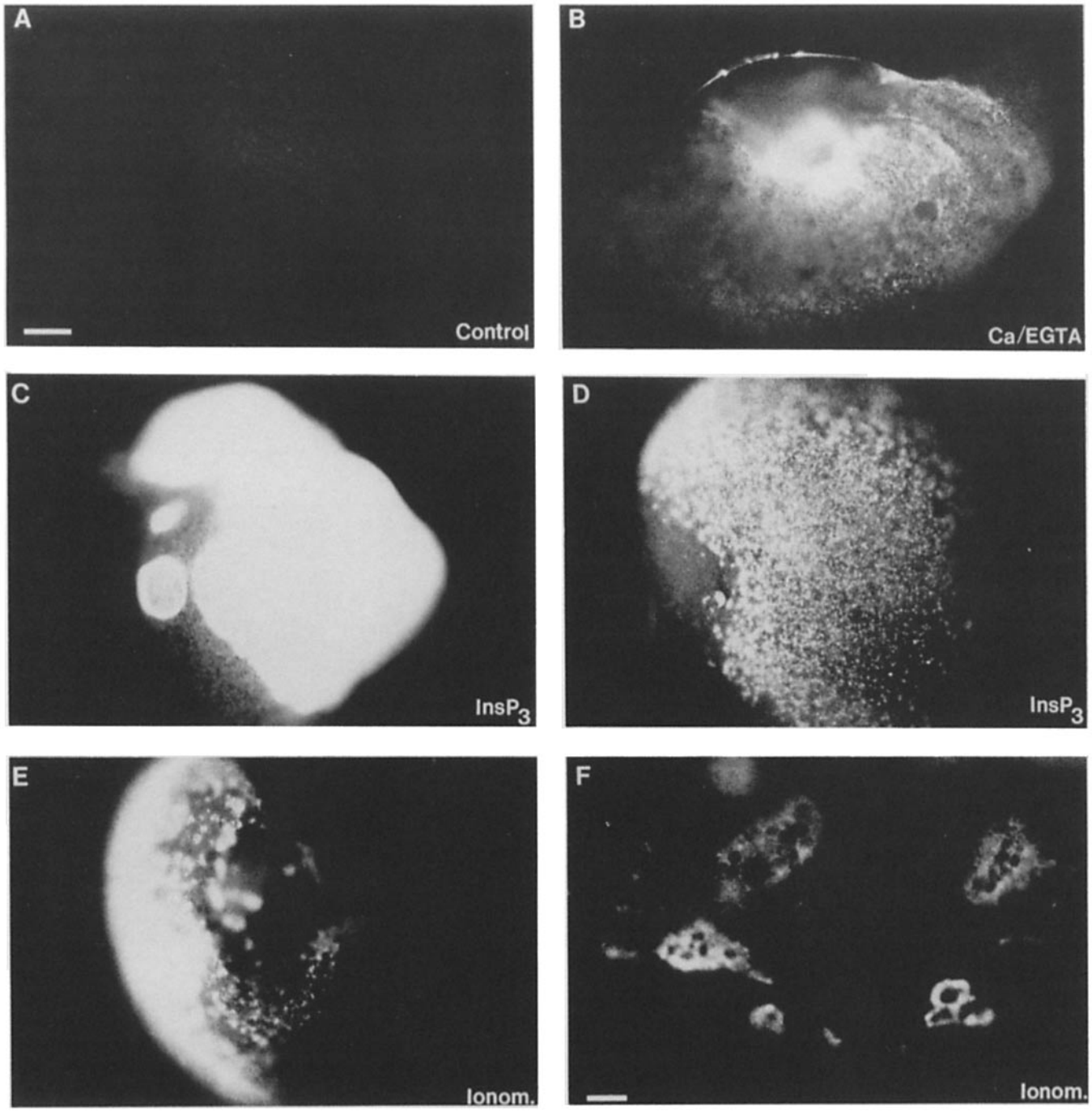
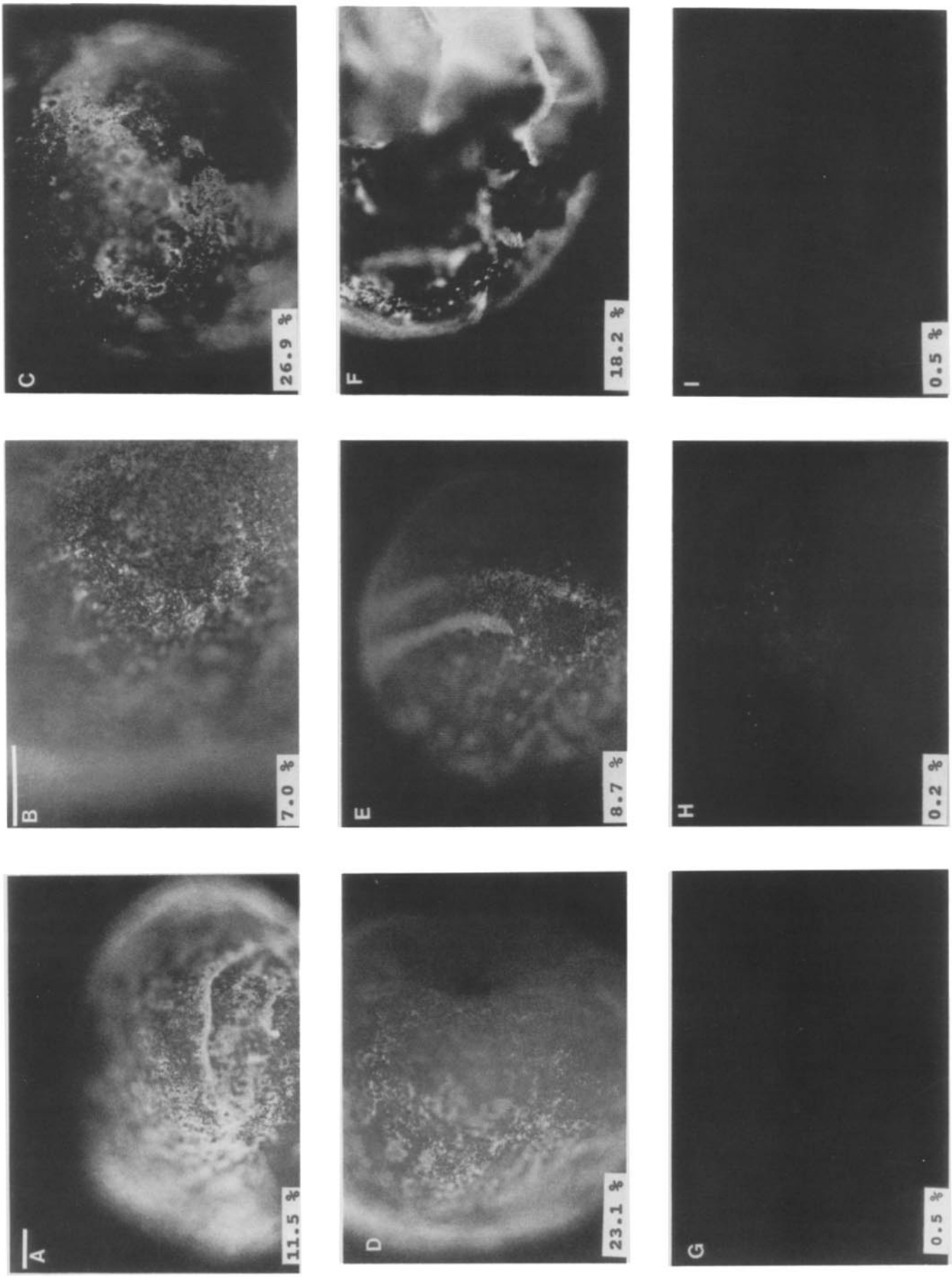


