Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 398–401, January 1987
Biochemistry

Phosphorylation by protein kinase C of a 20-kDa cytoskeletal polypeptide enhances its susceptibility to digestion by calpain

(myosin light chains/phorbol esters/neutrophil activation)

S. PONTREMOLI*, E. MELLONI*, M. MICHETTI*, B. SPARATORE*, F. SALAMINO*, O. SACCO*, AND B. L. HORECKER†

*Institute of Biological Chemistry, University of Genoa, Genoa, Italy; and †Department of Biochemistry, Cornell University Medical College, 1300 York Avenue, New York, NY 10021

Contributed by B. L. Horecker, October 3, 1986

ABSTRACT **Incubation of the cytoskeletal fraction from human neutrophils with the proteolytically activated form of protein kinase C results in the phosphorylation of several components, including a 20-kDa polypeptide, probably consisting of myosin light chains. The 20-kDa polypeptide is also specifically phosphorylated by activated protein kinase C in a solubilized 20-kDa/80-kDa complex that was obtained after sonication of the insoluble cytoskeletal fraction. Phosphorylation of this polypeptide, in either the insoluble cytoskeletal fraction or the soluble 20-kDa/80-kDa complex, greatly enhances its susceptibility to digestion by the Ca^{2+}-requiring proteinase (calpain, EC 3.4.22.17) of human neutrophils. Thus, signals that activate calpain by mobilizing intracellular calcium would lead to proteolytic activation of protein kinase C, phosphorylation of cytoskeletal proteins, and remodeling of the cytoskeleton by proteolysis of at least one cytoskeletal component.**

Stimulation of human neutrophils with low concentrations of phorbol 12-myristate 13-acetate (PMA) elicits a set of biochemical responses that appear to be mediated by the activated membrane-bound form of protein kinase C (see ref. 1 and references cited therein). These responses include the production of oxygen radicals and the release into the medium of a neutral, membrane-associated, serine proteinase (1, 2) and are correlated with the phosphorylation of membrane proteins (3, 4). On the other hand, stimulation of neutrophils with formylmethionylleucylphenylalanine (fMLF) results mainly in a degranulation response, with little production of active oxygen species and a negligible release of the neutral serine proteinase (1). The degranulation response appears to involve activation of an intracellular Ca^{2+}-requiring proteinase (calpain, EC 3.4.22.17) and the proteolytic conversion of protein kinase C to the proteolytically modified form (5) that is active in the absence of Ca^{2+} and phospholipids and no longer binds to the neutrophil membrane (6, 7).

During the course of studies on the mechanism of the degranulation response, we observed that the cytoskeletal fraction isolated from cells stimulated with high concentrations of PMA contained reduced quantities of a 20-kDa polypeptide component. Phosphorylation of the 20-kDa myosin light chains of human platelets by an endogenous kinase was described in 1973 by Adelstein *et al.* (8) and has recently been shown to be induced by phorbol esters and to modulate the release of serotonin induced by PMA (9, 10). Evidence for the participation of protein kinase C in the PMA-stimulated release of lysosomal enzymes from rabbit neutrophils has also been reported (11), and in these experiments, lysosomal enzyme release appeared to be related to the phosphorylation of a 50-kDa polypeptide; phosphorylation of a 20-kDa polypeptide was not reported.

We report here that the 20-kDa polypeptide in the isolated cytoskeletal fraction of human neutrophils is phosphorylated by calpain-activated protein kinase C and that phosphorylation greatly enhances its susceptibility to digestion by endogenous calpain.

MATERIALS AND METHODS

Materials. Human neutrophils (6) and neutrophil calpain (12) were prepared as previously described. The proteolytically activated protein kinase C was partially purified from human neutrophils stimulated by PMA following the procedure used for native protein kinase C (7). This modified form of protein kinase is fully active in the absence of Ca^{2+} and phospholipids (5). The specific activity of the enzyme was 6×10^4 units/mg of protein, when assayed in the presence of 5 mM EDTA (7). PMA, phenylmethylsulfonyl fluoride, ATP, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), and the standard proteins for $NaDodSO_4$/polyacrylamide gel electrophoresis were from Sigma. [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) and $H_3[^{32}P]PO_4$ (10 mCi/ml) were obtained from Amersham. Triton X-100 was purchased from Rohm and Haas.

