Phosphatidic acid mimicks the muscarinic action of acetylcholine in cultured bovine chromaffin cells

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In cultured bovine chromaffin cells, acetylcholine as well as muscarine stimulated the $^{32}P_i$ incorporation into phosphatidic acid, induced the efflux of $^{45}Ca^{2+}$ from prelabelled cells, and, in parallel, elevated intracellular cyclic GMP content. Phosphatidic acid added to the medium also stimulated the efflux of $^{45}Ca^{2+}$ and the synthesis of cyclic GMP in the cells in the same fashion as muscarinic agents, whereas it did not induce the secretion of catecholamines indicating that the effect of phosphatidic acid is specific to muscarinic action. The result supports the hypothesis that phosphatidic acid produced during phosphatidylinositol turnover is linked to the regulation mechanism of Ca^{2+} mobilization and cyclic GMP synthesis by muscarinic stimulation.

Phosphatidic acid *Ca^{2+} mobilization* *Cyclic GMP* *Acetylcholine* *Muscarinic receptor* *Chromaffin cell*

1. INTRODUCTION

It has been demonstrated that acetylcholine increases the intracellular cyclic GMP content [1,2] and also stimulates the turnover of phosphatidylinositol [3] by acting on a muscarinic receptor in a variety of tissues. However, the relationship between the synthesis of cyclic GMP and the turnover of phosphatidylinositol has not been elucidated. Recently we have shown that exogenous as well as endogenous phosphatidic acid, an intermediate produced during phosphatidylinositol turnover, stimulated the uptake of $^{45}Ca^{2+}$ and elevated the intracellular cyclic GMP content in neuroblastoma N1E 115 cells [4]. On the other hand, it has been reported [5] that cholinergic agents stimulated the efflux of $^{45}Ca^{2+}$ and increased the intracellular cyclic GMP content in pancreatic acinar cells. It is of essential importance to study which of the influx or efflux of Ca^{2+} is correlated with the increase of cyclic GMP content, and whether or not the effect of phosphatidic acid on Ca^{2+} mobilization and cyclic GMP synthesis is universal to a number of cells including normal tissues. Bovine chromaffin cells would offer a good model for the study of this issue, because they respond to both muscarinic and nicotinic stimulation of acetylcholine, by either the elevation of cyclic GMP [6,7] or the secretion of catecholamines [8], respectively. Here, we report that in cultured bovine chromaffin cells, exogenous phosphatidic acid stimulates the efflux of prelabelled $^{45}Ca^{2+}$ and the synthesis of cylic GMP in the same fashion as muscarinic agents.

Abbreviations: DMEM, Dulbecco-Vogt modified Eagle's minimal essential medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ACh, acetylcholine; PA, phosphatidic acid; CA, catecholamine

2. MATERIALS AND METHODS

2.1. *Culture of bovine chromaffin cells*

Fresh bovine adrenal glands were obtained from local slaughterhouse and chromaffin cells were

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isolated by treatment with 0.05% collagenase as in [9]. The dispersed cells were suspended in DMEM supplemented with 7% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, 20 μg/ml 5-fluorodeoxyuridine, and 50 μg/ml uridine. The cells were inoculated into 35-mm Nunc plastic dish or Nunc 24 multiwell plate (18 mm) at a concentration of $5-10 \times 10^5$ cells/dish or $1-2 \times 10^5$/well in 1.5 or 0.5 ml of culture medium, respectively. The cells were cultured in a humidified atmosphere of 7% CO_2 and 93% air at 37°C. Cells were used for experiment after 3–9 days of culture.

