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Polycation Inhibition of Exocytosis from Sea Urchin Egg Cortex

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Summary. The Ca^{2+}-stimulated release of vesicle contents from cortical fragments prepared from sea urchin eggs is an in vitro model for exocytosis. Cortical fragments have been isolated either in suspension (cell surface complex, CSC preparation), or attached to polycation-coated surfaces (cortical lawn, CL preparation). CL, but not CSC, have been reported to undergo a rapid "aging" process whereby they fail to respond to micromolar free Ca^{2+}. Since, in principle, the only difference between the two preparations is the use of polycations in the CL preparation, polycations were suspected of being inhibitory. This hypothesis was tested by evaluating the effects of polycation-containing buffers on the Ca^{2+} threshold, rate, and extent of exocytosis in CL prepared from the eggs of *Strongylocentrotus purpuratus*. A sensitive microphotometric assay, based on light scattering by the individual cortical vesicles in the CL, was used to quantitate the exocytotic response. Buffers containing polylysine were found to be potent inhibitors of cortical exocytosis. The Ca^{2+} threshold of CL that had been treated for 15 min at room temperature with 50 μg/ml of polylysine was more than three orders of magnitude greater than that of freshly prepared CL. The other polycations tested (protamine, spermine and neomycin) were also found to be inhibitory, but to a lesser degree than polylysine. Two lines of evidence suggested that the polycations used in the preparation of CL are responsible for the rapid "aging" phenomenon: (i) CSC fragments that had been affixed to polylysine-coated coverslips were shown to aquire "aging" characteristics similar to the CL preparations; control CSC that had been maintained in suspension did not. (ii) Radiolabeled poly-L-lysine was shown to dissociate from coated coverslips and redistribute onto CL.

Key Words exocytosis · polycations · cortical lawn · calcium · sea urchin

Introduction

Exocytosis of stored secretory products in response to a stimulus at the cell surface is a general feature of many eukaryotic cells. The stimulus is transduced into a rise in cytoplasmic free calcium (Ca^{2+}), which has been shown to be the intracellular signal for exocytosis (Baker & Knight, 1978; Kelly, Deutsch, Carlson & Wagner; 1979; Baker, Knight & Whitaker, 1980; Rubin, 1982). Cortical fragments prepared from the sea urchin egg provide a unique in vitro model that allows studies of the molecular mechanisms of exocytosis to be conducted in the absence of the panoply of calcium-requiring processes present in the whole cell.

Upon fertilization, the sea urchin egg, like most other vertebrate and invertebrate eggs, undergoes a massive Ca^{2+}-triggered exocytotic event. The cortical secretory vesicles (CVs) of the mature egg are firmly attached to the cytoplasmic aspect of the plasma membrane. This ultrastructural specialization allows isolation of cortical fragments (egg cortices) with an intact exocytotic apparatus.

Egg cortices have been isolated, either bound to polycation-coated surfaces, or in suspension (Vacquier, 1975; Detering, Decker, Schmell & Lennarz, 1977, respectively). Both preparations respond to micromolar free Ca^{2+} by a process considered to be exocytosis, based on morphological and biochemical criteria (Steinhardt, Zucker & Schatten, 1977; Baker & Whitmaker, 1978; Decker & Lennarz, 1979; Schon & Decker, 1981; Haggerty & Jackson, 1983; Moy, Kopf, Gache & Vacquier, 1983; Whitaker & Baker, 1983; Crabb & Jackson, 1985).

Egg cortices isolated on polycation-coated surfaces (referred to here as cortical lawns, CL) have been reported to undergo a rapid "aging" process resulting in a progressive loss of sensitivity to micromolar free Ca^{2+} (Baker & Whitaker, 1978; Moy, et al., 1983). This rapid loss of Ca^{2+} sensitivity (on the order or minutes) is not observed in egg cortices prepared in suspension (referred to here as cell surface complex, CSC) (Jackson, Ward & Haggerty, 1985). These observations prompted us to study the effects of the polycations used for attachment in the CL preparation. We report here that polylysine causes a dramatic time and temperature-dependent shift in the Ca^{2+} concentrations required for exocytosis, and our data strongly indicate that the aging

phenomenon is caused by the polycations used for CL attachment.

Materials and Methods

Materials

Strongylocentrotous purpuratus and *Lytechinus pictus* were purchased from Marinus (Ingelwood, CA). *S. purpuratus* were maintained at 12–15°C and *L. pictus* were maintained at 15–17°C in a refrigerated aquarium containing Instant Ocean seawater from Aquarium Systems (Mentor, OH). Poly-L-lysine hydrobromide (avg. mol wt 25, 180, 200, 240, 300, 540 kD), protamine sulfate, spermine tetrahydrocholoride, neomycin sulfate, piperazine-N,N'-bis(2-ethane sulfonic acid) (PIPES), ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), potassium-D-gluconate, glycine and N-ethylmaleimide (NEM) were purchased from Sigma Chemical (St. Louis, MO). [^{14}C]formaldehyde and Aquasol® were obtained from New England Nuclear (Boston, MA). Sodium cyanoborohydride was purchased from Aldrich Chemical (Milwaukee, WI). All other reagents were of the highest grade available.

Preparation of Cortical Lawns and Cell Surface Complex

Eggs were shed into 0.45 μm filtered (Millipore, Bedford, MA) seawater by intracoelomic injection of 0.5 M KCl, and filtered through a nylon screen of 125 (*S. purpuratus*) or 210 μm (*L. pictus*) mesh (Small Parts, Miami, FL). For CL preparation, eggs dejellied by two washes with 502 mM NaCl, 10 mM KCl, 2.5 mM $NaHCO_3$, 25 mM EGTA, pH 8.0, were maintained on ice as a 10% suspension in either filtered seawater (*L. pictus*), or PKME buffer (50 mM PIPES, 425 mM KCl, 10 mM $MgCl_2$, 10 mM EGTA, pH 6.8) (*S. purpuratus*). Unless otherwise stated, results were obtained with *S. purpuratus*.

Cortical lawns were prepared at room temperature, as follows: poly-L-lysine (1 mg/ml) was applied to a 22 mm^2, #1 coverslip (Corning Glass Works, Corning, NY) for 3 min then rinsed with a jet of GGE buffer (10 mM PIPES, 500 mM glycine, 250 mM potassium-D-gluconate, 10 mM NaCl, 10 mM $MgCl_2$, 10 mM EGTA, pH 6.8) from a hand-held wash bottle. The egg slurry was applied to the treated coverslip and allowed to settle and attach for 3 min. The coverslip with attached egg monolayer was gently rinsed, then sheared with GGE buffer from the wash bottle, leaving cortical fragments of the eggs attached to the coverslip. The resulting CL were inverted and immediately placed on a sample chamber constructed of an ordinary microscope slide fitted with two shims (a thin strip of coverslip glued to each of two edges with epoxy glue). The chamber was filled with GGE buffer plus or minus polycations at various temperatures, as indicated (the volume of the chamber is 30–40 μl). After incubation the chamber was washed with 100 μl of GGE buffer at the indicated temperature. All washings and buffer exchanges were done by carefully placing a filter circle (2.3 cm #2, Whatman, Clinton, NJ) on one end of the sample chamber and evenly perfusing 100 μl by capillary action with a micropipette from the opposite side of chamber. The chamber containing the CL was then assayed in the microphotometric assay (described in the following section) by application of GGE buffer containing the desired free Ca^{2+} concentration. Free Ca^{2+} concentrations were calculated as previously described (Haggerty & Jackson, 1983).

