Volume 304, number 2,3, 207–210 FEBS 11154 June 1992

A protein factor extracted from murine brains confers physiological Ca^{2+} sensitivity to exocytosis in sea urchin eggs

Hajime Sasaki

Bio-Science Laboratory Inc. and DRBC, 5-34-5 Shironishi, Yamagata 990, Japan

Received 11 March 1992; revised version received 22 April 1992

Exocytosis in sea urchin eggs can be reconstituted in vitro using the cell ghosts (the isolated cortices). When the isolated cortices were handled in the medium primarily composed of non-chaotropic ions, exocytosis can be induced by a micromolar level of Ca^{2+}. However, when the cortices are exposed to chaotropic anions such as Cl^-, it is induced only at higher Ca^{2+} concentrations of 10^{-5} to 10^{-4} M, due to the chaotropic anionic effect, by which a specific protein(s) is dissociated from the cortex. The dissociated protein can be added back to the cortex to restore the original Ca^{2+} sensitivity [(1984) *Dev. Biol.* 101, 125–135]. A protein which has the similar effect on the isolated cortex was also found in the extract of murine brain. This protein was neither calmodulin, a G-protein or a kinase. The data suggest the general regulatory mechanism of the Ca^{2+} sensitivity of exocytosis by a protein factor widely distributed among cells.

Exocytosis; Sea urchin egg; Brain; Calcium; Protein

1. INTRODUCTION

Cortical exocytosis of sea urchin eggs is strictly regulated by Ca^{2+}, as many pieces of evidence show [1–7]. To study its regulatory mechanism, the isolated egg cortex is used as a simplified system [4,5,8–11]. Exocytosis in the isolated cortex, membrane fusion between the cortical granules (the secretory vesicles) and the plasma membrane, is induced by increasing the Ca^{2+} concentration in a bathing solution to a micromolar level, similar to that seen at the Ca^{2+} transient at fertilization [5,8–11]. No other factors, such as adenine and guanine nucleotides, are required [5,8–12]. Therefore, this system is more simplified than other systems such as those using mast cells, in which both guanine nucleotides and Ca^{2+} are required as mediators [13,14].

Previously, I described that the Ca^{2+} sensitivity of exocytosis in the isolated cortex shifts from 10^{-6} to 10^{-4} M by exposure of the cortex to chaotropioc anions, due to dissociation of a specific protein(s) from the cortex, and the dissociated protein can be added back to the cortex to restore the original level of Ca^{2+} sensitivity [8]. In this report, I describe that the restoration activity was found in the murine brain, indicating that the similar protein is distributed among cells, and discuss that the exocytotic system of sea urchin eggs may be dissected into the skeletal apparatus, which retains minimal requirements to respond to Ca^{2+}, plus the calcium sensitizing factor (CSF), which is distributed among cells.

Correspondence address: H. Sasaki, c/o Dr. A. Hino, Department of Biological Sciences, Kanagawa University School of Science, Hiratsuka, Kanagawa 259-12, Japan. Fax: (81) (463) 58-9684.

2. MATERIALS AND METHODS

2.1. *Media*

Gly medium: 300 mM glycine, 250 mM potassium gluconate, 10 mM $MgCl_2$, 10 mM EGTA (ethyl glycol bis(1-aminoethyl ether)-*N,N'*-tetraacetic acid), 10 mM PIPES (piperazine-*N,N'*-bis(2-ethanesulfonic acid)), pH adjusted to 6.7 by KOH. *KCl medium:* 500 mM KCl, 10 mM $MgCl_2$, 10 mM EGTA, 10 mM PIPES, pH adjusted to 6.7 by KOH. *Media containing chaotropic anions:* 500 mM potassium acetate or KI, 10 mM $MgCl_2$, 10 mM EGTA, 10 mM PIPES, pH adjusted to 6.7 by KOH. Ca^{2+} concentrations were adjusted to micromolar levels in 10 mM Ca^{2+} buffer using the binding constants for EGTA at pH 6.7, of 6.10×10^5 for Ca^{2+} and 18.4 for Mg^{2+} [3]. All media were kept on ice.