2.2. *Measurement of $^{32}P_i$ incorporation into phosphatidic acid*

Cells were washed with phosphate-free HEPES-buffered Krebs–Ringer solution (pH 7.3) containing 2.2 mM $CaCl_2$ and 0.2% bovine serum albumin (HEPES buffer). After the cells were preincubated with the phosphate-free HEPES buffer containing 5 μCi of $^{32}P_i$ at 37°C for 1 h, acetylcholine was added to the medium at a final concentration of 0.1 mM. After the incubation periods indicated, the medium was removed by aspiration and 1 ml of 10% trichloroacetic acid was added to terminate the reaction, followed by centrifugation at 1500 × *g* for 15 min. Phospholipids were extracted from the precipitate with 3 ml of chloroform/methanol (2:1). The amounts of $^{32}P_i$ incorporated into phosphatidic acid was measured on thin-layer chromatography in the upper phase of ethyl acetate/*iso*-octane/acetic acid/water (90:50:20:100) as in [4].

2.3. *Measurement of $^{45}Ca^{2+}$ efflux*

Cells were preincubated with 1 ml of DMEM supplemented with 7% fetal bovine serum and 2 μCi of $^{45}CaCl_2$ overnight. After the cells were rapidly washed 3-times with 2 ml of HEPES buffer, 1 ml of HEPES buffer containing either acetylcholine or phosphatidic acid was added to the cells. After the incubation time indicated, the medium was transferred into a test tube in ice and centrifuged at 1500 × *g* for 15 min. The radioactivity in the supernatant was determined by a liquid scintillation counter.

2.4. *Measurement of $^{45}Ca^{2+}$ uptake*

The uptake of $^{45}CaCl_2$ by cultured chromaffin cells was measured as in [4]. In brief, after cells were preincubated in 0.9 ml HEPES buffer at 37°C for 30 min, the reaction was started by the addition of 0.1 ml HEPES buffer containing 2 μCi $^{45}CaCl_2$ and the agents to be tested. One min later, the medium was removed by aspiration and the cells were rapidly washed 3-times with 2 ml of HEPES buffer containing 0.1 mM $LaCl_3$. The washed cells were suspended in 0.1% deoxycholate and the radioactivity was determined.

2.5. *Determination of catecholamine secretion*

Secretion of catecholamines from cultured chromaffin cells was determined by a slight modification of the method in [10]. Cells cultured in multiwell plate were preincubated with 1 ml of HEPES buffer at 37°C for 30 min. After the medium was removed, 1 ml of HEPES buffer containing the agent to be tested was added to the well and incubation was continued for 5 min at 37°C when the medium was removed and chilled in ice. The cells were detached from dish, homogenized with 1 ml of 0.4 N perchloric acid and centrifuged at 1500 × *g* for 10 min. The supernatant was neutralized with 2 N KOH. Catecholamines (norepinephrine and epinephrine) in the medium and in the cells were assayed as in [11] by use of Amberite CG-50 Na^+ column (0.4 × 2.5 cm). The results are expressed as percent of total catecholamines.

2.6. *Determination of cyclic GMP content*

Cyclic GMP content in the cells was determined by the radioimmunoassay using Yamasa assay kit as in [12]. Protein was determined as in [13].

3. RESULTS AND DISCUSSION

When cultured chromaffin cells were incubated with 0.1 mM acetylcholine, the incorporation of $^{32}P_i$ into phosphatidic acid was rapidly increased (at 15 s, P 0.05) reaching a maximum level in 1 min (fig.1A). This reaction was faster than that in [14]. In parallel with the labelling of phosphatidic acid, $^{45}Ca^{2+}$ efflux from prelabelled cells and intracellular cyclic GMP content increased 2- and 7-fold, respectively (fig.1B,1C). Muscarine also stimulated these reactions in the same manner (not shown) indicating that the labelling of phosphatidic acid, $^{45}Ca^{2+}$ efflux and

