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INCREASED FORMATION OF PHOSPHATIDIC ACID INDUCED WITH VASOPRESSIN OR Ca^{2+} IONOPHORE A23187 IN RAT HEPATOCYTES

TADAOMI TAKENAWA,* YOSHIMI HOMMA and YOSHITAKA NAGAI†
Institute of Basic Medical Sciences, University of Tsukuba, Niihari-gun, Ibaraki-ken 305, and
†Institute of Medical Science, University of Tokyo, Shiroganedai, Minato-ku, Tokyo 108, Japan

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Abstract—The effects of vasopressin and Ca^{2+} ionophore A23187 on phospholipid metabolism were investigated in rat hepatocytes. Vasopressin stimulated the incorporation of [^{32}P]P_i into phosphatidic acid within 2 min but then it returned to control level after 10 min. On the other hand, the stimulation of the incorporation of [^{32}P]P_i into phosphatidylinositol continued with incubation times up to 20 min. The Ca^{2+} ionophore A23187 also increased the ^{32}P-labeling in phosphatidic acid, although it had no effect on [^{32}P]P_i incorporation into phosphatidylinositol. Concerning the incorporation of [^{3}H]glycerol, vasopressin did not enhance its incorporation into phosphatidic acid and phosphatidylinositol. The Ca^{2+} ionophore A23187 increased the incorporation into phosphatidic acid without significant effects on that into phosphatidylinositol. In the hepatocytes prelabeled with [^{3}H]arachidonic acid, stimulated degradation of phosphatidylinositol with the addition of vasopressin and resultant formation of phosphatidic acid were observed within 5 min. The transient accumulation of diacylglycerol, the product of phosphatidylinositol hydrolysis, also occurred within 5 min with vasopressin. On the other hand, with the Ca^{2+} inophore A23187, stimulated degradation of triacylglycerol to diacylglycerol and the consequent formation of phosphatidic acid were observed. The Ca^{2+} ionophore A23187 caused a significant release of free [^{3}H]arachidonic acid, although vasopressin had no effect.

A vast number of hormones and neurotransmitters stimulate phosphatidylinositol turnover in various mammalian tissues [1, 2]. The initial reaction in stimulated phosphatidylinositol turnover has been thought to be phosphatidylinositol breakdown to diacylglycerol.

The hepatocyte shows a phosphatidylinositol response to hormones, such as vasopressin, epinephrine and angiotensin [3–5]. These hormones cause receptor-mediated enhancement of phosphatidylinositol turnover (increase of specific radioactivity of phosphatidylinositol labeled with [^{32}P]P_i or *myo*-[^{3}H]inositol in a unit period) by the enhancement of calcium ion influx which results in physiological functions such as activation of glycogen phosphorylase. However, a significant increase in the incorporation of [^{32}P]P_i into phosphatidic acid with vasopressin has not been demonstrated, although it stimulated the incorporation of [^{32}P]P_i into phosphatidylinositol [6]. In addition, the Ca^{2+} ionophore A23187 was found to be effective in activation of glycogen phosphorylase, while a stimulatory effect on phosphatidylinositol turnover was not detected [4, 5].

We demonstrated that vasopressin and Ca^{2+} ionophore A23187 stimulated the synthesis of phosphatidic acid in a different manner and, also, suggested that the transient accumulation of phosphatidic acid induced by vasopressin plays an important role in Ca^{2+} influx.

* Author to whom correspondence should be addressed.

MATERIALS AND METHODS

Chemicals. Collagenase and arginine-vasopressin were obtained from the Sigma Chemical Co., St. Louis, MO. [^{32}P]P_i (carrier free), [^{3}H]arachidonic acid (72.2 Ci/mmole) and [2-^{3}H]glycerol (2.5 Ci/mmole) were from the New England Nuclear Corp., Boston, MA. A23187 was a gift from Eli Lilly & Co., Indianapolis, IN.

Isolation of rat hepatocytes. Hepatocytes were isolated from male rats (200–250 g) by collagenase perfusion as described by Seglen [7].