2.2. *Isolation of the cortex and assay of exocytosis*

Eggs of the sea urchin, *Hemicentrotus pulcherimus*, were washed with a solution containing 500 mM NaCl, 10 mM $MgCl_2$, 10 mM EGTA and 10 mM HEPES, pH 6.7, then stacked onto a glass surface coated with protamine sulfate [8]. The stacked eggs were sheared by a jet of isolation medium kept on ice. The cortices retained on the glass surface were used as the isolated cortices. When incubation was necessary for treatment of the cortices, the glass slides with the isolated cortices were placed on culture dishes kept on ice. The assay of exocytosis was performed as previously described [4,5,8,9]. The cortex isolated with either Gly or KCl medium was designated as the Gly or KCl cortex, respectively.

2.3. *Extraction of proteins from sea urchin eggs and guinea pig tissues*

Sea urchin eggs and guinea pig tissues were homogenized in an equal volume of Gly medium, and centrifuged by $10,000 \times g$ for 20 min then at $100,000 \times g$ for 60 min. The final supernatant was used as an extract.

2.4. *Treatment of the guinea pig brain by enzymes*

The guinea pig brain extract in 500 μl of Gly medium (1.0 mg/ml protein) was treated with 20 μg of trypsin (Sigma, type III), DNase-I (Sigma, type I) and RNase (Worthington) at 20°C for 30 min. The reaction of trypsin was stopped by adding 40 μg of soybean trypsin inhibitor (STI, Sigma, type I-S). As a control, both trypsin and STI were added to the extract.

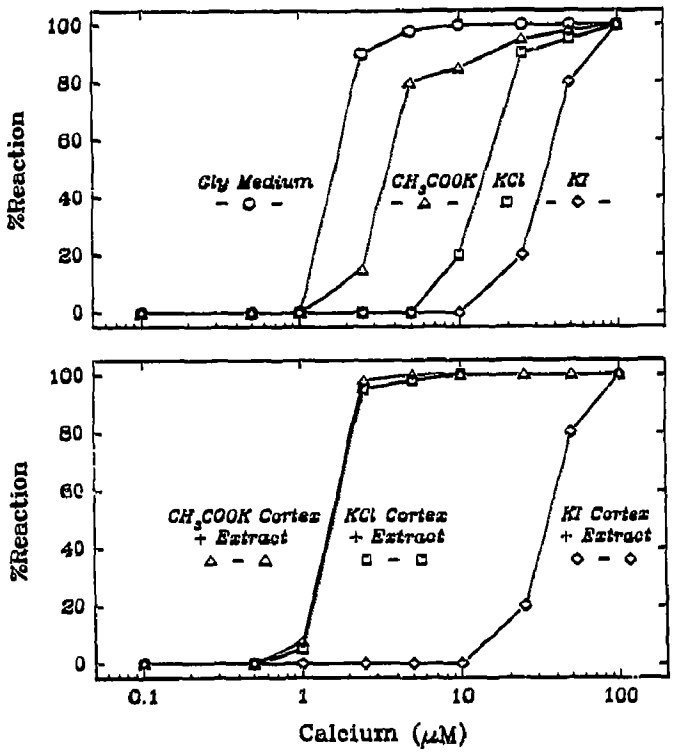

Fig. 1. (Top) The Ca^{2+} sensitivity of exocytosis in the cortices isolated from various media. The Ca^{2+} sensitivity of exocytosis decreases depending on the anion, the degree of which correlates to the chaotropic series, $CH_3COO^- > Cl^- > I^-$ [6–8]. The cortices were isolated in a medium containing the indicated anion at 500 mM, then exocytosis was induced by addition of Ca^{2+} buffers of various free Ca^{2+} concentrations in Gly medium. (Bottom) Restoration of the sensitivity of exocytosis in the isolated cortex to a micromolar level of Ca^{2+} by addition of the sea urchin egg extract. The cortex isolated in KCl medium (KCl cortex) can be sensitized from 25 μM to 2.5 μM Ca^{2+} by adding the sea urchin egg extract (KCl cortex + extract), while the cortex isolated in the medium containing 500 mM KI shows the same Ca^{2+} sensitivity in the presence and absence of extract (KI cortex ± extract). For comparison, the Ca^{2+} sensitivity of the Gly cortex is also shown (Gly cortex).

