ATP-dependent regulation of phospholipase C in permeabilized 3T3 cells

Kyoichiro Higashi and Hiroshi Ogawara

Department of Biochemistry, Meiji College of Pharmacy, Nozawa-1, Setagaya-ku, Tokyo 154, Japan

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Regulation of phospholipase C (PLC) coupled with a G-protein was studied with Swiss 3T3 cells permeabilized by digitonin. In permeabilized cells, activation of phospholipase C required millimolar concentrations of ATP in addition to a G-protein activator, AlF_4^- or nonhydrolysable GTP analogues. To determine the mechanism of the action of ATP, we examined the effects of ATP analogues. ATPγS directly activated phospholipase C in the presence or absence of AlF_4^-. On the other hand, neither β,γ-methylene ATP nor adenyl-5′-yl imidodiphosphate nor ADPβS could support the AlF_4^--dependent activation of phospholipase C. The action of ATPγS was not through the substrate supply for phospholipase C, because ATPγS did not augment the levels of PIP_2 or PIP in permeabilized cells. These results suggested the significance of the γ-phosphate group of ATP and/or phosphorylation by ATP in the activation of phospholipase C by a putative G-protein.

ATPγS, Phospholipase C; G-protein, Cell permeabilization

1. INTRODUCTION

It is widely accepted that the Ca^{2+}-mobilizing receptor stimulates phosphoinositide hydrolysis and provides two potent second messengers, i.e. inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DG) [3,4]. Recent studies suggest that the coupling of receptor function to phospholipase C is mediated by unidentified GTP-binding protein(s) (G-protein) [5,6]. However, there is no evidence of how a putative G-protein is able to activate phospholipase-C.

In this paper, we describe the procedures of permeabilization of 3T3 cells with responsiveness to G-protein-mediated activation of phospholipase C, and suggest the involvement of ATP in G-protein-mediated and/or direct activation of phospholipase C.

2. MATERIALS AND METHODS

2.1. *Materials*

Adenosine 5′-*O*-[3-thiotriphosphate], and adenosine 5′-*O*-[2-thiodiphosphate] were purchased from Boehringer Mannheim, and other nucleotides were from Sigma. Myo[^{3}H]inositol was purchased from Amersham. All other reagents used in this study were of the highest grades available.

Correspondence address: K. Higashi, Department of Biochemistry, Meiji College of Pharmacy, Nozawa-1, Setagaya-ku, Tokyo 154, Japan

Abbreviations: G-protein, GTP-binding protein; $AppCH_2p$, β,γ-methylene adenosine 5′-triphosphate; AppNHp, adenyl 5′-yl imidodiphosphate; ATPγS, adenosine 5′-*O*-[3-(thio)triphosphate]; ADPβS, adenosine 5′-*O*-[2-(thio)diphosphate]; GppNHp, guanyl-5′-yl imidodiphosphate; GTPγS, guanosine 5′-*O*-[3-(thio)triphosphate]; IP_3, inositol trisphosphate; IP_2, inositol bisphosphate; EGTA, [ethylenebis(oxyethylene nitrilo)]tetraacetic acid; PI, phosphatidyl inositol; PIP, phosphatidyl inositol 4-monophosphate; PIP_2, phosphatidyl inositol 4,5-bisphosphate; PBS, phosphate-buffered saline

2.2. *Permeabilization and stimulation of 3T3 cells*

Swiss 3T3 cells were grown in 50-mm dishes (Nunc) with Dulbecco's modified Eagle's medium supplemented by 10% newborn calf serum, in humidified 5% CO_2 air at 37°C. Subconfluently grown cells were labeled with myo[^{3}H]inositol (2 μCi/dish) for 24 h in the inositol-depleted Eagle's minimal essential medium. Thereby, cell permeabilization was performed according to Erusalimsky et al. [7] with minor modifications. Cells were washed once with 2 ml of phosphate-buffered saline without calcium or magnesium (PBS^-), and 0.75 ml of the permeabilization medium was quickly added to the dish. The composition of the permeabilization medium was as follows: 120 mM KCl, 10 mM NaCl, 20 mM Hepes-Na, 5 mM $MgCl_2$, 3 mM EGTA-Na, 0.6 mM $CaCl_2$, 20 μM digitonin, 1 mM ATP-Mg (pH 7.0). The concentration of free Ca^{2+} was calculated as 50 nM at pH 7.0 from the equations of Bartfai [8]. Under the conditions used here, cell morphology showed a smooth and flattened shape as normally quiescent 3T3 cells for at least an hour.

