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Vasopressin Induces Arachidonic Acid Release Through Pertussis Toxin-Sensitive GTP-Binding Protein in Aortic Smooth Muscle Cells: Independence From Phosphoinositide Hydrolysis

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Abstract We previously reported that pertussis toxin (PTX) had little effect on arginine vasopressin-induced formation of inositol trisphosphate (IP_3) in rat aortic smooth muscle cells [Kondo et al.: *Biochemical and Biophysical Research Communications* 161:677–682, 1989]. In the present study, we investigated the mechanism of vasopressin-induced arachidonic acid release in rat aortic smooth muscle cells. Vasopressin stimulated both the release of arachidonic acid and the formation of IP_3 dose dependently in the range between 10 pM and 1 μM. The effect of vasopressin on arachidonic acid release was more potent than that on the formation of IP_3. Quinacrine, a phospholipase A_2 inhibitor, significantly suppressed the vasopressin-induced arachidonic acid release but had little effect on the formation of inositol phosphates. NaF, a GTP-binding protein activator, mimicked vasopressin by stimulating the arachidonic acid release. The arachidonic acid release stimulated by a combination of vasopressin and NaF was not additive. PTX partially but significantly suppressed the vasopressin-induced arachidonic acid release. In the cell membranes, PTX catalyzed ADP-ribosylation of a protein with an *Mr* of about 40,000. Pretreatment of membranes with 0.1 μM vasopressin in the presence of 2.5 mM $MgCl_2$ and 100 μM GTP markedly attenuated this PTX-catalyzed ADP-ribosylation of the protein in a time-dependent manner. These results strongly suggest that PTX-sensitive GTP-binding protein is involved in the coupling of vasopressin receptor to phospholipase A_2 in primary cultured rat aortic smooth muscle cells.

Key words: arachidonic acid, phospholipase A_2, phosphoinositide, phospholipase C, GTP-binding protein, pertussis toxin

It is well-recognized that proliferation of aortic smooth muscle cells plays a crucial role in the pathogenesis of hypertension and atherosclerosis [Schwartz, 1983; Ross, 1986]. It is well-known that arginine vasopressin stimulates proliferation of aortic smooth muscle cells [Altura and Altura, 1977; Campbell-Boswell and Robertson, 1981; Hamada et al., 1990]. Vasopressin stimulates phosphoinositide (PI) hydrolysis by phospholipase C through its binding to V_1 receptor in aortic smooth muscle cells [Nabika et al., 1985; Aiyar et al., 1986; Vallotton et al., 1986; Grillone et al., 1988], resulting in the formation of diacylglycerol and inositol phosphates. Among these products, diacylglycerol and inositol trisphosphate (IP_3) serve as messengers for the activation of protein kinase C and the mobilization of intracellular Ca^{2+}, respectively [Berridge and Irvine, 1984; Nishizuka, 1986]. In addition, it is generally recognized that GTP-binding protein(s) is involved in the coupling of the receptor to phospholipase C as well as the adenylate cyclase-cAMP system [Gilman, 1987]. In several types of cells [Gilman, 1987; Birnbaumer et al., 1990], PI hydrolysis can be inhibited by pertussis toxin (PTX), which catalyzes ADP-ribosylation of the α-subunit of GTP-binding protein, including G_i and G_o [Sternweis and Robishaw, 1984; Ui, 1984]. It has been reported that vasopressin-induced PI hydrolysis is inhibited by PTX in rat aortic smooth muscle cell line, A-10 [Aiyar et al., 1986; Xuan et al., 1987], and this suggests that a GTP-binding protein is involved in the vasopressin-induced PI hydrolysis in A-10

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cells. In contrast, we have previously shown that vasopressin-induced formation of IP_3 is not affected by PTX in primary cultured rat aortic smooth muscle cells [Kondo et al., 1989].