Figure 1. Appearance of dopamine-β-hydroxylase on the surface of *Xenopus laevis* oocytes. Each of the oocytes in the figure was injected with either purified chromaffin granule membranes (*A–D*), a lysed P_2 fraction (*E*), or an intact P_2 fraction (*F*). Oocytes were then incubated for 15 min (unless otherwise indicated) and were then processed to detect dopamine-β-hydroxylase on the oocyte surface. (*A*) Purified chromaffin granule membranes (0.14 μg protein) suspended in 10 mM EGTA, 10 mM Hepes, pH 7.2, were injected into the oocyte which was then incubated in MBSH. (*B*) Purified chromaffin granule membranes (0.14 μg protein) suspended in 9.93 mM $CaCl_2$, 10 mM EGTA (free Ca^{2+} $\sim$18 μM), and 10 mM Hepes, pH 7.2, were injected into the oocyte which was then incubated in MBSH. (*C*) Purified chromaffin granule membranes (0.14 μg protein) suspended in 100 μM Ins-(1,4,5)P_3, and 10 mM Hepes, pH 7.2, were injected into the oocyte bathed in Ca^{2+}-free MBSH. The oocyte was then incubated in Ca^{2+}-free MBSH. (*D*) Purified chromaffin granule membranes (0.10 μg protein) suspended in 100 μM Ins-(1,4,5)P_3, 20 mM 2,3 diphosphoglycerate and 10 mM Hepes, pH 7.2, were injected into the oocyte which was then incubated for 30 min in MBSH. (*E*) A lysed P_2 fraction (0.15 μg protein) suspended in 1 mM DTT and 10 mM Hepes, pH 7.2, was injected into the oocyte. Immediately after injection, the oocyte was incubated for 15 min in MBSH containing 10 μM ionomycin. (*F*) Intact P_2 fraction (0.2 μg protein) suspended in 290 mM sucrose, 1 mM DTT, and 10 mM Hepes, pH 7.2, was injected into an oocyte preincubated for 1 h in 340 mOsM MBSH. Immediately after injection the oocyte was incubated for 15 min in 340 mOsM MBSH containing 10 μM ionomycin. Bars: 100 μm (*A–E*); 20 μm (*F*).



