Vol. 215, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 4, 1995 Pages 75-81

# $Ca^{++}$-CALMODULIN-DEPENDENT PHOSPHORYLATION AND DEPHOSPHORYLATION OF RAT PAROTID SECRETION GRANULES

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Received August 25, 1995

In our studies on the control of fusion of secretion granules with the plasma membrane which occurs during exocytosis, we have recently found that $Ca^{++}$ and calmodulin stimulated the fusion of isolated parotid secretion granules with isolated inside-out plasma membrane vesicles. We are now examining the possibility that they do so by stimulating protein phosphorylation. Secretion granules were isolated from rat parotid by differential and gradient centrifugation and incubated with [$\gamma^{32}$P]ATP. The granules were solubilized with sodium dodecylsulfate and the proteins resolved on a 7-15% polyacrylamide gel. Calmodulin plus $Ca^{++}$, but neither alone, stimulated phosphorylation of four proteins with molecular masses of 64, 58, 55 and 31 kDa, and decreased the phosphorylation of a 36 kDa protein. Trifluoperazine and calmidazolium inhibited these effects. The results suggest that $Ca^{++}$ and calmodulin may facilitate fusion of secretion granules with the plasma membrane by changing the phosphorylation state of one or more secretion granule proteins.



It is well established that in many secretory cells, $Ca^{++}$ is an important second messenger in the process of exocytosis. Whether or not $Ca^{++}$ plays such a role in the secretion of amylase which results after ß-adrenergic stimulation of the parotid gland is more controversial. Although some investigators have reported results which argue against a role for $Ca^{++}$, the bulk of the evidence supports the hypothesis that $Ca^{++}$ is an intermediate in the stimulation of amylase secretion from the parotid gland (1-6).

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0006-291X/95 $12.00

The exact role $Ca^{++}$ may play in exocytosis from the parotid is unknown. However we have recently been studying the fusion of isolated rat parotid secretion granules with isolated inside-out plasma membrane vesicles and have found that $Ca^{++}$ markedly stimulated this interaction (to be published). Furthermore, we also found that in the presence of $Ca^{++}$, the addition of the $Ca^{++}$-binding-protein, calmodulin, increased the interaction of the secretion granules and plasma membrane vesicles beyond that produced by $Ca^{++}$ alone; the addition of the calmodulin antagonist, trifluoperazine, in the presence of $Ca^{++}$ and calmodulin, inhibited the interaction (to be published). These results thus suggested that in the parotid gland a $Ca^{++}$-calmodulin complex may be involved in the fusion of the secretion granule membrane with the plasma membrane which takes place during exocytosis.

In many other systems the $Ca^{++}$-calmodulin complex has been shown to act by stimulating protein kinase activity (7-9). Since there are numerous reports of protein phosphorylation occurring on stimulation of amylase secretion from the parotid (10-17), it seemed possible that the $Ca^{++}$-calmodulin stimulation of secretion granule-plasma membrane interaction we observed may involve protein phosphorylation. In testing this possibility we have found thus far that the $Ca^{++}$-calmodulin complex stimulates the phosphorylation of several secretion granule proteins, and at the same time stimulates the dephosphorylation of one secretion granule protein as well.

## MATERIALS AND METHODS

The [$\gamma^{32}$P]ATP, with a specific activity of 7000 Ci/mmol, was purchased from ICN Biomedicals Inc. Sucrose, ultrapure, was purchased from Beckman, and methyl alcohol and acetic acid from J.T. Baker. Most of the high purity reagents needed for gel electrophoresis were purchased from Biorad (sodium dodecyl-sulfate, bromphenol blue, ammonium persulfate, and N,N,N',N'-tetramethylethylenediamine) or Gibco BRL (acrylamide and N,N'-methylenebisacrylamide). All other reagents were purchased from the Sigma Chemical Co.