Vasopressin also stimulates the synthesis of prostaglandins such as prostacyclin (PGI_2) and prostaglandin E_2 (PGE_2), which are metabolites of arachidonic acid, in aortic smooth muscle cells [Hassid and Williams, 1983; Vallotton et al., 1986]. Two major pathways of arachidonic acid release are generally accepted [Smith, 1989]. One is the activation of phospholipase A_2 which causes liberation of arachidonic acid directly from the esterified stores of phospholipids, and the other is the sequential PI hydrolysis by phospholipase C and glycerol lipases. Accumulating evidence suggests that GTP-binding protein is also involved in the activation of phospholipase A_2 and that the GTP-binding protein is PTX-sensitive in several types of cells [Birnbaumer et al., 1990]. It has been reported that PTX has little effect on the vasopressin-induced arachidonic acid release in aortic smooth muscle cell line, A-10 [Grillone et al., 1988]. We have previously shown that vasopressin stimulates arachidonic acid release and the synthesis of PGI_2 and PGE_2, and that vasopressin also has a suppressive effect on DNA synthesis in primary cultured rat aortic smooth muscle cells by inhibiting the progression from the late G_1 into S phase of the cell cycle through the synthesis of PGI_2 and PGE_2, and that protein kinase C acts as an amplifier of this mechanism [Murase et al., 1992]. However, the exact mechanism of the vasopressin-induced arachidonic acid release in aortic smooth muscle cells has not yet been clarified.

In the present study, we investigated the mechanism of vasopressin-induced arachidonic acid release in primary cultured rat aortic smooth muscle cells. Our results strongly suggest that PTX-sensitive GTP-binding protein is involved in the coupling of vasopressin receptor to phospholipase A_2 in primary cultured rat aortic smooth muscle cells.

MATERIALS AND METHODS

Materials

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (209.0 Ci/mmol) and *myo*-[2-^{3}H]inositol (81.5 Ci/mmol) were purchased from Amersham Japan (Tokyo, Japan). [α-^{32}P]NAD (800 Ci/mmol) was purchased from Du Pont/New England Nuclear (Boston, MA). Arginine vasopressin was purchased from Peptide Institute Inc. (Minoh, Japan). Essentially fatty acid-free bovine serum albumin (BSA), NaF and quinacrine were purchased from Sigma Chemical Co. (St. Louis, MO). PTX was purchased from Funakoshi Pharmaceutical Co. (Tokyo, Japan). The protein assay reagent kit was purchased from Pierce (Rockford, IL). Other materials and chemicals were obtained from commercial sources.

Cell Culture

Aortic smooth muscle cells were obtained from thoracic aorta of male Sprague-Dawley rat by the explantation method as described [Kariya et al., 1987]. The cells (1×10^5) were seeded into 35-mm diameter dishes and maintained in 2 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were used between the third and sixth passage. After 6 days, the medium was exchanged for 2 ml of serum-free Dulbecco's modified Eagle's medium. The cells were used for experiments after 48 h. In experiments for the formation of inositol phosphates, the medium was exchanged for 2 ml of inositol-free Dulbecco's modified Eagle's medium. When indicated, the cells were pretreated with quinacrine for 20 min or PTX for 24 h prior to the stimulation by vasopressin.

Assay for Arachidonic Acid Release

The cultured cells were labeled with [^{3}H]arachidonic acid (0.5 μCi/dish) for 24 h. The labeled cells were washed four times with 1 ml of an assay buffer [5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 150 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.8 mM $MgSO_4$ and 1 mM $CaCl_2$]. The cells were subsequently preincubated with 1 ml of the assay buffer containing 0.1% essentially fatty acid-free BSA at 37°C for 20 min and the cells were then stimulated by various doses of vasopressin or NaF. After the indicated periods, the medium was collected and the radioactivity of the medium was determined.

Measurement of the Formation of Inositol Phosphates

The cultured cells were labeled with *myo*-[^{3}H]inositol (3 μCi/dish) for 48 h. The labeled cells were pretreated with 10 mM LiCl at 37°C for 10 min in 1 ml of the assay buffer containing 0.01% BSA. The cells were then stimulated by various doses of vasopressin. After the incubation for 5 min, the reaction was terminated by

adding 1 ml of 30% trichloroacetic acid. The acid supernatant was treated with diethyl ether to remove the acid and neutralized with 0.1 M NaOH. The supernatant was applied to a column of Dowex AG1-X8 formate form. The radioactive inositol monophosphate (IP_1), inositol bisphosphate (IP_2) and IP_3 were separated by successive elution of the column with 8 ml each of 0.1 M formic acid containing 0.2, 0.4 and 1 M ammonium formate, respectively [Berridge et al., 1983, 1984].

