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INVOLVEMENT OF TYPE 2C PHOSPHATASE IN THE DEPHOSPHORYLATION OF 26 kDa PHOSPHOPROTEIN IN RAT PAROTID ACINAR CELLS

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SUMMARY: Posphorylation of three particulate proteins with molecular masses of 34, 26, and 22 kDa was stimulated in the presence of cyclic AMP / 3-isobutyl-1-methylxanthine in saponin-permeabilized rat parotid acinar cells. When the particulate fraction isolated from the cells labeled with [γ-^{32}P]ATP was incubated at 30°C, dephosphorylation of the 26 kDa phosphoprotein occurred in the presence of Mg^{2+} or Mn^{2+}. Okadaic acid had no effect on the Mg^{2+}-dependent dephosphorylation of the 26 kDa phosphoprotein. Addition of the recombinant type 2C phosphatase, Mg^{2+}-dependent and okadaic acid-insensitive phosphatase, caused a remarkable dephosphorylation of the 26 kDa phosphoprotein. These observations strongly suggest type 2C phosphatase is involved in the dephosphorylation of the 26 kDa phosphoprotein.

In the rat parotid gland, stimulation of the β-adrenergic receptors evokes amylase secretion through the elevation of the intracellular cyclic AMP and cyclic AMP-dependent phosphorylations (1-4). The β-adrenergic receptor stimulation in the rat parotid gland is accompanied by a specific phosphorylation of three particulate proteins of molecular masses of 35, 26, and 22 kDa (3, 4). It appears that these protein phosphorylation/dephosphorylation reactions may be the molecular mechanism involved in regulating exocytosis from the rat parotid gland via a cyclic AMP-mediated process (3, 5-7). While the 35 kDa protein has been identified as the ribosomal protein

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Abbreviations: IBMX, 3-isobutyl-1-methylxanthine; PMSF, phenylmethylsulfonyl fluoride; CaM, calmodulin.

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S6 (8, 9), the other two proteins, 26 kDa and 22 kDa proteins, have not yet been identified. The 26 kDa protein is an integral membrane protein (10, 11) and the 22 kDa protein is located in the rough endoplasmic reticulum (12). Quissell et al. have suggested the 26 kDa protein may be involved directly in exocytosis, since the rapid phosphate turnover of the 26 kDa protein coincided closely with a change of cellular cyclic AMP and cyclic AMP-dependent protein kinase activity during β-adrenergic receptor stimulation (10, 13). If so, the phosphatase which dephosphorylates the 26 kDa phosphoprotein may play an important role in amylase secretion in the cells.

The serine/threonine protein phosphatases have been classified to four types, type 1, type 2A, type 2B and type 2C, based on their sensitivity to heat stable inhibitory proteins and the metal ion requirement (14, 15). It is well known type 2C phosphatase requires Mg^{2+} for full activity (16) and is insensitive to okadaic acid (17). The type 2C phosphatase is also activated by Mn^{2+} (18). In the parotid gland, only a few phosphatase studies have been reported (19, 20). Phosphatases involved in the specific phosphoproteins, which are phosphorylated via the cyclic AMP-mediated process, have not yet been identified.

Studies were done to identify the phosphatase which is involved in the dephosphorylation of 26 kDa phosphoprotein. The results in this paper show that type 2C phosphatase is involved in the dephosporylation of the 26 kDa phosphoprotein in the rat parotid gland.

MATERIALS AND METHODS

Materials: [γ-^{32}P]ATP (6000 Ci/mmol) was obtained from Du Pont-New England Nuclear Corp. Trypsin, trypsin inhibitor and cyclic AMP were from Sigma. Collagenase was from Boehringer Mannheim GmbH (Germany). Okadaic acid was from Wako Chemicals (Osaka, Japan). A cDNA containing the entire coding sequence of rat type 2C phosphatase was expressed in E. *coli* as described previously (21). The recombinant type 2C phosphatase was partially purified by following steps, $(NH_4)_2SO_4$ participation, DEAE-cellulose column and Sephadex G-100 gel filtration (22). Specific activity of the enzyme prepared was 21 uints/mg protein with phosphohistone as a substrate. This enzyme did not have any other phosphatase activity.

