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Annexin-I inhibits phospholipase A_2 by specific interaction, not by substrate depletion

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Abstract

Annexin-I is a calcium dependent phospholipid binding and phospholipase A_2 (PLA_2) inhibitory protein. A 'substrate depletion' model has been proposed for the mechanism of PLA_2 inhibition by annexin-I in studies with 14 to 18 kDa PLA_2s. Herein, we have studied the inhibition mechanism using 100 kDa cytosolic PLA_2 from porcine spleen. The inhibition has been measured at various substrate and calcium ion concentrations. The pattern of PLA_2 inhibition by annexin-I was consistent with a 'specific interaction' mechanism rather than the 'substrate depletion' model. Apparent contradiction with previous studies can be explained by the calcium-dependent binding of annexin-I to the substrate.

Key words: Phospholipase A_2; Annexin-I; Inhibition mechanism; Substrate depletion; Specific interaction

1. Introduction

Annexins are widely distributed calcium- and phospholipid-binding proteins that have been implicated in various physiological roles including phospholipase A_2 (PLA_2) inhibition, membrane fusion, anti-inflammation, anti-coagulation, differentiation, cell adhesion, exocytosis and interaction with cytoskeletal proteins (see [1–3] for references). However, a well-defined biological function has not been determined for any of the annexins.

Annexin-I has been shown to be a major substrate for epidermal growth factor receptor kinase [4] and implicated in the regulation of signal transduction pathways of various mitogenic signals which are thought to be involved with PLA_2 inhibition in vivo [5–7].

The mechanism of PLA_2 inhibition by annexin-I has not been fully understood. Detailed in vitro studies on the effect of annexins on a 14 kDa PLA_2 from porcine pancreas have indicated that PLA_2 inhibition by annexin-I is due to depletion of the substrate [8,9]. Since the binding of annexin-I to the substrate is calcium ion-dependent [10] the inhibition of PLA_2 by annexin-I may also be calcium ion-dependent. The 14 kDa PLA_2 which has been extensively studied requires millimolar Ca^{2+} concentration for activation. Therefore, it has not been possible to investigate inhibition by annexin-I under a wide range of calcium ion concentrations using the 14 kDa PLA_2.

Recently, intracellular forms of PLA_2s with apparent sizes of 80 to 100 kDa have been found in a variety of mammalian cells [11–15]. In contrast to the 14 kDa PLA_2, these enzymes are activated under micromolar Ca^{2+} concentrations and selective to arachidonate-containing phospholipid.

In this contribution, the PLA_2 inhibition by annexin-I was studied using a 100 kDa cytosolic PLA_2 from porcine spleen to investigate whether inhibition by annexin-I is due to 'substrate depletion' or to 'specific interaction' with PLA_2.

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Abbreviations: PLA_2, phospholipase A_2; 2-AA-PC, L-3-phosphatidylcholine, 2-arachidonoyl; 2-AA-PE, L-3-phosphatidylethanolamine, 2-arachidonoyl.

2. Materials and methods

2.1. Materials

1-Stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-p hosphocholine (2-AA-PC) (56.0 mCi/mmol) and 1-acyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphoe thanolamine (2-AA-PE) (57.0 mCi/mmol) were purchased from Amersham (Buckinghamshire, UK) and used as substrates. Unlabeled 2-AA-PC, purchased from Sigma (St. Louis, MO, USA) was also used. Scintillation fluid (Aquasol-2) was obtained from NEN Research Products (Boston, MA, USA) and Fura-2 was from Molecular Probe (Eugene, OR, USA). All other reagents were analytical reagent grade or better.

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2.2. Purification of Annexin-I and PLA_2

Annexin-I cDNA was cloned and expressed in *E. coli.* The protein was purified essentially according to the methods as described [16,17]. An intracellular form of PLA_2 with an apparent size of 100 kDa was purified to near homogeneity from porcine spleen essentially according to the methods described in [18].

2.3. Standard assay for PLA_2 inhibition

A stock solution of the substrate was prepared as follows: 10–20 nmol of labeled phospholipid was dried under nitrogen, then suspended in 0.5–1.0 ml of distilled water by sonication at 25°C in a bath-type sonicator (Ultrasonik 300, NEY). Sonication was performed for 10 s, three times.

