Vasopressin Induces V₁ Receptors to Activate Phosphatidylinositol- and Phosphatidylcholine-specific Phospholipase C and Stimulates the Release of Arachidonic Acid by At Least Two Pathways in the Smooth Muscle Cell Line, A-10*

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Lisa R. Grillone, Mike A. Clark, Richard W. Godfrey, Frans Stassen, and Stanley T. Crooke
From the Smith Kline & French Laboratories, Philadelphia, Pennsylvania 19101

The rat thoracic aortic smooth muscle cell line, A-10, expresses vasopressin receptors of the V₁ subtype. Vasopressin treatment of these cells stimulated the release of arachidonic acid and the formation of diacylglycerol and phosphocholine. These responses to vasopressin were inhibited by the V₁-specific antagonist SK&F 100273, indicating that these were receptor-mediated phenomena.

The mechanisms by which V₁ receptors mediate arachidonic acid release appeared to be unaffected by cycloheximide or actinomycin D, suggesting that the release is independent of protein and RNA synthesis. The V₁ receptors also appeared to be coupled to a phospholipase C which can hydrolyze phosphatidylycerol, a possible source of the released arachidonic acid. Phosphocholine and diacylglycerol were also generated. The release of arachidonic acid, phosphocholine, or diacylglycerol was not affected by prior treatment of the cells with pertussis toxin (islet-activating protein). Thus, the release of these second messengers is not mediated by the guanine second nucleotide-binding protein G or other pertussis toxin-sensitive substrates.

We conclude that V₁ receptors induce the release of arachidonic acid and the formation of diacylglycerol and phosphocholine via the activation of both a phosphatidylinositol- and phosphatidylcholine-specific phospholipase C.

Vasopressin regulates water transport in the kidney (1) and pressor activity in vasculature (2) via interaction with V₂ and V₃ receptors, respectively. Modulation of the response to vasopressin by prostaglandins has been demonstrated in kidney (3, 4) and vascular smooth muscle cells (5). These studies suggest that the induction of prostaglandin (PG) V₁ biosynthesis by vasopressin may function as a negative regulatory pathway of vasopressin effects. Arachidonic acid is esterified to the cellular phospholipids and is released and metabolized in many cell types in response to a variety of agents (6–8), including vasopressin (3, 4, 8, 9). These metabolites include several biologically active eicosanoids which are synthesized via the lipoxygenase or cyclooxygenase pathways.

It has been suggested that, in some systems, Ca²⁺ mobilization and phosphatidylinositol (PI) metabolism are coupled to the vasoconstrictive response induced by the binding of vasopressin (V₁) to its receptors (9–11), and, in addition, the contractile effect of vasopressin may be regulated by the negative feedback control of PG biosynthesis (12). We have previously shown that vasopressin receptors in the A-10 cell line are coupled to a guanine nucleotide-binding regulatory protein (G protein), and pretreatment with islet-activating protein (IAP) partially inhibited vasopressin-induced PI turnover (11), indicating that a G protein associated with vasopressin receptors in A-10 cells may function in mediating phosphoinositide metabolism.

Diacylglycerol (DAG), another second messenger, activates protein kinase C, which in turn mediates a variety of cellular events. The generation of DAG as a result of receptor-ligand interactions is classically thought to be the result of phosphatidylinositol hydrolysis by phospholipase C (13, 14). It has also been suggested that DAG may be generated from phospholipids other than phosphoinositides (15, 16). Recently, investigators have reported that DAG may also be produced by the hydrolysis of phosphatidylycerol by a phosphatidylycholine-prefering phospholipase C (17, 18). Phospholipase C with a similar substrate specificity has been purified from dog heart (19) and human monocytes (20). In contrast to PI-specific phospholipase C, the importance of PC-specific phospholipase C in mediating receptor-ligand interactions remains to be elucidated.

