Identification of Protein Kinase Ca as an Essential, but Not Sufficient, Cytosolic Factor for Ca\(^{2+}\)-induced \(\alpha\)- and Dense-core Granule Secretion in Platelets*

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Akira Yoshioka‡, Ryutaro Shirakawa‡, Hiroaki Nishioka, Arata Tabuchi, Tomohito Higashi, Harunobu Ozaki, Akitsugu Yamamoto§, Toru Kita, and Hisanori Horiuchi∥

From the Department of Geriatric Medicine, Graduate School of Medicine, Kyoto University, Kyoto, 606-8507, Japan and the ∥Department of Physiology, Kansai Medical University, Moriguchi, 570-8506, Japan

Upon activation, platelets release many active substances. Here, we have analyzed the mechanism governing Ca\(^{2+}\)-induced secretion of von Willebrand factor stored in \(\alpha\)-granules and 5-hydroxytryptamine in dense-core granules in permeabilized human platelets. Both secretions were dependent on ATP and cytosol. An essential factor for both granule secretions was purified from rat brain cytosol and identified to be protein kinase Ca (PKCa) by partial amino acid sequencing. Purified PKCa efficiently stimulated both secretions in the presence of cytosol, whereas PKCa alone did not support the secretion of either type of granules, suggesting that PKCa is not a sufficient factor. Finally, in human platelet cytosol fractionated by a gel filtration column, the stimulatory activity for dense-core granule secretion paralleled with the concentration of PKC, suggesting that PKC could also be such a stimulatory factor in platelet cytosol. Thus, we identified PKCa as an essential, but not sufficient, cytosolic factor for the Ca\(^{2+}\)-induced secretions of both \(\alpha\)- and dense-core granules in platelets.

Certain types of cells contain specialized granules which are released in response to extracellular stimuli. The process is known as regulated exocytosis, which is often triggered by increased concentration of intracellular calcium ions, followed by docking and fusion of granule membrane with plasma membrane (1). Recent advances have revealed that the complex formation of SNARE proteins that bridge both sides of membranes is crucial for the docking/fusion process (2, 3). Vesicle docking is also regulated by Rab small GTPases at a step upstream of the SNARE complex formation (4, 5).

Platelets possess dense-core granules, \(\alpha\)-granules, and lysosomes, which are secreted upon activation of platelets (6, 7). Dense-core granules contain small molecules such as 5-hydroxytryptamine (5-HT)\(^1\) and \(\alpha\)-granules contain protein factors such as von Willebrand factors (vWF). Despite the biological significance of the secretions in platelet function (7), the molecular mechanisms governing the secretions remain unclear. N-Ethylmaleimide-sensitive factor and \(\alpha\)-SNAP, which are required for priming SNARE proteins in other types of cells (8), have also been shown to play important roles in granule secretions in platelets (9, 10). Furthermore, syntaxin 2 and SNAP 23, which are members of the SNARE family, are essential for the dense-core granule secretion (10), and syntaxin 4 and SNAP 23 function in the \(\alpha\)-granule secretion (11). Recently, we have identified small GTPase Rab4 as an essential regulator of \(\alpha\)-granule secretion, but not of dense-core granule secretion, using the assay system used in this investigation (12).

In addition to membrane-associated factors such as SNARE proteins and Rab small GTPases, cytosolic factors also play critical roles in intracellular membrane docking/fusion (13). For example, Rab5-regulated membrane docking/fusion of early endosomes in vitro is cytosol-dependent and the rate-limiting factor in cytosol was demonstrated to be a protein complex composed of a Rab5 effector, Rabaptin-5, and a Rab5 GDP/GTP exchange factor, Rabex-5 (14). The Ca\(^{2+}\)-induced norepinephrine release from neuroendocrine cells also requires cytosolic factors, including the phosphatidylinositol transfer protein (15), phosphatidylinositol-4-phosphate 5-kinase (16) and Ca\(^{2+}\)-dependent activator protein for secretion (CAPS/17, 18), a mammalian homologue of Caenorhabditis elegans unc31. However, cytosolic factors involved in the platelet secretion have not been identified.