beyond the resolution of the microscope. Very small speckles may correspond to the fusion of discrete chromaffin granules or chromaffin granule ghosts which would insert membrane with a diameter of ~0.5 μm.

The Appearance of DBH on the Surface of Oocytes Is Correlated with Secretion of [³H]Norepinephrine

If the appearance of DBH epitope on the surface of the oocyte upon stimulation with Ca^{2+}-raising manipulations reflects exocytosis, then there should be a strong correlation between the appearance of DBH on the oocyte surface and release of chromaffin granule contents. Therefore, an independent measure of exocytosis based upon secretion of the granule contents was devised based upon the release of catecholamine. Chromaffin granules containing [³H]norepinephrine were injected into oocytes. Oocytes were incubated for 20 min in the presence and absence of ionomycin. The appearance of DBH on the oocyte surface was detected immunocytochemically and the release of [³H]norepinephrine into the medium was measured for individual oocytes. There was an excellent correlation between the immunocytochemical detection of DBH on the oocyte surface and the release of [³H]norepinephrine. Some examples of responses are shown in Fig. 2 for oocytes incubated with ionomycin. The percentage on each photograph represents the fraction of the total injected radioactivity which was secreted from each oocyte. Oocytes with strong immunocytochemical responses released a much greater fraction of their [³H]norepinephrine (Fig. 2, *A–F*) than oocytes with little or no response (Fig. 2, *G–I*). The data from 37 oocytes were analyzed from two experiments and are summarized in Table I. The average catecholamine released from oocytes with little or no DBH expressed on the oocyte surface was 1.0% in contrast to the 10.1% release from oocytes with strong expression of DBH on the surface.

There was a definite ionomycin dependency for both the expression of DBH and [³H]norepinephrine release. Only 1 of 18 oocytes incubated in the absence of ionomycin gave a strong immunocytochemical response compared to 8 of 19 oocytes incubated in the presence of ionomycin. Ionomycin increased the number of oocytes which released >2% of the injected radioactivity from 2 of 18 oocytes to 11 of 19 oocytes (Fig. 3). The one oocyte that had a strong immunocytochemical response in the absence of ionomycin released 9% of its [³H]norepinephrine.