The standard incubation system (200 μl) for assay of PLA_2 contained 0.33 nmol (1.65 μM) of radioactive substrate (approximately 39,000 cpm), 10 ng of purified porcine spleen PLA_2, and 200 μg of fatty acid-free bovine serum albumin in 75 mM Tris-HCl (pH, 7.4). The reaction was started by the addition of the enzyme to the reaction mixture to give a final volume of 200 μl. Assays were then incubated at 37°C for 1 h (or as indicated), then stopped by the addition of 1.25 ml of Dole's reagent (2% N-H_2SO_4, 20% *n*-heptane, 78% isopropanol) [19]. The hydrolyzed radioactive free fatty acid was extracted and measured by liquid scintillation counting according to the methods described in [20]. For experiments in which the substrate concentration dependence was determined, unlabeled phospholipid was added to labeled phospholipid to give a designated final concentration. For accurate control of Ca^{2+} concentration, a $CaCl_2$/EGTA buffering system was used [21]. The final calcium concentration was verified by using Fura-2 [22]. In all analyses, samples were tested in more than duplicates and adjusted for nonspecific release by subtracting a control value in which preparation the enzyme was omitted. For inhibition assays, 5–100 ng of annexin-I was added to the reaction mixture.

2.4. Immunoprecipitation studies

1 μg of the 100 kDa porcine spleen PLA_2 was incubated with or without 100 μg of annexin-I in 200 μl of buffer containing 75 mM Tris-HCl (pH, 7.4), 1 mg/ml bovine serum albumin, and 0.1 μM Ca^{2+}. After 10 min incubation at 4°C, samples were incubated with anti-annexin-I polyclonal antibody, then immunocomplexes were precipitated with Protein-A as described [23]. PLA_2 activity remaining in the supernatant was determined in standard buffer containing 5 mM $CaCl_2$.

3. Results

3.1 Effect of calcium on the activity of PLA_2 from porcine spleen

Characteristic features of PLA_2 from porcine spleen used in this study, such as pH dependence, calcium dependence, and substrate specificity, were similar to those of bovine platelet cytosolic enzyme (data not shown) [20]. Fig. 1 shows the effect of calcium on enzymatic activity. The PLA_2 was sensitive to free calcium ion change especially from 0.1 μM to 1 μM with nearly full activity at 1–5 μM. When compared to 2-AA-PE as a substrate, the 2-AA-PC hydrolyzing activity was less under all calcium concentrations.

3.2. Calcium dependence of the PLA_2 inhibition by annexin-I

In order to investigate the mechanism of PLA_2 inhibition by annexin-I in detail, assays were carried out under various concentrations of annexin-I and calcium ion. Inhibition of PLA_2 by annexin-I was determined using 2-AA-PE as a substrate at 0.1, 1, 10, 100 μM and 1 mM calcium ion concentrations. The substrate concentration

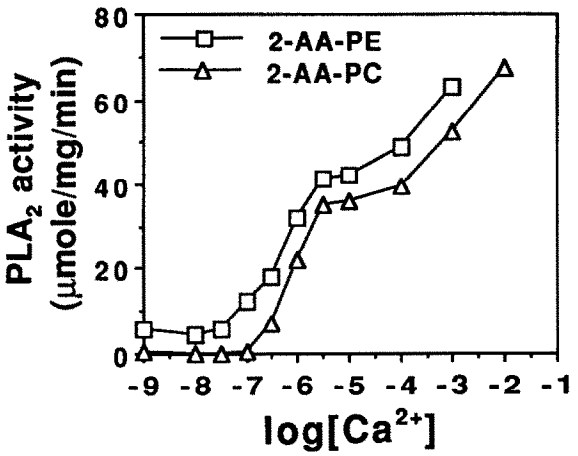

Fig. 1. Calcium dependence of phospholipase A_2 activity. 10 ng (0.5 nM) of purified phospholipase A_2 (PLA_2) from porcine spleen was incubated with 0.33 nmole (1.65 μM) of 1-stearoyl-2-[1-^{14}C]-*sn*-glycero-3-phosphocholine (2-AA-PC) and 1-acyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phospho-ethanolamine (2-AA-PE) (approximately 39,000 cpm) in 75 mM Tris/HCl (pH 7.4) in the presence of various concentrations of free Ca^{2+} for 1 hr at 37°C in a volume of 200 μl. △, 2-AA-PC; □, 2-AA-PE.