To investigate the involvement of PC-specific phospholipase C in signal transduction processes as well as to understand the mechanism of arachidonic acid release induced by vasopressin, we used the smooth muscle cell line, A-10. Previously, our laboratory has shown that this cell line expresses vasopressin receptors of the V₁ subtype (21) and responds to vasopressin by increased PI hydrolysis and calcium mobilization (11). In this paper, we present data which suggest that activation by vasopressin of the V₁ receptors in smooth muscle cells stimulates the release of arachidonic acid and synthesis of several metabolites. We also demonstrate that vasopressin receptor-ligand interaction results in the activation of a PC-specific phospholipase C which rapidly generates phosphocholine (P-choline), as well as the activation of PI-specific phospholipase C. Our data suggest that there is sufficient P-choline available soon after cell stimulation with vasopressin.
to account for DAG and arachidonic acid produced and that at least part of this response is a result of phospholipase C hydrolysis of phosphatidylcholine.

**EXPERIMENTAL PROCEDURES**

**Materials**—[5,6,8,9,11,12,14,15-3H]Arachidonic acid, [methyl-3H]cholesterol chloride, and [1-14C]oleic acid were obtained from Du Pont-New England Nuclear. Nonradioactive standards used in thin layer chromatography were obtained from Sigma. 8-Ariginine vasopressin (AVP) was purchased from Bachem (Torrance, CA). The vasopressin analog used was desamino(CH2),Tyr(Me)AVP: A selective V, antagonist ([3H]AVP was synthesized at Smith Kline & French Laboratories. Cycloheximide and actinomycin D were purchased from Sigma. IAP was purchased from List Biological Laboratories (Campbell, CA). Fatty acid-free bovine serum albumin was purchased from Miles Scientific (Naperville, IL). Silica Gel G thin layer chromatography plates were purchased from Analtech, Inc. (Newark, DE); Redi-Cota 2D thin layer chromatography plates were purchased from Supelco, Inc. (Belleville, PA). All agonists and antagonists were dissolved in Puck’s saline containing 10 mM HEPES (GIBCO), and 0.1% bovine serum albumin, pH 7.2, or in Dulbecco’s phosphate-buffered saline, containing 15 mM HEPES, pH 7.4 (buffer solution), just prior to use.

**Cell Culture**—Rat thoracic aortic smooth muscle cells (A-10; ATCC CRL 1476) were cultured in Dulbecco’s modified Eagles medium plus 20% fetal calf serum (DMEM/FCS). The cells were subconfluent to ensure a monolayer cell population. All experiments were performed with cells passed once a week for no more than 4 months. Culture wells (35 mm diameter, 6-well Linbro plates; Flow Laboratories Inc., McLean, VA) were seeded with 1 ml of medium containing 7.5 × 10⁴ cells; 100-μm Petri dishes (Nunc) were seeded with 10 ml of medium containing 6.0 × 10⁶ cells; 60-μm Petri dishes were seeded with 5 ml of medium containing 2.25 × 10⁹ cells. The cells were allowed to grow in culture for 5 days.

**Incorporation with Radioisotope**—In all experiments, cells were radiolabeled on day 2 of cell culture and incubated for an additional 18–22 h at 37 °C to allow for incorporation of radioactivity.

[3H]Arachidonic acid, 100 μCi in ethanol (specific activity 83.8 Ci/mmol), and 100 μCi in ethanol (specific activity 40 μCi/ml). Immediately before use this concentrated [3H]arachidonic acid was diluted in DMEM/FCS to 25 μCi/ml, and 100 μl of this solution was added to 1 ml of DMEM/FCS/well in 6-well plates. [3H]Choline chloride, 400 μCi in ethanol (specific activity 50 μCi/mmol), was dried down on a Speed Vac concentrator (Savant) and redissolved in 40 μl of 70% ethanol.

Immediately before use this concentrated [3H]arachidonic acid was diluted in DMEM/FCS to 25 μCi/ml, and 100 μl of this solution was added to 1 ml of DMEM/FCS/well in 6-well plates. [3H]Choline chloride, 400 μCi in ethanol (specific activity 50 μCi/mmol), was dried down on a Speed Vac concentrator (Savant) and redissolved in 40 μl of 70% ethanol. Immediately before use this was diluted to 166.7 μCi/ml in DMEM/FCS, and cells in either 60- or 100-μm Petri dishes were labeled with 4.2 μCi/ml. [3C]Oleic acid, 50 μCi in ethanol (specific activity 54.0 μCi/mmol), was diluted to 10.0 μCi/ml in DMEM/FCS, and cells in each well of a 6-well plate were labeled with 400 μl.

