Biochemical and Biophysical Research Communications **260,** 42–47 (1999)
Article ID bbrc.1999.0869, available online at http://www.idealibrary.com on IDEAL®

Antibodies against the PH Domain of Phospholipase C-δ1 Inhibit Ins(1,4,5)P_3-Mediated Ca^{2+} Release from the Endoplasmic Reticulum

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Received May 14, 1999

The pleckstrin homology domain (PH domain) is now well known as a structural module for the binding of inositol compounds. In the present study, polyclonal antibodies against the peptide KVKSSS-WRRERFYK, derived from the N-terminal of the PH domain of phospholipase C-δ1 (PLC-δ1), were raised in rabbits. These were then tested for their ability to inhibit the binding of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] to the binding proteins including the receptor molecule. The Fab fragment of the antibodies but not the whole molecule inhibited the binding of Ins(1,4,5)P_3 not only to PLC-δ1 but also to the Ins(1,4,5)P_3 receptor, indicating that the antibodies raised recognized the binding site for Ins(1,4,5)P_3 in the receptor. Rat basophilic leukemic cells were permeabilized with saponin and assayed for Ins(1,4,5)P_3-mediated Ca^{2+} release. Pretreatment of permeabilized RBL cells with the Fab fragment of the antibodies diminished the release of Ca^{2+} caused by Ins(1,4,5)P_3, and further absorption experiments using a variety of synthetic peptides suggested that the tripeptide KVK is the epitope of the antibodies. Structural information about KVK will help in screening for Ins(1,4,5)P_3 antagonists. © 1999 Academic Press

Studies in this laboratory and that of Rebecchi and colleagues have demonstrated that phospholipase C-δ1 (PLC-δ1) has a high affinity and high specificity for both D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2]. Also the deletion mutant, limited proteolysis and synthetic peptide experiments have shown that the binding site is situated in the N-terminal 30–43 residues in the molecule (1–7). The major part of these studies were done before the emergence of the term "pleckstrin homology domain (PH domain), which was first found in pleckstrin, a major substrate for protein kinase C (8, 9), and has been identified in more than 100 signaling or cytoskeletal proteins to date (10–12). We showed that the binding site for Ins(1,4,5)P_3/PtdIns(4,5)P_2 was mapped in the PH domain of PLC-δ1 (5, 6, 12). In the same year (1994), Harlan *et al.* (13) reported that the PH domains derived from a variety of proteins bound PtdIns(4,5)P_2, identifying several basic amino acids involved in the binding by NMR spectrophotometry. The location of these basic amino acids in the primary sequence corresponded to residues 30–43 in PLC-δ1, thereby confirming that the 30–43 peptide is responsible for the binding of PLC-δ1 to Ins(1,4,5)P_3/PtdIns(4,5)P_2 (5, 6). Therefore, we raised polyclonal antibodies against the synthetic peptide in rabbits, and revealed that Fab fragments of the antibody inhibited the binding of PLC-δ1 to Ins(1,4,5)P_3, whilst the whole antibody molecule inhibited the binding of the peptide, but not the native PLC-δ1, to Ins(1,4,5)P_3 (6).

Using a different approach, the binding site of the Ins(1,4,5)P_3 receptor molecule was first reported by Mignery and Südhof (14), who showed that the N-terminal 650 amino acids in the molecule were involved in the binding. Miyawaki *et al.* (15) and Newton *et al.* (16) supported this observation by showing that the N-terminal 576 amino acids fused to glutathione S-transferase specifically bound Ins(1,4,5)P_3 with a high affinity, whereas further N- or C-terminal dele-

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Abbreviations used: Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; PtdIns(4,5)$_2$, phosphatidylinositol 4,5-bisphosphate; PH domain, pleckstrin homology domain; PLC-δ1, δ1-isozyme of phospholipase C; RBL cells, rat basophilic leukemic cells; IgG, immunoglobulin G.