Measurement of [^{32}*P*]P_i *or* [^{3}*H*]*glycerol incorporation into phospholipids*. Isolated hepatocytes were suspended in low phosphate (1 mM) containing Krebs–Ringer bicarbonate buffer and incubated with 50 μCi/ml of [^{32}P]P_i or 5 μCi/ml of [^{3}H]glycerol at 37° for various periods under gassing with O_2/CO_2 (19:1). After the preincubation, 0.5 ml of the cell suspension (containing 5×10^5 cells) was transferred to siliconized tubes. Tubes were rapidly gassed and incubated with or without vasopressin or Ca^{2+} ionophore A23187 for the indicated time. Reaction was terminated with 3 ml of chloroform–methanol (1:1, v/v). After the addition of 0.5 ml of 1 M KCl, the phases were separated, and the lower phase was evaporated to dryness, dissolved in a small amount of chloroform, and spotted on thin-layer plates (precoated silica gel G plate, Merck). The plates were developed by chloroform–methanol–28% aqueous ammonia (65:35:5, by vol.) for the first dimension and by chloroform–acetone–methanol–acetic acid–water (10:4:2:2:1, by vol.) for the second dimension. Lipids on the plate were visualized by exposing

the plate to iodine vapor. The spots were scraped off, and the radioactivity in each spot was measured.

Measurement of the breakdown of [^{3}H]arachidonic acid-labeled lipids. Hepatocytes suspended in Krebs–Ringer bicarbonate buffer were incubated with 2 μCi/ml of [^{3}H]arachidonic acid at 37° for 1 hr under gassing with O_2/CO_2 (19:1) and then were washed twice with fresh buffer. Washed hepatocytes were suspended again in fresh buffer (5×10^5 cells/0.5 ml), and 0.5 ml of the suspension was transferred to siliconized tubes. Incubation was carried out for the indicated time in the presence or absence of vasopressin or Ca^{2+} ionophore A23187. Phospholipids were separated by two-dimensional thin-layer chromatography as described above. Neutral lipids and fatty acids were separated by one-dimensional thin-layer chromatography (petroleum ether–ethyl ether–acetic acid, 50:50:1, by vol.). Extraction and separation of phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5-diphosphate were carried out according to the method of Jolles *et al.* [8] except that the lipids were separated with chloroform–methanol–4.3 M NH_4OH (90:65:20, by vol.).

Other analytical methods. The method of Lowry *et al.* [9] was used to measure protein concentration. Bovine serum albumin was used as the standard. Phospholipid phosphorus was measured by the method of Rouser *et al.* [10].

RESULTS

Influence of vasopressin and Ca^{2+} ionophore A23187 on the incorporation of [^{32}P]P_i or [^{3}H]glycerol into phospholipids. Incubation of rat hepatocytes for 2 min with vasopressin or Ca^{2+} ionophore A23187 activated glycogen phosphorylase activity. In this case, the concentrations of vasopressin or Ca^{2+} ionophore A23187 which cause the maximum activation were found to be 2 nM and 10 μM respectively. Therefore, these concentrations were used to investigate the effect on phospholipid metabolism.

Vasopressin stimulated [^{32}P]P_i incorporation into phosphatidylinositol (Fig. 1A). A significant effect was observed after 2 min of incubation with vasopressin. On the other hand, Ca^{2+} ionophore A23187 had no significant effect on [^{32}P]P_i incorporation into phosphatidylinositol. These results are consistent with other reports described previously [4, 11]. We found that vasopressin also enhanced [^{32}P]P_i incorporation into phosphatidic acid within 2 min (Fig. 1B). This stimulation reached a maximum at 2 min and leveled off with further incubation. since maximum activation of glycogen phosphorylase with vasopressin was observed at 2–5 min after the addition of vasopressin, the accumulation of phosphatidic acid was considered to have occurred during the same period in which physiological responses took place. Furthermore, Ca^{2+} ionophore A23187 stimulated the labeling of phosphatidic acid very strongly without activating the incorporation of [^{32}P]P_i into phosphatidylinositol.

On the other hand, the incorporation of [^{32}P]P_i into other phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin, lysophosphatidylcholine and lysophosphatidylethanolamine was not enhanced with vasopressin or Ca^{2+} ionophore A23187. Ca^{2+} ionophore A23187 instead inhibited [^{32}P]P_i incorporation into phosphatidylcholine and phosphatidylethanolamine.