Preparation of permeabilized acinar cells: To disperse acinar cells, rat parotid glands were minced and digested with trypsin and collagenase as described previously (23). The dispersed cells were suspended in 25mM Hepes (pH 7.2), 100mM KCl, 20 mM NaCl, 5 mM $MgCl_2$, 0.96 mM NaH_2PO_4, 0.435 mM $CaCl_2$ (free [Ca^{2+}], 140 nM), 1mM EGTA, 11.1mM glucose, 2 % bovine serum albumin and 0.01% soybean trypsin inhibitor, and incubated with saponin (20 μg /ml) at 37°C for 5min to prepare the permeabilized acinar cells.

Preparation of the labeled particulate fractions: The phosphorylation was carried out at 37°C for 20 min in a final volume of 1 ml. The permeabilized acinar cells (5×10^7 / ml) were stimulated with [γ-^{32}P]ATP (0.2 mCi/ml) in the presence of 1 mM cyclic AMP and 0.1 mM 3-isobutyl-1-methylxanthine, IBMX. After the reaction mixture was removed, the cells were homogenized with buffer A (20mM Hepes-Tris buffer (pH 7.2), 2mM EDTA, 0.1mM EGTA, 0.3M sucrose, and 1mM PMSF) in a Potter-Elvehjem type homogenizer with a Teflon pestle. The homogenate was centrifuged at 750 x g for 10 min. The supernatant was centrifuged at 100,000 x g for 60 min. The resulting pellet was suspended in buffer A and used as the particulate fraction.

Dephosphorylation of phosphoproteins: The dephosphorylation of the phosphoproteins was measured by mixing 5 µl of the particulate fraction diluted in 20 µl of 50 mM Tris-HCl (pH 7.0). The reaction mixture was incubated for 0-15 min at 30°C with or without other indicated additions. The reaction was stopped by the addition of SDS-sample buffer. After samples were boiled for 5 min, the proteins were analyzed on 15% SDS-PAGE gels using the method of Laemmli (24). Autoradiograms were analyzed by an imazing analyzer (Fuji BAS 2000, Tokyo, Japan).

RESULTS AND DISCUSSION

Effect of divalent metal ions on the dephosphorylation of 26 kDa phosphoprotein

In permeabilized acinar cells of the rat parotid gland, labeling of three particulate proteins, molecular masses of 34, 26, and 22 kDa, with [γ-^{32}P]- ATP was stimulated in the presence of cyclic AMP and IBMX. Namely these proteins are endogeneous substrates for the cyclic AMP-dependent protein kinase. Three proteins were indicated with arrows in Fig. 1. These proteins correspond to the 35, 26, and 22 kDa proteins reported by Jahn et al., respectively (3). During incubation of the particulate phosphoproteins, the dephosphorylation of 34 kDa phosphoprotein occurred, whereas the dephosphorylation of the 26 kDa and 22 kDa phosphoproteins was not detected (Fig. 1).

To examine the dephosphorylating condition of the 26 kDa phosphoprotein, the effect of metal ions was examined. 0.1 mM EGTA or 1 mM EDTA did not effect on the dephosphorylation of this protein (Fig. 1). However, 10 mM Mg^{2+} in the presence of 0.1 mM EGTA markedly stimulated the dephosphorylation process (Fig. 1 and Fig.

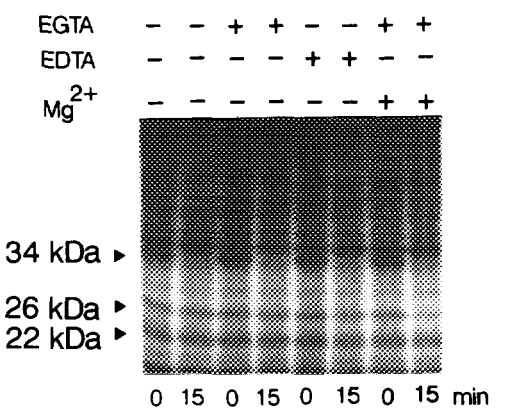

Fig. 1. Effect of divalent metal ions on the dephosphorylation of 26 kDa phosphoprotein.