0006-291X/99 $30.00

tion completely abolished the binding. Furthermore, Mourey *et al.* (17) labeled residues 471–501 in this region with a photoaffinity ligand. These results indicated that the Ins(1,4,5)P_3 binding site of the receptor is localized within the N-terminal 576 amino acids. Yoshikawa *et al.* (18) performed more systematic experiments using point-mutagenesis to narrow the binding site in the receptor. They found that the N-terminal 226–576 amino acids were sufficient and close enough to the minimal region for specific Ins(1,4,5)P_3 binding, and that 10 basic amino acids, of the 41 basic amino acids present within this region, were a critical requirement for the binding (18).

In the present study, we examined whether antibody against the 30–43 residues of PLC-δ1 inhibit binding of the Ins(1,4,5)P_3 receptor, and whether they affect the process of Ins(1,4,5)P_3-mediated Ca^{2+} release from the endoplasmic reticulum of cells. Furthermore, absorption experiments, in which the antibody was pretreated with synthetic peptides comprizing sections of the 30–43 residues of PLC-δ1, revealed that KVK could be an epitope of the antibody.

MATERIALS AND METHODS

Synthesis of the peptide and its conjugation with carrier proteins for immunization. A peptide corresponding to residues 30–43 of PLC-δ1 [PLC-δ1(30–43)], KVKSSSWRRERFYK, was synthesized and purified as described (6). A scrambled peptide of PLC-δ1(30–43), WKSRFKSERYKSRV, was also synthesized and purified as the control. The peptides used were supplemented with a cystein residue at the amino terminal to facilitate coupling with carrier protein. Conjugation of the peptide with carrier proteins such as keyhole limpet hemocyanin was carried out using the bifunctional reagent *m*-maleimidobenzoyl-N-hydroxysuccinimide ester, as described (19). The coupling ratio was determined by amino acid analysis. Four peptides, KVK [PLC-δ1(30–32)], KVKSSSWR [PLC-δ1(30–37)], RRER [PLC-δ1(37–40)] and RRERFYK [PLC-δ1(37–43)] were also synthesized for absorption experiments.

Immunization and purification of specific antibody against the peptide. Immunization was performed as described previously (6). Whole immunoglobulin G (IgG) specific for the peptide PLC-δ1(30–43) was purified by applying the antiserum to a peptide-coupled Sepharose 4B resin which was prepared according to the method described (19). Fab fragments from whole IgG were prepared and purified as follows: Rabbit IgG was isolated from immune serum by ammonium sulfate fractionation. Following dialysis against 0.1 M sodium acetate buffer (pH 5.5), digestion with papain was carried out in the presence of 1 mM EDTA, 50 mM L-cystein and 1% (w/w) of papain at 37°C for 16 h and stopped by adding iodoacetamide to give a final concentration of 75 mM. Fab fragments thus produced were separated from undigested IgG by applying the mixture to a HiLoad 26/60 Sephacryl S-100 column. A fraction containing Fab fragments, as assessed by SDS-polyacrylamide gel electrophoresis, was then applied to a peptide-coupled Sepharose 4B column to obtain Fab fragments specific for the peptide. Characterization of the anti-peptide antibody has been performed previously (6), i.e., the antibody recognized the full-length PLC-δ1, but not thrombin-treated PLC-δ1 lacking the N-terminal 60 residues, and the Fab fragments of the antibody inhibited the binding of [^{3}H]Ins(1,4,5)P_3 to native PLC-δ1.

Preparation of type I-Ins(1,4,5)P_3 receptor and assay for binding to [^{3}H]Ins(1,4,5)P_3. The Ins(1,4,5)P_3 receptor was purified to apparent homogeneity from rat cerebellum, as described (1). The purity was checked by SDS polyacrylamide gel electrophoresis (not shown). The binding of [^{3}H]Ins(1,4,5)P_3 to the receptor was determined by the polyethyleneglycol precipitation method, as described (20). Briefly, the purified receptor was incubated with 1.3 nM [^{3}H]Ins(1,4,5)P_3 in a solution (0.45 ml) containing 50 mM Tris-HCl buffer (pH 8.3), 2 mM EDTA and 0.2% Triton X-100 for 15 min on ice. Fifty μl of bovine γ-globulin (10 mg/ml) was added to this mixture, followed by the addition of 0.5 ml of 30% polyethylene glycol. After centrifugation, the precipitate was dissolved in 1 ml of 0.1 N NaOH and the radioactivity counted. For the binding assay using the native form of the Ins(1,4,5)P_3 receptor, rat cerebellar membrane fraction which was prepared by homogenization and sequential centrifugation, was also used. The assay mixture was the same as for the purified sample except for the omission of Triton X-100. After incubating on ice, the membrane fraction was precipitated by centrifugation and the radioactivity was analyzed. In both cases, non-specific binding (about 200 dpm) was determined in the presence of 10 μM Ins(1,4,5)P_3 and was subtracted from that in its absence to determine the specific binding.