was 1.65 μM, which was a large excess over the enzyme (10 ng/200 μl or 0.5 nM) and inhibitor (between 5 ng/200 μl (0.7 nM) and 100 ng/200 μl (14 nM)) concentrations. Fig. 2A shows the percent remaining 2-AA-PE hydrolyzing activity of PLA_2 in the presence of 0.7–14 nM annexin-I. The PLA_2 activity decreased in the presence of annexin-I, suggesting that enzymatic activity was inhibited. Inhibition of PLA_2 was observed at 0.7 nM annexin-I which was slightly higher than the enzyme concentration (0.5 nM). Inhibition increased as inhibitor concentration increased. Percent remaining activity was smallest, and therefore, percent inhibition was largest at 0.1 μM Ca^{2+} concentration. It decreased with increasing Ca^{2+} concentration, and became negligible at 1 mM Ca^{2+} concentration.

Fig. 2B shows a replotting of data in Fig. 2A. The percentage of remaining PLA_2 activity in the presence of annexin-I was plotted against the logarithmic value of the calcium concentration. At each annexin-I concentration the plot was essentially linear.

Experiments to determine the PLA_2 inhibition by annexin-I were carried out using 2-AA-PC as a substrate in the same manner as experiments with 2-AA-PE. As shown in Fig. 2C and D, similar PLA_2 inhibitory activity was observed under 1, 10 and 100 μM Ca^{2+} concentrations. At 0.1 μM [Ca^{2+}], the 2-AA-PC hydrolyzing activity of PLA_2 was very small (Fig. 1) and the inhibitory activity was not measurable. Nevertheless, patterns of inhibition were similar between assays with 2-AA-PE and 2-AA-PC as substrates.

In summary, Fig. 2 demonstrates that annexin-I inhibits PLA_2 in the presence of an excess amount of either

