Neurochemical Research, Vol. 7, No. 2, 1982

SPECIFIC PROTEOLYSIS OF A BRAIN MEMBRANE PHOSPHOPROTEIN (B-50): Effects of Calcium and Calmodulin

H. ZWIERS[1], W. H. GISPEN[1], L. KLEINE[2], AND H. R. MAHLER[2]

[1] *Division of Molecular Neurobiology*
Rudolf Magnus Institute for Pharmacology
Institute of Molecular Biology
University of Utrecht
Padualaan 8, 3508 TB
Utrecht, The Netherlands
[2] *Department of Chemistry*
Indiana University
Bloomington, Indiana, USA

Accepted September 22, 1981

The membrane bound phosphoprotein B-50 (MW 48K) was isolated from rat brain tissue. The fraction containing the highest endogenous B-50 phosphorylating activity (ASP 57–82%) contains protease activity. In the absence of calcium a time-dependent decrease of the protein B-50 is observed. Under these conditions another phosphoprotein B-60 (MW 46K) appears in the incubation medium. Addition of calcium and/or calmodulin enhances the protease activity whereas the substrate specificity is lost. Results of both isoelectric focussing and peptide mapping indicate that B-50 and B-60 are related proteins. These data support our hypothesis that the recently isolated behaviorally active peptide PIP (MW approx. 1600 D) is the smaller cleavage product of the proteolytic degradation of B-50 to B-60.

INTRODUCTION

Previously we have described the isolation and purification of the phosphoprotein B-50 from rat brain synaptosomal plasma membranes (SPM) (1, 2). Its endogenous phosphorylation was found to be insensitive to cAMP or cGMP but inhibited by peptides related to ACTH (3–6). Further

0364-3190/82/0200-0127$03.00/0

characterization revealed that its phosphorylation was stimulated by low concentrations of calcium and calmodulin (7, 8). Depending on the PAGE system used its apparent M_R was estimated to be 48K (2) or 54K (8) with an IEP of 4.5. We reported that the isolation procedure used also gave rise to a small biologically active peptide (PIP; M_R = 1.6K) that inhibited the B-50 protein kinase and we suggested that the formation of PIP resulted from the proteolytic conversion of B-50 to B-60, a protein with a lower M_R (apparent M_R = 46K), by an endogenous protease (5). The present communication lends further support for the structural similarity of B-50 and B-60 and describes some of the features of the endogenous proteolytic activity.

EXPERIMENTAL PROCEDURE

Fractionation of the ACTH-Sensitive, Membrane-Bound Protein Kinase and its Substrate B-50. The purification of this enzyme and one of its substrate proteins (B-50) has been described in detail (see 2). The procedures used here were similar, except that none of the buffers used contained calcium, unless specified otherwise. In short, crude SPM fractions were prepared from rat cortex (27 g wet weight). The proteins were partly solubilized with 0.5% Triton X-100 in 75 mM KCl (1) and then subjected to column chromatography over DEAE-cellulose using a NaCl gradient to elute the endogenous B-50 phosphorylating activity (2). The active eluate fractions were pooled (DEAE pool) and treated with ammonium sulphate (57% saturation). Precipitated proteins (ASP 0–57%) were removed by centrifugation and to the supernatant more ammonium sulphate was added till 82% of saturation. This precipitate (ASP 57–82%) was again collected by centrifugation and was redissolved in 200 μl buffer A (10 mM Tris-HCl, 0.1 mM dithiothreitol, pH 7.4) and dialysed for 5 hrs against one l buffer A. Among other proteins, the freshly prepared ASP 57–82% contains one protein kinase and one major phosphoprotein (B-50) (2). If the ASP 57–82% fraction was dialysed for longer periods of time or was allowed to remain at 4°C for one or two days, an extra phosphoprotein band became manifest (B-60) (5).

Endogenous Phosphorylation Assay. The B-50 protein kinase activity was assayed under the following conditions: 15 μl ASP 57–82%, 10 mM Na-acetate, 10 mM Mg-acetate, 1 mM Ca-acetate, 7.5 μM ATP, 1 μCi [γ-^{32}P]ATP (approx. 3000 Ci/mmol, Amersham, UK) pH 6.5, final volume 20 μl. After preincubation for 5 min at 30°C, the reaction was initiated by the addition of the [γ-^{32}P]ATP in 5 μl. After one min at 30°C the reaction was terminated by addition of 12.5 μl of a denaturing solution, containing 6% SDS (3). Previously, we had demonstrated that the phosphorylation of B-50 under these conditions was linear with time for at least 15–20 min (2).