Assay of Ca^{2+} release by Ins(1,4,5)P_3. Rat basophilic leukemic (RBL) cells were harvested 4 ~ 5 days after plating, and then were permeabilized with saponin, as described for peritoneal macrophages (21, 22). Ca^{2+} uptake and release by saponin-permeabilized RBL cells was assayed as follows: permeabilized RBL cells (1×10^6 cells/ml) were incubated in a solution (1 ml) containing 130 mM KCl, 20 mM Hepes buffer (pH 7.2), 5 mM $MgCl_2$, 5 mM NaN_3 and 1 μM fura 2. Ca^{2+} uptake by the endoplasmic reticulum was initiated by the addition of 5 mM ATP, and monitored with a fluorescent spectrometer at a wavelength of 380 nm and 510 nm for excitation and emission, respectively. Seven min after the addition of ATP, when the Ca^{2+} uptake reached a plateau, various concentrations of Ins(1,4,5)P_3 were added cumulatively to the mixture to observe the Ca^{2+} release. In order to examine the effect of the antibody, cells were preincubated with the antibody at room temperature for 10 min before the addition of ATP. Assays were carried out at room temperature (20–25°C).

Chemicals. [^{3}H]Ins(1,4,5)P_3 (specific radioactivity: 777 GBq/mmol) was obtained from DuPont New England Nuclear (Boston, MA). HiLoad 26/60 Sephacryl S-100 column and 1,6-diaminohexane-Sepharose 4B (EAH-Sepharose 4B) were purchased from Pharmacia Biotechnol Inc (Uppsala, Sweden). *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester and keyhole limpet hemocyanin were obtained from Pierce (Rockford, IL) and Sigma (St. Louis, MO), respectively. Fura 2 was purchased from Dojindo Laboratories (Kumamoto, Japan). All other reagents were of the highest grade available.

RESULTS

Figure 1 shows the effect of the antibody against the peptide comprizing PLC-δ1(30–43) on the binding of [^{3}H]Ins(1,4,5)P_3 to the Ins(1,4,5)P_3 receptor. The binding of [^{3}H]Ins(1,4,5)P_3 to the receptor was not inhibited by the IgG purified on the peptide-immobilized column. However, the Fab fragments isolated from the IgG by papain digestion inhibited the binding in a dose-dependent manner, indicating that the antibody against the Ins(1,4,5)P_3 binding peptide on the PH domain of PLC-δ1 recognized the Ins(1,4,5)P_3 binding site on the receptor molecule. We previously showed the same results with native PLC-δ1 (6). Failure of the whole antibody to inhibit binding could be because an antibody with divalent antigenic determinants does not have as much access to the native Ins(1,4,5)P_3 receptor binding site as to the native PLC-δ1 binding site, probably because of sterical hindrance.