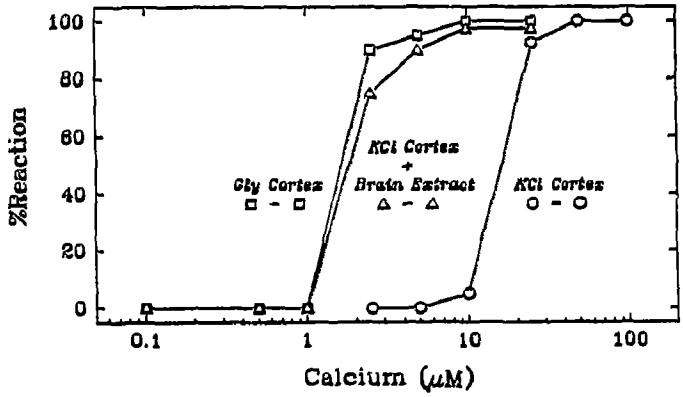

Fig. 2. Sensitization of the cortex isolated in KCl medium (KCl cortex) by the extract of guinea pig brain. The KCl cortex, which responds to 25 μM Ca^{2+}, was treated for 1 min with the extract of guinea pig brain in Gly medium, excess proteins were washed off with Gly medium, then the Ca^{2+}-EGTA buffer in Gly medium was added. Exocytosis in the treated cortex was induced at 2.5 μM Ca^{2+} (KCl cortex + extract), showing the same level of Ca^{2+} sensitivity as the Gly cortex (Gly cortex).

3. RESULTS AND DISCUSSION

Exocytosis in the isolated sea urchin egg cortex is induced by simply raising the Ca^{2+} concentration in a bathing solution without guanine or adenine nucleotides, which are required in some other systems such as those using mast cells [13,14] or platelets [15]. The Ca^{2+} concentration required to induce exocytosis in the isolated cortex varies depending on ionic components in a bathing solution [8]. Indeed, when the cortex was isolated with Gly medium, the medium mimicking the intracellular ionic condition, exocytosis was induced at 1.0–2.5 μM Ca^{2+}, but when it was isolated with KCl medium, the simplified medium containing 500 mM KCl, exocytosis was induced at 10–25 μM (Fig. 1, top). The ten-fold shift of the Ca^{2+} sensitivity by KCl was attributed to the chaotropic effect of Cl^-, since other anions such as CH_3COO^- or I^- similarly induced the downward shift of Ca^{2+} sensitivity (Fig. 1, top), the extent of which varied according to the potency of the chaotropic anions ($I^- > Cl^- > CH_3COO^-$) [16–18].

The protein(s), extracted from the cortex by chaotropic anions, can be added back to the cortex to restore the original Ca^{2+} sensitivity, showing that a specific cytoplasmic protein(s) confers the sensitivity to a micromolar level of Ca^{2+} to the cortex [8]. This was also confirmed here. The cortex isolated with KCl or CH_3COOK restored the original Ca^{2+} sensitivity, when incubated with the egg extract (Fig. 1, bottom). However, the Ca^{2+} sensitivity of the cortex isolated with KI stayed at the same level even in the presence of the egg extract (Fig. 1, bottom). This is probably due to that I^- is more chaotropic than Cl^- and CH_3COO^- [16–18]. I^- might have induced dissociation of more substances.

The activity to induce exocytosis at a micromolar level of Ca^{2+} was also found in the extract of murine brain. The tissues of guinea pig, brain, pancreas, spleen, adrenal, stomach and intestine, were homogenized, and the soluble fractions were added to the sea urchin egg cortex isolated in KCl medium. Among these tissues, the Ca^{2+} sensitizing activity was found in the extract from the brain (Fig. 2). Titer, dilution-fold to give 50% exocytosis at 2.5 μM Ca^{2+}, of the extract containing 5.2 mg/ml protein was 8. The brain extract diluted and applied to the cortex decreased the restorative activity accordingly (Fig. 3, top). The similar activity was also found in brain of rat and mouse. The Ca^{2+} concentration inducing exocytosis in the cortex treated with the appropriately diluted extract of guinea pig brain was between those for the Gly and KCl cortices (Fig. 3, bottom). When the brain extracts were added to the cortex isolated with CH_3COOK, the Ca^{2+} sensitivity was restored to the level identical to that of the cortex