Isoelectric focussing (IEF) and SDS-Polyacrylamide Gel Electrophoresis (PAGE). The ASP 57–82% protein fraction was subjected to IEF as detailed in ref. 2. The SDS-PAGE (polyacrylamide 11%) as first dimension was carried out essentially as described in ref. 3, whereas the procedure for SDS-PAGE as second dimension after IEF, was identical to that described in (2). After SDS-PAGE, the gels were stained for proteins, using Fast Green and destained. The protein pattern of the wet gels was scanned by densitometry at 650 nm using a Zeiss M4-QII spectrophotometer with KM3 chromatogram attachment.

Finger-Print Analysis by Partial Proteolysis. Enzymatic digestions with papain, α-chy-

motrypsin or trypsin in a 4% acrylamide spacer gel and separation on 15% polyacrylamide gels were performed by the method of Cleveland et al. (9).

Preparation of Calmodulin. Calmodulin was prepared from calf brain by the method of Cheung et al. (10).

Protein concentration was determined by the method of Lowry et al. (11).

RESULTS AND DISCUSSION

Freshly prepared ASP 57–82% proteins were incubated in buffer A at 4°C or 30°C for various periods of time, at the end of which all proteolysis was stopped by adding the SDS protein-denaturing solution. Subsequently, the samples were subjected to SDS-PAGE. As can be seen in Figure 1A, at time zero the ASP 57–82% fraction contains several proteins, one of which is the B-50 protein. In such preparations hardly any B-60 can be detected. At the end of a 8 hr incubation at 30°C, the protein pattern has undergone a specific change in that there is now less B-50 and substantially more B-60 as compared to time zero. No other protein bands seem to change in quantity. This confirms our previous experiments on the production of PIP and presumptive B-60 in ASP-protein preparations (5). Figure 1B demonstrates that endogenous B-50 degradation is temperature-dependent, since at 30°C the amount of B-60 after 8 hrs exceeded that recovered after 60 hrs at 4°C, without qualitative differences between the two protein profiles. Thus, a disappearance of B-50 protein is paralleled by the appearance of B-60.

Such a parallel is also evidenced by the pattern obtained after subjecting the ASP 57–82% fraction before and after incubation for 8 hrs at 30°C to a two dimensional separation using IEF followed by SDS-PAGE (Figure 2). The decrease in B-50 is accompanied by an increase in the B-60 protein with an M_R of 46K and an IEP in the range of 4.2. The isoelectric focussing data strengthen the hypothesis that B-60 and B-50 are related.

Additional evidence for this relationship was obtained by fingerprint analysis after partial proteolysis of the two proteins in the ASP 57–82% fraction, resulting from dialysis for 48 hrs at 4°C. Such an ASP preparation is relatively rich in B-60 protein. The dialysed ASP 57–82% was incubated with [γ-^{32}P]ATP resulting in the incorporation of labeled phosphate into both B-50 and B-60. These labeled phosphoproteins were separated on SDS-PAGE and excised from the stained dried gels. About equal amounts of B-50 and B-60 (1 μg) were applied to separate slots of 15% polyacrylamide gels and were covered prior to electrophoresis with a solution containing the different proteases used (papain 0.05 μg; trypsin 2 μg; α-chymotrypsin 2 μg). Figure 3 displays the autoradiographs of the peptide