Membrane Preparations

Crude membranes were prepared at 4°C as described [Nishimoto et al., 1989] with a minor modification. In brief, the cultured cells were scraped from 90-mm diameter dishes with a rubber policeman into 20 mM Tris-HCl, pH 7.5, containing 5 mM $MgCl_2$, 1 mM ethylenebis (oxyethylenenitrilo) tetraacetic acid, 1 mM dithiothreitol and 0.1 TIU/ml aprotinin, homogenized by passing through a 22-gauge syringe, and centrifuged at 100*g* for 5 min. The supernatant was subsequently centrifuged at 20,000*g* for 60 min and the pellet was resuspended at a final protein concentration of 20 mg/ml in 50 mM HEPES, pH 7.4, containing 0.1 mM EDTA and 1 TIU/ml aprotinin. After sonication for 30 s, the crude membranes were used for assays. When we examined the effect of PTX pretreatment, the cultured cells were treated with 1 μg/ml PTX or vehicle for 24 h and harvested as described above.

ADP-Ribosylation of Membrane Proteins by PTX

Preactivation of PTX was performed by incubating with 50 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol and 1 mM ATP at a concentration of 50 μg/ml at 30°C for 15 min. The crude membranes were solubilized with 1% sodium cholate, and the cholate extracts (100 μg of protein) were incubated at 30°C for 60 min with 10 μg/ml preactivated PTX in 100 μl of an assay buffer [100 mM Tris-HCl, pH 8.0, containing 2.5 μM [α-^{32}P]NAD (1,000–2,000 cpm/pmol), 10 mM thymidine, 1 mM EDTA, 1 mM dithiothreitol and 1 mM L-α-dimyristoyl phosphatidylcholine]. The reaction was terminated by adding 400 μl of 25 mM Tris-HCl, pH 8.8, containing 192 mM glycine and 0.1% sodium dodecyl sulphate (SDS). The sample was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [11% polyacrylamide] as specified [Laemmli, 1970] and processed by autoradiography using Kodak X-Omat film. When the effect of vasopressin on ADP-ribosylation was examined, fresh cell membranes were previously incubated in 50 μl of 100 mM HEPES, pH 7.5, containing 2.5 mM $MgCl_2$, 100 μM GTP, 120 mM NaCl, 5 mM KCl, 15 mM sodium acetate, 1 mM EDTA, 10 mM dextrose, and 0.5% BSA with 0.1 μM vasopressin at 37°C for the indicated periods. These membranes were then combined with 50 μl of the reaction mixture containing 200 mM Tris-HCl, pH 8.0, 5 μM [α-^{32}P]NAD (1,000–2,000 cpm/pmol), 20 mM thymidine, 1 mM EDTA, 2 mM dithiothreitol, 2 mM L-α-dimyristoyl phosphatidylcholine and 20 μg/ml preactivated PTX, and incubated at 30°C for another 60 min. For detection of radioactive proteins, BAS 2000 (Fuji) equipped with imaging plates was used [Amemiya and Miyahara, 1988].

Determination

The radioactivity of ^{3}H-samples was determined with a Beckman LS 5000TD liquid scintillation spectrometer. Protein concentrations were determined by using a protein assay reagent kit with BSA as a reference protein.

Statistical Analysis

The data were analyzed by Student's *t* test and a $P < 0.05$ was considered significant. All data are represented as the mean ± S.D. of triplicate determinations.

RESULTS

Effects of Vasopressin on Arachidonic Acid Release and Formation of IP_3

First, we compared the dose-dependency of vasopressin-induced arachidonic acid release with that of vasopressin-induced formation of IP_3 in rat aortic smooth muscle cells. Vasopressin stimulated both the arachidonic acid release and the formation of IP_3 in a dose-dependent manner in the range between 10 pM and 1 μM (Fig. 1). The effect of vasopressin on the arachidonic acid release was more potent than that on the formation of IP_3 (Fig. 1).