Cell surface complex was prepared as described by Jackson et al. (1985), with the following modifications: GGE buffer replaced PKME buffer and GGE_{25} (same as GGE, but with 25 instead of 10 mM EGTA) replaced PKE for the homogenization and first two washes. Centrifugations were slowed to 204 × *g*, 3 min, followed by 204 × *g*, 2.5 min.

Cell surface complex lawns were prepared by allowing approximately 100 μl of suspended CSC to attach to coverslips coated with poly-L-lysine as in CL preparation, and shearing with a jet of GGE buffer to produce flat lawns. CSC were sometimes maintained in 1.5 mM diisopropylfluorophosphate during "aging" since it has been observed to enhance the attachment to the coverslips during subsequent lawn preparation. This treatment in no way affected the exocytotic reactivity of the preparation.

Microphotometric Assay

This assay is similar to that described by Zimmerberg, Sardet and Epel (1985), with modifications. The sample chamber containing the CL to be assayed was positioned on the stage of a Nikon Labophot microscope equipped with a trinocular head and 10 and 20× achromat phase contrast objectives (Don Santo Corp., Natick, MA). A photodiode assembly, machined into an aluminum block, rested on the top of the camera tube and consisted of an FPT100A photodiode/transistor (Fairchild, Mountainview, CA) in conjunction with an RCA 3130 operational amplifier used in the emitter-follower mode, powered by two 5.4 V mercury batteries (constructed by the electronics laboratory of Dartmouth Medical School). This is known as a short-circuit current and is linear with increasing light (Oriel, Stamford, CN). The photodiode output was displayed on a potentiometric chart recorder, typically set to 1000 mV full scale and 1 cm/min (LKB Instruments, Rockville, MD, or Soltec, Sun Valley, CA).

Using darkfield optics with the 10× objective, a field which was free of debris (which might scatter light), was chosen visually in the CL. The trinocular head was then rotated to expose the field to the photodiode and the recorder response was set to approximately 950 mV by adjusting the intensity of the light source. The response was allowed to stabilize (approximately 30 sec) and the test buffer containing the desired free Ca^{2+} concentration was applied as described. The $t_{1/2}$ of buffer exchange was 0.34 ± 0.045 sec as determined on dummy chambers flushed with a 0.1% suspension of latex spheres (1.0 μm diameter, Dow Diagnostics, Indianapolis, IN) under darkfield optics.

Quantitation of the microphotometric response is described in Results.

Photography of CL

Cortical lawns were photographed on Panatomic X film (Kodak) using a Zeiss universal microscope equipped with the same objectives used for the microphotometric assay and employing darkfield optics. Measurements were made using a stage micrometer.

Poly-L-Lysine Labeling

Poly-L-lysine was labeled by reductive methylation with [^{14}C]formaldehyde in the presence of sodium cyanoborohydride by a

modification of the procedure of Dottavio-Martin and Ravel (1978). Briefly, 325 μl of 20-mg/ml poly-L-lysine (avg. mol wt 200 kD in 40 mM PIPES, pH 7.0) was added to an ampuole containing 50 μCi [^{14}C]formaldehyde in H_2O. 100 μl of freshly prepared $NaBH_3CN$ (6 mg/ml) in 40 mM PIPES, pH 7.0) was added, and the reaction mixture was incubated for 1 hr at 25°C with gentle shaking every 15 min. The reaction mixture was diluted with 2 ml of 40 mM PIPES, pH 7.0, dialyzed overnight *vs.* 1.0 liter of the diluent at 4°C, then exhaustively dialyzed against dH_2O. The resulting preparation had a specific activity of 9.2 μCi/mg poly-L-lysine, which corresponds to methylation of 12% of the lysine residues.

[^{14}C] Poly-L-Lysine Binding, Dissociation, and Redistribution

Coverslips were cut to 22 × 10 mm and coated with 50 μl [^{14}C] poly-L-lysine (1 mg/ml in dH_2O) for 3 min, then rinsed with a jet of GGE buffer as described for preparation of CL. For comparative purposes coverslips were also coated for 3 min, then rinsed with dH_2O and dried, essentially as described by Vacquier (1975). The amount of bound polylysine was determined by counting the [^{14}C]poly-L-lysine-coated coverslips in 10 ml Aquasol® in a liquid scintillation spectrometer (Beckman Instruments, Fullerton, CA). Dissociation of poly-L-lysine from coverslips was assessed by immersing [^{14}C]poly-L-lysine coated coverslips in a scintillation vial containing 5.0 ml of GGE buffer. After incubation for the desired period of time at room temperature, the coverslips were removed and counted. Comparison with coverslips identically coated but not incubated in buffer yielded the amount of radiolabel lost during the incubation.

In order to determine if coverslip-bound poly-L-lysine is capable of dissociating from the glass and depositing on a CL, the following experiment was performed. CL were prepared on 22 × 10 mm poly-L-lysine coated coverslips as described above. These CL were then placed in a 12-mm wide trough made by gluing 2 shims (2 coverslips thick) on a microscope slide with epoxy glue. [^{14}C]poly-L-lysine-coated coverslips were prepared on coverslips cut to approximately 22 × 15 mm and laid over the trough containing the CL. This made a sandwich with the CL and [^{14}C]poly-L-lysine coated coverslip facing each other, separated by a thin channel (approximately 0.15 mm) filled with GGE buffer. After the experimental interval, the CL-coverslip and [^{14}C]poly-L-lysine coated coverslip were briefly rinsed to remove unbound radiolabel (by dipping into a beaker of GGE buffer), then counted in 10 ml Aquasol®. This is actually a competition experiment: the [^{14}C]poly-L-lysine competes with unlabeled poly-L-lysine (used in the attachment of the CL) for sites on the exposed surface of the CL. Nevertheless, it can be used qualitatively to determine whether or not poly-L-lysine can redistribute from the glass surface to that of the CL.

Results

The Microphotometric Assay is Sensitive and Linear with Respect to Cortical Exocytosis

In our initial attempts to study the effects of polycations on the CSC we used the turbidimetric assay developed in this laboratory (Haggerty & Jackson, 1983). It was discovered, however, that polylysine interfered with the turbidimetric assay, through aggregation of both intact CSC and released CV contents. Hence, we employed a microscopic assay based on light scattering by the individual CVs in a CL, as described in Materials and Methods (microphotometric assay).