**Arachidonic Acid Release**—Following incubation with [3H]arachidonic acid, 50-μl samples were taken from the supernatant for measurement of unincorporated [3H]arachidonic acid. We determined that greater than 90% [3H]arachidonic acid was incorporated in the cellular lipid. Cells were rinsed at room temperature 2 times with Puck’s saline and then incubated for 10 min in this buffer to stabilize the cells. Subsequently, the saline was replaced with saline containing AVP, a vasopressin antagonist, or a combination of AVP and antagonist. The incubation was continued for 4 min at room temperature. The quantity of [3H]arachidonic acid released in response to AVP and AVP analogs was determined by taking 200-μl samples from the cell supernatant. Released [3H]arachidonic acid was measured by counting in Ready Solv® HP/b scintillation mixture on a LS6000 counter (Beckman Instruments, Inc.).

**Diacylglycerol Generation**—Following incubation with radiolabel, the cells were rinsed with buffer twice and allowed to equilibrate for 10 min at room temperature with buffer. Subsequently, the buffer was removed and the cells were treated with AVP, antagonist, AVP and antagonist, or buffer alone. The reaction proceeded at room temperature and was terminated, as indicated timed, by removing the supernatant from the cells followed by the rapid addition of methanol. Methanol extracts from cells plated in 6-well plates were dried under N₂, and the resulting residue was redissolved in 50 μl of methanol before being applied to TLC plates. The mobile phase consisted of ethyl acetate:acetic acid:water (70:20:1). The separated lipids were visualized by staining with iodine vapor, and the diglyceride bands were identified by their co-migration with authentic standards included in each lane. The bands were scraped from the TLC plates into liquid scintillation vials containing methanol (0.5 ml), and the amount of radioactivity in each vial determined by liquid scintillation spectroscopy, or radiolabel was quantified using the Bioscan 200 TLC plate scanner (Bioscan, Washington, D.C.).

**Phosphocholine Formation**—Following incubation with radiolabel, the cells were rinsed twice with buffer and allowed to equilibrate for 10 min at room temperature prior to the start of the experiment. Subsequently, the buffer was replaced with buffer containing AVP, antagonist, AVP and antagonist or buffer alone. The reaction proceeded at room temperature for the indicated times at which point aliquots of 200 μl were taken and counted. The remaining supernatant was lyophilized and applied to a TLC plate to determine the percent radioactivity counts released from the cells that co-migrated with authentic P-choline standard (17).

**Two-dimensional Thin Layer Chromatography**—Cells were labeled overnight with [3H]arachidonic acid as described above. Cells were then rinsed twice with buffer and allowed to equilibrate for 10 min at room temperature prior to the start of the experiment. Cells were treated with or without 100 nM AVP, and cellular lipids were extracted using the method previously described by Bligh and Dyer (22). The mobile phase for the first dimension was chloroform:methanol: NH₄OH (65:26.5:5) and for the second dimension was chloroform:acetone:methanol:acetic acid/water (3:4:1:3.5). Authentic standards were added to the cellular lipids to enhance their visualization by iodine vapor.

**Analysis of Products of Arachidonic Acid Metabolism**—[3H]Arachidonic acid and its metabolites released by AVP stimulation were identified and quantitated by reverse phase high performance liquid chromatography. The cells were cultured in T-100 culture flasks, and supernatant was prepared as the [3H]arachidonic acid release studies. After treatment, the supernatant was acidified with 50 μl of phosphoric acid and extracted twice with 10 μl of ethyl acetate. The ethyl acetate extracts were combined and dried under N₂ at 25 °C. Extracts were reconstituted with 250 μl of methanol and chromatographed on a 5-μm C₁₈ Nova-Pak column (Waters Associates, Milford, MA) using a Beckman 421 binary system with a series 112 solvent delivery system. Aliquots of 200 μl of the samples were applied to the column at a solvent ratio of 84% solvent I (83:16.1, water:acetonitrile:acetic acid) and 16% solvent II (100:0.1, acetonitrile:acetic acid) using a flow rate of 1.7 ml/min. This system was run isocratically for 10 min before initiating a 5-min linear gradient to a ratio of 70% solvent I and 30% solvent II. This ratio was run isocratically for 5 min before starting a linear gradient of 25 min to a final ratio of 10% solvent I and 90% solvent II. This ratio was maintained isocratically for 15 min. Analyses of radioisotope were processed by the Ramona D radioactivity monitor (INUS, Fairfield, NJ) and recorded to an Apple II Plus computer.