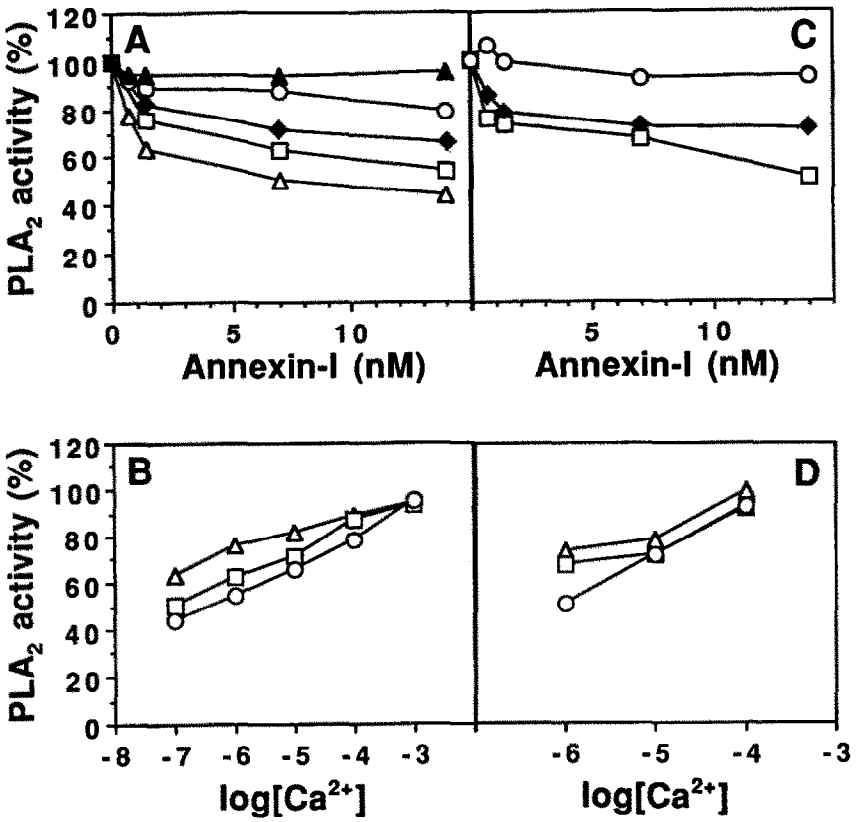

Fig. 2. Calcium concentration dependence of the phospholipase A_2 inhibition by annexin-I. The inhibition of PLA_2 by annexin-I was determined using 2-AA-PE (A,B) and 2-AA-PC (C,D) as a substrate at 0.1, 1, 10, 100 μM and 1 mM Ca^{2+} concentrations. The reaction was carried out in 75 mM Tris-HCl (pH, 7.4) containing 1 mg/ml bovine serum albumin for 1 h at 37°C. Concentrations of PLA_2 and substrate were 0.5 nM (10 ng/200 μl) and 1.65 μM, respectively. The annexin-I concentration was varied from 0.7 nM (5 ng/200 μl) to 14 nM (100 ng/200 μl). PLA_2 activity with or without the inhibitor was determined and percentage of remaining activity in the presence of the inhibitor was calculated. Symbols (A,C): △, 0.1 μM; □, 1 μM; ◆, 10 μM; ○, 100 μM; ▲, 1 mM in Ca^{2+} concentration; (B,D) △, 1.4 nM; □, 7.0 nM; ○, 14 nM in annexin-I concentration.

2-AA-PE or 2-AA-PC as substrate, even though the [I]/[E] value may be as low as 1.4 and the [S]/[I] value as high as 2300. Percent inhibition was calcium ion-dependent and plots of percent inhibition against log[Ca^{2+}] were linear.

Since the substrate concentration was in large excess over the inhibitor, the inhibition of PLA_2 observed in this experiment is not likely due to 'substrate depletion' by the inhibitor. Instead, the inhibition may be through a 'specific interaction' between annexin-I and PLA_2. This result is consistent with the following interpretation. Binding of the substrate to annexin-I is calcium ion-dependent. At 0.1 μM [Ca^{2+}], substrate binding by annexin-I is minimal, and therefore, annexin-I is available for PLA_2 inhibition by direct interaction. Substrate binding by annexin-I increases with increasing Ca^{2+} concentration, which results in the reduction of free annexin-I for PLA_2 inhibition. At 1 mM [Ca^{2+}], annexin-I is depleted by binding to the substrate and is unavailable for PLA_2 inhibition. This interpretation is also consistent with the previous studies which were carried out at 1 mM calcium where no PLA_2 inhibition by annexin-I was observed in the presence of a large excess of substrate [8,10].

3.3. *Substrate concentration dependence of the PLA_2 inhibition by annexin-I*

To eliminate the possibility that the PLA_2 inhibition by annexin-I is due to the substrate depletion, the substrate concentration was varied from 1.65 μM to 38.3 μM while holding other components constant. As shown in Fig. 3, inhibition of PLA_2 by annexin-I was essentially independent of substrate concentration. This result is in sharp contrast to the results from previous studies in which PLA_2 inhibition by annexin-I at a limiting substrate concentration was abolished with increasing amount of substrate [8,10].

Table 1
Comparison of PLA_2 activity in supernatants after immunoprecipitation

Immunoprecipitation	PLA_2 activity (μmol/mg/min)[a]
PLA_2 + antibody[b]	1.087 ± 0.11
PLA_2 + Annexin I + antibody[b]	0.185 ± 0.07

[a] PLA_2 activity remaining in the supernatant after immunoprecipitation with anti-annexin-I antibody.
[b] Anti-annexin-I polyclonal antibody.

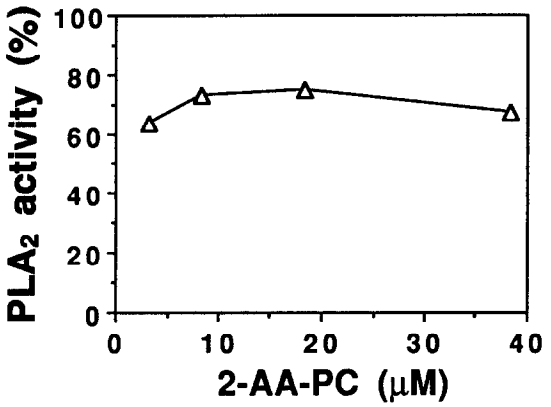

Fig. 3. Substrate concentration dependence of the PLA_2 inhibition by annexin-I. The reaction was performed as in Fig. 2 except that the Ca^{2+} and annexin-I concentrations were kept constant at 1 μM and 7 nM, respectively, and the substrate (2-AA-PC) concentration was varied from 1.65 μM to 38.3 μM.

3.4. *Immunoprecipitation studies*

To demonstrate the direct interaction between PLA_2 and annexin-I, co-precipitability of PLA_2*annexin-I complex, if stably exist in solution, by anti-annexin-I antibody was investigated. As shown in Table 1, the PLA_2 activity in the supernatant decreased after immunoprecipitation with anti-annexin-I antibody when annexin-I was co-present. Therefore, the presence of PLA_2*annexin-I complex which was co-precipitated by anti-annexin-I antibody was demonstrated.