Protein kinase C (PKC) family members are important signaling molecules (19, 20). Conventional PKCs have regulatory Ca\(^{2+}\)- and phorbol ester-binding domains (19, 20). The function of PKC as a signaling molecule has been analyzed mainly pharmacologically using small cell-permeable compounds of inhibitors and stimulators such as phorbol esters. Recently, Munc13-1, which contains a phorbol ester-binding domain, has been demonstrated to play an important role in membrane docking/fusion in exocytosis in synapses (21, 22). Specifically, we do not understand whether the effects of phorbol ester are mediated by Munc13 and/or PKC. Therefore, the interpretation of the earlier reports with experiments using phorbol ester has become complicated especially concerning vesicle transport. Furthermore, since PKC increases intracellular Ca\(^{2+}\) concen-

\(^{1}\) The abbreviations used are: 5-HT, 5-hydroxytryptamine; vWF, von Willebrand factor; \(\alpha\)-SNAP, \(\alpha\)-soluble N-ethylmaleimide-sensitive factor-attachment protein; SNARE, SNAP receptor; SNAP 23, synaptosome-associated protein of 23 kDa; PKC, protein kinase C; SLO, streptolysin-O; GST, glutathione S-transferase; JAM, junctional adhesion molecule; PAGE, polyacrylamide gel electrophoresis.
tration through modulation of Ca^{2+} channels in the plasma membrane in neurons (23, 24), it remains a question whether PKC acts upstream and/or downstream of increase of Ca^{2+}.

In platelets, PKC inhibitors have been shown to inhibit platelet aggregation and granule release, and phosphol 12-myristate 13-acetate, a phosphol ester, have been shown to stimulate both functions (25–27). Therefore, PKC has been considered to be an important signaling molecule in platelet activation (7). However, with reasons described above, further characterization of PKC function in platelet activation is required especially in the granule secretions. Here, we have demonstrated that the Ca^{2+}-induced secretions of α- and dense-core granules in platelets are ATP- and cytosol-dependent and that PKCα is an essential, but not sufficient, cytosolic factor for both secretions.

**EXPERIMENTAL PROCEDURES**

**Assay for Secretion of α- and Dense-core Granules**—The standard assay method was essentially as described previously (12). Briefly, freshly obtained washed platelets (5 × 10^6 platelets per assay, counted with Coulter counter) were incubated with [3H]-H5-HT (Amersham Pharmacia Biotech) to allow uptake into dense-core granules. After washing the platelets, the platelet plasma membrane was permeabilized in Buffer A (50 mM Hepes/KOH, pH 7.2, 78 mM KCl, 4 mM MgCl_2, 0.2 mM CaCl_2, 2 mM EGTA, 1 mM dithiothreitol, the calculated free calcium ion concentration was ~20 mM) containing 4 mg/ml bovine serum albumin and 0.6 µg/ml SLO as described (12). Approximately, more than 80% of the cytosolic lactate dehydrogenase was recovered in the media by the procedure, measured with lactate dehydrogenase-cytoxic test kit (Wako Chemical, Osaka, Japan). Usually, the permeabilized platelets per assay contained ~20,000 cpm of [3H]-H5-HT. Unless otherwise specified, the platelets were incubated in Buffer A containing 4 mg/ml bovine serum albumin with an ATP regeneration system containing 8 mM MgCl_2, 50 µM creatine phosphokinase, and 1 mM ATP, cytosol, and/or others at 4°C for 60 min followed by further incubation at 30°C for 5 min. Finally, the platelets were stimulated with 20 µM Ca^{2+} by addition of CaCl_2 at 30°C for 1 min. Then, after removing the platelets by centrifugation, aliquots of the supernatant containing any secreted [3H]-H5-HT and vWF were measured for the presence of [3H]-H5-HT by a liquid scintillation counter (Beckman) and of vWF by Western blot analysis after immunoprecipitation with anti-vWF antibody (Sigma) followed by quantification using densitometric software. Assay methods for [3H]-ATP and [3H]-vWF were performed for the characterization of the human platelet cytosol, the cytosol was separated by Superdex 200 gel filtration column in the same way as described above.