Ionomycin did not induce [³H]norepinephrine release from oocytes injected with [³H]norepinephrine in the absence of a P_2 fraction (data not shown).

Table I. Relationship between Immunofluorescence Detection of Dopamine-β-Hydroxylase and Release of [³H]Norepinephrine

Dopamine-β-hydroxylase immunofluorescence	[³H]Norepinephrine release Percent of total radioactivity injected
No or weak response	1.0 ± 0.2 (n = 18)
Moderate response	2.0 ± 0.6 (n = 10)
Strong response	10.1 ± 2.5* (n = 9)

The data are from the experiments in Fig. 2. Each oocyte was evaluated for immunofluorescence without information concerning incubation condition or radioactivity released. Weak immunofluorescence indicates low density and sparse fluorescence covering a small fraction (<10%) of the total surface. Moderate response indicates easily detectable areas of dense patchy fluorescence covering no more than ~10% of the area. A strong response indicates patchy fluorescence covering >10% of the total surface area. Strong responses were intense and sometimes covered 40% or more of the surface area of the oocyte. The data were from 18 oocytes incubated in the absence of ionomycin and 19 in the presence of ionomycin. One oocyte incubated without ionomycin gave a strong response. Eight oocytes incubated with ionomycin gave strong responses.
* $P < 0.001$ vs no or weak response.

Discussion

The experiments with injected P_2 fraction or purified chromaffin granule membranes demonstrate: (*a*) an excellent correlation between the expression of DBH on the oocyte surface and [³H]norepinephrine release; (*b*) a strong Ca^{2+} dependency for the surface expression of DBH; and (*c*) a strong Ca^{2+} dependency for [³H]norepinephrine release. The data provide compelling evidence for Ca^{2+}-dependent exocytosis of injected chromaffin granules and chromaffin granule membranes.

Secretory vesicles usually if not always contain in addition to stored hormones or neurotransmitters other substances such as nucleotides, peptides and proteins. Chromaffin granules normally contain approximately 500 mM catecholamine (epinephrine and norepinephrine), 125 mM ATP, acidic proteins (chromogranins A and B), proenkephalin, and peptides including leu- and met-enkephalin (see 20, 26 for reviews). The experiments with highly purified chromaffin granule membranes demonstrate for the first time that the secretory vesicle contents are unnecessary for exocytosis. The secretory vesicle membrane without vesicle contents is competent to undergo the sequence of events leading to exocytosis. Thus, a soluble protein within the granule is not necessary for exocytosis. Furthermore, a change in the osmotic state of the very nonideal mixture of neurotransmitters, ATP and proteins of the granule (16) is unnecessary for exocytosis, a conclusion consistent with previous work in chromaffin cells (10, 11) and mast cells (1, 28).

Figure 2. The appearance of DBH on the surface of oocytes is correlated with the release of [³H]norepinephrine. Chromaffin granules labeled with [³H]norepinephrine were injected into oocytes. Oocytes were washed within 2 min with 5 and 1 ml of 340 mOsM MBSH and then incubated individually in the presence or absence of 10 μM ionomycin in 340 mOsM MBSH. After 20 min the medium was removed and the radioactivity in the medium was determined. Each oocyte was rapidly fixed and processed for immunocytochemistry to detect DBH. The percentage in each panel corresponds to the fraction of the total radioactivity which was released during the 20-min incubation. The data are examples of responses from two experiments in which either 10,000 cpm (0.16 μg protein) or 16,000 cpm (0.18 μg protein) were injected per oocyte and incubated with ionomycin. 8 of 19 oocytes had strong surface expression of DBH in the presence of ionomycin whereas only 1 of 18 oocytes had strong surface expression of DBH in the absence of ionomycin. Note that the absence of surface expression of DBH (*G–I*) is associated with little [³H]norepinephrine release in contrast to the substantial release in other oocytes which expressed DBH on the surface (*A–F*). Table I and Fig. 3 give a complete analysis of the responses in the presence and absence of ionomycin. The scale bars represent 100 μm. The bar in *A* provides the scale for all panels except for *B*.