**Treatment with Inhibitors**—IAP was reconstituted with distilled water to a stock concentration of 100 μg/ml. Final concentrations of IAP from 0.1 to 500 ng/ml were prepared in buffer solution. Cells were rinsed once with buffer solution and incubated with IAP for 4 h at 37 °C. IAP was removed and cells rinsed twice with buffer solution before treatment with 100 nM AVP or with buffer alone as described above.

Cycloheximide was prepared as a stock solution of 10 mg/ml in DMEM/FCS or Puck’s saline. Cycloheximide was used at a final concentration of 50 μM and was administered to a 5-μm C₁₈ Nova-Pak column (Waters Associates, Milford, MA) using a Beckman 421 binary system with a series 112 solvent delivery system. Aliquots of 200 μl of the samples were applied to the column at a solvent ratio of 84% solvent I (83:16.1, water:acetonitrile:acetic acid) and 16% solvent II (100:0.1, acetonitrile:acetic acid) using a flow rate of 1.7 ml/min. This system was run isocratically for 10 min before initiating a 5-min linear gradient to a ratio of 70% solvent I and 30% solvent II. This ratio was run isocratically for 5 min before starting a linear gradient of 25 min to a final ratio of 10% solvent I and 90% solvent II. This ratio was maintained isocratically for 15 min. Analyses of radioisotope were processed by the Ramona D radioactivity monitor (INUS, Fairfield, NJ) and recorded to an Apple II Plus computer.

**RESULTS**

**Time Course of AVP-stimulated Arachidonic Acid Release**—AVP stimulated the release of [3H]arachidonic acid from cultured smooth muscle cells in a time- and concentration-dependent manner. As shown in Fig. 1A, 1 μM AVP stimulated the release of [3H]arachidonic acid within 1 min, and by 4 and 8 min, at the same concentration, the release was 70% above control levels. Based on these results, a treatment time of 4 min was used in subsequent experiments at room temperature.
Vasopressin Induces Arachidonic Acid, Diacylglycerol, Phosphocholine Release

**Time Course of AVP-stimulated DAG Release**—AVP stimulated the release of DAG from cultured smooth muscle cells. As shown in Fig. 1B, treatment of smooth muscle cells with 100 nM AVP resulted in an increase in the production of DAG. The maximal effect, 35% above control levels, was observed 4 min after the addition of AVP (at room temperature). This stimulation was not dissimilar from that seen by others (17). In addition, in a comparison of the efficacy of this technique for DAG extraction with another more commonly used system (22), we found that our method was at least 70% as efficient.

**Time Course of AVP-stimulated P-choline Release**—One possible explanation for the generation of DAG is the activation of a phospholipase C-mediated hydrolysis of phosphatidylcholine and the subsequent release of phosphocholine. To evaluate this possibility, cells prelabeled with [3H]choline chloride were treated with 100 nM AVP. AVP stimulated the release of P-choline from smooth muscle cells in a time-dependent manner. As shown in Fig. 1C, 100 nM AVP stimulated the release of labeled P-choline within 5 s, and the release reached a maximal level by about 4 min. The results from TLC analyses demonstrated that greater than 90% of the total radioactivity co-chromatographed with P-choline (data not shown).

**Dose Response of AVP-stimulated Arachidonic Acid Release**—Release of [3H]arachidonic acid was dependent on the concentration of AVP employed (Fig. 2). Samples were taken 4 min after stimulation with AVP. Stimulation was detectable at a concentration of 0.1 nM and reached a plateau at 100 nM.