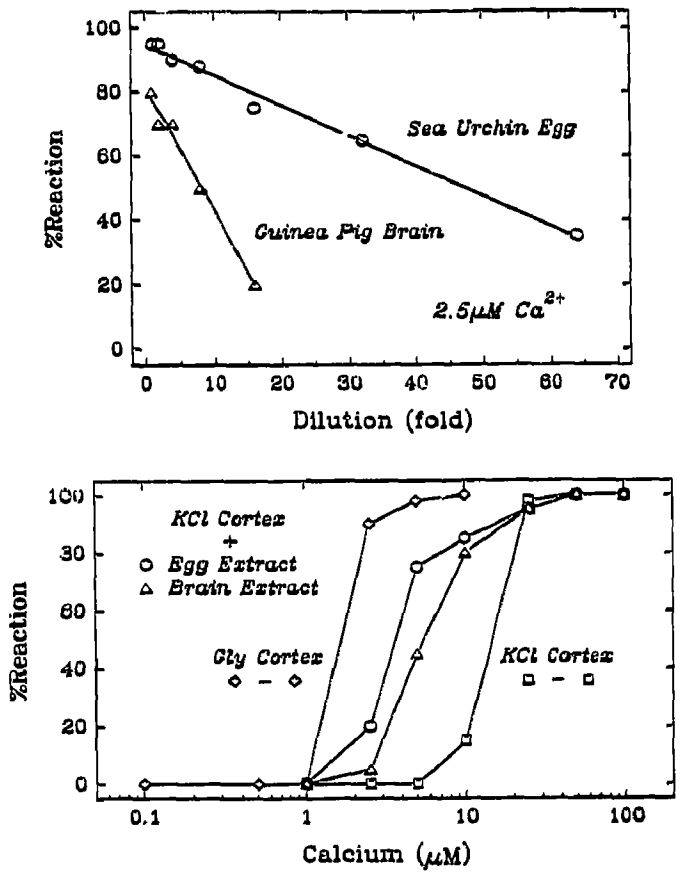

Fig. 3. (Top) The restorative activities of the extracts either of sea urchin eggs or guinea pig brain linearly decrease with dilution of the extracts. The extracts of sea urchin eggs and guinea pig brain, containing protein of 7.3 and 5.2 mg/ml, respectively, were diluted in Gly medium, and applied to the KCl cortex. After washing off excess proteins, the cortex was exposed to 2.5 μM Ca^{2+} in Gly medium. The reactivity of the cortex decreases with the dilution. (Bottom) The Ca^{2+} sensitivity of exocytosis in the isolated cortex shifts when the extract of either sea urchin eggs or guinea pig brain was diluted. The extract of either sea urchin eggs or guinea pig brain was diluted 70- or 20-fold, respectively, and applied to the KCl cortex. The Ca^{2+} sensitivity increased to the level between the Gly and KCl cortices.

isolated with Gly medium. However, in the cortex isolated with KI, no restoration was observed (not shown). These data indicate that the activity in the guinea pig brain is similar to that of the sea urchin egg extract, and that CSF is not unique to sea urchin eggs, but is distributed among cells.

The CSF in the brain extract was a protein(s), since the activity was inactivated by trypsin, not by nucleotidases (Table I). However, CSF was not calmodulin, since the activity in the brain extract was heat-labile (100°C, 1 min), contrary to the property of calmodulin [19]. CSF was neither a G-protein nor a kinase, since there were no immediate requirements of guanine or adenine nucleotides for induction of exocytosis in the isolated cortex. Moreover, the protein was active even after extensive dialysis to Gly medium or after partial purification by chromatography (not shown), excluding possible effects of contaminated nucleotides.

These data indicate that the cortex, from which CSF has been dissociated, undergoes exocytosis even if its Ca^{2+} sensitivity is not physiological, and that the physiological Ca^{2+} sensitivity of exocytosis in the isolated cortex is restored by CSF. Therefore, the sea urchin egg may consist of the basic machinery of exocytosis which exerts exocytosis in response to a sub-millimolar level of Ca^{2+}. To this machinery, CSF may be added to restore the physiological Ca^{2+} sensitivity. In other words, considered that exocytosis is a catalytic reaction of membrane fusion, the molecules responsible for the reaction at a sub-millimolar level might basically associate with the cortex, and CSF increases the Ca^{2+} sensitivity, for example, by increasing the accessibility of Ca^{2+} to these molecules. The data that CSF activity is found in guinea pig brain suggest that this aspect is applicable to other systems.

Recently, it was found that the isolated cortex retains a portion of the endoplasmic reticulum, from which Ca^{2+} may be released at fertilization as the Ca^{2+} transient [20–22]. This structure is well preserved when the cortex is isolated with media similar to Gly medium, whereas it is disrupted when exposed to media containing high concentrations of chloride [22]. Because of its behavior in the chloride-containing media, it is interesting to consider its role in the Ca^{2+} regulation of exocytosis. There might be some relation between this structure and CSF.