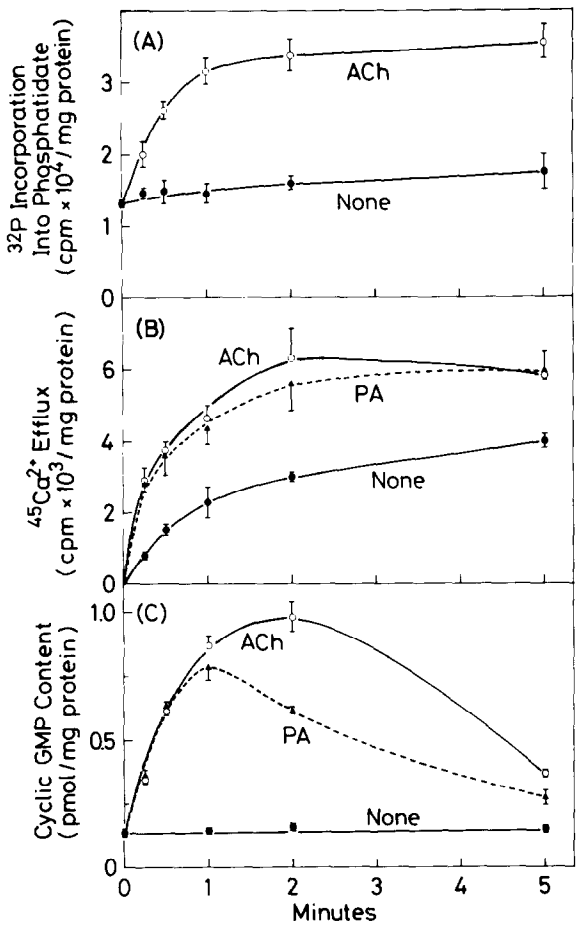

Fig.1. Effect of acetylcholine and phosphatidic acid on the $^{32}P_i$ incorporation into phosphatidic acid (A), efflux of $^{45}Ca^{2+}$ (B), and cyclic GMP content (C), in cultured bovine chromaffin cells. Cells were incubated for the time indicated in the absence (●), or in the presence of 0.1 mM acetylcholine (○) or 100 μg/ml of phosphatidic acid (▲). Points and bars indicate the means and S.E. of 4 or 5 samples.

cyclic GMP synthesis were induced by the stimulation of muscarinic receptor. The increase of cyclic GMP content by acetylcholine was abolished by removing Ca^{2+} from the medium, whereas the $^{32}P_i$ labelling of phosphatidic acid by acetylcholine was observed in the absence as well as in the presence of extracellular Ca^{2+} (not shown).

The addition of phosphatidic acid to the medium stimulated the efflux of $^{45}Ca^{2+}$ from prelabelled chromaffin cells and increased the synthesis of cyclic GMP in the cells in the same fashion as acetylcholine (fig.1B,1C). In the absence of extracellular Ca^{2+}, phosphatidic acid failed to elevate the intracellular cyclic GMP content (not shown) indicating that the presence and the mobilization of Ca^{2+} are required for the synthesis of cyclic GMP induced by phosphatidic acid as well as by acetylcholine. On the other hand, the addition of cyclic GMP or dibutyryl cyclic GMP to the medium stimulated neither the $^{32}P_i$ incorporation into phosphatidic acid nor the efflux of $^{45}Ca^{2+}$ from the cells (not shown), thus suggesting that the elevation of intracellular cyclic GMP is not a causal event to, but presumably a consequence of phosphatidic acid turnover and Ca^{2+} mobilization.

To study whether phosphatidic acid also mimicks the nicotinic action of acetylcholine on chromaffin cells, the uptake of $^{45}Ca^{2+}$ and the secretion of catecholamines from the cells were measured (fig.2). Acetylcholine (0.1 mM) and nicotine (0.01 mM) stimulated the uptake of $^{45}Ca^{2+}$ and the secretion of catecholamines 10- and 12-fold over the unstimulated cells, respectively. In contrast, muscarine (0.1 mM) induced neither $^{45}Ca^{2+}$ uptake nor catecholamine secretion from the cells. Phosphatidic acid exogenously added to the medium failed to stimulate catecholamine