Concerning the incorporation of [^{3}H]glycerol into phospholipids, vasopressin did not increase the incorporation into phosphatidylinositol or phospha-

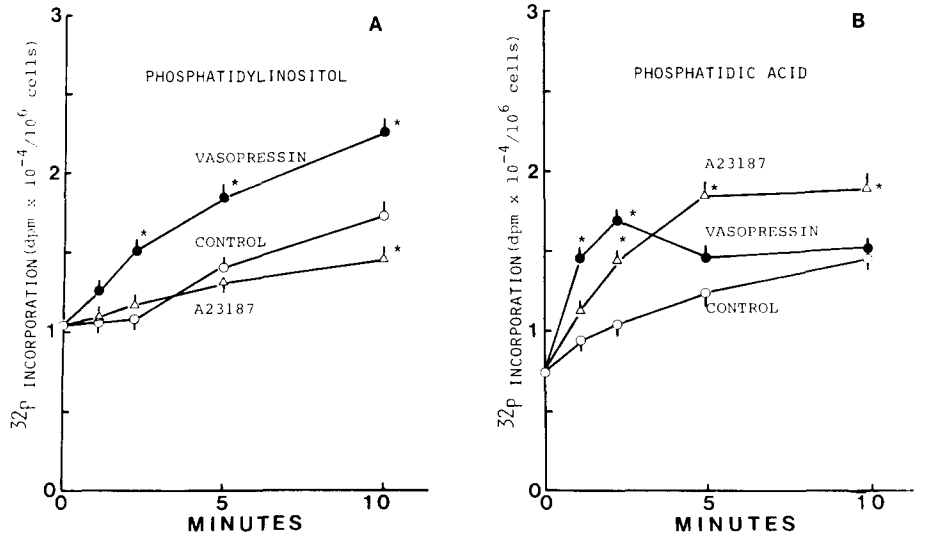

Fig. 1. Effect of vasopressin or Ca^{2+} ionophore A23187 on [^{32}P]P_i incorporation into phosphatidylinositol (A) and phosphatidic acid (B). After hepatocytes were incubated with [^{32}P]P_i (50 μCi/ml) for 20 min at 37°, reaction was started by the addition of vasopressin (2 nM) or Ca^{2+} ionophore A23187 (10 μM). Basal values at zero time for ^{32}P-incorporation into phosphatidylinositol and phosphatidic acid were 27.0 and 17.5 pmoles/10^6 cells respectively. Results are means ±S.E. of triplicate determinations. Key: (*) significantly different from control ($P < 0.05$).

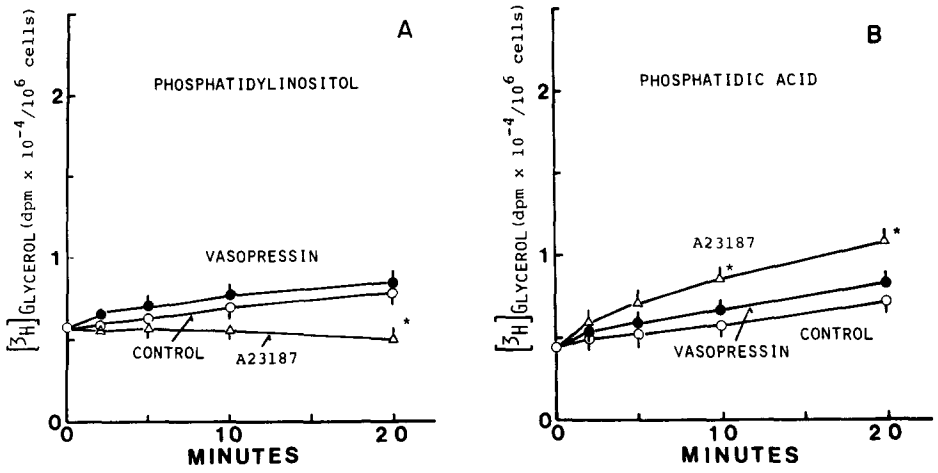

Fig. 2. Effect of vasopressin or Ca^{2+} ionophore A23187 on the incorporation of [^{3}H]glycerol into phosphatidylinositol (A) and phosphatidic acid (B). After hepatocytes were incubated with [^{3}H]glycerol (5 μCi/ml) for 40 min at 37°, reaction was started by the addition of vasopressin (2 nM) of Ca^{2+} ionophore A23187 (10 μM). Results are means ±S.E. of triplicate determinations. Key: (*) significantly different from control ($P < 0.05$).