Parotid secretion granules were isolated by differential and gradient centrifugation in sucrose using a modification of the methods of Williams et al (18) and Castle et al (19). Virus-free rats weighing 125-150 g were obtained from Harlan Sprague Dawley, Inc., Indianapolis, IN, and were sacrificed by anesthetizing with ether and then removing the

heart. Parotid glands weighing between 1 and 1.5 g were removed from three rats, minced, and homogenized in a solution of 0.3 M sucrose containing 5 mM imidazole buffer pH 7.0, 5 mM EDTA, and 5 mM EGTA. Homogenization consisted of 16 strokes in a loose fitting glass homogenizer with a Teflon plunger. The homogenate was centrifuged at 270 x g for 5 min. The pellet was resuspended in 2 ml of the same buffered sucrose-EDTA-EGTA solution and recentrifuged. The combined supernatants were then divided in half, both aliquots were centrifuged at 1930 x g for 9 min, and the combined pellets were resuspended in 6 ml of the buffered sucrose-EDTA-EGTA solution. The suspension was then divided into two aliquots of 3 ml each, and each aliquot was diluted with an equal volume of the buffered sucrose-EDTA-EGTA solution and layered over 4 ml of 1.6 M sucrose containing 5 mM each of imidazole pH 7.0, EDTA, and EGTA. The latter in turn was layered over 1 ml of 2.2 M sucrose. The gradients were centrifuged at 51,000 x g for 24 min. in a Beckman SW 41 rotor, the granule bands at the 1.6-2.2 M interface were removed, combined, diluted with 0.3 M buffered sucrose without EDTA or EGTA, and centrifuged for 20 min at 14,600 x g. The pelleted granules were washed by resuspending and recentrifuging. The final pellet was resuspended in 1 ml of the buffered 0.3 M sucrose without chelators and either used the same day or stored at - 20°C.

Phosphorylation of specific secretion granule proteins was studied by the method of Schulman and Greengard (20). The reaction mixture contained 200-300 µg of granule protein, 40 mM HEPES buffer pH 7.0, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 3.4 x $10^{-4}$ mM ATP containing 20 µCi [$\gamma^{32}$P]ATP, and other additions as indicated, in a final volume of 250 µl. The reaction was started by adding the secretion granules. After 3 min incubation at room temperature the reaction was stopped by adding 250 µl of a solution of 0.125 M Tris, pH 6.8, containing 4% sodium dodecylsulfate, 20% glycerol, 10% 2-mercaptoethanol and 10% bromphenol blue, followed by immersion in a boiling water bath for 3 min. The $^{32}$P-labeled proteins in a 100 µl aliquot were resolved on a 7-15% sodium dodecylsulfate-polyacrylamide vertical slab gel as previously described (21). The gels were stained, dried, wrapped in plastic wrap and exposed to X-ray film at -80°C with an intensifying screen. The molecular masses of the phosphorylated proteins were determined from a molecular mass calibration curve with ß-galactosidase (116 kDa), phosphorylase B (92 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

## RESULTS

Figure 1 shows that when parotid secretion granules were incubated in the basic phosphorylation reaction mixture without additions, several proteins of varying molecular masses became phosphorylated (lane 1). The addition of $10^{-4}$M $Ca^{++}$ (lane 2) had little or no effect, but the addition of 2.4 x $10^{-6}$M calmodulin along with $10^{-4}$ M $Ca^{++}$ (lane 3) resulted in the appearance of three major new phosphorylated proteins with molecular masses of

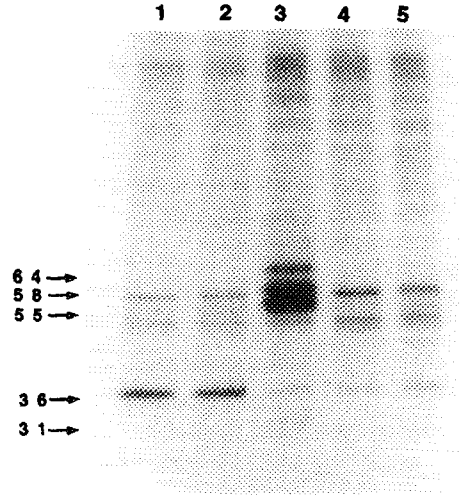


**FIG. 1.** Effect of $Ca^{++}$ and calmodulin on phosphorylation of parotid secretion granule proteins. Lane 1-control without additions; lane 2-$Ca^{++}$ added; lane 3-$Ca^{++}$ and calmodulin added; lane 4-$Ca^{++}$, calmodulin and trifluoperazine added; lane 5-$Ca^{++}$, calmodulin and calmidazolium added. Final concentrations: 0.1 mM $Ca^{++}$, 2.4 μM calmodulin, 0.5 mM trifluoperazine, 50 μM calmidazolium.