The permeabilized acinar cells were labeled with [γ-^{32}P]ATP in the presence of 1 mM cyclic AMP and 100 µM IBMX at 37°C for 20 min. The labeled particulate fractions were obtained as described in the " Materials and Methods ". Final concentrations of additions used were 0.1 mM EGTA, 1 mM EDTA and 10 mM $MgCl_2$. Incubation times were indicated at the bottom of a figure. Zero time was just prior to further incubation. Proteins were analyzed by SDS-PAGE and autoradiograms of the gel was presented.

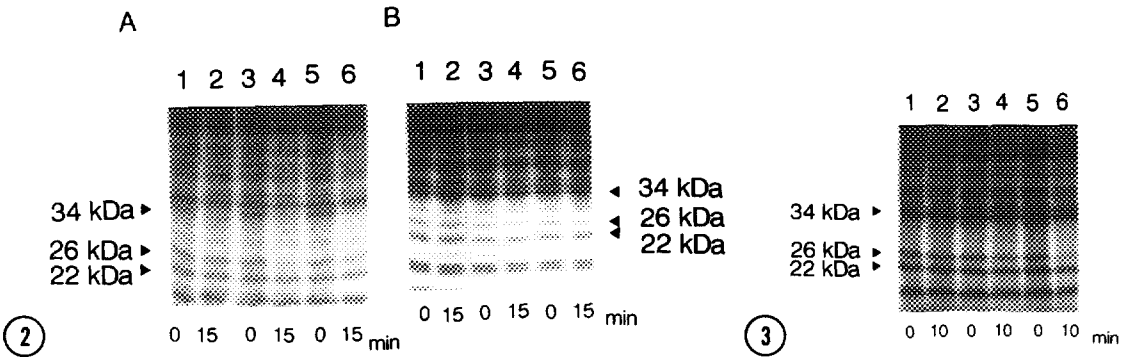

Fig. 2. Effect of metal ion and okadaic acid on the dephoshorylation of 26 kDa phosphoprotein.
The particulate fractions were obtained as described in the " Materials and Methods ". The labeled particulate fractions were further incubated in the absence (A) or presence (B) of 2.5 μM okadaic acid. (A) Lanes 1 and 2, control; lanes 3 and 4, 10 mM $MgCl_2$ plus 0.1 mM EGTA; lanes 5 and 6, 10 mM $MgCl_2$, 0.1 mM EGTA plus 20 mM NaF. (B) Lanes 1 and 2, control; lanes 3 and 4, 10 mM $MgCl_2$ plus 0.1 mM EGTA; lanes 5 and 6, 2 mM $MnCl_2$. Incubation times were indicated at the bottom of figures. Zero time was just prior to further incubation.

Fig. 3. Effect of the recombinant type 2C phosphatase on the dephosphorylation of 26 kDa phosphoprotein.
The labeled particulate proteins were obtained as described in the " Materials and Methods " and for this experiment the particulate protein fraction was washed several times with buffer A. The dephosphorylation of particulate 26 kDa phosphoprotein was done in the presence of 2.5 μM okadaic acid. Lanes 1 and 2, control; lanes 3 and 4, 10 mM $MgCl_2$; lanes 5 and 6, 10 mM $MgCl_2$ plus recombinant type 2C phosphatase (2.5 μg). Incubation times were indicated at the bottom of a figure. Zero time was just prior to further incubation.

2A, lanes 3 and 4). These data showed Mg^{2+} is required for the dephosphorylation of the 26 kDa phosphoprotein. In contrast to the 26 kDa phosphoprotein, 34 kDa phosphoprotein was dephosphorylated in any conditions. NaF, an inhibitor of serine/threonine phosphatases, showed less inhibition on the dephosphorylation of the 26 kDa phosphoprotein (Fig. 2A, lanes 5 and 6).

Another divalent metal ion, 2 mM Mn^{2+}, also stimulated the dephosphorylation of this protein (Fig. 2B, lanes 5 and 6). From these results this dephosphorylation is mediated by a Mg^{2+} or Mn^{2+}-dependent phosphatase. Furthermore, bovine brain CaM-stimulated phosphatase, type 2B phosphatase, is not involved in the dephosphorylation of the 26 kDa phosphoprotein, since the dephosphorylation of this protein could not observed by the addition of type 2B phosphatase with either Ca^{2+}/CaM or Ni^{2+}/CaM (data not shown).