Figure 1 shows the microphotometric response of CL triggered with 1 mM free Ca^{2+}. Photographs *A* and *B* show a microscopic field in the CL before and after addition of Ca^{2+} buffer and correspond to points *A* and *B* of the recorder tracing. The bright appearance of the unreacted CL is due to light scattering by the individual CVs. Addition of the Ca^{2+}-containing buffer results in rapid discharge of the CVs and a concomitant reduction in the amount of scattered light available to the photodiode. At this Ca^{2+} concentration the reaction is virtually complete, with refractory CVs remaining almost exclusively at the torn edge of each individual cortical fragment. The addition of water to the reacted CL causes a further, albeit slight, response due to lysis of refractory CVs.

Since we were interested in quantitative changes in the reactivity of CL, we felt that it was important to establish that the output voltage of the photodiode apparatus was linearly proportional to the extent of reaction in the CL. Thus, the microphotometric response was quantitated with respect to the extent of cortical exocytosis, as follows: Dejellied eggs of *S. purpuratus* were split into two aliquots. One aliquot was left untreated and the other was treated with 10 mM NEM for 5 min at room temperature, followed by aspiration and resuspension in ice-cold NEM-free PKME buffer. CL prepared from eggs which had been NEM-treated by this procedure were virtually unresponsive to 1 mM free Ca^{2+}.

The NEM-inactivated eggs were mixed with untreated eggs in various proportions, and CL were prepared from these mixtures. The CL were then assayed in the microphotometric assay by triggering with 1 mM free Ca^{2+}. This resulted in 100% reaction in the untreated cortical fragments while the NEM-treated fragments remained unreacted (Fig. 2). The photodiode response was recorded, and the trinocular head of the microscope was rotated back for visual inspection of the field. The total number of unreacted cortical fragments were counted before and after Ca^{2+} addition so the percentage of cortical fragments that had reacted could be correlated with the decrease in output voltage recorded from the same field. For this particular experiment fields were chosen that had cortical fragments of uniform size. The data were subjected to a linear regression analysis to determine the linearity of the assay and correlation coefficient.

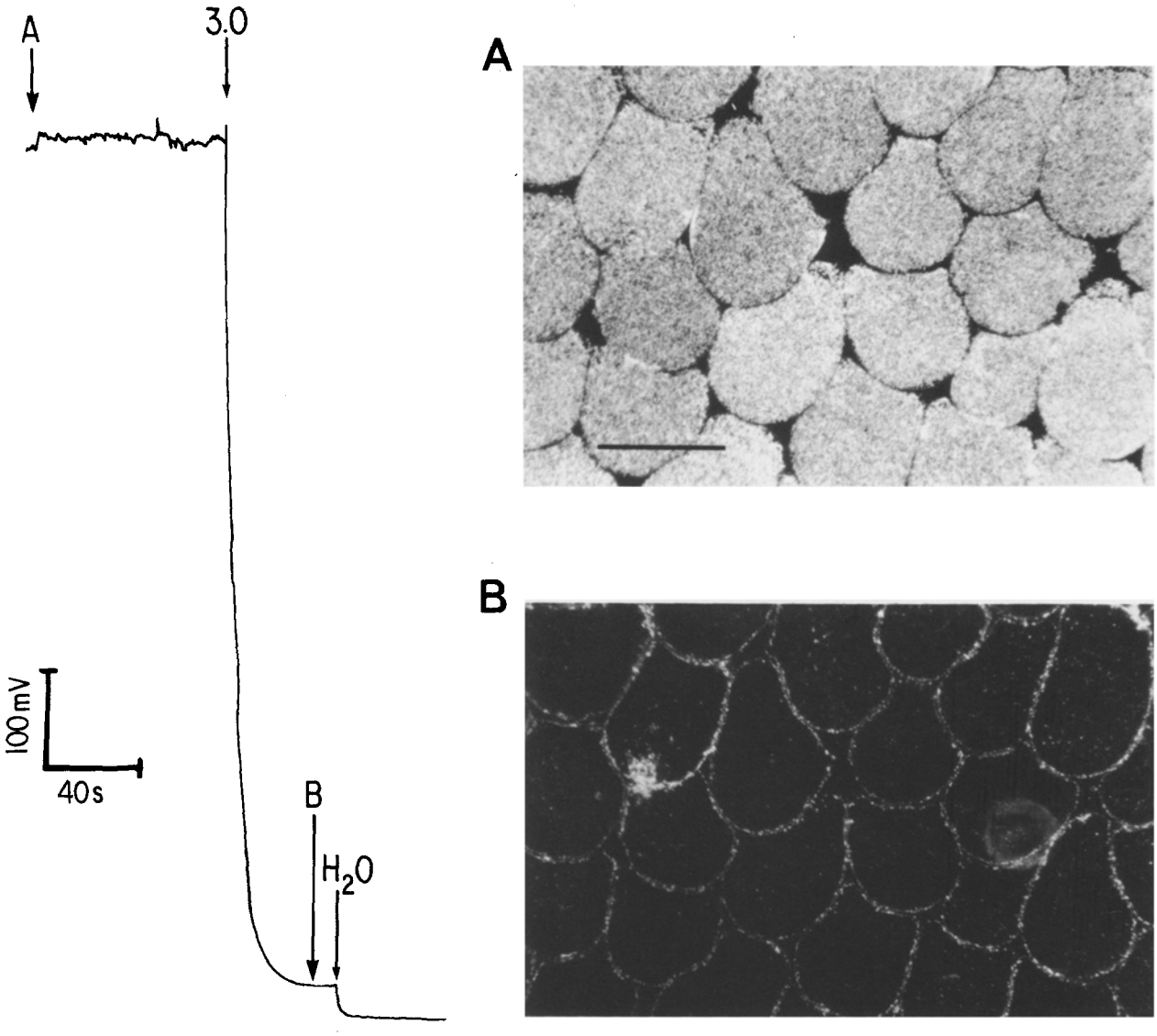

Fig. 1. Microphotometric assay of CL exocytosis. CL were prepared and assayed as described in Materials and Methods. Micrographs *A* and *B* correspond to points *A* and *B* on the recorder tracing. Ca^{2+}-containing buffer ($pCa = 3.0$) was applied as indicated by the arrow (3.0), and the response was allowed to plateau. Distilled water was added where indicated to lyse any remaining cortical secretory vesicles. Bar, 100 μm

Figure 3 shows the accumulated results of several experiments to quantitate the microphotometric response. The dashed line shows a theoretical correlation between microphotometric response and percentage of reacted cortical fragments. The solid line depicts the "best fit" line drawn from the statistical analysis of the data. The microphotometric response provides a very linear measure of the extent of cortical exocytosis (correlation coefficient = 0.98).