64, 58, and 55 kDa, as well as a minor one with a molecular mass of 31 kDa. Additionally, the degree of phosphorylation of a protein with a molecular mass of 36 kDa was greatly diminished by the addition of $Ca^{++}$ and calmodulin. It should be noted that the same changes were observed by the addition of 10 μM $Ca^{++}$ in the presence of calmodulin (data not shown). As can also be seen, the presence of either of the calmodulin antagonists, trifluoperazine (lane 4) or calmidazolium (lane 5), along with $Ca^{++}$ and calmodulin, inhibited the phosphorylation of the 64, 58, 55 and 31 kDa proteins, and partially prevented the decrease in the degree of phosphorylation of the 36 kDa protein.

## DISCUSSION

Considerable evidence exists to suggest that phosphorylation and dephosphorylation of proteins are important in the regulation of amylase secretion from the parotid gland. Stimulation of amylase secretion by ß-adrenergic receptor activation is accompanied by phosphorylation of several proteins of various sizes (10-17), and it has been reported that the time course of

phosphorylation paralleled the time course of secretion (13). The proteins which are phosphorylated on stimulation of secretion have been reported to be associated with various cell fractions including secretion granules (13, 16, 22). Additionally a decrease in the degree of phosphorylation of a protein with a molecular mass of 13.6 kDa (10) and a 34 kDa protein, identified as ribosomal protein S6 (23), has been reported.

In most cases evidence was presented that the reported protein phosphorylation was due to the action of either the cAMP-dependent protein kinase or protein kinase C. Although there is evidence that calmodulin, along with $Ca^{++}$, is involved in amylase secretion (24-27), very little has been done on the possible involvement of $Ca^{++}$-calmodulin-dependent phosphorylation and/or dephosphorylation. There have been reports of the presence of $Ca^{++}$-calmodulin dependent protein phosphatase in guinea pig parotid (28) and the isolation of such a phosphatase from bovine parotid (29). Additionally, a $Ca^{++}$-calmodulin dependent protein kinase which acts on a number of exogenous substrates, primarily galactosyltransferase (30), has been isolated from proliferating rat parotid cells chronically stimulated with isoproterenol. However, no evidence of a role for any of these in amylase secretion has been presented.

The results described here clearly show, for the first time, that parotid secretion granules contain both $Ca^{++}$-calmodulin-dependent protein kinase(s) and $Ca^{++}$-calmodulin-dependent protein phosphatase(s) as well as substrates on which they act. The importance of calmodulin in the phosphorylation and dephosphorylation shown in Fig. 1 is indicated by two findings. First, the addition of calmodulin along with $Ca^{++}$ was necessary; addition of $Ca^{++}$ alone did not produce these effects. Secondly, both trifluoroperazine, a phenothiazine which interferes with the action of calmodulin, and calmidazolium, a more potent and specific antagonist of calmodulin, inhibited the phosphorylation and dephosphorylation stimulated by $Ca^{++}$-calmodulin. At the same time $Ca^{++}$ is likewise essential; the requirement for $Ca^{++}$ is indicated by the fact that calmodulin in the presence of EGTA in place of $Ca^{++}$ was ineffective (data not shown).

It is important to note that the increase in the phosphorylation produced by added $Ca^{++}$-calmodulin is seen only if

the sucrose used to prepare the secretion granules contains both EGTA and EDTA to remove endogenous $Ca^{++}$ and calmodulin. When the preparation was made using only EDTA as described by Castle et al (19), no bands showed any enhancement on adding $Ca^{++}$ and calmodulin. The use of both chelators to remove endogenous calmodulin from isolated plasma membranes of adrenocortical cells was also found to be necessary in order to show calmodulin binding (31).

In view of the likelihood that [$^{32}$P]ATP does not penetrate the secretion granule membranes, these studies suggest that the protein kinase(s), the protein phosphatase(s) and their substrate(s) are located on the surface of the secretion granules. In this site they could conceivably be involved in the $Ca^{++}$-calmodulin stimulation of the fusion of the secretion granule membranes and the plasma membrane which we have shown occurs. If so, whether the dephosphorylation of the 36 kDa protein or the increased phosphorylation of one of the other proteins is the critical step in the action of $Ca^{++}$-calmodulin on the fusion remains to be determined.