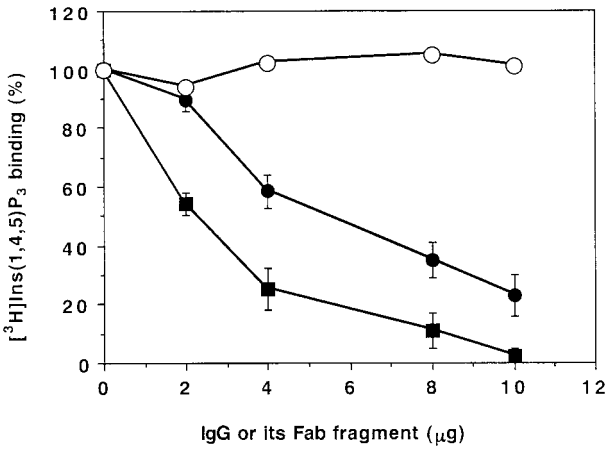

FIG. 1. Effect of antibody against the N-terminal portion of the PH domain of PLC-δ1 on [^{3}H]Ins(1,4,5)P_3 binding to the Ins(1,4,5)P_3 receptor. Ins(1,4,5)P_3 receptor (10 μg, ■; 20 μg, ○, ●) purified from rat cerebellum was preincubated with various concentrations of whole IgG (○) or the Fab fragment (●, ■) against PLC-δ1(30–43) peptide at 4°C for 20 min and then assayed for [^{3}H]Ins(1,4,5)P_3 binding. Each point represents the mean ± SE of four determinations. The mean value of 100% binding by Ins(1,4,5)P_3 receptor was 3524 dpm at 20 μg and 1997 dpm at 10 μg.

We then examined whether the antibody inhibits the process initiated by the receptor molecule. For this purpose, permeabilized RBL cells were incubated in the presence of ATP and NaN_3, an inhibitor of mitochondrial Ca^{2+} accumulation, and in the absence of EGTA and exogenously added Ca^{2+}. Thus, the endoplasmic reticulum of permeabilized cells would accumulate contaminating Ca^{2+} as a result of ATP hydrolysis. The amount of extra-endoplasmic reticulum Ca^{2+} was monitored by the changes in the fluorescence of fura 2. The addition of ATP together with Mg^{2+} caused a decrease in fura 2 fluorescence due to a decrease in the Ca^{2+} concentration, as shown in Fig. 2A. Neither ATP nor Mg^{2+} could be omitted from the mixture for the decrease in the fluorescence, indicating that it must be due to the accumulation of Ca^{2+} by permeabilized cells as a result of ATP hydrolysis. The sequential addition of Ins(1,4,5)P_3 from low concentrations caused a stepwise increase in fluorescence, indicating that Ins(1,4,5)P_3 causes the release of Ca^{2+} accumulated in the endoplasmic reticulum of permeabilized cells in a dose-dependent manner. After addition of highest concentration of Ins(1,4,5)P_3, there was no further increase in fluorescence, even after the addition of 1 μM ionomycin. Also, if ionomycin was added to the assay cuvette before or during the addition of Ins(1,4,5)P_3, the fluorescence increased up to the level of that after the addition of the highest concentration of Ins(1,4,5)P_3, indicating that all of the Ca^{2+} accumulated after the addition of ATP was in the Ins(1,4,5)P_3-sensitive pool (Fig. 2B).

When the permeabilized cells were incubated with the Fab fragment of the antibody, there was no difference in the amount of Ca^{2+} accumulated in control and pretreated cells after the addition of ATP, therefore indicating that the Fab fragment has no effect on the Ca^{2+} accumulating process. The amount of Ca^{2+} released after the addition of Ins(1,4,5)P_3 decreased, however, depending on the amount of antibody used (Fig. 2C). The addition of ionomycin released the remaining Ca^{2+} in the permeabilized cells, indicating that the antibody had inhibited the process of Ins(1,4,5)P_3-mediated release of Ca^{2+}. Fab fragments unrelated to the antibody against PLC-δ1(30–43), that is, the fraction that passed through the peptide-immobilized column (see "Materials and Methods"), was ineffect in the inhibition of Ca^{2+} release (results not shown). Taken together with the results in Fig. 1, where the antibody inhibited the binding of Ins(1,4,5)P_3 to the receptor, the inhibition of Ca^{2+} release mediated by Ins(1,4,5)P_3 was considered to be due to the inhibition of ligand binding. However, inhibition of Ca^{2+} release was not complete even when a very high dose of the antibody was used, although the binding process could be completely inhibited.