Isolation of Neutrophil Cytoskeleton. The cytoskeletal fraction was isolated by a modification of the procedure reported by Yassin *et al.* (13). Neutrophils (2×10^8 cells) were incubated at 0°C for 10 min in 10 ml of an ice-cold solution containing 1% Triton X-100, 160 mM KCl, 50 mM Tris·HCl at pH 7.0, 10 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride. The Triton-insoluble material was collected by centrifugation at 8000 × *g* for 10 min and washed twice with 10 ml of 50 mM Tris·HCl, pH 7.0, containing 160 mM KCl and 10 mM EDTA. The cytoskeletal fraction was finally suspended in 2.5 ml of 50 mM sodium borate, pH 7.5, containing 0.1 mM EDTA.

Phosphorylation of Cytoskeletal Proteins by Proteolytically Activated Protein Kinase C. Cytoskeletal fractions containing 0.4 mg of protein were incubated with continuous shaking at 30°C in 1 ml of 50 mM sodium borate buffer, pH 7.5, containing 5 mM $MgCl_2$, 5 μM [γ-^{32}P]ATP (0.25 mCi/mmol), and 1 mM EDTA, in the presence of 5 units of activated protein kinase C (see above). After 10 min the mixture was cooled to 4°C and centrifuged at 8000 × *g* for 10 min at 4°C. The insoluble cytoskeletal fraction was collected, washed with 5 ml of 50 mM sodium borate, pH 7.5, containing 0.1 mM EDTA, and suspended in 0.5 ml of the same buffer.

Digestion of Cytoskeletal Proteins with Calpain. Aliquots (0.1 mg of protein) of the cytoskeletal fraction either untreat-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: PMA, phorbol 12-myristate 13-acetate.

ed or phosphorylated with ATP and protein kinase C were incubated at 30°C in 0.5 ml of 50 mM sodium borate, pH 7.5, containing 0.1 mM $CaCl_2$ and 5 units of purified neutrophil calpain. As indicated, 0.1-ml aliquots were removed from the incubation mixture and the reaction was stopped by addition of 7% trichloroacetic acid (final concentration). The acid-soluble material was used to measure the amount of free amino groups liberated (14) and the acid-insoluble fraction was analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. The precipitated proteins were washed with acetone and dissolved in 0.06 M Tris·HCl, pH 6.8, containing 1% $NaDodSO_4$, 10% (vol/vol) glycerol, and 2% (vol/vol) 2-mercaptoethanol. The samples were boiled for 2 min and 80 μg of protein was applied to each lane. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed as described (15) except that the running gel consisted of an 8–18% acrylamide gradient. The optical density of the protein bands in autoradiographs was measured by scanning each lane with a Cellosystem densitometer (Ciampolini).

Isolation of an 80-kDa/20-kDa Protein Complex from the Cytoskeletal Fraction. The cytoskeletal fraction (5.0 mg of protein) was suspended in 2 ml of 50 mM sodium borate, pH 7.5, containing 1 mM EDTA and 0.5 M KCl and sonicated (Branson B15, six pulses for 10 s each). The material that remained insoluble was discarded by centrifugation at 15,000 $\times$ *g*. Approximately 85–90% of the total protein was recovered in the clear supernatant solution. This solution (1.5 ml) was loaded on a Sephadex G-200 column (2 $\times$ 100 cm) previously equilibrated with 50 mM sodium borate, pH 7.5, containing 1 mM EDTA and 0.5 M KCl. The flow rate was 12 ml/hr and 2-ml fractions were collected. Aliquots (0.1 ml) of the eluted fractions were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. The fractions containing the 20-kDa protein (fractions 31 to 41) corresponding to an apparent molecular mass of 250–300 kDa were pooled, concentrated to 2 ml by ultrafiltration on an Amicon UM-10 membrane, and dialyzed against 50 mM sodium borate, pH 7.5, containing 0.1 mM EDTA.