The isolated cortex of sea urchin eggs is unique in such a way that it can be isolated as a well-organized structure for exocytosis, which primarily consists of the cortical granules, the plasma membrane and the vitellin membrane. The cortical granules and the plasma membrane, between which membrane fusion occurs, are tightly associated via an amine-sensitive mechanism [23], so that the cortex is frozen at the step immediately before membrane fusion just waiting for the Ca^{2+} signal to proceed to the next step. Therefore, the studies on the regulatory mechanism of exocytosis in sea urchin eggs can be concentrated into the machinery responsible for

Table I

Inactivation of the Ca^{2+} sensitizing activity in the guinea pig brain extract by trypsin

Enzyme	Exocytosis[a]
None	62
Trypsin	0
Trypsin + STI	56
DNase	59
RNase	51

[a] The cortex isolated with KCl medium, originally not reactive to 2.5 μM Ca^{2+}, was incubated with the enzyme-treated extracts, and exocytosis was induced by 2.5 μM Ca^{2+}. The activity was expressed by the percentages of exocytosis.

the Ca^{2+} induction of exocytosis. Since CSF may be involved in the intracellular Ca^{2+} sensitization, studies on identification of CSF and its interaction with the isolated cortex should help elucidate the general molecular mechanism of the Ca^{2+} regulation of exocytosis.

REFERENCES

[1] Chambers, E.L., Pressman, B.C. and Rose, B. (1974) Biochem. Biophys. Res. Commun. 60, 126–132.
[2] Steinhardt, R.A. and Epel, D. (1974) Proc. Natl. Acad. Sci. USA 71, 1915–1919.
[3] Steinhardt, R.A., Zucker, R.S. and Schatten, G. (1977) Dev. Biol. 58, 185–196.
[4] Vacquier, V.D. (1975) Dev. Biol. 43, 62–74.
[5] Baker, P.F. and Whitaker, M.J. (1978) Nature 276, 513–515.
[6] Hamaguchi, Y. and Hiramoto, Y. (1981) Exp. Cell Res. 134, 171–179.
[7] Whitaker, M.J. and Steinhardt, R.A. (1982) Q. Rev. Biophys. 15, 593–666.
[8] Sasaki, H. (1984) Dev. Biol. 101, 125–135.
[9] Crabb, J.H. and Jackson, R.C. (1985) J. Cell Biol. 101, 2263–2273.
[10] Moy, G.W., Kopf, G.S., Gache, C. and Vacquier, V.D. (1983) Dev. Biol. 100, 267–274.
[11] Whalley, T.D. and Whitaker, M.J. (1988) Biosci. Rep. 8, 335–343.
[12] Sasaki, H. and Epel, D. (1983) Dev. Biol. 98, 327–337.
[13] Howell, T.W., Cockcroft, S. and Gomperts, B.D. (1987) J. Cell Biol. 105, 191–197.
[14] Barrowman, M.M., Cockcroft, S. and Gomperts, B.D.J. (1986) Nature 319, 504–507.
[15] Knight, D.E. and Scutton, M.C. (1984) Nature 309, 66–68.
[16] Hamaguchi, Y. and Geiduschek, E.P. (1962) J. Am. Chem. Soc. 84, 1329–1338.
[17] Hatefi, Y. and Hanstein, W.G. (1969) Proc. Natl. Acad. Sci. USA 62, 1129–1136.
[18] Hatefi, Y., Hanstein, W.G., Davis, K.A. and You, K.S. (1974) Ann. NY Acad. Sci. 227, 504–520.
[19] Kakiuchi, S., Yamazaki, R., Teshima, Y. and Uenishi, K. (1973) Proc. Natl. Acad. Sci. USA 70, 3526–3530.
[20] Terasaki, M. and Jaffe, L.A. (1991) J. Cell Biol. 114, 929–940.
[21] Terasaki, M. and Sardet, C. (1991) J. Cell Biol. 115, 1031–1037.
[22] Terasaki, M., Henson, J., Begg, D., Kaminer, B. and Sardet, C. (1991) Dev. Biol. 148, 398–401.
[23] Hylander, B.L. and Summers, R.G. (1981) Dev. Biol. 86, 1–11.