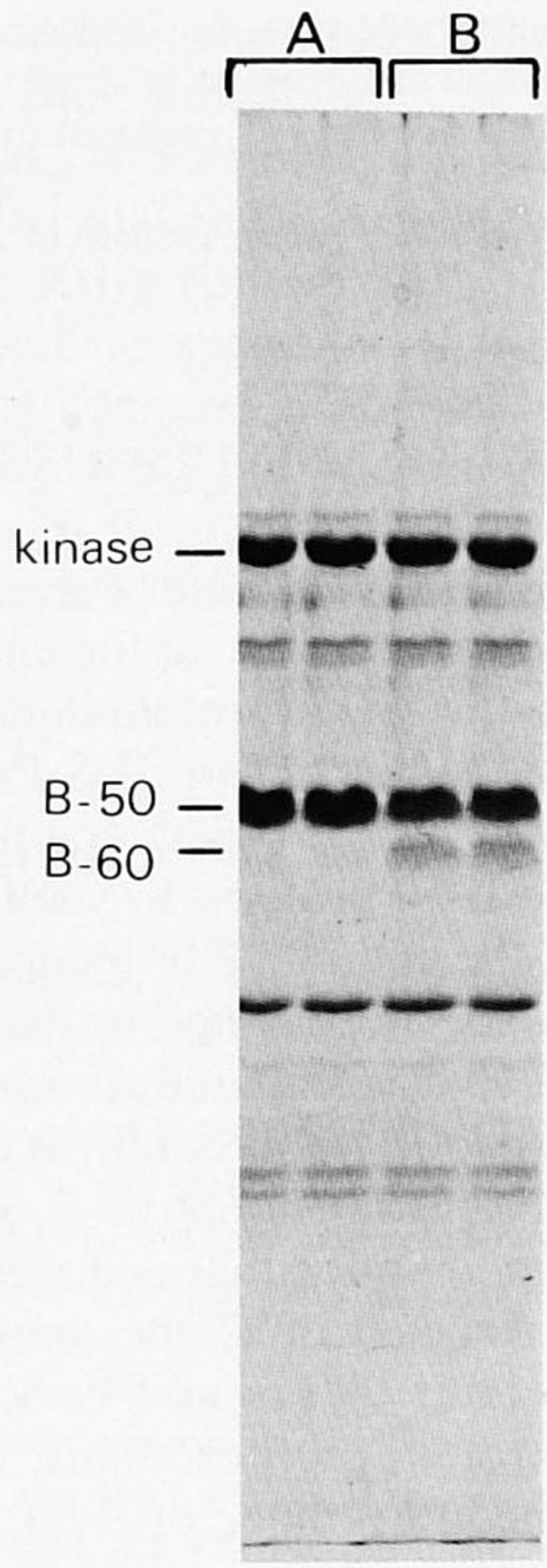

FIG. 1A. Protein staining pattern of ASP 57–82% proteins. Duplicate samples (3 μg of total protein, volume 30 μl, containing 1 mM EGTA), were incubated for 8 hrs (part B) or 0 hr (part A) at 30°C. Incubation was terminated by the addition of 15 μl of a stop mixture containing SDS. Proteins were separated by SDS-PAGE.

maps from the labeled B-50 and B-60. The results demonstrate that the peptide maps of B-50 and B-60 are similar but there is a consistent increase in mobility of all fragment peptides in the B-60 maps as compared to those in B-50. This shift downwards may be the result of a consistently larger segment (e.g. PIP) retained in all peptides of B-50 after proteolysis. The papain maps differ markedly from the chymotrypsin and trypsin maps, but the similarity of B-50 and B-60 is clear. Other phosphorylated proteins both from SPM and purified kinase show a completely different digestion pattern (8).

From previous (5) and present results we therefore conclude that B-60 is a degradation product of B-50. Preliminary evidence shows that this conversion of B-50 to B-60 may also take place in SPM (Zwiers, unpub-