RESULTS

Effect of Phosphorylation on the Rate of Digestion by Calpain of Proteins in the Cytoskeletal Fraction. The composition of proteins in the cytoskeletal fraction of human neutrophils was similar to that previously reported for rabbit neutrophils (16), with major bands in $NaDodSO_4$/polyacrylamide gel electrophoresis corresponding to molecular masses of 80, 65, 48, 40, 36, 29, 25, and 20 kDa (Fig. 1). This fraction was relatively resistant to proteolysis by purified calpain, but the rate of proteolysis was increased by approximately 3-fold after phosphorylation with protein kinase C (Fig. 2).

Characterization of the Phosphorylated Polypeptide(s) Modified by Calpain. To determine which of the components of the cytoskeletal fraction were degraded by calpain, the undigested and digested ^{32}P-labeled cytoskeletons were analyzed by autoradiography after $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 3). In the undigested samples the major phosphorylated peak was in the position corresponding to the 20-kDa polypeptide, with smaller peaks corresponding to peptides with molecular masses of 65, 48, 29, and 25 kDa (Fig. 3*A*). In the digested sample there was a marked decrease in the quantity of 20-kDa phosphorylated peptide, accompanied by the appearance of a new radioactive band with molecular mass of 15 kDa (Fig. 3*B*).

Specificity of the Phosphorylation Signal for Proteolysis of the Cytoskeletal Components. The increased rate of calpain proteolysis of the phosphorylated 20-kDa cytoskeletal protein could be attributed to a change in its accessibility in the cytoskeletal fraction or more directly to a signal-directed proteolysis involving the 20-kDa protein. Support for the latter hypothesis was obtained with a fraction containing the 20-kDa polypeptide isolated by Sephadex G-200 chromatography from the solubilized cytoskeleton. The peak emerging near the void volume (apparent molecular mass, 250–300 kDa) was found to contain two components with molecular masses of 80 kDa and 20 kDa (see Fig. 5*A*). Phosphorylation of this fraction increased its susceptibility to digestion with calpain nearly 10-fold (Fig. 4). Analysis by $NaDodSO_4$/polyacrylamide gel electrophoresis revealed that only the 20-kDa component was digested, with the appearance of a 15-kDa fragment (Fig. 5*B*). It must be emphasized that incubation of the 20-kDa/80-kDa complex with [γ-^{32}P]ATP and protein kinase C results in labeling of only the 20 kDa-component (Figs. 3 and 5) and that the 80-kDa component is not susceptible to digestion by calpain. Unfortunately, we have thus far been unable to separate the native forms of the 80- and 20-kDa components of the complex.

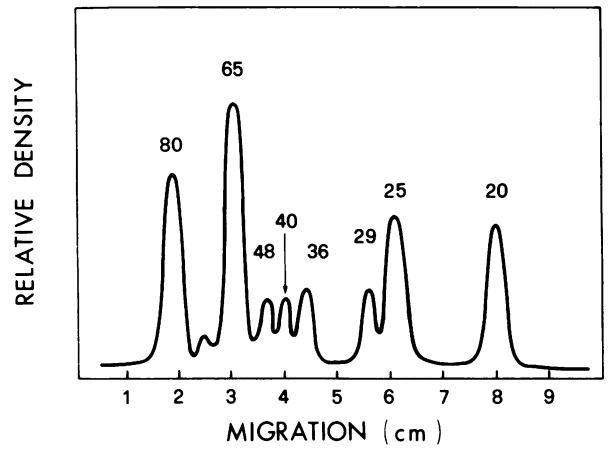

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of the cytoskeletal fraction from human neutrophils. The cytoskeletal fraction (50 μg) was dissolved in 0.1 ml of 0.06 M Tris·HCl, pH 6.8, containing 1% $NaDodSO_4$, 10% glycerol, and 2% 2-mercaptoethanol and analyzed by polyacrylamide gel electrophoresis. The numbers above the peaks are molecular masses, in kDa.