**Assay for PKC Activation in the Platelets**—The cytosolic domain of junctional adhesion molecule (JAM) has been demonstrated to be phosphorylated by PKC (30). The glutathione S-transferase (GST)-tagged partial cytosolic domain of JAM (JAM290) (20 µM) containing the PKC phosphorylation site (30) was incubated for 1 min at 30°C with 1 µM [γ-32P]ATP (300 cpm/µmol), 1 µM okadaic acid, and 20 µM Ca^{2+} in the presence or absence of permeabilized platelets, purified PKCα, and phosphol 12-myristate 13-acetate. Then, the GST-JAM290 was collected with glutathione-Sepharose beads (Amersham Pharmacia Biotech). The phosphorylation levels of the JAM290 were analyzed by autoradiography after SDS-PAGE.

**Antibodies, Materials, and Others**—Anti-vWF rabbit polyclonal antibody was purchased from Sigma, anti-PKCα rabbit polyclonal antibody used for Western blot analysis was from Santa Cruz, anti-PKCα mouse monoclonal antibody used for immunodepletion was from Transduction Laboratories, and a control mouse IgG from Zymed Laboratories Inc. Horseradish peroxidase-labeled anti-mouse and anti-rabbit IgG monoclonal antibodies were from Amersham Pharmacia Biotech, which were used as secondary antibodies for Western blot analysis visualized by the enhanced chemiluminescence method (Amersham Pharmacia Biotech). Unless otherwise specified, all the chemicals were purchased from Sigma, except G66850 (bisindolylmaleimide 1) which was from Calbiochem Co. and streptolysin-O (SLO) from Dr. Bhakdi, Mainz University, Mainz, Germany. Protein concentrations were determined by Bradford’s method (31) (Bio-Rad) or densitometric scanning of the Coomassie Blue-stained band of SDS-PAGE, using bovine serum albumin as a standard.

**TABLE I**

| Step | Fraction | Protein | Specific activity | Total activity | Purification | Recovery |
|------|----------|---------|------------------|---------------|--------------|----------|
|      |          | (mg units)/mg | units × 10⁻² | -fold | %          |
| 1    | Cytosol  | 1400    | 135             | 189           | 1.0          | 100.0    |
| 2    | Ammonium sulfate 35–55%  | 455     | 156             | 71            | 1.2          | 37.7     |
| 3    | Mono-Q    | 51      | 1110            | 57            | 8.3          | 30.2     |
| 4    | Mono-S    | 10      | 610             | 6.0           | 4.5          | 3.2      |
| 5    | Superdex 200  | 2.0     | 2870            | 5.7           | 21.1         | 3.0      |
| 6    | Resource PHE | 0.6     | 8620            | 5.1           | 64.3         | 2.7      |

* One unit was defined as secretion of 1% [3H]H5-HT from platelets per assay per min.
RESULTS

Ca2+-induced Secretions of α- and Dense-core Granules in SLO-permeabilized Platelets Were ATP- and Cytosol-dependent—We established an in vitro assay system using SLO-permeabilized platelets by monitoring secreted vWF stored in α-granules and [3H]5-HT in dense-core granules (12). It is well known that agonists drive the granule secretions by increasing intracellular calcium ion concentrations in platelets (32). Since the calcium ion concentration inside the platelets would be the same as that of the outside following permeabilization, we used calcium chloride as a stimulus. Therefore, we analyzed the secretion mechanism triggered by increased Ca2++. In the assay, secretions of α- and dense-core granules appeared physiologically, since the characterization of the secretions revealed similar time course and Ca2+ sensitivity to those in intact platelets (12).