tidic acid, as shown in Fig. 2. However, Ca^{2+} ionophore A23187 caused the increased incorporation of [^{3}H]glycerol into phosphatidic acid although it rather inhibited the incorporation of [^{3}H]glycerol into phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine.

Influence of vasopressin and Ca^{2+} ionophore A23187 on [^{3}H]arachidonic acid-labeled hepatocytes. After hepatocytes were labeled with [^{3}H]arachidonic acid, the effects of vasopressin and Ca^{2+} ionophore A23187 on the metabolism of arachidonic acid-associated lipids were examined. Vasopressin caused a small but significant breakdown of phosphatidylinositol (Fig. 3A), although the Ca^{2+} ionophore did not enhance the degradation. Concerning phosphatidic acid, significant accumulation with the addition of vasopressin or Ca^{2+} ionophore A23187 was demonstrated (Fig. 3B). In this case, the accumulation of phosphatidic acid with vasopressin was transient. After a 20-min incubation, the stimulated accumulation disappeared. When hepatocytes were treated with Ca^{2+} ionophore A23187, however, the stimulated accumulation continued with the incubation time up to 20 min.

As shown in Fig. 4A, a small but significant increase of [^{3}H]arachidonic acid-labeled diacylglycerol was demonstrated with vasopressin or Ca^{2+} ionophore A23187. When hepatocytes were stimulated with vasopressin, the effect was maximum at 2 min and, thereafter, declined to the control level. On the other hand, [^{3}H]arachidonic acid-labeled triacylglycerol was diminished with Ca^{2+} ionophore but not with vasopressin (Fig. 4B). With regard to the release of free [^{3}H]arachidonic acid, the accumula-

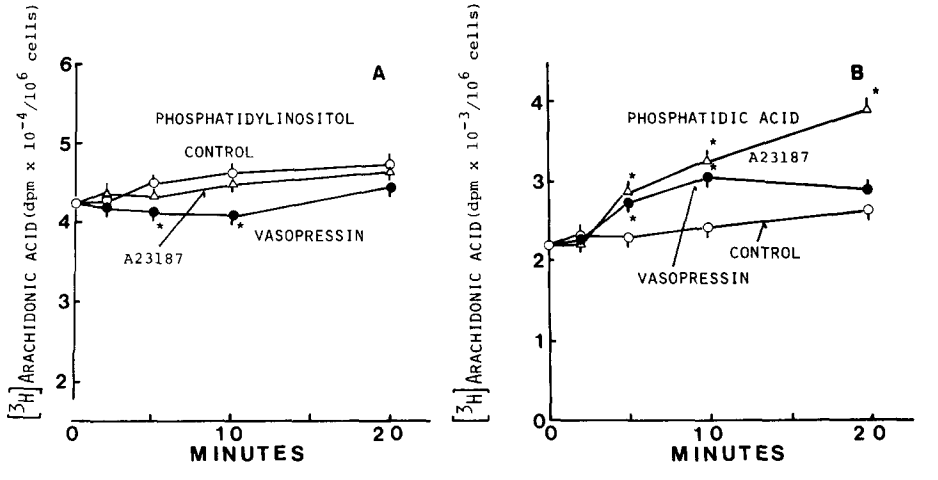

Fig. 3. Effect of vasopressin or Ca^{2+} ionophore A23187 on [^{3}H]arachidonic acid-labeled phosphatidylinositol (A) and phosphatidic acid (B). Hepatocytes prelabeled with [^{3}H]arachidonic acid were incubated with 2 nM vasopressin or 10 μM Ca^{2+} ionophore A23187 for the indicated time at 37°. Results are means ±S.E. of triplicate determinations. Key: (*) significantly different from control ($P < 0.05$).