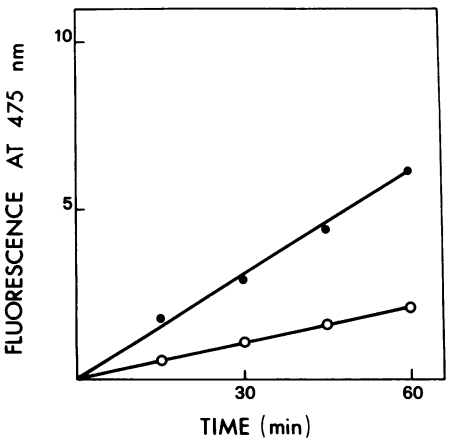

FIG. 2. Susceptibility of the phosphorylated and nonphosphorylated cytoskeletal fractions to digestion by neutrophil calpain. Aliquots of native (○) or phosphorylated (●) cytoskeletal fractions, each containing 0.5 mg of protein, were incubated at 37°C in 1 ml of 50 mM sodium borate, pH 7.5, containing 0.1 mM Ca^{2+} and 5 units of purified calpain. At the times indicated aliquots (0.2 ml) were treated with trichloroacetic acid (7% final concentration). The appearance of $-NH_2$ groups in the supernatant solution was determined with fluorescamine (14).

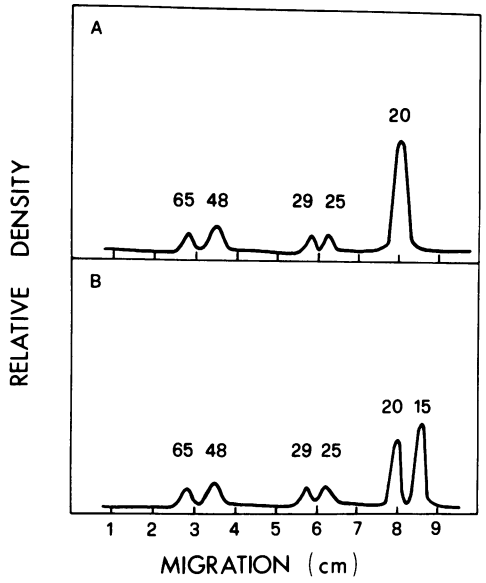

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis of ^{32}P-labeled cytoskeletal proteins. The phosphorylated cytoskeletal fraction was digested with calpain as described in Fig. 2. The phosphorylated proteins (80 μg) were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis. Densitometric scans of autoradiographs are shown. (*A*) Labeled cytoskeletal proteins before digestion with calpain. (*B*) Labeled cytoskeletal proteins after digestion with calpain for 30 min as described for Fig. 2.

DISCUSSION

Stimulation of human neutrophils by external agents elicits a variety of responses, including production of active oxygen species (17), release of a membrane-bound serine proteinase (2), and release of contents of subcellular granules (18). While the first two responses appear to be mediated by a series of events involving tight binding of protein kinase C to the plasma membrane (19–24) resulting in its activation and the phosphorylation of membrane proteins (3, 4, 25, 26), degranulation appears to be related to mobilization of intracellular Ca^{2+} (1, 27–29), followed by activation of calpain. The active proteinase promotes proteolytic conversion of the membrane-bound protein kinase C into a soluble irreversibly activated form with substrate specificity different from that of the native kinase (4, 6, 7). This activated form of the kinase is presumably involved in the phosphorylation of a number of intracellular proteins, including components of the cytoskeletal network. Phosphorylation, particularly of 40- and 20-kDa polypeptides, is frequently associated with the activation of platelets (9, 10, 30–33) and neutrophils (11), including cells activated by PMA (9–11, 33). The identification of myosin light chain as the 20-kDa polypeptide phosphorylated by protein kinase C is supported by the observation that myosin light chains purified from chicken gizzard muscle are phosphorylated by this kinase (34). The results reported here indicate that, at least in neutrophils, phosphorylation of a cytoskeletal 20-kDa polypeptide, probably myosin light chains, is catalyzed by the proteolytically activated form of protein kinase C. These results provide a mechanism involving calpain in such intracellular responses of activated neutrophils as the release of granule contents. Activated calpain is responsible for the proteolytic conversion of protein kinase C (6, 7) and also, as shown here, for a reorganization of the cytoskeletal architecture. Phosphorylation of the 20-kDa polypeptide increases by at least 10-fold its susceptibility to digestion by calpain, with the appearance in the cytoskeletal fraction of a 15-kDa product.