The linearity of the microphotometric assay permitted the direct comparison of data from samples assayed at increasing free Ca^{2+} concentrations and thus comparison of the relative Ca^{2+} sensitivity of CL treated under various conditions. Such a Ca^{2+} sensitivity plot is shown in Fig. 4, where the left panel shows the microphotometric response of freshly isolated CL tested with buffers containing free Ca^{2+} from 0-513 μM ($pCa = 3.29$). In each sample the microphotometric response was allowed to plateau after the addition of the Ca^{2+}-containing buffer. The right panel shows a plot of the relative extent of exocytosis versus the pCa of the test buffer. Here, 50% reaction occurred at approxi-

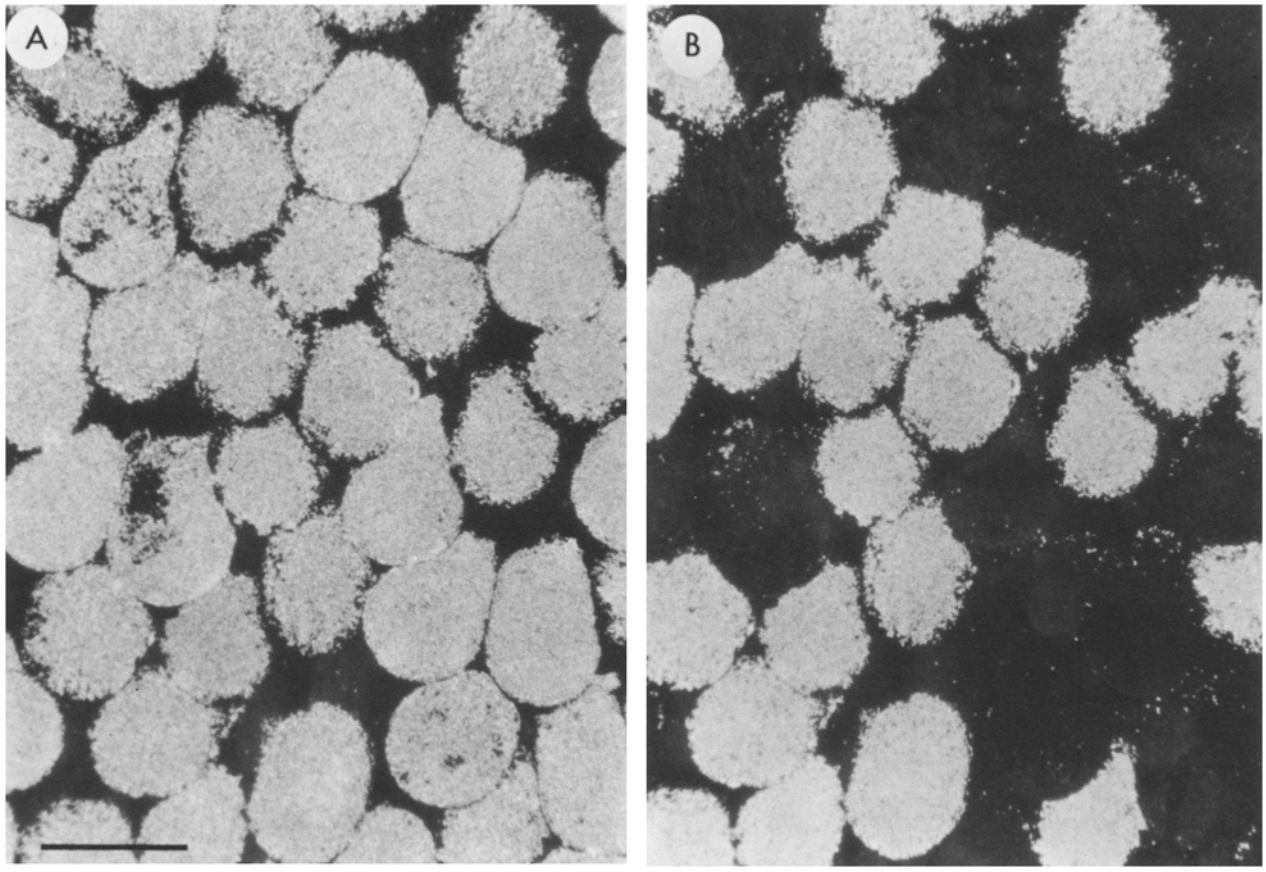

Fig. 2. Exocytosis from CL prepared from a mixture of NEM-inactivated and control eggs. Dejellied eggs were inactivated with 10 mM NEM, mixed with an equal volume of control eggs, and used to prepare a CL, as described in Materials and Methods. Darkfield micrographs of the lawn were taken before (*A*) and after (*B*) the addition of GGE buffer containing 1.0 mM free Ca^{2+}. Bar, 100 μm

mately 1 μM free Ca^{2+}. This value remained consistent throughout the season for freshly isolated *S. purpuratus* CL.

Polylysine Inhibits Exocytosis in CL

Since the rate of "aging" is rapid in CL preparations (Baker & Whitaker, 1978; Moy et al., 1983) but slow in CSC preparations (Jackson et al., 1985), we suspected that the polycationic substances used for attachment in the CL preparations were inhibitory. Support for this hypothesis was obtained by showing that exogenously added aqueous polycations were capable of inhibiting CL in a dose-dependent manner. Figure 5 shows the result of incubating *S. purpuratus* CL with poly-L-lysine-containing buffers for 15 min at room temperature. Control CL responded to 1 mM free Ca^{2+} with a rapid and complete reaction. CL that had been treated with 25 μg/ml poly-L-lysine (average mol wt, 240 kD) then rinsed (as described in Methods), reacted more slowly, yet virtually to completion. CL that had been treated with 50 μg/ml poly-L-lysine were totally unresponsive, despite repetitive rinses with a buffer containing 1 mM free Ca^{2+}, but reacted promptly upon addition of buffer containing 10 mM free Ca^{2+} (pCa = 2.0). The failure of repetitive washes to reduce the Ca^{2+} requirement of inhibited lawns suggests that, under these conditions, poly-L-lysine inhibition is essentially irreversible.

Further characterization of the polylysine inhibition is shown by the Ca^{2+} sensitivity plots in Fig. 6, where CL were treated with aqueous phase poly-L-lysine as above, at room temperature and 0°C. It is evident that a dose-dependent shift in the relative Ca^{2+} sensitivity occurred when CL were incubated in the presence of aqueous poly-L-lysine. The shift was most dramatic (three to four orders of magnitude) when the incubation was carried out at room temperature, but even at 0°C, a marked shift occurred. Poly-L-lysines with molecular weights rang-

ing from 25,000 to 540,000 were all inhibitory (*data not shown*).