4. Discussion

To investigate the mechanism of PLA_2 inhibition in detail, especially to learn whether the inhibition is through 'specific interaction' or by 'substrate depletion', we carried out an array of assays under various substrate, calcium ion, and inhibitor concentrations. Prior studies have indicated that PLA_2s from several sources were not inhibited by annexins in the presence of an excess amount of substrate. That is, PLA_2 inhibition by annexins at a limiting substrate concentration was abolished with increasing substrate concentration [8,10]. In contrast, the results of this study showed that inhibition of a 100 kDa PLA_2 from porcine spleen by annexin-I was independent of substrate concentration.

The major differences in the assay conditions between previous studies and this study are (1) 100 kDa cytosolic enzyme from porcine spleen was used in this study whereas PLA_2s from porcine pancreas (14 kDa), snake venom (14 kDa), and a mouse macrophage-like cell line (18 kDa) were used in previous studies; (2) the Ca^{2+} concentration was between 0.1 μM and 1 mM in this study while in all previous studies assays were performed at 1 mM [Ca^{2+}]; (3) the concentrations of the enzyme, substrate, and inhibitor were different. The assay conditions are summarized in Table 2. The range of [S]/[I] values in this study was 120 to 55,000, as compared with 2.5 to 100 or 36 to 360 in previous studies [8,10]. PLA_2 inhibition was observed at an [S]/[I] value as high as 55,000 in this study whereas the inhibition was abolished at an [S]/[I] value of 50, or negligible at an [S]/[I] value of 360, in previous studies [8,10]. It is, therefore, evident that PLA_2 inhibition in this study is not due to substrate depletion. The range of [I]/[E] values in this study were 1.4–28 as compared with 196 in the previous study [8], which further supports the specific interaction model. In conclusion, the data presented in Table 2 clearly demonstrates that PLA_2 inhibition was observed in this study even though the [I]/[E] value was lower and the [S]/[I] value was much higher than in previous studies.

The discrepancy in results between this and previous studies is most likely due to differences in assay conditions, especially the Ca^{2+} concentration. As shown in Fig. 2, inhibition of the 100 kDa PLA_2 by annexin-I was very sensitive to the Ca^{2+} concentration, and inhibition was negligible at 1 mM [Ca^{2+}]. Therefore, at 1 mM [Ca^{2+}], PLA_2 inhibition by 'specific interaction' is small, and 'substrate depletion' may be a major inhibition mechanism. Experiments designed analogously to previous studies resulted in data similar to that previously observed and interpreted as 'substrate depletion' (data not shown) [8,10]. At 1 mM [Ca^{2+}], the 100 kDa PLA_2 also follows the substrate depletion mechanism similar to the 14 kDa PLA_2 (data not shown).

Both 100 kDa and 14 kDa PLA_2, which exist in many cell types, are thought to be differently regulated [24].

Table 2
Comparison of assay conditions from previous studies and this study

Component	This study	Prior study[a]	Prior study[b]
Enzyme	Porcine spleen 100 kDa PLA_2	Porcine pancreatic 14 kDa PLA_2	Macrophage-like cell
[E]	0.5 nM	0.51 nM	N/A
Substrate	2-AA-PC	*E. coli* membrane	2-AA-PC
[S][c]	1.65–38.3 μM	0.25–10 μM	10 μM
Inhibitor	Annexin-I	Annexin-II	Annexin-I
[I]	0.7–14 nM	0.1 nM	28–280 nM
[Ca^{2+}]	0.1 μM–1 mM	1 mM	1 mM
[I]/[E]	1.4–28	196	N/A
[S]/[I]	120–55,000	2.5–100	36–360
$\{[S]/[I]\}_{max}$[d]	55,000	50	360

[a] Deduced from reference 8.
[b] Deduced from reference 10.
[c] Substrate concentration was calculated as a phospholipid monomer concentration without considering the actual status of the liposome.
[d] Maximum substrate concentration at which PLA_2 inhibition was observed.

Since the calcium concentrations required for activation are different between 100 kDa and 14 kDa enzymes, it is reasonable to assume that they work by different mechanisms in vivo.