Effects of Quinacrine on Vasopressin-Induced Arachidonic Acid Release and Formation of Inositol Phosphates

To clarify whether the activation of phospholipase A_2 is involved in the vasopressin-induced arachidonic acid release, we examined the effect of quinacrine, known to be a phospholipase A_2 inhibitor [Lapetina et al., 1981], on the release in rat aortic smooth muscle cells. Pretreatment with quinacrine, which by itself had little effect

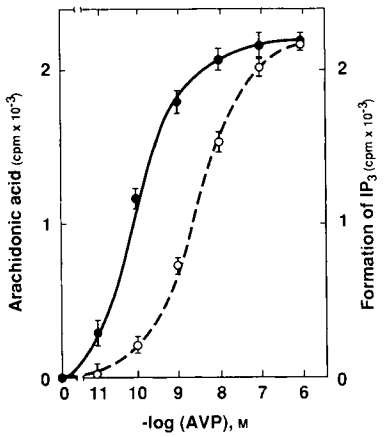

Fig. 1. Dose-dependent effects of arginine vasopressin (AVP) on the arachidonic acid release and the formation of IP_3 in rat aortic smooth muscle cells. The [^{3}H]arachidonic acid-labeled cells were stimulated by vasopressin for 30 min, and the release of arachidonic acid (●) was then determined. The [^{3}H]inositol-labeled cells were stimulated by vasopressin for 5 min, and the formation of IP_3 (○) was then determined. Values are expressed as a net increase compared with unstimulated cells. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

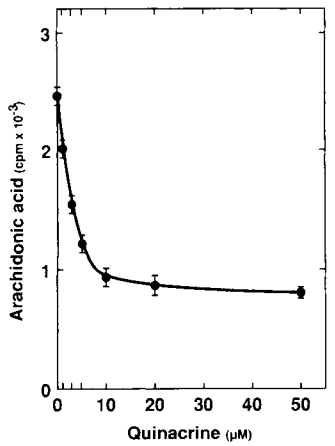

Fig. 2. Effect of quinacrine on the arginine vasopressin-induced arachidonic acid release in rat aortic smooth muscle cells. The [^{3}H]arachidonic acid-labeled cells were pretreated with various doses of quinacrine for 20 min, then stimulated by 0.1 μM vasopressin for 30 min. Values are expressed as a net increase compared with unstimulated cells. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

on arachidonic acid release (data not shown), significantly inhibited the vasopressin-induced arachidonic acid release in a dose-dependent manner in the range between 1 and 50 μM (Fig. 2). However, quinacrine had little effect on the vasopressin-induced formation of IP_1, IP_2, or IP_3 (Fig. 3).

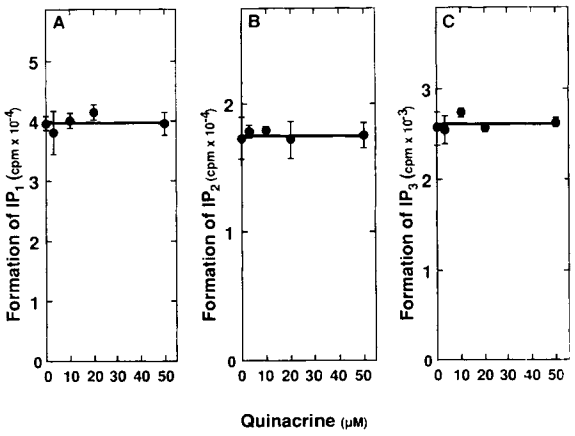

Fig. 3. Effect of quinacrine on the arginine vasopressin-induced formation of inositol phosphates in rat aortic smooth muscle cells: A) IP_1; B) IP_2; C) IP_3. The [^{3}H]inositol-labeled cells were pretreated with various doses of quinacrine for 20 min, then stimulated by 0.1 μM vasopressin for 5 min. Values are expressed as a net increase compared with unstimulated cells. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

Effect of NaF on Vasopressin-Induced Arachidonic Acid Release

To clarify whether GTP-binding protein is involved in the vasopressin-induced arachidonic acid release, we next examined the effect of NaF, known as a GTP-binding protein activator [Gilman, 1987], on the release. NaF stimulated the arachidonic acid release dose dependently in the range between 10 and 40 mM (data not shown). The effect of 20 mM NaF was similar to that of 0.1 μM vasopressin (Fig. 4). The release of arachidonic acid stimulated by a combination of 0.1 μM vasopressin and 20 mM NaF was not additive (Fig. 4).

Effect of PTX on Vasopressin-Induced Arachidonic Acid Release

The pretreatment with PTX (1 μg/ml), which by itself had little effect on arachidonic acid release (data not shown), partially but significantly inhibited the vasopressin-induced arachidonic acid release (Fig. 5). The value of inhibitory effect of PTX (1 μg/ml) on the arachidonic acid release induced by 0.1 μM vasopressin was about 50%.