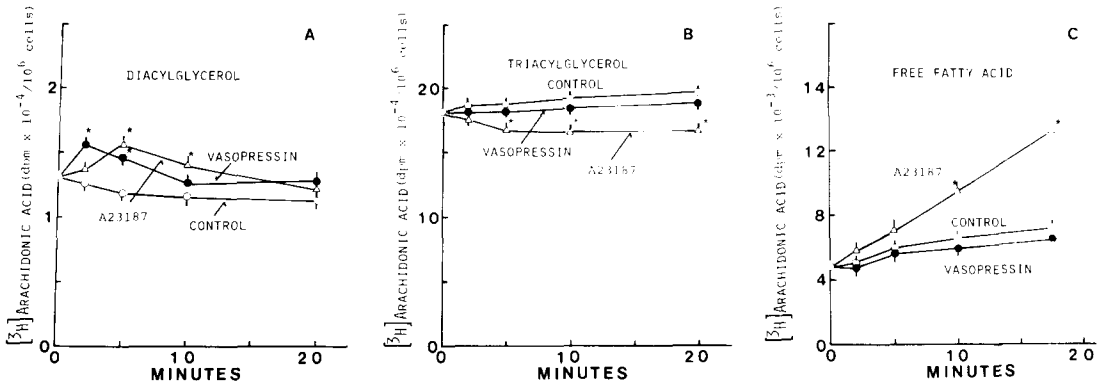

Fig. 4. Effect of vasopressin or Ca^{2+} ionophore A23187 on the production of [^{3}H]arachidonic acid-labeled diacylglycerol (A), triacylglycerol (B) and fatty acid (C). Experimental conditions are the same as described in the legend of Fig. 3. Results are means ±S.E. of triplicate determinations. Key: (*) significantly different from control ($P < 0.05$).

tion of free [^{3}H]arachidonic acid was observed with the stimulation by Ca^{2+} ionophore A23187. However, vasopressin did not enhance the release (Fig. 4C).

The participation of phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5-diphosphate as sources of [^{3}H]arachidonic acid may be neglected because the amount of [^{3}H]arachidonic acid incorporated into them was relatively small.

DISCUSSION

Vasopressin stimulated the incorporation of [^{32}P]P_i into phosphatidylinositol. This stimulation continued with a rising time course over 60 min. Although Ca^{2+} ionophore A23187 did not enhance the incorporation of [^{32}P]P_i into phosphatidylinositol, it stimulated the activity of glycogen phosphorylase as described previously [4, 11]. This finding supports the hypothesis that vasopressin-induced turnover of phosphatidylinositol is not secondary to an elevation of intracellular calcium. We found that vasopressin stimulated the breakdown of phosphatidylinositol and the subsequent accumulation of phosphatidic acid. On the other hand, Ca^{2+} ionophore A23187 stimulated the breakdown of triacylglycerol to diacylglycerol and the subsequent accumulation of phosphatidic acid in addition to the *de novo* synthesis of phosphatidic acid. Thus, it is clear that vasopressin activates the pathway, phosphatidylinositol → diacylglycerol → phosphatidic acid → phosphatidylinositol and that Ca^{2+} ionophore A23187 activates the pathways, *de novo* synthesis of phosphatidic acid from glycerol 3-phosphate and triacylglycerol → diacylglycerol → phosphatidic acid. In this case, vasopressin activated the pathway from phosphatidic acid to phosphatidylinositol. But Ca^{2+} ionophore A23187 did not enhance the conversion of phosphatidic acid to phosphatidylinositol. This difference may have been due to the fact that CDP-diacylglycerol : inositol transferase, which catalyzes the final step of phosphatidylinositol synthesis, is very sensitive to Ca^{2+} [12, 13]. Therefore, Ca^{2+} ionophore A23187 may inhibit the recovery synthesis of phosphatidylinositol through an elevation of Ca^{2+}.