The plot of the control samples, where CL were incubated for 15 min at room temperature in buffers containing no added poly-L-lysine (Fig. 6, top pannel, ●—●), illustrates the "aging" phenomenon. The Ca^{2+} sensitivity of these aged CL was markedly shifted relative to freshly isolated CL (Fig. 6, top panel, ▲---▲). CSC, on the other hand, do not exhibit a comparable "aging" phenomenon. The

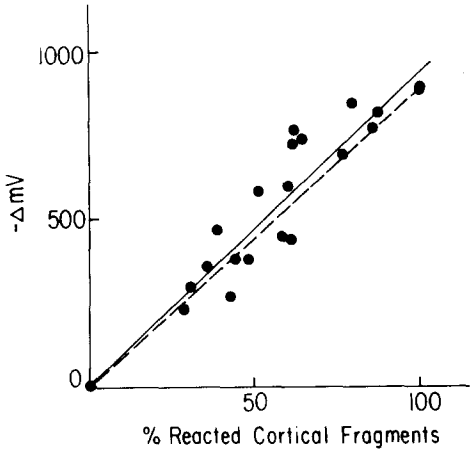

Fig. 3. Linearity of the microphotometric assay. CL were prepared from mixtures containing various ratios of NEM-treated to control eggs and microphotometrically assayed for cortical exocytosis at 1.0 mM free Ca^{2+} as described in Materials and Methods (employing a 20× instead of a 10× objective). The MP response was plotted as the change in millivolts (−Δ mV) versus the percentage of reacted cortical fragments in the same field. The dashed line shows the theoretical correlation. The solid line is a "best fit" line drawn from a linear regression analysis of the data. (Correlation coefficient = 0.98)

Ca^{2+} threshold of CSC suspensions maintained at room temperature and assayed by the turbidimetric procedure increases, but at a much slower rate, on the order of hours (Jackson et al., 1985).

In addition to polylysine, we found that protamine, as well as smaller polycations (spermine and neomycin) were also capable of increasing the Ca^{2+} threshold of CL, but to a lesser degree than polylysine. Protamine-coated surfaces have been prepared by incubation with 10 mg/ml of protamine sulfate (Vacquier, 1975), versus 1 mg/ml polylysine. Like CL prepared on polylysine-coated surfaces, CL prepared on protamine-coated surfaces have been reported to exhibit the "aging" phenomenon (Moy et al., 1983).

The inhibition data for protamine are shown in Fig. 7. CL were prepared using polylysine-coated coverslips, as described in Methods, and incubated, at 0°C, with GGE buffer containing 1.0 mg/ml protamine. The purpose of the 0°C incubation was to minimize the effect of the solid phase polylysine during the incubations (Fig. 6, bottom panel), allowing accurate assessment of the effects of the aqueous phase protamine. These conditions also minimize the effect of the added protamine; nevertheless, as shown in Fig. 7 the inclusion of 1 mg/ml protamine sulfate does increase the Ca^{2+} threshold, but to a smaller extent than polylysine. Spermine and neomycin increased the Ca^{2+} threshold of CL to approximately the same extent as protamine, when assayed under the same conditions (*data not shown*).

Lytechinus pictus CL were also tested for inhibition by polylysine. A dramatic shift in the Ca^{2+} sensitivity was also observed in these preparations. The magnitude of inhibition closely paralleled that observed in *S. purpuratus* CL (*data not shown*).

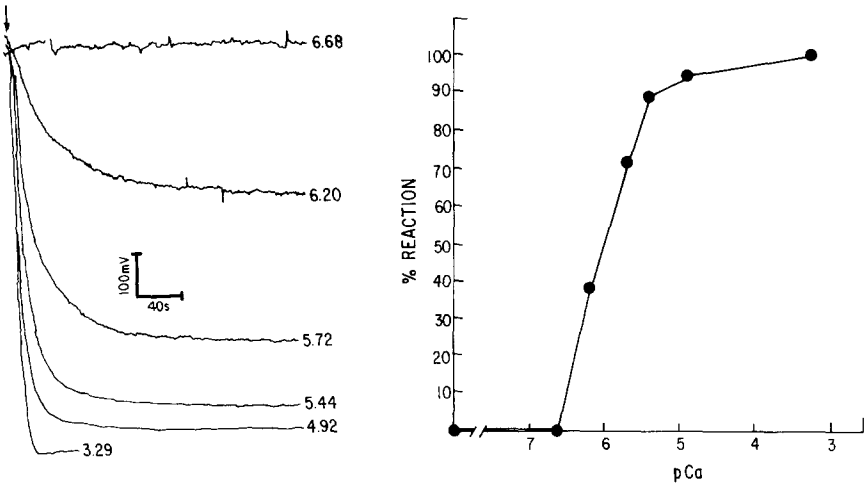

Fig. 4. Microphotometric response of CL as a function of free Ca^{2+} concentration. *Left panel:* microphotometric responses of CL assayed with GGE test buffers containing the indicated concentrations of free Ca^{2+} (pCa). *Right panel:* Ca^{2+} sensitivity plot of the data. The microphotometric responses were normalized to the percent maximal response (at pCa = 3.29), and plotted versus the corresponding pCa value

Inactivation Is Not Assay Dependent

Although we suspected that the rapid "aging' of CL was caused by the dissociation of polylysine from the coverslip, the discrepancy in the rates of inactivation of the CL and CSC preparations could also have arisen from differences in the microphotometric and turbidimetric assays.

We investigated this possibility by assaying freshly prepared and aged (5 hr) preparations of both CL and CSC with the microphotometric assay. CSC, prepared in GGE buffer, as described in Methods, was maintained in suspension during the incubation period, while CL (also in GGE) were incubated, in parallel, on polylysine-coated coverslips. Immediately prior to assay, the CSC was attached to polylysine-coated coverslips and sheared with a jet of buffer (exactly as in CL preparation) to produce CSC lawns. Photomicrographs of a CSC lawn, taken before and after the addition of a buffer containing 1 mM free Ca^{2+}, demonstrate the reactivity of CSC lawns (Fig. 8) and their similarity to CL (Fig. 1). This technique permits the direct comparison of the Ca^{2+} threshold of CL and CSC. Fig. 9 shows that CSC that had been maintained in suspension for a period of 5 hr, and assayed as CSC lawns, was only slightly less active than freshly prepared CSC (t = 0); whereas a 5-hr incubation increased the Ca^{2+} threshold of CL by approximately three orders of magnitude.

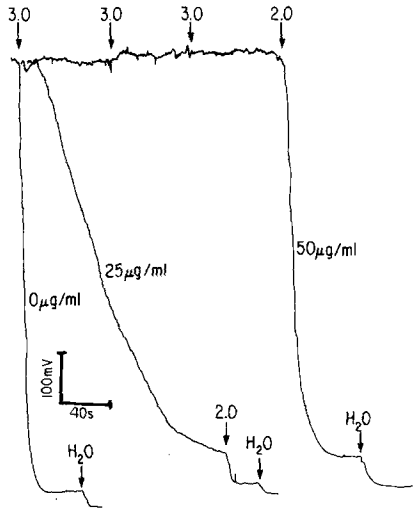

Fig. 5. Effect of polylysine on the kinetics of cortical exocytosis. CL were incubated for 15 min at room temperature with GGE buffer containing 0, 25, or 50 μg/ml of polylysine and washed with polylysine-free GGE. The CL were then assayed for cortical exocytosis by the addition of GGE buffer with a pCa of 3.0 (1 mM) or 2.0 (10 mM) as indicated by the arrows

CSC Lawns are Inhibited by Polylysine-Containing Buffers and Display the Aging Phenomenon

While, in principle, the only difference between the CL and CSC preparations is the use of polylysine in the CL preparation, other differences, arising from the different manipulative procedures used in the two preparations, could not be excluded. In order to conclude that the use of polylysine was responsible for the rapid inactivation of CL it was necessary to establish that the CSC preparation could also be inhibited by polylysine. However, as stated previously, polylysine is incompatible with assays of cortical exocytosis performed on suspended CSC, so we assessed the dose-dependent effects of polylysine on CSC lawns.