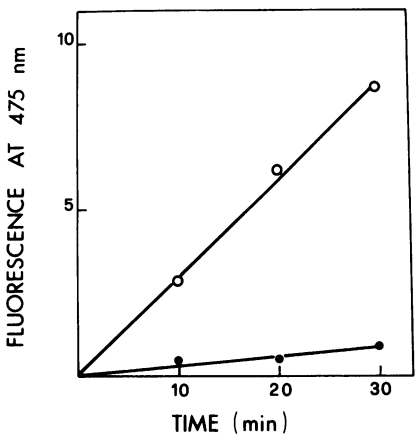

FIG. 4. Effect of phosphorylation of the 20-kDa/80-kDa complex on its susceptibility to digestion with calpain. The solubilized 20-kDa/80-kDa complex was phosphorylated as described in *Materials and Methods* for the insoluble cytoskeletal fraction. Samples of the untreated (●) and phosphorylated (○) complex (0.5 mg each) were then treated with purified calpain as described in the legend to Fig. 2.

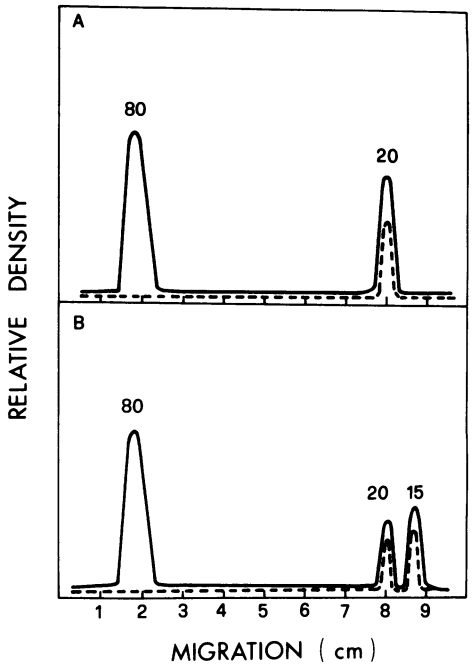

FIG. 5. $NaDodSO_4$/polyacrylamide gel electrophoresis of the 20-kDa/80-kDa complex after phosphorylation and digestion with calpain. The trichloroacetic acid precipitate from the 30-min sample in the experiment in Fig. 4 was solubilized in buffered $NaDodSO_4$ and aliquots (100 μg of protein) were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis as described in the legend to Fig. 3. (*A*) Before digestion with calpain. (*B*) After digestion with calpain for 30 min. —, Coomassie blue staining; – – –, ^{32}P radioactivity.

The present data provide support for an involvement of cytoskeletal proteins in exocytosis and specifically for the concept that reorganization of the cytoskeletal network facilitates the access of granules to the plasma membranes (35).

In yeast, proteolytic degradation of fructose-1,6-bisphosphatase after its phosphorylation in response to the addition of glucose to starved cells represents an important mechanism for metabolic regulation (36, 37). But, to our knowledge, the phosphorylation of a mammalian protein acting as a signal for its proteolysis has not been reported previously.

This work was supported in part by a grant from the Consiglio Nazionale delle Ricerche, Progetto Finalizzato Oncologia, Sottoprogetti Biologia Molecolare and Cellulare, and from the Association Italiana Ricerche Cancro.