**Incorporation of [3H]Arachidonic Acid into Cellular Lipids**—Results obtained from two-dimensional TLC demonstrate that when smooth muscle cells are incubated with [3H]arachidonic acid overnight, greater than 85% is incorporated by the cells. The distribution of label among various cellular lipids is shown in Table I. Stimulation of these cells with 100 nM AVP resulted in the loss of arachidonic acid from PC, phosphatidylethanolamine, phosphatidic acid, phosphatidylserine, and PI (Table I). These data suggest that there exist multiple sources for the release of arachidonic acid.

**Effect of Vasopressin Antagonists on Arachidonic Acid, DAG, and P-choline Release**—The specific V1 antagonist desamino(CH2)6Tyr(Me)AVP, added simultaneously with 10 nM AVP, blocked the release of [3H]arachidonic acid 86 and 100%...
The distribution of $[^{3}H]$arachidonic acid in cellular lipids before and after stimulation with AVP

Smooth muscle cells were labeled overnight with $[^{3}H]$arachidonic acid (2.5 μCi/ml). This resulted in >85% of the exogenously added $[^{3}H]$arachidonic acid being cell-associated. Cells were then treated as described under "Experimental Procedures" either with control buffer or 100 nM AVP (4 min) prior to extraction of cellular phospholipids. The phospholipids were then separated by two-dimensional TLC, and the amount of $[^{3}H]$arachidonic acid associated with various phospholipids was determined. The values shown are disintegrations per minute from a representative experiment. PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid.

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TABLE I

The distribution of $[^{3}H]$arachidonic acid in cellular lipids before and after stimulation with AVP

|          | PC  | PE  | PI  | PS  | PA  | DAG |
|----------|-----|-----|-----|-----|-----|-----|
| Control  | 69,266 | 232,489 | 18,581 | 15,559 | 1,514 | 4,744 |
| AVP      | 63,563 | 184,987 | 7,558  | 8,090  | 1,223 | 6,561 |
| %Δ       | -8.5 | -20 | -59  | -30   | -19  | +37  |

**FIG. 3.** Effect of V₁-specific antagonist on AVP-stimulated $[^{3}H]$arachidonic acid release from smooth muscle cells. Effect of the specific V₁ antagonist desamino(Gly₂,Tyr(Me)₃)AVP on AVP-induced $[^{3}H]$arachidonic acid ($[^{3}H]$AA) release. At concentrations of $10^{-6} \text{ M (I)}$ and $10^{-5} \text{ M (II)}$, the antagonist abolished the AVP-stimulated response (+) as compared to the control (C) and AVP (10 nM) (III) only. Data represent the percent stimulation of $[^{3}H]$arachidonic acid release and are mean values ± S.E. derived from three experiments each with six determinations.

at concentrations of 10 nM and 1 μM, respectively (Fig. 3). These data correlate with the relative affinity of this antagonist for the V₁ receptor. In the rat liver, the Kᵦ of desamino(Gly₂,Tyr(Me)₃)AVP is 0.5 ± 0.1 nM as compared with AVP, which is 0.6 nM (21). Thus, we conclude that the $[^{3}H]$arachidonic acid release observed is mediated by V₁ receptors.

To determine whether the AVP-stimulated events leading to DAG generation and phosphocholine release were also receptor-mediated, the V₁-specific antagonist was used. This antagonist added simultaneously with AVP completely blocked the AVP-stimulated generation of DAG when assayed at 4 min. The antagonist by itself had no effect on basal levels of DAG generation (Table II). Under these same conditions, this antagonist inhibited the AVP-stimulated release of phosphocholine by 74% (Table II).

**Analysis of the Products of Arachidonic Acid Metabolism**—High performance liquid chromatography analyses of arachidonic acid metabolites produced in response to stimulation by 10 nM AVP are shown in a representative high performance liquid chromatogram (Fig. 4). In response to AVP, $[^{3}H]$arachidonic acid was released and converted to several metabolites including prostaglandins. However, the production of particular prostaglandins was variable and nonselective. Control chromatographs (not included) show negligible production of arachidonic acid metabolites.