Without addition of ATP, Ca2+ did not induce α-granule secretion (vWF) or dense-core granule secretion ([3H]5-HT) (data not shown) (33, 34), indicating that ATP is essential for both secretions. The permeabilized platelets were extensively depleted of cytosol since more than 80% of lactate dehydrogenase, a cytosolic protein, was recovered in the media following permeabilization (data not shown). Under this condition, Ca2+ did not induce α- (Fig. 1A) or dense-core (Fig. 1B) granule secretion without addition of exogenous cytosol, even in the presence of ATP. On the other hand, the Ca2+-induced secretions of both types of granules were efficiently reconstituted by the presence of ATP and cytosol (data not shown), as shown previously (35). These results indicated that both ATP and cytosolic factor(s) are essential for the Ca2+-induced secretions in platelets.

The cytosol dependence indicated the presence of essential cytosolic factor(s) for the secretions. Cytosols of human fibroblast cell line, HeLa cells, and human T-cell cell line, Jurkat cells, also supported the Ca2+-induced secretions of both granules (data not shown), suggesting that the essential cytosolic factor(s) are expressed ubiquitously among various species and tissues.

Purification and Identification of PKCa as the Essential Cytosolic Factor in Rat Brain Cytosol—The cytosol dependence prompted us to identify the essential factor(s) in the cytosol. The Ca2+-induced secretions of both vWF and [3H]5-HT were almost maximal in the presence of rat brain cytosol at 2–5 mg of protein/ml (Figs. 6 and 7). In order to maintain minimal secretory activity, the assays during the purification procedure were performed in the presence of rat brain cytosol at 0.5 mg of protein/ml, which supported ~5–10% of both secretions induced by Ca2+ (Figs. 6 and 7).

We first separated rat brain cytosol by Superdex 200 gel filtration column (Fig. 2A). We detected a potent peak of secretion supporting activity between markers 44 and 158 kDa (Bio-Rad) (Fig. 2A). Notably, the peaks for α- and dense-core granule secretions were almost completely overlapped, suggesting that the factor is common for both secretions.

Then, we scaled up the purification procedure. The purification was performed by ammonium sulfate precipitation and sequential column chromatography with Mono-Q, Mono-S, Superdex 200, and Resource PHE columns as described under “Experimental Procedures.” During each procedure (Table I), the most potent activity for either α- or dense-core granule secretion was recovered in a single peak. Then, the peaks of both α- and dense-core granule secretions were almost completely overlapped in each column chromatography (data not shown). In Superdex 200 gel filtration column chromatography, the peak fraction emerged at a position between 44 and 158 kDa (data not shown), similar to the results in experiments of the direct separation of the cytosol by Superdex 200 (Fig. 2A).
FIG. 3. The Ca2+-induced secretions of α- (A) and dense-core (B) granules were affected by Gö68650, a PKC inhibitor. The permeabilized platelets were first incubated for 60 min at 4 °C with the ATP regeneration system in the presence or absence of Gö68650 and rat brain cytosol (2 mg of protein/ml) as indicated in the figure. The platelets were then incubated with 20 nM (lanes 1) or 20 μM Ca2+ (lanes 2–5) for 1 min at 30 °C and released vWF (A) and [3H]5-HT (B) were measured as described under “Experimental Procedures.” The data shown were mean ± S.E. in five independent experiments. The inset immunoblot (A) is one of the typical results for measuring the released vWF.

FIG. 4. The purified PKCα sample immunodepleted with anti-PKCα antibody (A) lost the stimulatory activity of the Ca2+-induced secretion of α- (B) and dense-core (C) granules. A, the purified PKCα samples (lane 1) were immunodepleted with an anti-PKCα monoclonal antibody (lane 2) or a control mouse IgG (lane 3). Then, the samples were analyzed by Western blot with anti-PKCα polyclonal antibody. B and C, the permeabilized platelets were incubated for 60 min at 4 °C in the presence of 0.5 mg of protein/ml rat brain cytosol and the ATP regeneration system with the purified PKCα (40 nm) (lane 2), the purified PKCα after immunodepletion as shown in A (lanes 3 and 4) or without purified PKC (lane 1). The platelets were then stimulated with 20 μM Ca2+ for 1 min at 30 °C and released vWF (B) and [3H]5-HT (C) were measured as described under “Experimental Procedures.” The data shown were mean ± S.E. in five independent experiments. The inset immunoblot (B) is one of the typical results for measuring the released vWF.