Figure 10 shows that when aqueous phase polylysine is incubated with CSC lawns for 15 min at room temperature, a dose-dependent shift in the Ca^{2+} threshold occurs. This parallels the results obtained when the same experiment was performed on the CL preparations (Fig. 6). The primary difference between the CL and CSC results is that in the absence of exogenously added polylysine, the Ca^{2+} threshold of the CSC preparation (at t = 15 min) is

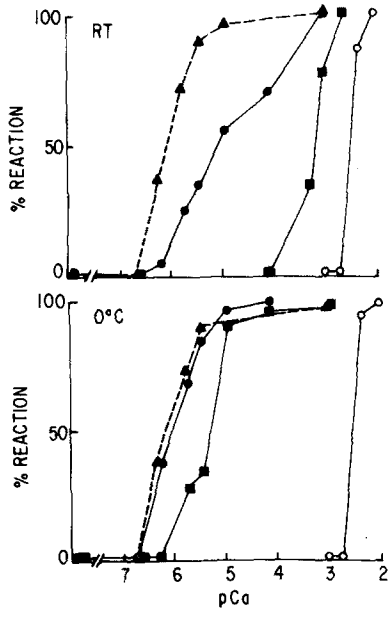

Fig. 6. Polylysine increased the Ca^{2+}-threshold of CL. CL were prepared and incubated for 15 min with GGE buffer containing 0 (filled circles), 25 (filled squares), or 50 μg/ml (open circles) of polylysine, and microphotometrically assayed for cortical exocytosis. The Ca^{2+} sensitivity of freshly isolated CL was also determined (filled triangles). *Top panel:* incubations performed at room temperature (approx. 22°C). *Bottom panel:* incubations performed at 0°C

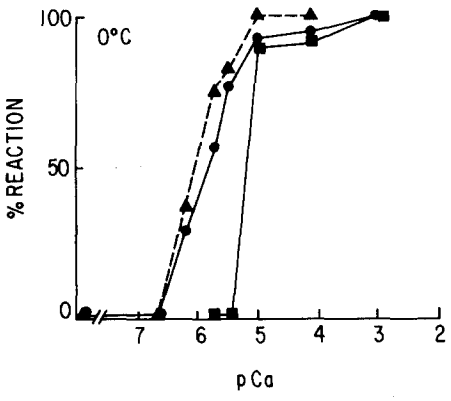

Fig. 7. Protamine also increased the threshold Ca^{2+} concentration required for exocytosis. CL were prepared and assayed as described in Materials and Methods. Prior to the microphotometric assay, the CL were incubated for 15 min at 0°C with 1 mg/ml protamine sulfate in GGE buffer (filled squares). The Ca^{2+} sensitivity of freshly isolated CL (filled triangles) and those incubated for 15 min at 0°C in the absence of protamine was also determined (filled circles)

virtually unchanged (Fig. 10, ●—●) whereas that of CL is significantly increased (Fig. 6, top panel, ●—●). We believe that this reflects the fact that in a CSC lawn substantial portions of the individual CSC fragments are not attached to the polylysine substratum. The extracytoplasmic surface of these unattached portions of CSC may bind polylysine as it dissociates from the glass, delaying inactivation. Nevertheless, as shown in Fig. 11, CSC lawns that had been incubated in the absence of exogenously added polylysine were inactivated. In this experiment the activity of CSC lawns that had been incubated for 4 h at room temperature was compared to the activity of CSC lawns that were freshly prepared from CSC that had been incubated in suspension for 4 h at room temperature. Figure 11 shows that the activity of aged CSC lawns is decreased relative to that of CSC lawns freshly prepared from an aged suspension of CSC. Since both sets of lawns were prepared from the same batch of CSC, and incubated for the same period of time, prepara-

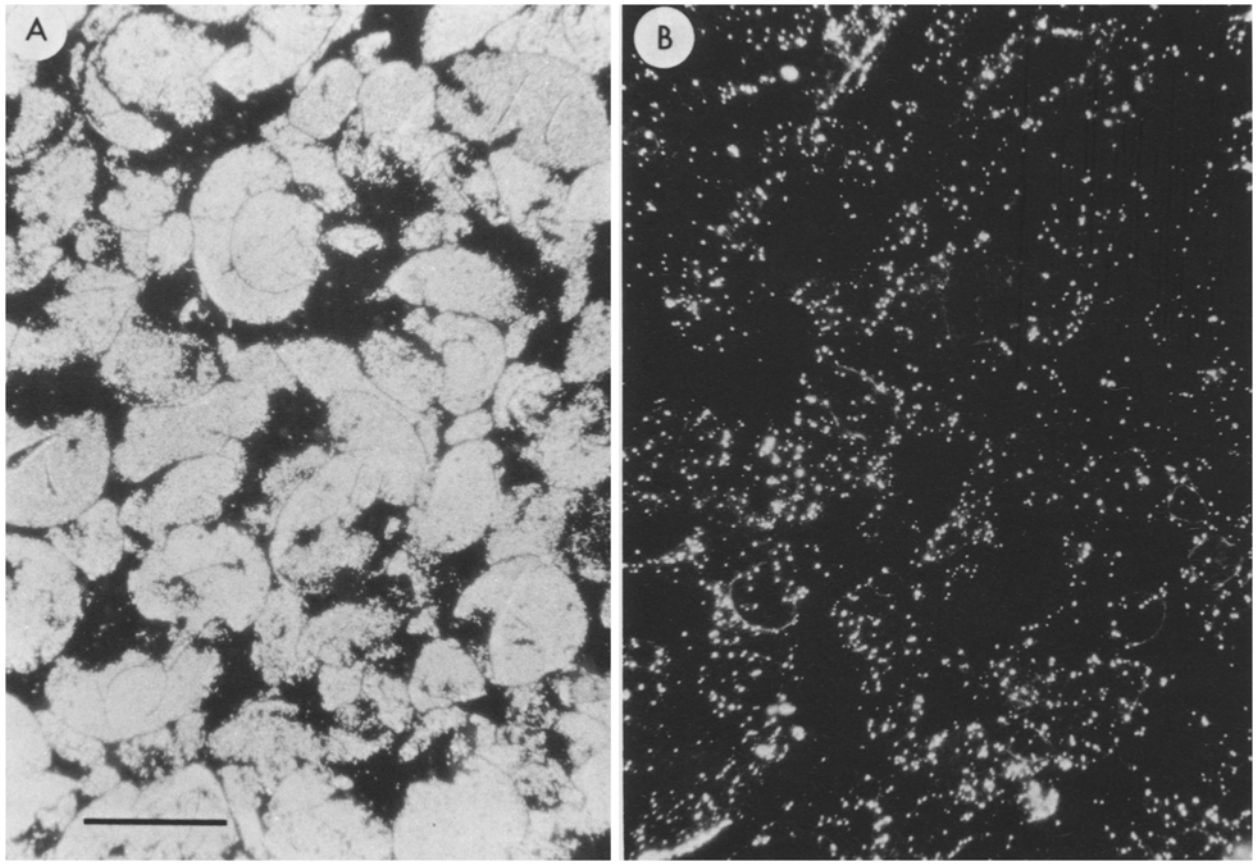

Fig. 8. Exocytosis in a CSC lawn. A CSC lawn was prepared by permitting CSC to attach to a polylysine-coated coverslip as described in Results. The lawn was photographed (*A*) before, and (*B*) after addition of GGE buffer containing 1 mM free Ca^{2+}. Bar, 100 μm