1. Pontremoli, S., Melloni, E., Michetti, M., Sacco, O., Salamino, F., Sparatore, B & Horecker, B. L. (1986) *J. Biol. Chem.* **261,** 8309–8313.
2. Pontremoli, S., Melloni, E., Michetti, M., Sacco, O., Sparatore, B., Salamino, F., Damiani, G. & Horecker, B. L. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 1685–1689.
3. Melloni, E., Pontremoli, S., Salamino, F., Sparatore, B., Michetti, M., Sacco, O. & Horecker, B. L. (1986) *J. Biol. Chem.* **261,** 11437–11439.
4. Pontremoli, S., Melloni, E., Salamino, F., Sparatore, B., Michetti, M., Sacco, O. & Horecker, B. L. (1986) *Arch. Biochem. Biophys.* **250,** 23–29.
5. Kishimoto, A., Kajikawa, N., Shiota, M. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258,** 1156–1164.
6. Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B., Salamino, F. & Horecker, B. L. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 6435–6439.
7. Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B. & Horecker, B. L. (1986) *J. Biol. Chem.* **261,** 4101–4105.
8. Adelstein, R. S., Conti, M. A. & Anderson, W., Jr. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 3115–3119.
9. Naka, M., Nishikawa, M., Adelstein, R. S. & Hidaka, H. (1983) *Nature (London)* **306,** 490–494.
10. Inagaki, M., Kawamoto, S. & Hidaka, H. (1984) *J. Biol. Chem.* **259,** 14321–14323.
11. White, J. R., Huang, C. K., Hill, J. M., Jr., Naccache, P. H., Becker, E. L. & Sha'afi, R. I. (1984) *J. Biol. Chem.* **259,** 8605–8611.
12. Pontremoli, S., Sparatore, B., Salamino, F., Michetti, M., Sacco, O. & Melloni, E. (1985) *Biochem. Int.* **11,** 35–44.
13. Yassin, R., Shefcyk, J., White, J. R., Tao, W., Volpi, M., Molski, T. F. P., Naccache, P. H., Feinstein, M. B. & Sha'afi, R. I. (1985) *J. Cell Biol.* **101,** 182–188.
14. Undenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leingruber, W. & Weigele, M. (1972) *Science* **178,** 871–872.
15. Andrews, P. C. & Krinsky, N. I. (1981) *J. Biol. Chem.* **256,** 4211–4218.
16. White, J. R., Naccache, P. H. & Sha'afi, R. I. (1983) *J. Biol. Chem.* **258,** 14041–14047.
17. Babior, B. M., Kipnes, R. S. & Curnutte, J. T. (1973) *J. Clin. Invest.* **52,** 741–744.
18. Hirsch, J. C. & Cohn, Z. A. (1960) *J. Exp. Med.* **12,** 1005–1014.
19. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, V. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257,** 7847–7851.
20. Kraft, A. S. & Anderson, W. B. (1983) *Nature (London)* **301,** 621–622.
21. Nishizuka, Y. (1984) *Nature (London)* **308,** 693–698.
22. Ashendel, C. L. (1985) *Biochim. Biophys. Acta* **822,** 219–242.
23. Wolf, M., LeVine, H., III, May, W. S., Jr., Cuatrecasas, P. & Sahyoun, N. (1985) *Nature (London)* **317,** 546–549.
24. Wolfson, M., McPhail, L. C., Nasrallah, V. N. & Snyderman, R. (1985) *J. Immunol.* **135,** 2057–2063.
25. Andrews, P. C. & Babior, B. M. (1983) *Blood* **61,** 333–340.
26. Andrews, P. C. & Babior, B. M. (1984) *Blood* **64,** 883–890.
27. Mottola, C. & Romeo, D. (1982) *J. Cell Biol.* **93,** 129–134.
28. Lagast, H., Pozzan, T., Wedvogel, F. A. & Lew, P. D. (1984) *J. Clin. Invest.* **73,** 878–883.
29. Gennaro, R., Pozzan, T. & Romeo, D. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1416–1420.
30. Haslam, R. J. & Lynham, J. A. (1977) *Biochem. Biophys. Res. Commun.* **77,** 714–719.
31. Bennett, W. F., Belville, J. S. & Lynch, G. (1979) *Cell* **18,** 1015–1023.
32. Lyons, R. M., Stanford, N. & Majerus, P. W. (1975) *J. Clin. Invest.* **56,** 924–936.
33. Bourguignon, L. Y. W., Walker, G. & Bourguignon, G. J. (1985) *J. Biol. Chem.* **260,** 11775–11780.
34. Endo, T., Naka, M. & Hidaka, H. (1982) *Biochem. Biophys. Res. Commun.* **105,** 942–948.
35. Southwick, F. S. & Stossel, T. P. (1983) *Semin. Hematol.* **20,** 305–321.
36. Muller, D. & Holzer, H. (1981) *Biochem. Biophys. Res. Commun.* **103,** 926–933.
37. Mazon, M. J., Gancedo, J. M. & Gancedo, C. (1982) *J. Biol. Chem.* **257,** 1128–1130.