ADP-Ribosylation by PTX of Membrane Proteins of Rat Aortic Smooth Muscle Cells

We next examined the existence of PTX substrate in the membranes of rat aortic smooth

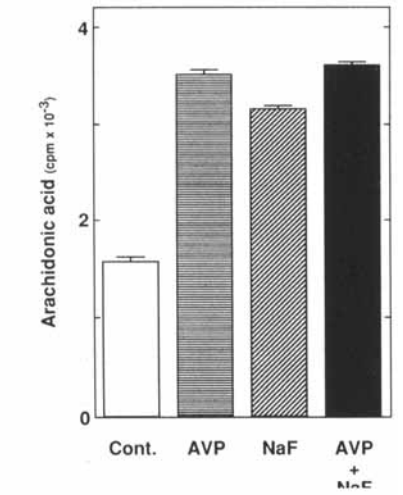

Fig. 4. Effect of NaF on the arginine vasopressin (AVP)-induced arachidonic acid release in rat aortic smooth muscle cells. The [^{3}H]arachidonic acid-labeled cells were stimulated by 0.1 μM vasopressin or 20 mM NaF for 30 min. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

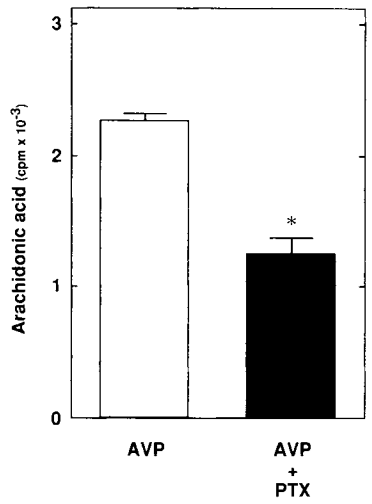

Fig. 5. Effect of PTX on the arginine vasopressin (AVP)-induced arachidonic acid release in rat aortic smooth muscle cells. The [^{3}H]arachidonic acid-labeled cells were pretreated with 1 μg/ml PTX for 24 h, then stimulated by 0.1 μM vasopressin for 30 min. Values are expressed as a net increase compared with unstimulated cells. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *$P < 0.05$ versus vasopressin alone.

muscle cells. Autoradiography after SDS-PAGE of the plasma membranes incubated with [^{32}P]NAD and preactivated PTX showed that this toxin ADP-ribosylated a protein with an *Mr* of about 40,000 (Fig. 6, lane 3). Since the inhibition by PTX of vasopressin-induced arachidonic

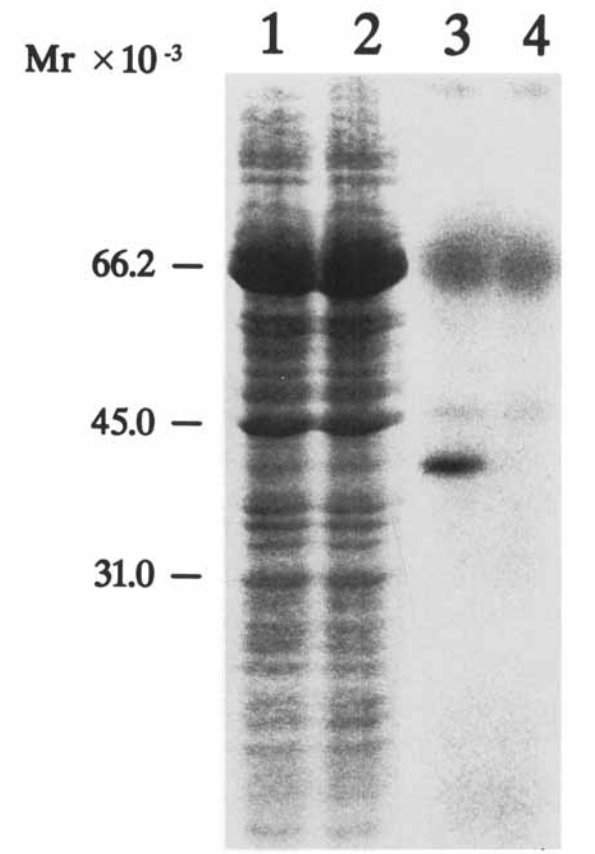

Fig. 6. The ADP-ribosylation of membrane proteins of rat aortic smooth muscle cells by PTX. The cultured cells were pretreated with 1 μg/ml PTX or vehicle for 24 h, and the membrane proteins were subjected to PTX-catalyzed ADP-ribosylation in the presence of [^{32}P]NAD. **Lanes 1, 2:** Coomassie Brilliant Blue stain. **Lanes 3, 4:** Autoradiography. Lanes 1, 3: Pretreated with vehicle. Lanes 2, 4: Pretreated with 1 μg/ml PTX.