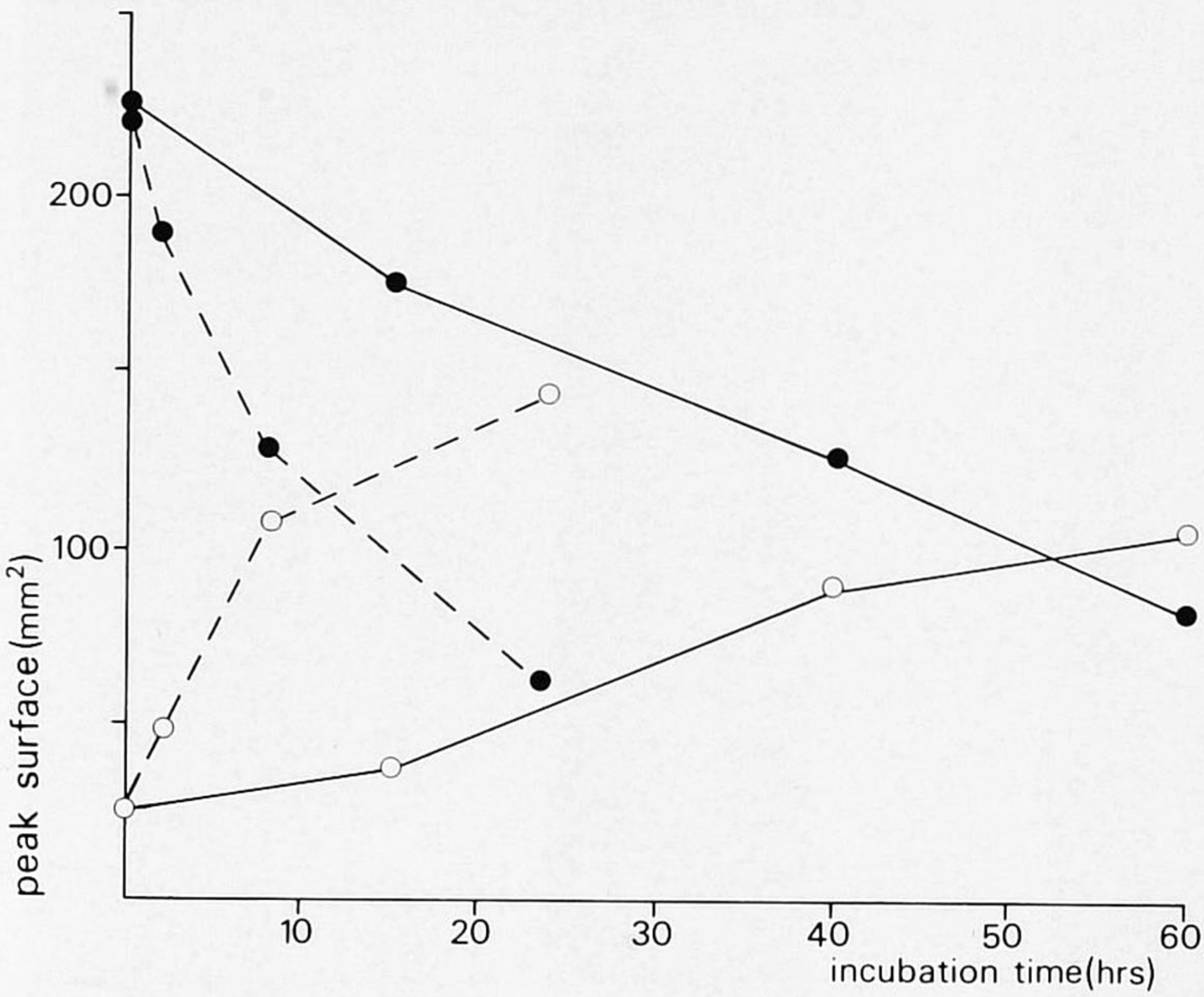

FIG. 1B. Time course of the disappearance of B-50 (●) and the appearance of B-60 (○) in the incubation medium. Incubation conditions are described in (a). Proteolysis was performed at 4°C (solid lines) or at 30°C (dashed lines). The amounts of B-50 and B-60 were determined by measuring peak surfaces (mm^2) of densitometric scans of the SDS-PAGE profiles.

lished). Interestingly, while a number of exogenous proteases can fragment B-50 or B-60 into several different peptides (Figure 3), the endogenous protease seems to cleave B-50 primarily into B-60 and PIP (5). These differences in cleavage patterns by endogenous and exogenous proteases are confirmed also by the observation that B-60 is not a major product of cleavage of B-50 by the exogenous enzymes and PIP is probably retained in the peptide fragments obtained (Figure 3). If this interpretation is correct the limit peptides—which are different for the proteases employed but identical for B-50 and B-60 in each instance—must all retain PIP.

When B-50 protein, isolated by means of IEF, was incubated in buffer A for 40 hrs at 30°C there was no detectable degradation and no appearance of B-60 (data not shown). Previously, we had shown that incubation after heating of the ASP fraction to 70°C for 20 min likewise did not result in the production of B-60 (5). Apparently, proteolytic activity resides in one of the ASP 57–82% proteins other than B-50.