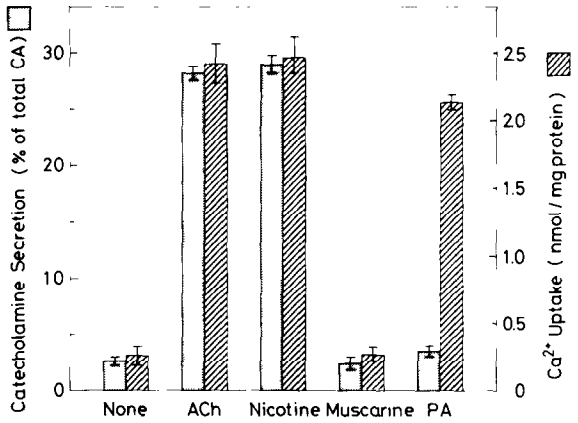

Fig.2. Effect of transmitters and phosphatidic acid on the uptake of $^{45}Ca^{2+}$ and the secretion of catecholamines. Cells were incubated with either acetylcholine (0.1 mM), nicotine (0.01 mM), muscarine (0.1 mM) or phosphatidic acid (100 μg/ml). Incubation was carried out for 1 and 5 min for the uptake of $^{45}Ca^{2+}$ and catecholamine secretion, respectively. Values represent the means of 4–6 samples and vertical bars indicate S.E.

secretion, although an apparent uptake of $^{45}Ca^{2+}$ into chromaffin cells was markedly enhanced.

This study shows that acetylcholine and muscarine stimulate the $^{32}P_i$ incorporation into phosphatidic acid, the efflux of $^{45}Ca^{2+}$, and the synthesis of cyclic GMP in cultured bovine chromaffin cells. Our observation is consistent with other reports [3,14–17] that acetylcholine stimulated the turnover of phosphatidylinositol in a variety of tissues via muscarinic receptor, and the reports [5,15,16] that muscarinic agents stimulated the efflux of $^{45}Ca^{2+}$ and increased cyclic GMP levels in pancreatic acinar cells, pituitary glands and chromaffin cells. We further demonstrate that phosphatidic acid exogenously added to cultured chromaffin cells mimicks the effect of acetylcholine in that it induces the efflux of $^{45}Ca^{2+}$ from prelabelled cells and elevates the intracellular cyclic GMP content, thus extending our recent finding on cloned neuroblastoma N1E 115 cells [4]. We have recently observed that the efflux of preloaded $^{45}Ca^{2+}$ from the neuroblastoma cells was stimulated by exogenous phosphatidic acid, acetylcholine, histamine and prostaglandin E_1 that elevate the intracellular cyclic GMP content in the cells, although an apparent uptake of $^{45}CaCl_2$ was not stimulated by the transmitters (Ohsako and Deguchi, unpublished). Taking these observations together, it is indicated that the efflux, rather than the influx of $^{45}Ca^{2+}$ is intimately correlated with the increased synthesis of cyclic GMP in tissues. In this and in previous reports, we observed that exogenous phosphatidic acid stimulated the apparent influx of $^{45}Ca^{2+}$ into neuroblastoma cells [4] and chromaffin cells. It could be that Ca^{2+}-phosphatidic acid is simply adsorbed on cell surface or that Ca^{2+} uptake induced by phosphatidic acid is mediated by the process which is not linked to catecholamine secretion.

It has been shown in many tissues that the elevation of intracellular cyclic GMP in response to neurotransmitters or hormones was dependent on extracellular Ca^{2+} [1,2], although the role of Ca^{2+} in the activation of guanylate cyclase has remained obscure. The omission of extracellular Ca^{2+} might deplete the intracellular Ca^{2+} pool needed for the cyclic GMP synthesis. Alternatively, Ca^{2+} attached to cell surface is partly taken up by the cells and in part liberated from the cells by stimulation of muscarinic receptor, thus resulting in an apparent efflux of prelabelled $^{45}Ca^{2+}$. Further evidence is required to elucidate the mechanism of Ca^{2+} mobilization and its involvement in cyclic GMP synthesis in response to muscarinic stimulation.

Recently much attention has been devoted to the involvement of membrane phospholipids in Ca^{2+} mobilization. These include phosphatidic acid [18–24], lysophosphatidic acid [24,25] and methylation of phosphatidylethanolamine [26]. Phosphatidic acid has been shown to mediate the translocation of Ca^{2+} across an artificial membrane [27,28] and to induce contraction of smooth muscle [18] and other biological responses [19–23]. This study together with our recent report [4], indicates that the action of muscarinic agents could be mediated by phosphatidic acid in membrane which induces Ca^{2+} mobilization, reflected on the efflux of preloaded $^{45}Ca^{2+}$, and as a consequence, enhances the synthesis of cyclic GMP in tissues.