**Effect of IAP on AVP-stimulated Arachidonic Acid and P-choline Release**—IAP was used to investigate the involvement of G protein(s) in AVP-stimulated $[^{3}H]$arachidonic acid release. In membrane preparations from the same cells IAP, at concentrations between 1 and 500 ng/ml, ADP-ribosylated a protein with a molecular mass between 37 and 39 kDa (data not shown). At concentrations from 0.1 to 500 ng/ml, IAP pretreatment for 4 h (37 °C) did not inhibit the AVP-stimulated $[^{3}H]$arachidonic acid (Table III). Pretreatment with 100 ng/ml IAP did not inhibit the AVP-stimulated release of phosphocholine (Table III).

**Effect of Cycloheximide and Actinomycin D on AVP-stimulated Arachidonic Acid Release**—Pretreatment of cells for 30 min at 37 °C with cycloheximide (100 μg/ml) or actinomycin D (10 μg/ml) inhibited the incorporation of $[^{3}H]$leucine or $[^{3}H]$uridine, respectively, into acid-precipitable counts by 85% (data not shown). Cells treated with either cycloheximide or actinomycin D were stimulated by the addition of 10 nM AVP.

**Fig. 4.** Representative chromatogram of AVP-stimulated production of arachidonic acid metabolites from smooth muscle cells. Representative chromatogram of reverse phase high performance liquid chromatography of supernatant from smooth muscle cells treated with $10^{-8} \text{ M AVP}$ for 4 min. Data are presented as dpm × 10⁻³. Products are indicated at the respective times for the standards. TXB₂, thromboxane B₂; A.A., arachidonic acid; LTB₄, leukotriene B₄; 5 HETE, 5-hydroxy-eicosatetraenoic acid.

**TABLE II**

|          | Diacylglyceride | Phosphocholine |
|----------|-----------------|----------------|
| AVP      | +35             | +180           |
| V₁-specific antagonist | -22             | +15            |
| V₁-specific antagonist + AVP | +6              | +42            |

**TABLE III**

|          | Diacylglyceride | Phosphocholine |
|----------|-----------------|----------------|
| AVP      | +35             | +180           |
| V₁-specific antagonist | -22             | +15            |
| V₁-specific antagonist + AVP | +6              | +42            |

**FIG. 3.** Effect of V₁-specific antagonist on AVP-stimulated $[^{3}H]$arachidonic acid release from smooth muscle cells. Effect of the specific V₁ antagonist desamino(Gly₂,Tyr(Me)₃)AVP on AVP-induced $[^{3}H]$arachidonic acid ($[^{3}H]$AA) release. At concentrations of $10^{-6} \text{ M (I)}$ and $10^{-5} \text{ M (II)}$, the antagonist abolished the AVP-stimulated response (+) as compared to the control (C) and AVP (10 nM) (III) only. Data represent the percent stimulation of $[^{3}H]$arachidonic acid release and are mean values ± S.E. derived from three experiments each with six determinations.
arachidonic acid is independent of protein and RNA synthesis were not cytotoxic as determined by trypan blue dye exclusion. Pretreated with cycloheximide release of arachidonic acid in a vascular smooth muscle cell Tyr(Et)Val-AVP) (data not shown) and was completely inhibiting proteolysis of phospholipases. Abolished by the V1-specific antagonist desamino(CH2),-D-[Tyr(Me)AVP. This antagonist, however, did not affect [3H]arachidonic acid release stimulated by the ionophore A23187 (data not shown). The vasopressin analog desaminoo-2-vasopressin did not induce [3H]arachidonic acid release (data not shown), thus providing evidence that stimulated release of [3H]arachidonic acid is not artifactual. Thus, activation of V1 receptors by vasopressin leads to arachidonic acid release from these smooth muscle cells. Arachidonic acid release in response to vasopressin was independent of protein or RNA synthesis as indicated by the results of experiments with cycloheximide and actinomycin D (Table IV). Thus, the mechanism of arachidonic acid release by vasopressin is different from the mechanism by which leukotriene D4 induces arachidonic acid release (23), which requires RNA and protein synthesis.