As shown in Fig. 2B, a band with apparent molecular mass at ~80 kDa was purified to homogeneity after the fifth step of Superdex 200 column chromatography (Table I). Furthermore, the active fractions were collected and fractionated by a hydrophobic interaction column, Resource PHE. The secretion-supporting activities for both granules again paralleled the 80-kDa band (data not shown). The molecular weight was consistent with the results in the Superdex 200 gel filtration column chromatography for direct separation of rat brain cytosol (Fig. 2A). Taken together, the 80-kDa protein was most likely the essential common factor for both secretions.

The partial amino acid sequences of the purified 80-kDa protein excised from the SDS-PAGE gel after the Resource PHE column chromatography (Fig. 2B, lane 6) were determined by the Edman’s method (29). The determined sequence IARFF, LIPMDFNGL, and IRSTLNPQW completely corresponded to the sequences of rat PKCα(s) 38–44, 182–191, and 214–222, respectively (36). Since all three sequences corresponded to parts of the rat PKCα sequence, the 80-kDa protein was determined to be PKCα which was a conventional type of PKC composed of 672 amino acids with a calculated molecular mass of 76.6 kDa (36).

Accordingly, Gö68650, a PKC inhibitor, concentration dependently inhibited the Ca2+-induced secretions of both types of granules in the permeabilized platelets (Fig. 3, A and B). Gö68650 at 10 μM almost completely inhibited both secretions (Fig. 3, A and B). Furthermore, when PKCα was immunodepleted from the purified samples with anti-PKCα monoclonal antibody-coated beads (Fig. 4A), the immunodepleted samples lost the secretion stimulatory activity for both types of granules (Fig. 4, B and C). Thus, PKCα was identified to be an essential factor in rat brain cytosol for the Ca2+-induced secretions of both α- and dense-core granules in platelets.

We then examined whether the added PKCα was activated in the assay. The PKC activity in the assay was monitored by phosphorylation levels of GST-JAM290, which contained a PKCα-dependent phosphorylation site (30) of JAM, a cell-cell adhesion molecule (37, 38). GST-JAM290 was hardly phosphorylated by the permeabilized platelets alone without the purified PKCα (Fig. 5, lane 3). On the other hand, the purified PKCα alone weakly phosphorylated GST-JAM290 (Fig. 5, lane 4). The phosphorylation level of GST-JAM290 was strongly enhanced in the presence of both purified PKCα and permeabilized platelets (Fig. 5, lane 5) to the similar level obtained in the experiments with the purified PKCα, permeabilized platelets, and phorbol 12-myristate 13-acetate, a PKC activator (Fig. 5, lane 1). These results suggested that the exogenously added PKCα was indeed activated in the assay by Ca2+ and the permeabilized platelets. Since PKCα is activated by Ca2+ in the presence of activators such as phosphatidylserine and diacylglycerol (19, 20), the permeabilized platelet membrane could substitute for the activators.

In another set of experiments, 10 μM Gö68650 completely inhibited Ca2+-induced phosphorylation of GST-JAM290 in the presence of permeabilized platelets (data not shown). Since Gö68650 at 10 μM also inhibited platelet secretion as shown in Fig. 3, catalytic activity of PKC could be required for secretion.

PKCα Alone Did Not Support the Ca2+-induced Secretions—We characterized the purified PKCα in the Ca2+-induced secretions in permeabilized platelets. First, we examined the amount of PKC remaining after permeabilization by Western blot analysis. Since we detected very little PKC associated with the permeabilized platelets after the procedure (data not shown), most of the PKC in the platelets appeared to be lost by diffusion through holes in the plasma membrane. Second, we carefully determined the PKC concentration in rat brain cy-
tosol by Western blot analysis using purified PKCα as a control. The PKC concentration in the rat brain cytosol preparation used in the assay (10 mg of protein/ml) was 250 nM.