No effect of okadaic acid on the dephosphorylation of 26kDa phosphoprotein

Okadaic acid, a potent specific inhibitor of type 1 and type 2A phosphatases, is a valuable tool with which to test the involvement of phosphatases in the cells. So, we examined the effect of okadaic acid on the dephosphorylation of the 26 kDa phosphoprotein. As shown in Fig. 2B, lanes 3 and 4, 2.5 μM okadaic acid did not

inhibit the Mg^{2+}-stimulated dephosphorylation of the 26 kDa phosphoprotein. Even a higher concentration of okadaic acid, 5 μM, did not effect on the dephosphorylation of the 26 kDa phosphoprotein (data not shown). Since 5 μM okadaic acid should inhibit type 1 and type 2A phosphatases completely (25), these phosphatases are not involved in the dephosphorylation of the 26 kDa phosphoprotein. These data suggest that okadaic acid-insensitive protein phosphatase is involved in the dephosphorylation of the 26 kDa phosphoprotein.

Based on these observations, the phosphatase which is involved in the dephosphorylation of the 26 kDa phosphoprotein is supposed to be type 2C phosphatase, since this phosphatase showed Mg^{2+}- or Mn^{2+}-requirement and okadaic acid-insensitivity.

Effect of recombinant type 2C phosphatase on the dephosphorylation of 26 kDa phosphoprotein

To further test the involvement of type 2C phosphatase in the dephosphorylation of 26 kDa phospoprotein, we studied the effect of recombinant type 2C phosphatase on this dephosphorylation reaction. Unpublished results suggested the exsistence of type 2C phosphatase activity in the cytosol fraction of the rat parotid acinar cells. So to display clearly the effect of exogeneous type 2C phosphatase on the dephosphorylation of 26 kDa phosphoprotein, the labeled particulate fraction was washed several times with a homogenizing buffer to remove the supernatant contamination. The results were shown in Fig. 3. Using densitometric analysis of the 26 kDa phosphoprotein, the addition of 10 mM Mg^{2+} caused 40% dephosphorylation of this protein (Fig. 3, lanes 3 and 4). Further addition of recombinant type 2C phosphatase showed 85% dephosphorylation of the 26 kDa phosphoprotein (Fig. 3, lanes 5 and 6). The recombinant type 2C phosphatase showed less effect on the dephosphorylation of 34 kDa and 22 kDa phosphoproteins. These results suggest the 26 kDa phosphoprotein is dephosphorylated by type 2C phosphatase.

The observation that the addition of Mg^{2+} in the washed particulate fraction caused the dephosphorylation of the 26 kDa phosphoprotein may suggest the presence of type 2C phosphatase in the particulate fraction in the rat parotid acinar cells. However, the possibility of contaminating of cytosol type 2C phosphatase can not be completely ruled out. This result differs from a previous report which showed the absence of type 2C phosphatase in microsomal fractions in the guinea-pig parotid gland (19). This difference may be due to the species difference or different approach used to analyze the phosphatases. Further study will make clear the localization of type 2C phosphatase in the rat parotid acinar cells.

The phosphorylation level of 26 kDa protein

The β-adrenergic receptor stimulation is accompanied by the phosphorylation of the 26 kDa protein (3, 4). The phosphorylation of the 26 kDa protein coincides with the elevation of intracellular cyclic AMP, suggesting the phosphorylation of this protein is catalyzed by a cyclic AMP-dependent protein kinase (3, 4). Quissell et al. have reported the dephosphorylation of the 26 kDa phosphoprotein occurred immediately

after β-adrenergic receptor blockade sebsequent to β–adrenergic receptor stimulation in the rat parotid and submandibular gland acinar cells (10, 13). Based on these results, it is considered that the phosphorylation/dephosphorylation reactions of 26 kDa protein is an intracellular signal transduction pathway linked to the β-adrenergic receptor. Also the phosphorylation level of 26 kDa protein may directly be involved in exocytotic amylase release due to the rapid phosphate turnover of the 26 kDa protein (10, 11,13). The increased phosphorylation of the 26 kDa protein following stimulation of β–adrenergic receptor should be regulated by protein phosphatases. However, the dephosphorylation process of 26 kDa phosphoprotein has not been elucidated. In this study, we showed the Mg^{2+}-dependent and okadaic acid-insensitive phosphatase, type 2C phosphatase, dephosphorylated the 26 kDa phosphoprotein in the rat parotid acinar cells. Future studies should be elucidated the relationship between the regulation of 26 kDa phosphoprotein and amylase secretion.