2.3. *Assay of the formation of inositol phosphates*

Permeabilized cells were stimulated with 5 mM NaF/20 μM $AlCl_3$ or with 1 mM ATPγS. In the case of ATPγS, ATP was omitted from the permeabilization medium. Reactions were stopped at indicated times by the addition of 0.5 ml of 10% TCA to the dishes. IP_2 and IP_3 in acid-soluble extracts were assayed by the method of Berridge et al. [9].

2.4. *Determination of [^{3}H]phosphoinositide levels*

Permeabilized cells were stimulated for 10 min at 37°C under the same condition as that for inositol phosphate formation. At the end of incubation, the medium was discarded, and the cells were fixed by the addition of 0.5 ml of 1 N HCl and immediately chilled on ice. [^{3}H]Inositol phospholipids were extracted and further separated by TLC following the method of Schacht [14]. Separated [^{3}H]PI, -PIP, and -PIP_2 were assigned by fluorography, and the corresponding portions of gel plates to each phosphoinositide were scraped and the radioactivity was measured by liquid scintillation counting.

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3. RESULTS AND DISCUSSION

Fig. 1 shows the activation of phospholipase C by NaF and $AlCl_3$ in intact 3T3 cells. Addition of NaF caused the accumulation of IP_2 and IP_3 in the cells with about 4 min of a lag period, which may be due to the permeation of NaF and/or activation of a G-protein coupled with phospholipase C. Because activation by NaF was enhanced by the addition of $AlCl_3$ (data not shown), and AlF_4^- formed from NaF and $AlCl_3$ is known as a potent activator of G-proteins, it seems likely that phospholipase C activation by NaF was mediated through a G-protein coupled with phospholipase C.

The activation of phospholipase C was further studied by permeabilizing the cells. In the digitonin-permeabilized 3T3 cells, addition of NaF plus $AlCl_3$ caused rapid augmentation of IP_2 and IP_3 (Fig. 2). The permeabilization of the cells resulted in extreme shortening of the lag-period of the activation. This suggests that in intact cells, the lag period of NaF-dependent activation was mainly due to permeation of the AlF_4^- ion into the cells.

The permeabilized cells required ATP for AlF_4^- activation. The permeabilization buffer used here contained 1 mM of ATP. Depletion of ATP from the permeabilization buffer caused loss of responsiveness to AlF_4^- in permeabilized 3T3 cells (Fig. 2). A similar concentration of GTP, in place of ATP, was less effective for the activation by AlF_4^- (data not shown).

Fig. 3 shows the activation of phospholipase C by GppNHp in permeabilized 3T3 cells. GppNHp activated the phospholipase C also in an ATP-dependent manner as in the case of AlF_4^- activation. The half-effective concentration of GppNHp for phospholipase C activation was about 10 μM. GTPγS also activated the phospholipase C in a similar manner (data not shown). These results suggest that the activation of phospholipase C by AlF_4^- or GTP analogues was mediated through G-protein activation, and the activation by the G-protein required millimolar concentrations of ATP.

Fig. 4 shows the effects of nucleotide analogues on phospholipase C activation. Neither β,γ-methylene adenosine 5′-triphosphate ($AppCH_2p$) nor adenyl-5′-yl imidodiphosphate (AppNHp), non-hydrolysable ATP analogues, could support the activation by AlF_4^-. On the other hand, ATPγS, a thiophosphorylating analogue of ATP, activated phospholipase C. Furthermore, ATPγS solely activated phospholipase C without activating a G-protein. ADPβS showed no effect on the activation of phospholipase C. This suggests that the (thio)phosphate of ATP or ATPγS may be directly implicated in the activation process of phospholipase C.

In the purinergic receptor (P_{2Y}) system, all of the nucleotides mentioned above are equally effective on

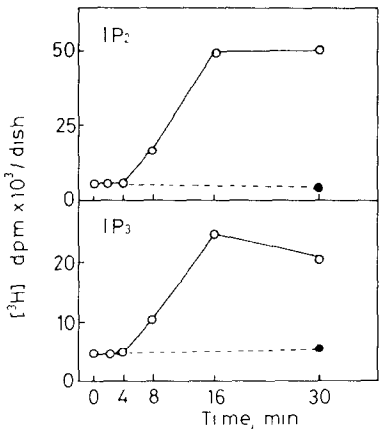

Fig 1. Activation of phospholipase C by AlF_4^- in intact 3T3 cells. Cells were preloaded with 50 μM of $AlCl_3$ for 30 min. After $AlCl_3$ loading, medium was changed to 0.7 ml of PBS containing Ca^{2+} and Mg^{2+}. Incubations were started by the addition of 30 mM NaF to the dish at 37°C. Open circles = plus 30 mM NaF; closed circles = without addition of NaF.

phospholipase C activation at the concentrations used here [10]. Furthermore, ATP for the activation by AlF_4^- was required only with permeabilized cells, but not with intact cells (Figs 1 and 2). This suggests that the site of the action of ATP, observed in this study, was inside the cells but not on the purinergic receptor of the cell surface.