tive differences were eliminated in this experiment. Therefore, the decreased activity of the aged CSC lawns can be directly attributed to incubation of polylysine-coated coverslips.

Radiolabeled Poly-L-Lysine Can Dissociate from Glass and Redistribute onto the Exposed Surface of the CL

A possible mechanism for the time-dependent "aging" of CL exocytosis is that the polycations attaching the CL to the coverslip may dissociate from the glass and redistribute onto the cytoplasmic face of the CL. We investigated the feasibility of this mechanism by examining the ability of radiolabeled polylysine to dissociate from coverslips and redistribute onto CL. Under the conditions used to prepare coated coverslips for CL production, 0.56 ± 0.11% (n = 7) of the applied [^{14}C]poly-L-lysine remained bound to the coverslips. During a 2 hr incubation in GGE buffer 13.3 ± 2.8% (n = 4) of the bound [^{14}C]poly-L-lysine dissociated. These values were virtually identical when the coverslips were prepared by either the GGE- or distilled H_2O-wash procedures. Time course experiments indicated that the maximal amount of dissociation occurred within 15 min. Finally, when [^{14}C]poly-L-lysine-coated coverslips were incubated in a chamber containing CL (as described in Materials and Methods), 2.37 ±

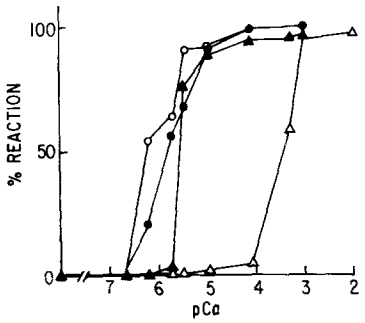

Fig. 9. Time-dependent increase in the Ca^{2+}-threshold of CL and CSC. CSC and CL were prepared as described in Materials and Methods. CSC lawns were prepared from a CSC suspension, either immediately (filled circles), or after a 5 -hr incubation at room temperature (filled triangles). CL, prepared from the same batch of eggs, were also assayed immediately upon preparation (open circles), or after 5 hr incubation at room temperature (open triangles)

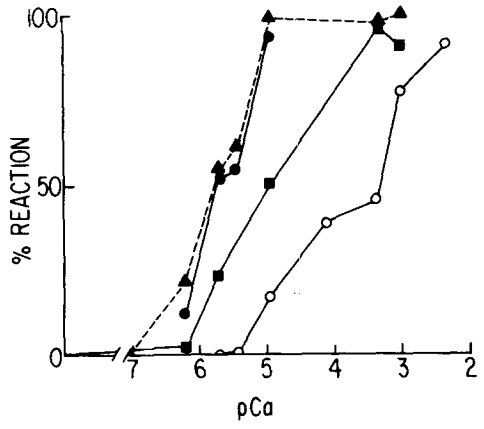

Fig. 10. Polylysine increases the Ca^{2+}-threshold of CSC lawns. CSC lawns were incubated at room temperature for 15 min with GGE buffer containing 0 μg/ml (filled circles), 25 μg/ml (filled squares), or 50 μg/ml (open circles) of polylysine, then microphotometrically assayed for cortical exocytosis. The Ca^{2+} sensitivity of freshly isolated CSC lawns was also determined (filled triangles) for comparison

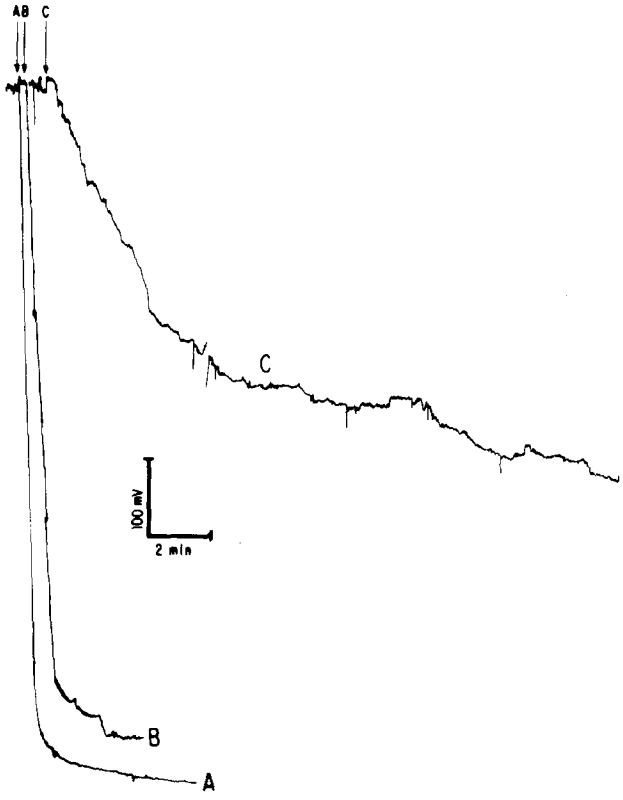

Fig. 11. Time-dependent inactivation of CSC lawns as compared to suspended CSC. CSC and CSC lawns were prepared as described in Materials and Methods. Freshly prepared CSC was used to prepare CSC lawns and assayed immediately in the microphotometric assay by the addition of 12 μM Ca^{2+} (A). CSC lawns were incubated at room temperature for 4 hr and assayed as above (C). Suspended CSC was incubated for 4 hr, after which lawns were prepared and assayed as above (B)

0.46% (n = 3) of the radiolabel was transferred to the CL after 2 hr.

Together with the observation that exogenous polylysine is inhibitory, these results strongly suggest that polylysine is responsible for the rapid "aging" of CL preparations.

Discussion

A decade has passed since the first description of the cortical lawn preparation for studies on Ca^{2+}-triggered exocytosis in the sea urchin egg (Vacquier, 1975). This preparation has the unique feature of being among the only in vitro models of exocytosis which closely approximates the in vivo process. Until recently, however, the only assay for in vitro cortical exocytosis has been microscopic observation of the disappearance of CVs. Quantitation of the extent of exocytosis was accomplished by counting CVs on micrographs taken before and after the addition of Ca^{2+}-containing buffers. Since this could practically be done only on a portion of an individual coritcal fragment per microscopic field, the assay was both extremely tedious and prone to large sampling errors. This problem has restricted the popularity of the sea urchin cortex as a model exocytotic system.