Concluding remarks

Our study shows the 26 kDa phosphoprotein is dephosphorylated by a Mg^{2+}-dependent and okadaic acid-insensitive phosphatase, type 2C phosphatase, in the rat parotid acinar cells. Type 2C phosphatase has no effect on the dephosphorylation of the other two phosphoproteins, 34 and 22 kDa proteins. The phosphorylation level of the 26 kDa protein is regulated by both cyclic AMP-dependent kinase and type 2C phosphatase in the rat parotid acinar cells.

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REFERENCES

1. Butcher, F. R., and Putney, J. W. Jr. (1980) Adv. Cyclic Nucleotide Res. 13, 215-249.
2. Quissell, D. O., Barzen K. A., and Lafferty, J. L. (1981) Am. J. Physiol. 241C, 76-85.
3. Jahn, R., Unger, C., and Söling. H. D. (1980) Eur. J. Biochem. 112, 345-352.
4. Thiel, G., Schmidt, W. E., Meyer, H. E., and Söling, H-D. (1988) Eur. J. Biochem. 170, 643-651.
5. Freedman, S. D., and Jamieson, J. D. (1982) J. Cell. Biol. 95, 903-908.
6. Baum, B. J., Freiberg, J. M., Ito, H., Rath, G. S., and Roth, C. R. (1981) J. Biol. Chem. 256, 9731-9736.
7. Baum, B. J., Colpo, F. T., and Filburn, C. R. (1981) Arch. Oral. Biol. 26, 333-337.
8. Freedman, S. D., and Jamieson, J. D. (1982) J. Cell. Biol. 95, 909-917.
9. Jahn, R., and Söling, H. D. (1983) FEBS lett. 153, 71-76.
10. Quissell, D. O., Deisher, L. M., and Barzen, K. A. (1985) Proc. Natl. Acad. Sci. USA. 82, 3237-3241.
11. Quissell, D. O., and Deisher, L. M. (1992) Arch. Oral. Biol. 37, 289-295.
12. Plewe, G., Jahn, R., Immelmann, A., Bode, C., and Söling, H. D. (1984) FEBS Lett. 166, 96-103.

13. Quissell, D. O., Deisher, L. M., and Barzen, K. A. (1983) Am. J. Physiol. G44-G53.
14. Ingebritsen, T. S., and Cohen, P. (1983) Eur. J. Biochem. 132, 255-261.
15. Ingebritsen, T. S., and Cohen, P. (1983) Science. 221, 331-338.
16. Tsuiki, S., Hiraga, A., Kikuchi, K., and Tamura, S. (1988) Methods Enzymol. 159, 437-446.
17. Cohen, P., Klumpp, S., and Schelling, D. L. (1989) FEBS Lett. 250, 596-600.
18. Hiraga, A., Kikuchi, K., Tamura, S., and Tsuiki, S. (1981) Eur. J. Biochem. 119, 503-510.
19. Mieshes, G and Sŏling, H. D, (1987) Eur. J. Biochem. 167, 377-382.
20. Yokoyama, N., Ozaki, I., Yamamoto, H., and Furuyama, S. (1989) Cell Calcium. 10, 457-466.
21. Tamura, S., Yasui, A., and Tsuiki, S. (1989) Biochem. Biophys. Res. Commun. 163, 131-136.
22. Fukunaga, K., Kobayashi, T., Tamura, S., and Miyamoto, E. (1993) J. Biol. Chem. 268, 133-137.
23. Sugiya, H., Tennes, K. A., and Putney, J. W. Jr. (1987) Biochem. J. 244, 647-653.
24. Laemmli, U. (1970) Nature. 227, 680-685.
25. Bialojan, C., and Takai, A. (1988) Biochem. J. 256, 283-290.