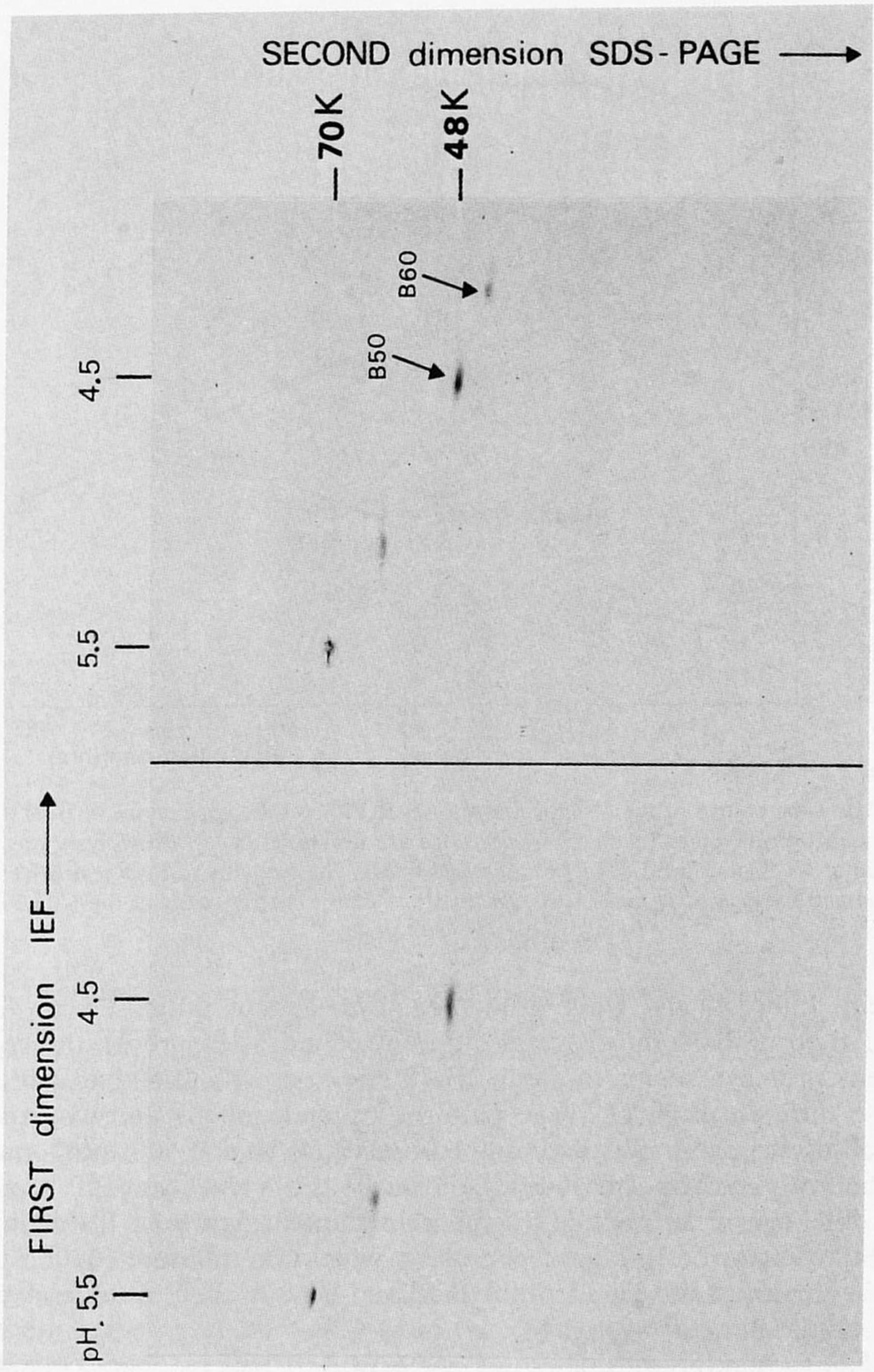

FIG. 2. Protein staining pattern after two dimensional separation of ASP 57–82% proteins. ASP proteins (3 μg), were incubated for 8 hrs (right part of the figure) or for 0 hr (left part) at 30°C. The incubation mixture contained 1 mM EGTA. Samples were subjected to an IEF slab gel. After running, the lower halves of the two tracks were mounted on a SDS gel for separation in the second dimension. At the top the pH of corresponding gel slices is indicated, at the right the positions of the kinase (70K) and B-50 (48K) in the SDS gel are indicated.