Next, we performed absorption experiments to see which part of the peptide is the antigenic epitope of the antibody. First, the inhibition of Ins(1,4,5)P_3-mediated Ca^{2+} release caused by the addition of the antibody was restored when the antibody was preincubated with the peptide of PLC-δ1(30–43), but not after pre-incubation with the scrambled peptide (Fig. 3A). The peptides of PLC-δ1(30–37) and (37–43) were both synthesized, and then served for the absorption experiments. Each peptide was pre-incubated with the antibody before analyzing the effect on Ca^{2+} release. The peptide of PLC-δ1(30–37) was effective in restoring the inhibition of Ca^{2+} release, but the peptide of PLC-δ1(37–43) was ineffective, indicating that the antibody must be epitope in PLC-δ1(30–37) (Fig. 3B). Furthermore, the peptide of KVK, PLC-δ1(30–32) was found to be effective in restoring the inhibition (Fig. 3B), whereas the peptide of RRER, PLC-δ1(37–40) was ineffective (results not shown). These peptides by themselves without Fab fragments had no effect on Ins(1,4,5)P_3-mediated Ca^{2+} release.

DISCUSSION

Antibody against the N-terminal portion of the PH domain of PLC-δ1 was effective in inhibiting the binding of Ins(1,4,5)P_3 to the receptor molecule, indicating that the antibody recognizes the binding site of the receptor molecule. Since the binding of neither PLC-δ1 nor the receptor to the ligand was inhibited by an antibody with divalent antigenic determinants, but, rather, by the Fab fragments with monovalent antigenic determinants, it could be assumed that the Ins(1,4,5)P_3 binding site of both PLC-δ1 and the receptor is sterically restricted to exclude bulky molecules.