acid release was partial (Fig. 5), we examined whether the PTX substrate had been completely ADP-ribosylated. The membranes from the cells pretreated with or without 1 μg/ml PTX were subjected to PTX-catalyzed ADP-ribosylation in the presence of [^{32}P]NAD. The incorporation of radiolabel into 40-kDa protein almost completely disappeared after the pretreatment with PTX (Fig. 6, lane 4).

Effect of Vasopressin on PTX-Catalyzed ADP-Ribosylation of 40-kDa Protein

To clarify the coupling between vasopressin receptor and the PTX substrate, we monitored alterations in PTX-catalyzed ADP-ribosylation of the 40-kDa protein after treatment with vasopressin. Preincubation of the membranes with 0.1 μM vasopressin in the presence of 2.5 mM $MgCl_2$ and 100 μM GTP markedly attenuated

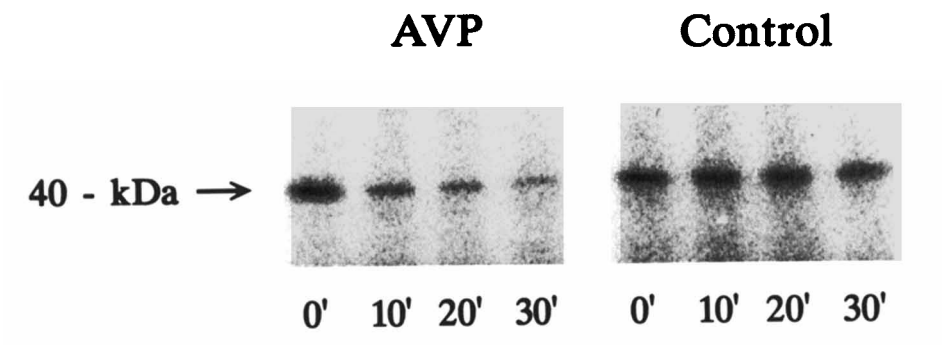

Fig. 7. Arginine vasopressin (AVP)-induced attenuation in PTX-catalyzed ADP-ribosylation of a 40-kDa protein in rat aortic smooth muscle cell membranes. Membranes were incubated with 0.1 μM vasopressin or vehicle in the presence of 2.5 mM $MgCl_2$ and 100 μM GTP for the indicated periods before ADP-ribosylation by PTX.

subsequent ADP-ribosylation of the 40-kDa substrate in a time-dependent manner up to 30 min (Fig. 7).

DISCUSSION

In a previous report [Kondo et al., 1989], we have shown that vasopressin-induced formation of IP_3 is not affected by PTX in primary cultured rat aortic smooth muscle cells. This finding suggests that PTX-insensitive GTP-binding protein is involved in vasopressin-induced activation of phospholipase C. We have also shown that vasopressin stimulates arachidonic acid release in these aortic smooth muscle cells [Murase et al., 1992]. Two major pathways of arachidonic acid release are generally accepted [Smith, 1989]. One is the activation of phospholipase A_2 and the other is the sequential PI hydrolysis by phospholipase C and glycerol lipases. We first compared the vasopressin-induced arachidonic acid release with the formation of IP_3 in dose-dependency and showed that the effect of vasopressin on arachidonic acid release was more potent than that on the formation of IP_3. Our findings lead us to speculate that vasopressin stimulates arachidonic acid release and PI hydrolysis via independent pathways in primary cultured rat aortic smooth muscle cells. In addition, we demonstrated that quinacrine, a phospholipase A_2 inhibitor [Lapetina et al., 1981], significantly inhibited the vasopressin-induced arachidonic acid release in aortic smooth muscle cells, while quinacrine had little effect on the vasopressin-induced formation of inositol phosphates in these cells. These results suggest that the major pathway of vasopressin-induced arachidonic acid release is the activation of phospholipase A_2 in primary cultured rat aortic smooth muscle cells.