Then, we examined whether PKCα alone was enough to support the Ca²⁺-induced secretions. The Ca²⁺-induced secretions of both types of granules were examined with various concentrations of the purified PKCα and rat brain cytosol (Fig. 6). The activities of the cytosol and the purified PKCα were compared based on concentrations of contained PKC (Fig. 6, B and C). Without addition of any cytosol or purified PKCα (see the points of PKC concentration at 0 nM in Fig. 6, B and C), Ca²⁺ did not induce either vWF or [³H]5-HT secretion over the levels obtained without Ca²⁺ stimulation (Fig. 6, B and C). Under these conditions, rat brain cytosol efficiently supported the Ca²⁺-induced secretions of both granules in a concentration-dependent manner (Fig. 6, B and C). The cytosol was saturated at 4 mg of protein/ml (containing 100 nM PKC) for the vWF secretion and at 2 mg of protein/ml (containing 50 nM PKC) for the [³H]5-HT secretion (Fig. 6, B and C). The concentrations of rat brain cytosol which supported half-maximal α- and dense-core granule secretion were 2.0 mg of protein/ml (containing 50 nM PKC) (Fig. 6B) and 1.2 mg of protein/ml (containing 30 nM PKC), respectively (Fig. 6C). On the other hand, addition of purified PKCα alone at comparable concentrations hardly stimulated either secretion (Fig. 6, B and C), indicating that PKCα is not a sufficient factor for the Ca²⁺-induced secretions in platelets.

Next, in another set of experiments, we examined the effects of the purified PKCα on the Ca²⁺-induced secretions of α- (Fig. 7, A and B) and dense-core granules (Fig. 7C) in the presence of various concentrations of rat brain cytosol. Rat brain cytosol alone (closed circles in Fig. 7, B and C) supported both secretions concentration dependently, as shown in Fig. 6. In the experiments with the cytosol plus 40 nM purified PKCα (open circles in Fig. 7, B and C), the purified PKCα strongly enhanced both secretions in the assay with low concentrations of the cytosol, indicating that PKCα is indeed a limiting factor for the secretions. Similar levels of the secretions with high concentrations of the cytosol in the absence or presence of 40 nM purified PKCα (Fig. 7, B and C) could reflect the saturated effects of PKCα in the assay.
The parallel emergence of the secretion supporting activity with PKC suggested that PKC is a core regulatory component also in platelet cytosol for the Ca\(_{2+}\)-induced secretion of dense-core granules.

**DISCUSSION**

We have established an assay system to analyze Ca\(_{2+}\)-induced secretions of \(\alpha\)- and dense-core granules in SLO-permeabilized platelets (12). In the system, both secretions were ATP- and cytosol-dependent, indicating the presence of essential cytosolic factor(s). We purified an essential factor for both ATP- and cytosol-dependent, indicating the presence of essential, common factor in cytosol for both secretions. We have shown that PKC and calmodulin are sufficient factors since either of the factors alone stimulated the secretion (40). Then, they have shown that their activities were additive and that the contributions of PKC and calmodulin were 33–44% and 13–22%, respectively, of the total activity of the cytosol (40). In the case of platelets, we have here demonstrated that PKC is an essential factor in cytosol. However, PKC alone supported very little secretion of either \(\alpha\)- or dense-core granules (Fig. 6, B and C), indicating that PKC is not a sufficient factor in platelet secretions. The difference from the results of Chen et al. (40) could be due to a different mechanism of regulation used by platelets and PC12 cells, or due to different assay conditions.

PKC has been demonstrated to increase intracellular Ca\(_{2+}\) concentration through modulation of Ca\(_{2+}\) channels in the plasma membrane in the case of neuronal cells (23, 24). Therefore, another question remains: whether PKC acts downstream and/or upstream of increased concentration of Ca\(_{2+}\). Here, we could safely say that PKC plays a critical role at least downstream of the increased Ca\(_{2+}\) in the granule secretions in platelets, since Ca\(_{2+}\) itself was the stimulus in our assay.