In conclusion, annexin-I inhibited a 100 kDa PLA_2 from porcine spleen by a mechanism which is consistent with a 'specific interaction' model. The Ca^{2+} concentration was a key factor in determining whether the mechanism of PLA_2 inhibition is 'substrate depletion' or 'specific interaction'.

The results presented here are potentially important since annexin-I specifically inhibited a 100 kDa PLA_2 near intracellular Ca^{2+} concentration. In view of the selectivity of the cytosolic PLA_2 for arachidonate-containing phospholipid used in this study, annexin-I may regulate several biological processes through regulation of PLA_2 activity as has been suggested previously [5–7].

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References

[1] Flower, R.J. (1990) in: Lipocortin Cytokines and Lipocortins in Inflammation and Differentiation, pp. 11–25, Wiley-Liss.
[2] Hirata, F. (1989) in: The Role of Lipocortins in Cellular Function as a Second Messenger of Glucocorticoids. Anti-inflammatory Steroid Action, pp. 67–95, Academic Press.
[3] Creutz, C.E. (1992) Science 258, 924–930.
[4] Fava, R.A. and Cohen, S. (1984) J. Biol. Chem. 259, 2636–2645.
[5] Colotta, F., Re, F., Muzio, M., Bertini, R., Polentarutti, N., Sironi, M., Dower, S.K., Sims, J.E. and Mantovani, A. (1993) Science 261, 472–475.
[6] Goulding, N.J. and Guyre, P.M. (1993) Curr. Opinion Immunol. 5, 108–113.
[7] Perretti, M. and Flower, R.J. (1993) J. Immunol. 150, 992–999.
[8] Davidson, F.F., Dennis, E.A., Powell, M. and Glenney, J.R., Jr. (1987) J. Biol. Chem. 262, 1698–1705.
[9] Ahn, N.G., Teller, D.C., Bienkowski, M.J., McMullen, B.A., Lipkin, E.W., de Haen, C. (1988) J. Biol. Chem. 263, 18657–18663.
[10] Davidson, F.F., Lister, M.D. and Dennis, E.A. (1990) J. Biol. Chem. 265, 5602–5609.
[11] Kim, D.K., Kudo, I. and Inoue, K. (1991) Biochim. Biophys. Acta 1083, 80–88.
[12] Takayame, K., Kudo, I., Kim. D.K., Nagata, K., Nozawa, Y. and Inoue, K. (1991) FEBS Lett. 282, 326–330.
[13] Leslie, C.C. (1991) J. Biol. Chem. 266, 11366–11371.
[14] Sharp, J.D., White, D.L., Chiou, X.C., Goodson, T., Gamboa, G.C., McClure, D., Burgett, S., Hoskins, J., Skatrud, P.L., Sportsman, J.R., Becker, G.W., Kang, L.H., Roberts, E.F. and Kramer, R.M. (1991) J. Biol. Chem. 266, 14850–14853.
[15] Clark, J.D., Lin, L-L., Keiz, R.W., Ramesha, C.S., Sultzman, L.A., Lin, A.Y., Milona, N. and Knopf, J.L. (1991) Cell 65, 1043–1051.
[16] Huang, K.S., Wallner, B.P., Mattaliano, R.J., Tizard, R., Burne, C., Frey, A., Hession, C., McGray, P., Sinclair, L.K., Chow, E.P., Browning, J.L., Ramachandran, K.L., Tang, J., Smart, J.E. and Pepinsky, R.B. (1986) Cell 46, 191–199.
[17] Weng, X., Luecke, H., Song, I.S., Kang, D.S., Kim, S.-H., Huber, R.(1993) Protein Sci. 2, 447–458.
[18] Kim, D.K., Suh, P.G. and Ryu, S.H., (1991) Biochem. Biophys. Res. Commun. 174, 189–196.
[19] Dole, V.P. and Meinertz, H. (1960) J. Biol. Chem. 235, 2595–2599.
[20] Horigome, K., Hayakawa, M., Inoue, K. and Nojima, S. (1987) J. Biochem. 101, 53–61.
[21] Durham, A.C. (1983) Cell. Calcium 4, 33–46.
[22] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[23] Raynal, P., Hullin, F., Ragab-Thomas, J.M.F., Fauvel, J. and Chap, H. (1993) Biochem. J. 292, 759–765.
[24] Piomelli, D. (1993) Curr. Opinion Cell Biol. 5, 274–280.