Evidence is accumulating that GTP-binding protein is involved in the activation of phospholipase A_2 in several types of cells [Birnbaumer et al., 1990]. In the present study, we showed that NaF, a GTP-binding protein activator [Gilman, 1987], mimicked vasopressin by stimulating the release of arachidonic acid in aortic smooth muscle cells and that the arachidonic acid release stimulated by a combination of vasopressin and NaF was not additive. These results suggest that GTP-binding protein is involved in vasopressin-induced arachidonic acid release in primary cultured rat aortic smooth muscle cells.

In addition, we demonstrated that the vasopressin-induced arachidonic acid release was significantly inhibited by PTX pretreatment. It is well-known that PTX catalyzes ADP-ribosylation of the α-subunit of certain GTP-binding proteins including G_i and G_o, and causes uncoupling of receptor to these GTP-binding proteins [Sternweis and Robishaw, 1984; Ui, 1984]. It has been reported that PTX ADP-ribosylates a protein with an *Mr* of about 40,000 in primary cultured rat aortic smooth muscle cells [Gaul et al., 1988]. We confirmed that PTX caused ADP-ribosylation of a protein with an *Mr* of about 40,000 in aortic smooth muscle cells and showed that this PTX-catalyzed ADP-ribosylation was markedly attenuated by pretreatment of the membranes with 0.1 μM vasopressin in the presence of 2.5 mM $MgCl_2$ and 100 μM GTP in a time-dependent manner. It is well-established that high molecular GTP-binding proteins (e.g., G_s, G_i, G_o, and transducin) are heterotrimers composed of α-, β-, and γ-subunits [Gilman, 1987]. Stimulation of GTP-binding protein-coupled receptors by agonist dissociates the GTP-binding proteins into α-subunit and βγ-subunits as a result of exchange of GDP for GTP at the

guanine nucleotide-binding sites in the presence of Mg^{2+} [Gilman, 1987]. It is well-known that PTX ADP-ribosylates the α-subunit of G_i, G_o, or transducin [Sternweis and Robishaw, 1984; Ui, 1984; Gilman, 1987] only when the GTP-binding protein is in the heterotrimeric form [Katada et al., 1986]. Namely, PTX does not ADP-ribosylate the α-subunit dissociated from βγ-subunits [Katada et al., 1986]. Reduction in the PTX substrate activity of GTP-binding protein is probable to reflect the dissociation of their subunits [Halenda et al., 1986; Rothenberg and Kahn, 1988]. Thus, our finding that vasopressin attenuated PTX-catalyzed ADP-ribosylation in aortic smooth muscle cells strongly suggests that vasopressin induces dissociation of the GTP-binding protein into its active state. These results suggest that vasopressin receptor is coupled to PTX-sensitive GTP-binding protein in primary cultured rat aortic smooth muscle cells.

On the other hand, it has been reported that vasopressin-induced PI hydrolysis is inhibited by PTX in rat aortic smooth muscle cell line, A-10 [Aiyar et al., 1986; Xuan et al., 1987] and that vasopressin-induced arachidonic acid release is not affected by PTX in A-10 cells [Grillone et al., 1988]. These discordances may be due to the difference between primary cultured smooth muscle cells and cell line.

In this study, we have demonstrated the coupling of vasopressin receptor with PTX-sensitive GTP-binding protein in primary cultured rat aortic smooth muscle cells, but PTX-caused inhibition of vasopressin-induced arachidonic acid release was partial. This seems not to be due to an incomplete ADP-ribosylation of the subunits by PTX, because the membranes from the cells pretreated with PTX could not be further ADP-ribosylated. It has been shown that PTX partially inhibits the carbachol-induced PI hydrolysis in CHO cells expressing M1 muscarinic acetylcholine receptor, and that the receptor couples to both PTX-sensitive and insensitive GTP-binding proteins [Ashkenazi et al., 1989]. In primary cultured rat aortic smooth muscle cells, it is possible that vasopressin-induced arachidonic acid release is mediated by distinct GTP-binding proteins of which one is PTX substrate and the other is not.

In conclusion, our results strongly suggest that PTX-sensitive GTP-binding protein is involved in the coupling of vasopressin receptor to phospholipase A_2 in primary cultured rat aortic smooth muscle cells.

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