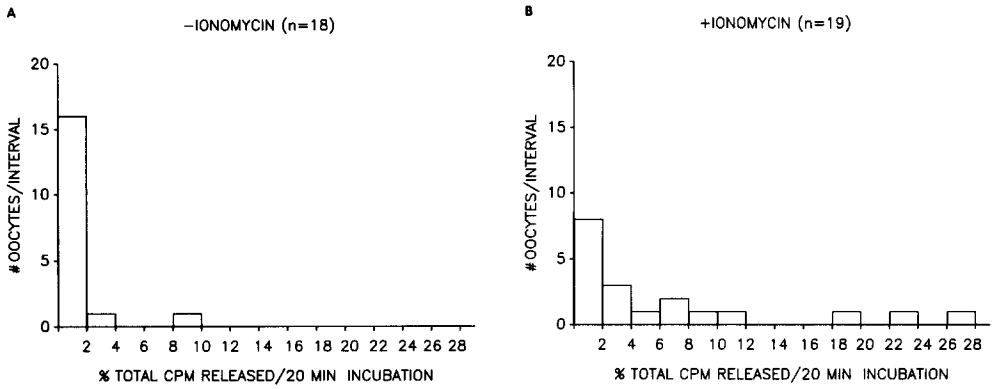


Figure 3. The effect of ionomycin on the release of radioactivity from oocytes injected with [^{3}H]norepinephrine-labeled chromaffin granules. The data are derived from the experiments described in Fig. 2 and Table I. Ionomycin (10 μM) caused a large reduction in the number of oocytes which released <2% of the injected radioactivity and a corresponding increase in the number of oocytes that released a larger percentage of radioactivity. Note that three oocytes in the presence of ionomycin released >18% of the injected radioactivity. The one oocyte that released 8–10% of its radioactivity in the absence of ionomycin was the one oocyte which had strong surface expression of DBH in the absence of ionomycin.

Secretory vesicles must interact with the plasma membrane and probably with cytosolic factors in order for exocytosis to occur. In these experiments a mammalian secretory vesicle was able to to utilize amphibian cellular machinery necessary for exocytosis. The interchangeability of mammalian and amphibian components suggests substantial biochemical conservation of the regulated exocytotic pathway during the evolutionary progression from amphibians to mammals. This conclusion is consistent with findings in the constitutive secretory pathway in which there is at least some interchangeability of components between yeast and mammals. SEC18p from yeast can substitute for mammalian N-ethylmaleimide sensitive fusion factor in the intra-Golgi transfer of proteins in mammalian cells (24).

An important issue is whether the Ca^{2+} sensitivity for exocytosis is a characteristic of the secretory vesicle membrane. We observed responses when chromaffin granule membranes were co-injected with solutions buffered at approximately 18 μM Ca^{2+} with EGTA (Fig. 1). This Ca^{2+} concentration causes the maximal extent of secretion in electropermeabilized (15) and digitonin-permeabilized chromaffin cells (6) and suggests that the Ca^{2+} sensitivity for chromaffin granule exocytosis is similar in frog oocytes and bovine chromaffin cells. In neutrophils the secretory granule seems to be an important determinant of Ca^{2+} sensitivity in exocytosis since the different secretory granule populations in the same cell appear to have different Ca^{2+} sensitivities for exocytosis (18). We are currently investigating whether the endogenous cortical granules undergo exocytosis with the same or different sensitivity to Ca^{2+} stimuli as exocytosis of injected chromaffin granules.

In experiments with intact chromaffin granules, some of the oocytes released 18% or more of their [^{3}H]norepinephrine upon incubation with ionomycin. Because this magnitude of response is comparable to secretion observed in cultured chromaffin cells, it is likely that a significant fraction of the injected chromaffin granules are competent to undergo exocytosis. The maintenance of function of exogenous secretory vesicles in frog oocytes may allow the investigation of components of the secretory vesicle membrane necessary for regulated exocytosis.

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