Although ATP may be required for the production of PIP_2 or PIP from phosphatidyl inositol, it is less possible that ATP and ATPγS only function as a source of the (thio)phosphate group in polyphosphoinositide. We examined the effects of ATP and ATPγS on the levels of PIP and PIP_2 in permeabilized 3T3 cells. Fig. 5 shows that ATPγS did not augment the levels of PIP or PIP_2, although ATP

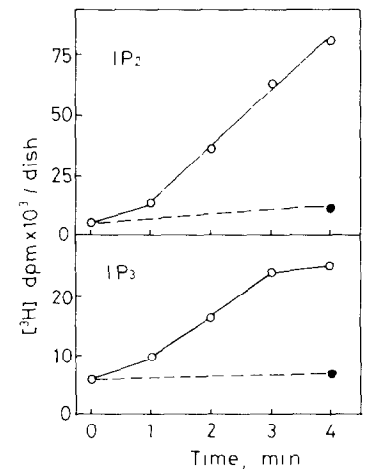

Fig. 2. Activation of phospholipase C by AlF_4^- in permeabilized 3T3 cells. Incubation of permeabilized cells was started by the addition of 5 mM NaF to the dish at 37°C. Permeabilization buffer also contained 20 μM of $AlCl_3$. Open circles = plus 1 mM ATP to the permeabilization buffer; closed circles = without addition of ATP.

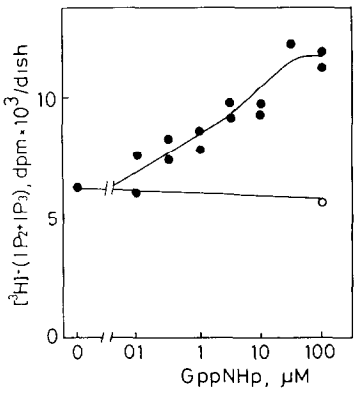

Fig. 3. Activation of phospholipase C by GppNHp in permeabilized 3T3 cells. Incubation of permeabilized cells was started by the addition of indicated concentration of GppNHp to the dish at 37°C. Open circles = plus 1 mM ATP to the permeabilization buffer; closed circles = without addition of ATP.

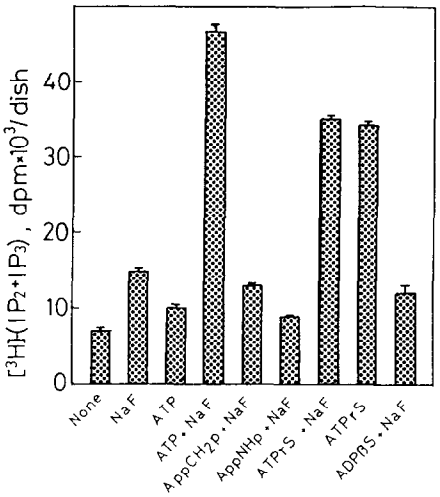

Fig. 4. Nucleotide requirements on the activation of phospholipase C in permeabilized 3T3 cells. Permeabilization buffer contained 20 μM $AlCl_3$ and 1 mM ATP or its analogue. Reaction was started by the addition of 5 mM NaF at 37°C. After 5 min of incubation, cells were fixed and IP_2 and IP_3 were separated as described in section 2. Values were expressed as means of duplicate determinations.

did slightly. If the accumulation of inositol phosphates was caused by overproduction of PIP_2 or by suppression of degradation of inositol phosphates, the level in PIP_2 should not decrease but rather increase. Decrease of PIP_2 level by ATPγS strongly suggests that ATPγS activates phospholipase C and that the activation by ATPγS is not due to the augmentation of substrate supply for phospholipase C.

The results presented here suggest that ATP, to be precise the γ-phosphate group of ATP, plays a central role in the G-protein-mediated activation of phospholipase C. The evidence that ATPγS by-passed the G-protein action in phospholipase C activation indicated that ATPγS and a factor mediating the action of ATPγS might be located downstream of the G-protein in the phospholipase C system. It seems likely that the action of ATPγS or ATP was mediated through hydrolysis of the (thio)phosphate group, because other non-hydrolysable analogues (AppNHp and $AppCH_2p$) failed to activate phospholipase C. ATPγS is a known donor of thiophosphate for protein thiophosphorylation [13]. Several kinds of protein kinases and polynucleotide kinases can utilize ATPγS and transfer the thiophosphate group to each acceptor molecule. It was reported that thiophosphorylated proteins such as the myosin light chain were more resistant to the action of protein phosphatase than phosphorylated forms [1,2,13].