**ACKNOWLEDGMENT**

This work was supported by a grant from the National Institute of Dental Research (DE09410).

**REFERENCES**

1. Argent, B.E., and Arkle, S. (1985) J. Physiol. 369, 337-353.
2. Arkle, S., Michalek, R., and Templeton, D. (1989) Biochem. Pharmacol. 38, 1257-1261.
3. Butcher, F.R. (1978) Adv. in Cyclic Nucleotide Res. 9, 707-721.
4. Gallacher, D.V., and Peterson, O.H. (1983) Intl. Rev. Physiol. 28, 1-52.
5. Horn, V.J., Baum, B.J., and Ambudkar, I.S. (1988) J. Biol Chem. 263, 12454-12460.
6. Putney, J.W., Jr., Weiss, S.J., Leslie, B.A., and Marier, S.H.(1977) J. Pharmacol. and Exp. Ther. 203, 144-155.
7. Colca, J.R., Brooks, C.L., Landt, M. and McDaniel, M.L. (1983) Biochem. J. 212, 819-827.
8. Johnson, J.D., and Mills, J.S. (1986) Med. Res. Revs. 6, 341-363.
9. Watkins, D.T. (1991) Diabetes 40, 1063-1068.
10. Baum, B.J., Frieberg, J.M., Ito, H., Roth, G.S., and Filburn, C.R. (1981) J. Biol. Chem. 256, 9731-9736.

11. Horio, B., Dowd, F., Watson, E., Mednieks, M., and Warren, J. (1984) J. Pharmacol. Exp. Ther. 229, 608-614 .
12. Jahn, R., Unger, C., and Söling, H.-D. (1980) Eur. J. Biochem. 112, 345-352.
13. Marino, C.R., Castle, J.D., and Gorelick, F.S. (1990) Am. J. Physiol. 259, G70-G77.
14. Plewe, G., Jahn, R., Immelmann, A., Bode, C., and Söling, H.-D. (1984) FEBS Letters 166, 96-103.
15. Quissel, D.O., Deisher, L.M., and Barzen, K.A. (1985) Proc. Natl. Acad. Sci. USA 82, 3237-3241.
16. Spearman, T.N., Hurley, K.P., Olivas, R., Ulrich, R.C., and Butcher, F.R. (1984) J. Cell Biol. 99, 1354-1363.
17. Takuma, T.(1990) J. Biochem. 108, 99-102, 1990.
18. Williams, M.A., Pratten, M.K., Turner, J.W., and Cope, G.H. (1979) Histochem. J. 11, 19-50.
19. Castle, J.D., Jamieson, J.D., and Palade, G.E. (1975) J. Cell Biol. 64, 182-210.
20. Schulman, H. and, Greengard, P. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 5432-5436.
21. Laemmli, U.K. (1970) Nature 227, 680-685.
22. Dowd, F., Watson, E.L., Lau, Y.-S., Pasieniuk, J., and Jacobson, K.L. (1987) J. Dent. Res. 66, 557-563.
23. Hara-Yokoyama, M., Sugiya, H., Furuyama, S., Wang, J.H., and Yokoyama, N. (1994) Biochem. and Molec. Biol. Intl. 34, 1177-1187.
24. Arkle, S., Pickford, P.D., Schofield, P.S., Ward, C., and Argent, B.E. (1986) Biochem. Pharmacol. 35, 4121-4124, 1986.
25. Kanagasuntheram, P. and, Teo, T.S. (1983) Biochem. Intl. 7, 511-518.
26. Ku, K.Y., and Butcher, F.R. (1980) Biochim. Biophys. Acta 631, 70-78.
27. Tojyo, Y., Matsumoto, Y., Okumura, K., and Kanazawa, M. (1989) Japan. J. Pharmacol. 50, 149-157.
28. Mieskes, G., and Söling, H-D. (1987) Eur. J. Biochem. 167, 377-382.
29. Yokoyama, N., Ozaki, T., Yamamoto, H., and Furuyama, S. (1989) Cell Calcium 10, 457-466.
30. Purushotham, K.R., Bologna, J., Nakagawa, Y., and Humphreys-Beher, M.G. (1992) Biochem. Cell Biol. 70, 250-255.
31. Widmaier, E.P., and Hall, P.F. (1987) Endocrinology 121, 914-923.