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REFERENCES

[1] Goldberg, N.D. and Haddox, M.K. (1977) Annu. Rev. Biochem. 46, 823–896.
[2] Rasmussen, H. and Goodman, D.B.P. (1977) Physiol. Rev. 57, 421–509.
[3] Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81–147.
[4] Ohsako, S. and Deguchi, T. (1981) J. Biol. Chem. 256, 10945–10948.
[5] Christophe, J.P., Fransen, E.K., Conlon, T.P., Krishna, G. and Gardner, J.D. (1976) J. Biol. Chem. 251, 4640–4645.
[6] Schneider, A.S., Cline, H.T. and Lemaire, S.(1979) Life Sci. 24, 1389–1394.
[7] Yanagihara, N., Isosaki, M., Ohuchi, T. and Oka, M. (1979) FEBS Lett. 105, 296–298.
[8] Schneider, A.S., Herz, R. and Rosenheck, K. (1977) Proc. Natl. Acad. Sci. USA 74, 5036–5040.

[9] Waymire, J.C., Waymire, K.G., Boehme, R., Noritake, D. and Wardell, J. (1977) in: Structure and Function of Monoamine Enzymes (Usdin, E. et al. eds) pp. 327–363, Marcel Dekker, New York.
[10] Wilson, S.P. and Viveros, O.H. (1981) Exp. Cell Res. 133, 159–169.
[11] Barchas, J., Erdelyi, E. and Angwin, P. (1972) Anal. Biochem. 50, 1–17.
[12] Deguchi, T., Saito, M. and Kono, M. (1978) Biochim. Biophys. Acta 554, 8–19.
[13] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[14] Fisher, S.K., Holz, R.W. and Agranoff, B.W. (1981) J. Neurochem. 37, 491–497.
[15] Young, P.W., Bicknell, R.J. and Schfield, J.G. (1979) J. Endocrinol. 80, 203–213.
[16] Oka, M., Isosaki, M. and Watanabe, J. (1983) in: Synthesis, Storage and Secretion of Adrenal Catecholamine: Dynamic Integration of Functions (Izumi, F. et al. eds) Pergamon, New York, in press.
[17] Azila, N. and Hawthorne, J.N. (1982) Biochem. J. 204, 291–299.
[18] Salmon, D.M. and Honeyman, T.W. (1980) Nature 284, 344–345.
[19] Putney, J.W., Jr., Weiss, S.J., Van De Walle, C.M. and Haddas, D.A. (1980) Nature 284, 345–347.
[20] Gerrard, J.M., Peterson, D.A. and White, J.G. (1978) Prostaglandins Med. 1, 387–396.
[21] Limas, C.J. (1980) Biochem. Biophys. Res. Commun. 95, 541–546.
[22] Clark, R.B., Salmon, D.M. and Honeyman, T.W. (1980) J. Cyclic Nucleotide Res. 6, 37–49.
[23] Barritt, G.J., Dalton, K.A. and Whiting, J.A. (1981) FEBS Lett. 125, 137–140.
[24] Lapetina, E.G., Billah, M.M. and Cuatrecasas, P. (1981) Nature 292, 367–369.
[25] Gerrard, J.M., Kindom, S.E., Peterson, D.A., Peller, J., Krantz, K.E. and White, J.G. (1979) Am. J. Pathol. 96, 423–438.
[26] Hirata, F. and Axelrod, J. (1980) Science 209, 1082–1090.
[27] Tyson, C.A., Vande Zande, H. and Green, D.E. (1976) J. Biol. Chem. 251, 1326–1332.
[28] Serhan, C., Anderson, P., Goodman, E., Dunham, P. and Weissman, G. (1981) J. Biol. Chem. 256, 2736–2741.