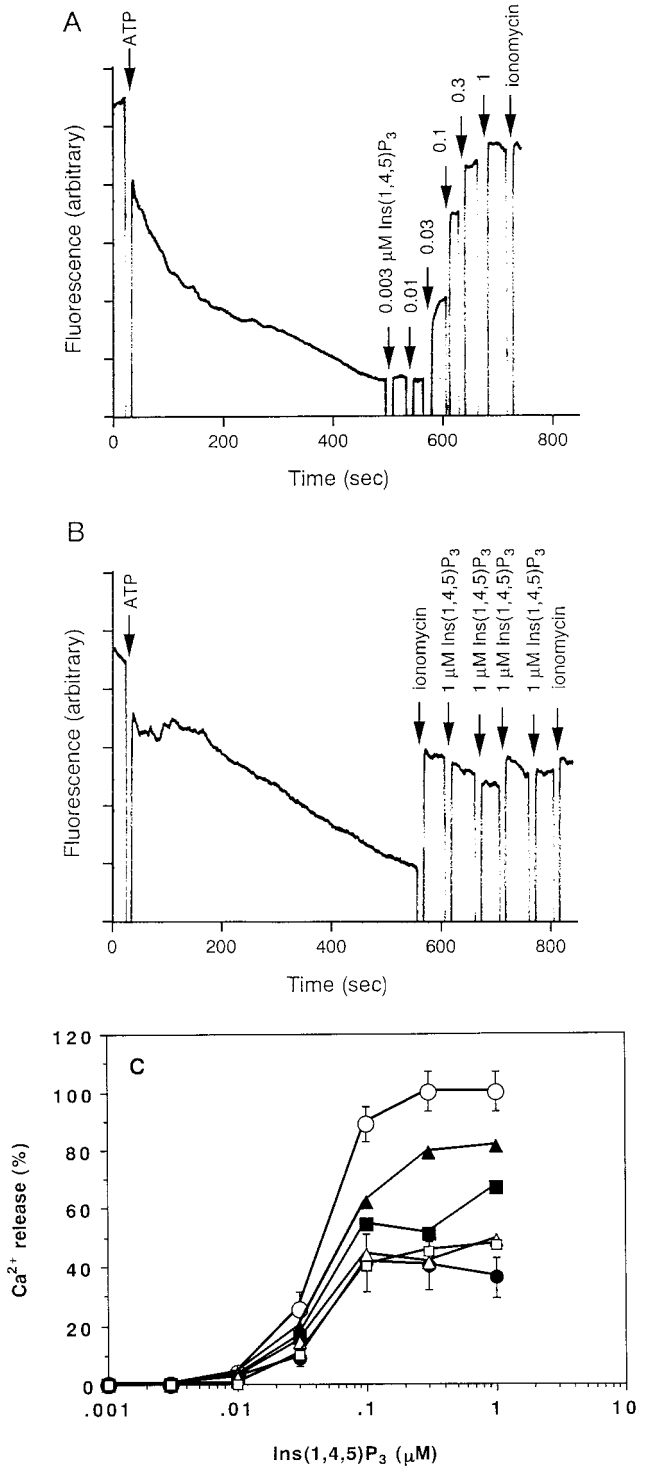

FIG. 2. Effect of Fab fragments of the antibody against the N-terminal portion of the PH domain of PLC-δ1 on $Ins(1,4,5)P_3$-mediated release of Ca^{2+} from permeabilized cells. (A and B) Ca^{2+} accumulation and release in permeabilized RBL cells was monitored with fura 2. Seven min after the addition of ATP, various concentrations of $Ins(1,4,5)P_3$ were sequentially added to observe the release of Ca^{2+}. Additions are indicated by arrows. (**C**) Permeabilized cells were pre-incubated with various concentrations of the Fab fragments for 10 min before the addition of ATP. ○, None; ▲, 1 μg Fab fragments; ■, 2 μg Fab fragments; △, 5 μg Fab fragments; ●, 10 μg Fab fragments; □, 20 μg Fab fragments. Each point represents the mean of two determinations using a different preparation of permeabilized cells. For the control cells and those pretreated with 10 μg Fab fragments, five determinations were made with different preparations of Fab fragments and permeabilized cells, and, therefore, the SE values are shown.

However, their 3D-structures are not similar to each other, because the $Ins(1,4,5)P_3$ binding region of the receptor does not have the same structure as that of the PH domain (23, 24). These results indicate that the antibody recognizes the alignment of the basic amino acids which are important for interacting with the negatively-charged phosphates of $Ins(1,4,5)P_3$. The absorption experiments using the synthetic peptides KVKSSSWR or KVK supports this view. The results that RRER was ineffective in absorption shows the importance of the alignment of the basic amino acids.

The Ca^{2+} accumulated into the Ca^{2+} pool of permeabilized cells was all released by the addition of high concentrations of $Ins(1,4,5)P_3$ and the level released was much the same as that by an ionophore, indicating that all of the Ca^{2+} accumulated after the addition of ATP was in the $Ins(1,4,5)P_3$-sensitive pool of the RBL cells. However, even if very high concentrations of Fab fragments of the antibody (up to 20 μg) were used, complete inhibition of the Ca^{2+} release mediated by $Ins(1,4,5)P_3$ at the micromolar range was not observed. On the other hand, high doses of the antibody completely inhibited the binding of 1.3 nM [^{3}H]$Ins(1,4,5)P_3$ to the receptor. The highest concentration of Fab fragments of the antibody was equal to about 0.4 nmole, while the concentration of $Ins(1,4,5)P_3$ required for the release of Ca^{2+} was around 1 nmole (~1 μM/ml), and for the binding assay was 0.65 pmole (1.3 nM/0.5 ml). Therefore it could be assumed that the higher concentration of $Ins(1,4,5)P_3$ displaced the Fab fragment of the antibody bound to the $Ins(1,4,5)P_3$ site on the receptor molecule, due to the lower affinity of the antibody. However this was unlikely because inhibition by the Fab fragment was much the same at both lower and higher concentrations of $Ins(1,4,5)P_3$. For example, even $Ins(1,4,5)P_3$ at 0.01 μM (equal to 0.01 nmole, a 40 times lower molar ratio than that of the Fab fragments) inhibited Ca^{2+} release to the same extent as that observed at 1 μM $Ins(1,4,5)P_3$. Alternatively, a difference in the receptor subtypes might be the reason for the difference observed. Molecular cloning studies have revealed the presence of three types of receptors, together with their splice variants, encoded by separate genes, although their similarity exceeds 62% and they shares the domain structures (ligand binding, coupling and membrane spanning) (25, 26). Rat cerebellar $Ins(1,4,5)P_3$ receptor was reported to be predominantly subtype I (Type I, more than 90%) (27, 28), whereas that of RBL cells was predominantly subtype