The function of PKC\(\alpha\) is presumed to phosphorylate its substrate proteins involved in the exocytosis machinery. Recently, syntaxin 4, a SNARE protein, has been shown to be phosphorylated by PKC upon thrombin stimulation in platelets (41). Since syntaxin 4 has been implicated in \(\alpha\)-granule secretion (11), a putative target molecule of PKC\(\alpha\) could be syntaxin 4. Interestingly, syntaxin 4 has very recently been shown to interact directly with Rab4 in pancreatic \(\beta\) cells (42), which we have shown to be an essential regulator for \(\alpha\)-granule secretion in platelets (12). The function of syntaxin 4 could be finely regulated by both PKC\(\alpha\) and Rab4 in \(\alpha\)-granule secretion in platelets. Furthermore, Munc18-1, a syntaxin-binding protein essential for neurotransmitter release in the synapses (43–45), has also been shown to be phosphorylated by PKC (46). Since a homologue of Munc18-1 is present in platelets (47), it could also be a substrate. Further investigation is required to elucidate how PKC\(\alpha\) regulates the Ca\(_{2+}\)-induced secretions of both \(\alpha\)- and dense-core granules in platelets.

Besides membrane-associated components such as SNARE proteins and RabGTPases, cytosolic factors have also been demonstrated to play critical roles in membrane docking/fusion (13, 15). However, only a few proteins have been identified so far in exocytosis. Ca\(_{2+}\)-induced norepinephrine release has been analyzed in vitro in the crackled-cell assay using PC12.
cells (17). Using the assay, Hay et al. (15) detected three peaks of secretion stimulating activities in rat brain cytosol in gel filtration column chromatography and two of these were identified to be phophatidylinositol transfer protein and phosphatidylinositol-4-phosphate 5-kinase (16). Furthermore, they have found the Ca\(^{2+}\)-dependent activator protein for secretion, a mammalian homologue of C. elegans unc31, as the cytosolic stimulatory factor in rat brain cytosol (17, 18).

However, we detected only one peak of the activity obtained with PKCa (Fig. 24), although we have shown that other cytosolic factor(s) are also required for secretions. In the assay used in the purification procedure, rat brain cytosol at 0.5 mg of protein/ml was added since the activity would not be detected if the essential factors were multiple and separated by column chromatography. With the results shown in Fig. 7, B and C, we speculated that the concentrations of rat brain cytosol which supported half-maximal secretions of both granules were 1.2–2.0 mg of protein/ml for PKCa, whereas it was 0.5–0.7 mg of protein/ml for other factor(s). Therefore, the rat brain cytosol at 0.5 mg of protein/ml might be limiting for PKC while it could contain relatively high concentrations of other factors, which could be essential, but not rate-limiting. This would not allow us to detect the stimulatory activity caused by the putative factors other than PKCa. Identification of other cytosolic factors is absolutely required for elucidation of the regulatory mechanism taking place in the cytosol for the granule secretions in platelets.