The advent of the CSC preparation (Detering et al., 1977) has lead to the development of rapid and quantitative assays for cortical exocytosis and has permitted large-scale screening for specific inhibitors (Haggerty & Jackson, 1983; Sasaki & Epel, 1983; Sasaki, 1984). Development of new assays for CL exocytosis, based on CV marker enzyme release (Moy et al., 1983) and most recently the light scattering-based microphotometric assay (Zimmerberg et al., 1985), has permitted more detailed analysis of CL exocytosis than was previously possible. These new developments have brought to light contradictory evidence with regard to "aging" of CL preparations and the role of metabolic energy in cortical exocytosis.

In this study it is shown that exogenously added polycations inhibit CL in a dose-dependent fashion, by slowing the rate (Fig. 5) and increasing the Ca^{2+} threshold of exocytosis (Figs. 6 and 7). The similarity of polycation inhibition (Figs. 5–7) to "aging" (Figs. 6 and 11) strongly suggests that polycations are responsible for "aging."

Although the biochemical mechanism of polycation inhibition of CL is presently unclear, at least three distinct modes of inhibition may be envisioned. First, the polycation substrate could be acting as an ion exchange medium, establishing an equilibrium such that factor(s) required for conferring Ca^{2+} sensitivity to the cortex are removed from the CL and deposited on the substrate. This postulates a loose association of the factor(s) with the cortex. In principle it should be possible to test this hypothesis by following the inactivation of CL on substrates where polycations are convalently attached. We attempted such experiments, using glass derivatized with alkylamine groups as well as on polystyrene which had been convalently modified with a +8 mV charge (Falcon Primaria, Becton Dickinson Co.) as the polycationic surface. However, we experienced difficulty in obtaining satisfactory attachment of the eggs to either surface.

Secondly, the physical attachment of CL and not the polycations, *per se*, could conceivably be responsible for the loss of Ca^{2+} sensitivity by decreasing membrane deformability, due to the strong ionic bonds formed between the polycationic substrate and the vitelline layer of the CL. It would seem likely, however, that such changes should occur rapidly (at the time of attachment) and should not exhibit the time dependence of the "aging" phenomenon. It is also difficult to reconcile this hypothesis with the observation that fully active CL can be prepared from eggs that have been attached to polylysine-coated coverslips and incubated for 2 hr (*data not shown*). Experiments in which polylysine was incubated with a suspension of intact eggs have shown that these eggs can elevate a fertilization envelope when treated with ionophore in Ca^{2+}-containing media, suggesting that polylysine cannot be exerting its inhibitory effects through binding to the extracytoplasmic face of the CL.

The third, and most likely, explanation is that during the incubation period polycations dissociate from the glass coverslip and deposit on the cytoplasmic face of CL, causing inhibition. We have determined that radiolabeled poly-L-lysine can dissociate from a coated coverslip and deposit onto the exposed surface of an adjacent CL. The notion that the CL cytoplasmic surface could be a sink for polycations dissociating from the glass is consistent with the time-dependence of the "aging" phenomenon. Also, buffers containing polylysine speed the inactivation of preformed CL in a dose-dependent manner (Figs. 5 and 6). Furthermore, when polylysine was incubated with the CSC in suspension and the resultant CSC aggregates observed microscopically, plus and minus Ca^{2+}, it was apparent that polylysine could exert its inhibitory effects in the absence of a solid substrate (*data not shown*).

The association of polylysine with the cytoplasmic surface of CL may be dependent on ionic interactions since polylysine is far less inhibitory when a chloride-containing buffer (PKME) is used in place of GGE. Indeed, we routinely utilize PKME buffer

in the preparation and handling of CL, as well as lawns reconstituted from isolated CVs and plasma membranes (Crabb & Jackson, 1985), in part to obviate the problems associated with the polycations.

Our results may also bear on the proposal that metabolic energy is required to maintain the Ca^{2+} sensitivity of CL preparations. ATP is not required for exocytosis in either CL or CSC preparations; however, it has been reported to slow the "aging" of CL preparations (Baker & Whitaker, 1978; Moy et al., 1983; Sasaki & Epel, 1983). In PKME-prepared CSC, ATP has no discernable effect on the extent or Ca^{2+} sensitivity of exocytosis (K.K.W. and R.C.J., *unpublished observations*).

The observation that polycations used in preparing CL are inhibitory suggests that data obtained regarding the inclusion of polyanions, e.g., ATP (Baker & Whitaker, 1978; Moy et al., 1983; Sasaki & Epel, 1983) or polyanionic proteins, e.g., calmodulin (Steinhardt & Alderton, 1982; Steinhardt & Alderton, 1983), during prolonged incubations with CL should be re-examined. Nucleotides and other polyanions have been shown to form stable complexes with polycations (Nakai & Glinsman, 1977*a*; Yip & Balis, 1980); therefore, inclusion of high concentrations of polyanions in CL buffers (5 mM ATP is typically used) can be expected to decrease polycation inhibition of exocytosis, through polyanion competition for the polycations released from the polycation-coated surfaces used to prepare CL. Conversely, caution must also be exercised in the use of polycationic proteins with either CL or CSC. Appropriate controls must be run to establish the specificity of any inactivation. These controls should include chemical or physical inactivation of the polycationic protein or enzyme in question.

Unfortunately, polycation inhibition does not provide a specific clue to the molecular mechanism of cortical exocytosis. Polycations exert a wide range of effects on cellular metabolic processes including: inhibition of calmodulin-regulated enzymes through formation of a polycation-calmodulin complex (Qi et al., 1983; Itano, Itano & Penniston, 1980); inhibition of phospholipase A_2, phospholipase C (bacterial), and phosphatidylinositol-specific phospholipase C, through alteration of the state of their phospholipid substrates (Seydel & Wasserman, 1976; Sechi et al., 1978; Hostetler & Matsuzawa, 1981; Nakas & Graff, 1982; Hostetler, 1984; Reasor, 1984); inhibition of Ca^{2+} and phospholipid-dependent protein kinase C (Qi et al., 1983); inhibition of proteases (Anderson, Harthill & Rahmatalla, 1980); and inhibition of various nucleotide-requiring enzymes (Nakai & Glinsman, 1977*b*). Polycations have also been shown to stabilize cellular membranes by binding anionic phospholipids (Ballas, Mohandas, Marton & Shohet, 1983). Recently, Whitaker and Aitchison (1985) have shown a correlation between neomycin inhibition of polyphosphoinositide-specific phospholipase C activity and inhibition of Ca^{2+}-triggered exocytosis in CL preparations.

Though one or more of the above-mentioned processes may potentially play a role in exocytosis, a coherent model will emerge only through a systematic consideration of these processes in view of all independent lines of evidence converging on this problem.

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