There is no evidence to explain how ATPγS activates phospholipase C at the present time. One of the possible explanations is that the activity of phospholipase C is regulated by phosphorylation and dephosphorylation of phospholipase C or its associated protein. In this model, the G-protein is able to activate phospholipase C by suppressing the dephosphorylation, and ATPγS is able to activate phospholipase C by introducing the thiophosphate group, which is resistant to the protein phosphatases [13].

According to the model described above, inhibition of the phosphatase or activation of protein kinases should lead to the activation of phospholipase C in the absence of G-protein activator. The putative protein kinase, if any, would be constitutively active, because ATPγS directly activated phospholipase C without other stimuli (Fig. 4). Cyclic AMP, cyclic GMP and a phorbol ester failed to activate phospholipase C (data not shown). It appears therefore that the candidate is

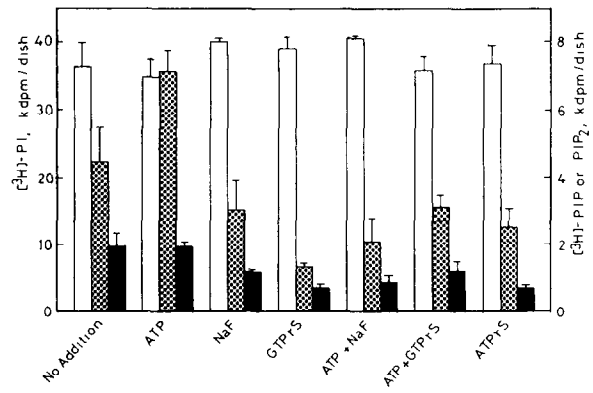

Fig. 5. Failure of augment of polyphosphoinositide levels by ATPγS. Permeabilization buffer contained 20 μM $AlCl_3$ and 1 mM each of indicated nucleotide. Reactions were started by the addition of 5 mM NaF at 37°C. After 10 min of incubation, medium was discarded and cells were immediately fixed with 0.5 ml of 1 N HCl on ice. Each ^{3}H-labelled phosphoinositide was separated and measured as described in section 2. Open columns = phosphatidylinositol; dotted columns = phosphatidylinositol monophosphate; closed columns = phosphatidylinositol bisphosphate. Values were expressed as means of duplicate determinations. Data were a typical one of three individual experiments.

neither cyclic-AMP nor cyclic-GMP nor calcium-phospholipid-dependent protein kinase. Furthermore, a potent protein phosphatase inhibitor, okadaic acid, did not activate phospholipase C in the ATP-dependent manner (data not shown). Okadaic acid inhibits protein phosphatase-2A, -1 and weakly -2B, but does not inhibit -2C [17]. Thus, phosphatase-1 or -2A are apparently not solely responsible for the phospholipase C activation described in this paper. Involvement of phosphatase-2B (i.e. Ca^{2+}, calmodulin-dependent phosphatase) is less likely, because of the low levels of Ca^{2+} (about 50 nM) in the permeabilization medium. We could not ascertain the involvement of phosphatase-2C. Rhee and his colleagues have presented evidence that EGF- or PDGF-dependent activation of phospholipase C II is associated with phosphorylation of phospholipase C II at a tyrosine residue by the receptor-associated tyrosine kinase [16]. The involvement of protein tyrosine phosphatase was examined by using vanadate plus molybdate, heparin or poly(Glu-Tyr), known inhibitors of protein tyrosine phosphatases [18]. These inhibitors did not promote the ATP-dependent activation of phospholipase C in the absence of AlF_4^- (data not shown).

Several types of G-proteins and PIP_2-specific phospholipase C were purified to homogeneity [15]. However, no reconstitution experiment between G-protein and phospholipase C has been successful [15]. Therefore, several investigators speculated that an unidentified factor may be implicated in the coupling between G-protein and phospholipase C. These ideas adequately support the data presented in this paper. More recently, Grinstein et al. [11] reported the ATPγS-dependent activation of superoxide anion production in electrically permeabilized neutrophils, and Wagner and Vu [12] showed the ATPγS-dependent activation of norepinephrine release from PC12 cells. Yet, neither author has mentioned the activation of PI-turnover. But it has been postulated that both responses were mediated through activation of the PI-response.

In this study, we have failed to obtain reliable AlF_4^- stimulation with the membrane preparation without artificial detergents such as deoxycholate. For further elucidation of the regulatory mechanism of phospholipase C, identification of enzymes involved in the action of ATP or ATPγS will be required.

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