The release of arachidonic acid and subsequent synthesis of metabolites in response to stimulatory agents has also been demonstrated in several other cell types including platelets (9, 24), macrophages (25, 26), renal medullary interstitial cells (8), and glomerular mesangial cells (4, 12, 27). The rate-limiting step in the synthesis of prostaglandins is the generation of free arachidonic acid from phospholipids (for review, see Ref. 28). It has been suggested that activation of the PI cycle by phospholipase C leads to the release of arachidonic acid and the production of eicosanoids when vasopressin receptors of platelets (9) or cultured rat renal mesangial cells (27) are stimulated. In our study, characterization of the arachidonic acid metabolites demonstrates that several products result from AVP stimulation of smooth muscle cells, including PGD2 and 6-keto-PGF1α (Fig. 4). Released arachidonic acid may also be derived from the sequential action of a phospholipase C and diacylglycerol lipase and monacylglycerol lipase (29,30), or by phospholipase A2 (31,32). Cooper and Malik (33) suggested that stimulation of isolated rat kidney cells by vasopressin causes mobilization of intracellular Ca2+ resulting in the activation of phospholipase A2 leading to arachidonic acid release and prostaglandin biosynthesis. More recently, a hormone-sensitive PC-specific phospholipase C has been demonstrated (17, 18). This too may be involved in the release of arachidonic acid. Although vasopressin induces PI turnover in A-10 cells (11) our data suggest that release of [3H]arachidonic acid and DAG may at least in part by independent of PI turnover.

It is well known that agonist stimulation in a variety of cell types leads to the formation of inositol 1,4,5-trisphosphate as well as DAG, a result of polyphosphoinositide metabolism (13, 15, 16, 34). The cellular response to various stimuli, in the form of DAG production, may be either sustained or transient, depending on cell type and/or stimulus. A transient response to thrombin in platelets (35) and vasopressin in hepatocytes (15) has been reported. In contrast, others have demonstrated a sustained increase in DAG formation in hepatocytes (16), in neutrophils (36), and in smooth muscle cells (34). It has been suggested that, following hormonal stimulation of hepatocytes, a portion of the DAG formed may result from the hydrolysis of another phospholipid, perhaps phosphatidylcholine (15, 18). Others have demonstrated the existence of a Ca2+-independent phospholipase C specific for phosphatidylcholine (38). Indeed, there may be two forms of PC-specific phospholipase C, a protein kinase C-regulated form and a protein kinase C-independent form (17). Recently, Daniel et al. (38) have suggested that phorbol ester stimulates a degradation and resynthesis of choline containing phosphoglycerides initiated by a PC-specific phospholipase C. Such an enzyme in mammalian tissue has recently been identified, isolated, and characterized as having a specificity for phosphatidylcholine (19, 20). Vasopressin has been shown to increase PC-specific phospholipase C activity in the liver by

### Table III

Effect of IAP on AVP-stimulated arachidonic acid and 1-choline release from smooth muscle cells

Smooth muscle cells were labeled with [3H]arachidonic acid (2.5 μCi/ml) or with [3H]choline chloride (4.2 μCi/ml) as described under "Experimental Procedures." Cells were pretreated with the indicated concentrations of IAP, or with buffer solution, for 4 h at 37 °C. Following IAP treatment, cells were exposed to AVP (100 nM) for 4 min. For AVP-stimulated [3H]arachidonic acid release, the data are the mean values of at least two determinations derived from two experiments. The values of IAP-treated cells did not significantly differ from the control values and paired t test based on actual disintegrations per minute values from AVP-stimulated groups showed no difference between these pretreated with IAP or buffer alone (p > 0.05). For the AVP-stimulated [3H]choline chloride release, the data are the mean values of at least two determinations from three experiments.

| [IAP] (ng/ml) | Arachidonic acid % | Phosphocholine % |
|---------------|-------------------|-----------------|
| 0             | 100               | 100             |
| 0.1           | 114               |                 |
| 1.0           | 106               |                 |
| 10.0          | 107               |                 |
| 100.0         | 96                | 100             |
| 500.0         | 89                |                 |

### Table IV

Effect of cycloheximide and actinomycin D on AVP-stimulated [3H]arachidonic acid release from smooth muscle cells

Smooth muscle cells were labeled with [3H]arachidonic acid (2.5 μCi/ml) as described under "Experimental Procedures." Cells were pretreated with cycloheximide (100 μg/ml) or actinomycin D (10 μg/ml) for 30 min at 37 °C, then exposed to AVP (10 nM) for 4 min, at which time samples were taken to determine [3H]arachidonic acid release. Data are the mean values ± S.E. of three determinations derived from three experiments.