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REFERENCES

1. Jahn, R., and Sudhof, T. C. (1999) Annu. Rev. Biochem. 68, 863–911
2. Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J. E. (1993) Nature 362, 318–324
3. Mayer, A. (1999) Curr. Opin. Cell Biol. 11, 447–452
4. Lian, J. P., Stone, S., Jiang, Y., Lyons, P., and Ferrus-Novick, S. (1994) Nature 372, 698–701
5. Sogaard, M., Tani, K., Ye, R. R., Geromanos, S., Tempst, P., Kirchhausen, T., Rothman, J. E., and Sollner, T. (1994) Cell 76, 907–948
6. Silverstein, R. L., and Febbraio, M. (1992) Blood 80, 1470–1475
7. Plow, E. F., and Ginsberg, M. H. (2000) in Hematolgy: Basic Principles and Practice (Hoffman, R. S., S., Benz, E. J., Furie, B., Cohen, H., and Silberstein, L. E., ed) 3rd Ed., pp. 1741–1752, Churchill Livingston, New York
8. Mayer, A., Wickner, W., and Haas, A. (1996) Cell 85, 83–94
9. Polgar, J., and Reed, G. L. (1999) Blood 94, 1313–1316
10. Chen, D., Bernstein, A. M., Lemons, P. P., and Whiteheart, S. W. (2000) Blood 95, 921–929
11. Flavumna, R., Croce, K., Chen, E., Furie, B., and Furie, B. C. (1999) J. Biol. Chem. 274, 2492–2501
12. Shirakawa, R., Yoshioka, A., Horiuchi, H., Nishinaka, T., Tabuchi, A., and Kita, T. (2000) J. Biol. Chem. 275, 33834–33849
13. Avery, J., Jahn, R., and Edwardsen, J. M. (1999) Annu. Rev. Physiol. 61, 777–807
14. Horiuchi, H., Lippe, R., McBride, H. M., Rubino, M., Woodman, P., Stenmark, H., Rybin, V., Wilm, M., Ashman, K., Mao, M., and Zerial, M. (1997) Cell 90, 1149–1159
15. Hay, J. C., and Martin, T. F. (1993) Nature 366, 572–575
16. Hay, J. C., Fisette, P. L., Jenkins, G. H., Fukami, K., Takenawa, T., Anderson, R. A., and Martin, T. F. (1995) Nature 374, 173–177
17. Walent, J. H., Porter, B. W., and Martin, T. F. (1992) Cell 70, 765–775
18. Ann, K., Kowalchyk, J. A., Loyet, K. M., and Martin, T. F. (1997) J. Biol. Chem. 272, 19637–19640
19. Nishizuka, Y. (1992) Science 258, 607–614
20. Newton, A. C., and Johnson, J. E. (1998) Biochim. Biophys. Acta 1376, 155–172
21. Betz, A., Ashery, U., Rickmann, M., Augustin, I., Neher, E., Sudhof, T. C., Rettig, J., and Brose, N. (1998) Neuron 21, 123–136
22. Augustin, I., Rosenmund, C., Sudhof, T. C., and Brose, N. (1999) Nature 400, 457–461
23. Swartz, K. J. (1993) J. Biol. Chem. 311, 305–329
24. Stea, A., Soong, T. W., and Snutch, T. P. (1995) Neuron 15, 929–940
25. Kubauchi, K., Takay, A., Sawamura, M., Hoshijima, M., Fojikura, T., and Nishizuka, Y. (1983) J. Biol. Chem. 258, 6701–6704
26. Siess, W., and Lapetina, E. G. (1988) Biochem. J. 255, 309–318
27. Gerrard, J. M., Beatte, L. L., Park, J., Israel, S. J., McNicol, A., Lint, D., and Cragoe, E. J. (1989) Blood 74, 2405–2411
28. Fabiato, A., and Fabiato, F. (1979) J. Physiol. (Paris) 75, 483–505
29. Edman, P. (1970) Mol. Biol. Biochem. Biophys. 8, 211–255
30. Ogita, K., Kikkawa, U., Ogita, K., and Nishizuka, Y. (1993) Nature 362, 807–810
31. Kuroki, T., Ashery, U., Rickmann, M., Augustin, I., Neher, E., Sudhof, T. C., Scheller, R. H., and Takai, Y. (1996) J. Biol. Chem. 271, 2501–2504
32. Gengyo-Ando, K., Kamiya, Y., Yamakawa, A., Kodaira, K., Nishiwaki, K., Neher, E., Sudhof, T. C., T. (2000) J. Biol. Chem. 275, 21157–21160
33. Betz, A., Ashery, U., Rickmann, M., Augustin, I., Neher, E., Sudhof, T. C., Rettig, J., and Brose, N. (1998) Neuron 21, 123–136
34. Augustin, I., Rosenmund, C., Sudhof, T. C., and Brose, N. (1999) Nature 400, 457–461
35. Suzuki, T., and Martin, T. F. (1997) J. Biol. Chem. 272, 2501–2504
36. Reed, G. L., Houng, A. K., and Fitzgerald, M. L. (1999) Science 286, 953–956
37. Reed, G. L., Houng, A. K., and Fitzgerald, M. L. (1999) Blood 93, 2617–2626
38. Reed, G. L., Houng, A. K., and Fitzgerald, M. L. (1999) Blood 93, 2617–2626