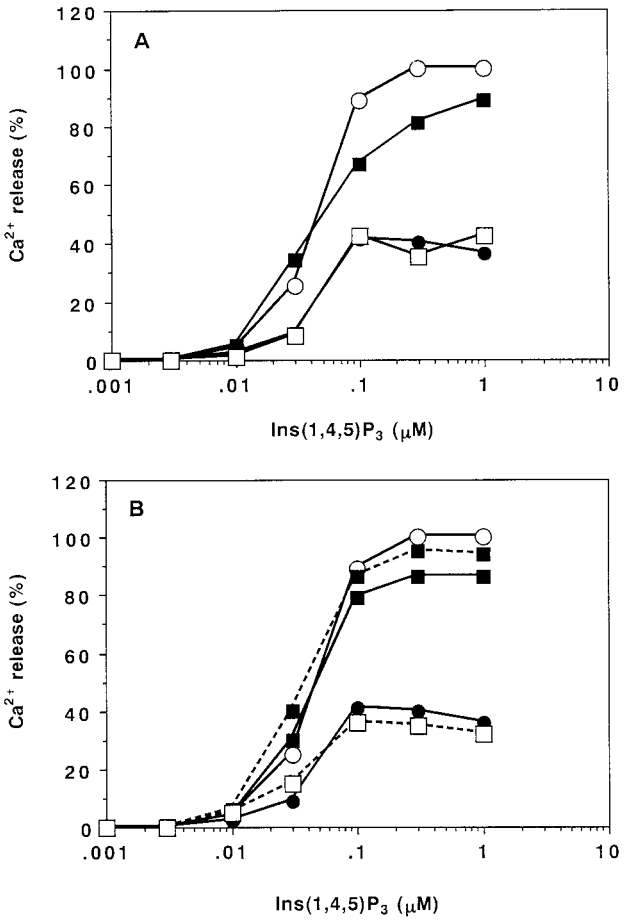

FIG. 3. Absorption experiments with synthetic peptides. The Fab fragments (10 μg) were pre-treated with various synthetic peptides (1 mg) before being added to permeabilized cells. Values of Ins(1,4,5)P_3-mediated Ca^{2+} release from the control cells (○) and from those pre-treated with 10 μg Fab fragments (●) were taken from Fig. 2C. (A) The effect of PLC-δ1(30–43) peptide (■) and the scrambled peptide (□, for the sequence see "Materials and Methods"). (B) Effect of shorter peptides of PLC-δ1(30–43). -■-, PLC-δ1(30–37); ---□---, PLC-δ1(37–43); ---■---, PLC-δ1(30–32).

II (Type I, 17%, Type II, 70% and Type III, 13%) (27–29). However this possibility is also unlikely, because 10 basic residues present in the ligand binding "core" of the type I receptor, some of which would be the site for the recognition of the antibody Fab fragments, are identical in all of the receptor subtypes as reported by Yoshikawa *et al.* (18). Furthermore, another cell type, COS cells, whose receptor subtype composition was reported to be Type I, 1%, Type II, 37% and Type III, 62%, exhibited very similar pattern of inhibition of Ins(1,4,5)P_3-mediated Ca^{2+} release by Fab fragments of the antibody (unpublished observations). Thus, we have no reasonable explanation for the incomplete inhibition of Ins(1,4,5)P_3-mediated release of Ca^{2+} by the antibody. It might be that even Fab fragments with monovalent antigenic determinants could not have access to all the sites for Ca^{2+} storage.

In conclusion, the results presented here could be summarized as follows: (i) an antibody against the peptide comprizing PLC-δ1(30–43), which resides in the N-terminal region of the PH domain of PLC-δ1, inhibits the binding of Ins(1,4,5)P_3 to the receptor molecule, and thus inhibits the release of Ca^{2+} from the Ins(1,4,5)P_3-sensitive Ca^{2+} pool. (ii) the minimal epitope of the inhibitory antibody was revealed to be the tripeptide, KVK. Structural information about KVK will be helpful in screening for Ins(1,4,5)P_3 antagonists.

ACKNOWLEDGMENTS

This work was funded by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (to HY, TK, and MH), Kyushu University Interdisciplinary Programs in Education and Projects in Research Development (to MH), the Naito Memorial Foundation (to MH) and the Uehara Memorial Foundation (to TK).

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