| Conditions | [3H]Arachidonic acid released | AVP-induced release |
|------------|-----------------------------|---------------------|
| Control    | 11,615 ± 1,116              |                     |
| AVP        | 20,114 ± 2,822              | 73.2 ± 10.3         |
| Cycloheximide | 9,144 ± 1,487           |                     |
| Cycloheximide + AVP | 20,486 ± 270          | 124.1 ± 20.2        |
| Actinomycin D | 7,610 ± 946              |                     |
| Actinomycin D + AVP | 12,371 ± 616          | 62.6 ± 7.8          |

for 4 min at room temperature, and [3H]arachidonic acid release was quantitated as described above. Under the experimental conditions used, cycloheximide and actinomycin D were not cytotoxic as determined by trypan blue dye exclusion. These studies show that the AVP-stimulated release of [3H]arachidonic acid is independent of protein and RNA synthesis (Table IV). In fact, cycloheximide appears to enhance the ability of AVP to induce arachidonic acid release, perhaps by inhibiting proteolysis of phospholipases.

### DISCUSSION

Our data show that V1 receptors mediates AVP-induced release of arachidonic acid in a vascular smooth muscle cell line. Vasopressin-stimulated release of [3H]arachidonic acid from the A-10 smooth muscle cells in culture was time- and dose-dependent. The reaction was rapid at 37 °C (data not shown) but proceeded more slowly at room temperature. AVP-stimulated [3H]arachidonic acid release was inhibited as much as 78% by the V1/V2 antagonist desaminot(Ch2)n-D-[Tyr(Et)Val-AVP] (data not shown) and was completely abolished by the V1-specific antagonist desamino(Ch2)n-Tyr(Me)AVP. This antagonist, however, did not affect [3H]arachidonic acid release stimulated by the ionophore A23187.
Vasopressin Induces Arachidonic Acid, Diacylglycerol, Phosphocholine Release

Figure 5. Diagram of model of possible pathways of arachidonic acid release following activation of vasopressin V₁ receptors. Schematic representation of documented and implied pathways involved in vasopressin-stimulated arachidonic acid (A.A.) release. Solid lines depict established pathways; dotted lines represent proposed pathways. Agonist-induced activation of the phosphoinositide cycle is thought to be coupled through GTP-binding protein (Gᵣ). Activation of this pathway by vasopressin stimulates release of arachidonic acid. We propose that there is another pathway which, when activated by an agonist such as vasopressin, results in arachidonic acid release. This is a PC-specific phospholipase C (PLC) which may be coupled through an unidentified G protein (Gᵢᵣ). PLA₂, phospholipase A₂; IP₃, inositol 1,4,5-trisphosphate; R, receptor; DGlipase, diacylglycerol lipase.

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approximately 20% (18). In A-10 cells, however, it appears as though this enzyme may be activated to a much greater extent, i.e., 160%.

Our data demonstrate that an IAP substrate (Gᵢ) is not involved in AVP activation of PC-specific phospholipase C, nor is it involved in the AVP-stimulated release of arachidonic acid. Activation of this enzyme in our system may involve another GTP-binding protein similar to that which has been suggested for activation in liver plasma membrane (18). It has been suggested that in some systems such as mast cells (6) and neutrophils (24), an IAP substrate may be involved in various steps in the signal transduction process, since treatment with IAP inhibits arachidonic acid release from these cells. However, in other systems IAP does not abolish the agonist-induced PI response (11, 39). In A-10 cells, prior treatment with IAP did not inhibit AVP-stimulated [¹H]arachidonic acid release, suggesting that this receptor-mediated event may activate phospholipase C by interaction with an as yet unidentified G protein.

The results presented here are summarized in the model depicted in Fig. 5 in which we speculate as to the mechanism of receptor-mediated vasopressin-stimulated arachidonic acid release from smooth muscle cells. We interpret our findings to indicate that such an event may result from activation of a PC-specific phospholipase C in addition to PI-specific phospholipase C and phospholipase A₂ and is independent of an IAP substrate.