Recently, Tolbert *et al.* [6] have reported that the stimulation of [^{32}P]P_i incorporation into phosphatidic acid by vasopressin is not significant, compared to that of phosphatidylinositol. This discrepancy could be explained by the fact that they measured the incorporation of [^{32}P]P_i into phosphatidic acid at 1 hr after the addition of vasopressin. Under such conditions, the enhanced incorporation of [^{32}P]P_i into phosphatidic acid has already leveled off to the control level, as shown in Fig. 1B. Therefore, it is important to measure the initial reaction 2–5 min after the addition of vasopressin since physiological responses appear at that time. Concerning the effect of vasopressin on the incorporation of the glycerol moiety, Kirk and Michell [14] have reported that vasopressin (23 nM) enhances the incorporation of [^{14}C]glycerol into phosphatidylinositol, although we did not detect increased incorporation by 2 nM vasopressin. This inconsistent result may be due to the different concentrations of vasopressin used.

The diacylglycerol formed from phosphatidylinositol upon stimulation of phospholipase C is an intermediate precursor of phosphatidic acid, which is formed by diacylglycerol kinase. Usually, newly formed diacylglycerol is primarily converted very rapidly to phosphatidic acid. Therefore, the accumulation of diacylglycerol is not as significant as that of phosphatidic acid. Alternatively, under certain conditions, such as stimulation with Ca^{2+} ionophore A23187 diacylglycerol which is formed by the activation of triacylglycerol lipase is degraded to monoacylglycerol and fatty acid by diacylglycerol lipase besides the formation of phosphatidic acid by diacylglycerol kinase. Participation of phospholipase A_2 in the production of free fatty acid may be neglected since the formation of lysophosphatidylcholine and lysophosphatidylethanolamine was not significant with Ca^{2+} ionophore.

It has been postulated that the enhancement of turnover of phosphatidylinositol is related to Ca^{2+} influx and that the resultant increase of Ca^{2+} causes physiological reactions to stimuli. But it is unclear how the stimulated breakdown is associated with Ca^{2+} influx. Recently, it has been found that phosphatidic acid behaves as a Ca^{2+} ionophore [15–18]. If that is the case, it is possible that a transient increase in phosphatidic acid in cellular membranes

increases the Ca^{2+} permeability of those membranes for short periods.

REFERENCES

1. R. H. Michell, *Biochim. biophys. Acta* **415**, 81 (1975).
2. R. H. Michell, *Trends biochem. Sci.* **4**, 128 (1979).
3. C. J. Kirk, T. R. Verrinder and D. A. Hems, *Fedn Eur. Biochem. Soc. Lett.* **83**, 267 (1977).
4. M. M. Billah and R. H. Michell, *Biochem. Soc. Trans.* **6**, 1033 (1979).
5. C. J. Kirk, L. M. Rodrigues and D. A. Hems, *Biochem. J.* **178**, 493 (1979).
6. M. E. M. Tolbert, A. C. White, K. Aspry, J. Cuttus and J. N. Fain, *J. biol. Chem.* **255**, 1938 (1980).
7. P. O. Seglen, *Meth. Cell Biol.* **13**, 29 (1976).
8. J. Jolles, L. H. Schrama and W. H. Gispen, *Biochim. biophys. Acta* **666**, 90 (1981).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
10. G. Rouser, S. Fleischer and A. Yamamoto, *Lipids* **5**, 494 (1970).
11. M. M. Billah and R. H. Michell, *Biochem. J.* **182**, 661 (1979).
12. K. Egawa, B. Sacktor and T. Takenawa, *Biochem. J.* **194**, 129 (1981).
13. K. Egawa, T. Takenawa and B. Sacktor, *Molec. cell. Endocr.* **21**, 29 (1981).
14. C. J. Kirk and R. H. Michell, *Biochem. J.* **194**, 155 (1981).
15. C. A. Tyson, H. V. Zande and D. E. Green, *J. biol. Chem.* **251**, 1326 (1976).
16. D. H. Salmon and T. W. Honeyman, *Nature, Lond.* **284**, 344 (1980).
17. G. J. Barrit, K. A. Dalton and J. A. Whiting, *Fedn Eur. Biochem. Soc. Lett.* **125**, 137 (1981).
18. C. Serhan, A. Anderson, E. Goodman, P. Dunham and G. Weissman, *J. biol. Chem.* **256**, 2736 (1981).