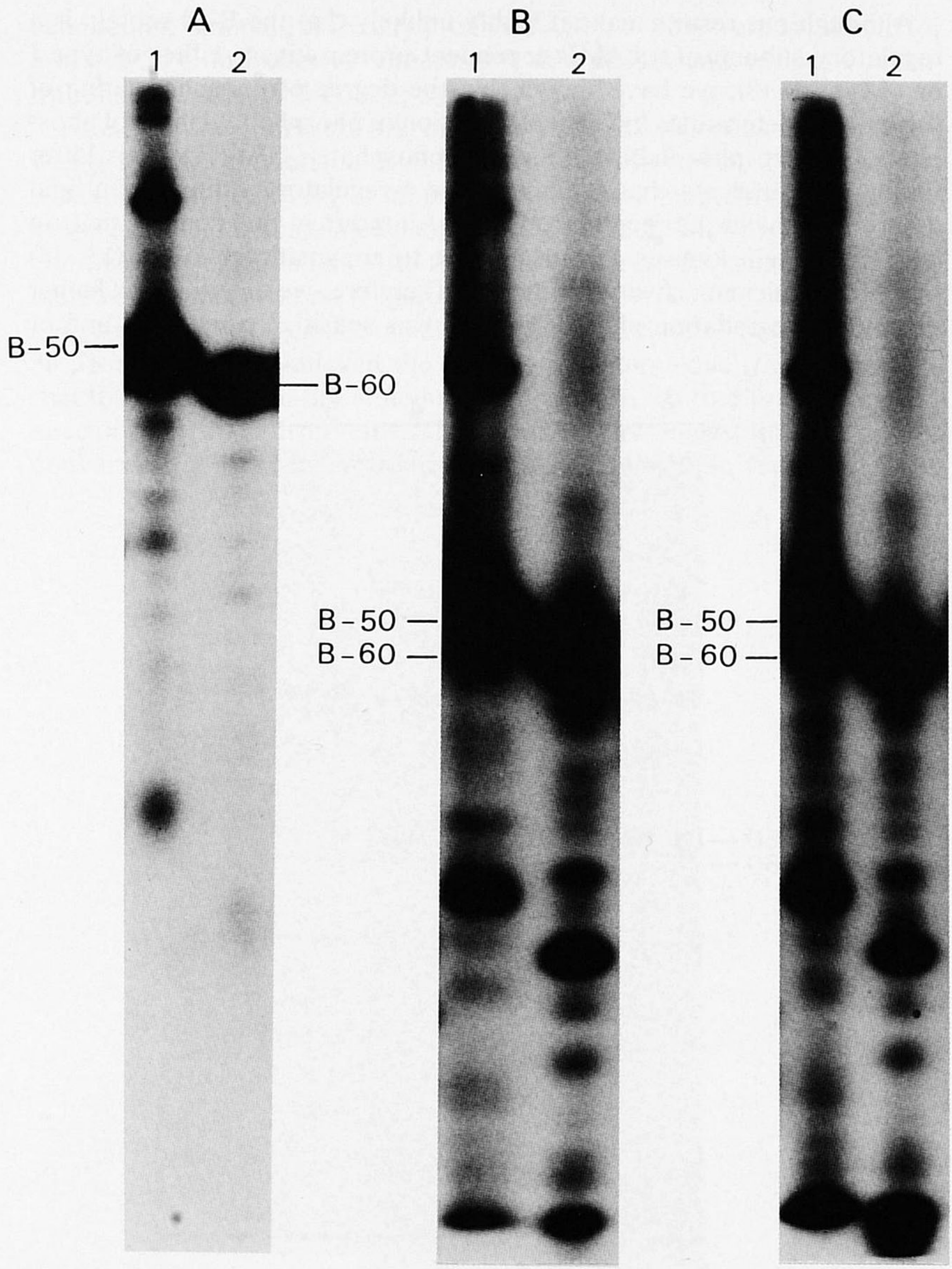

FIG. 3. Autoradiograph of the peptide maps obtained from [^{32}P]-labeled B-50 (1) and B-60 (2). Digestion was performed with the following proteases: A, 0.05 μg papain; B, 2 μg α-chymotrypsin; C, 2 μg trypsin. Samples of [^{32}P]-labeled B-50 and B-60 were excised from dried gels, incubated in stacking buffer for 30 min, placed in sample wells of a second SDS-polyacrylamide slab gel and overlayed with protease. Electrophoresis was started at 4 mA/slab for 10 hr and completed at 25 mA/slab.

Although our results make it highly unlikely that the B-50 protein is a regulatory subunit of a cAMP-dependent protein kinase either of type I or of type II (8), we have shown that the degree of phosphorylation of this protein determines the phosphorylation of phosphatidyl inositol phosphate (DPI) to phosphatidyl inositol biphosphate (TPI) (12). This latter finding may indicate that B-50 could be a regulatory subunit of a lipid kinase present in the membrane. It is of interest in this context that, in general, protein kinases are susceptible to enzymatic cleavage (13, 14) by specific calcium-activated proteases. Therefore, we determined whether or not the degradation of B-50 to B-60 was sensitive to calcium and or

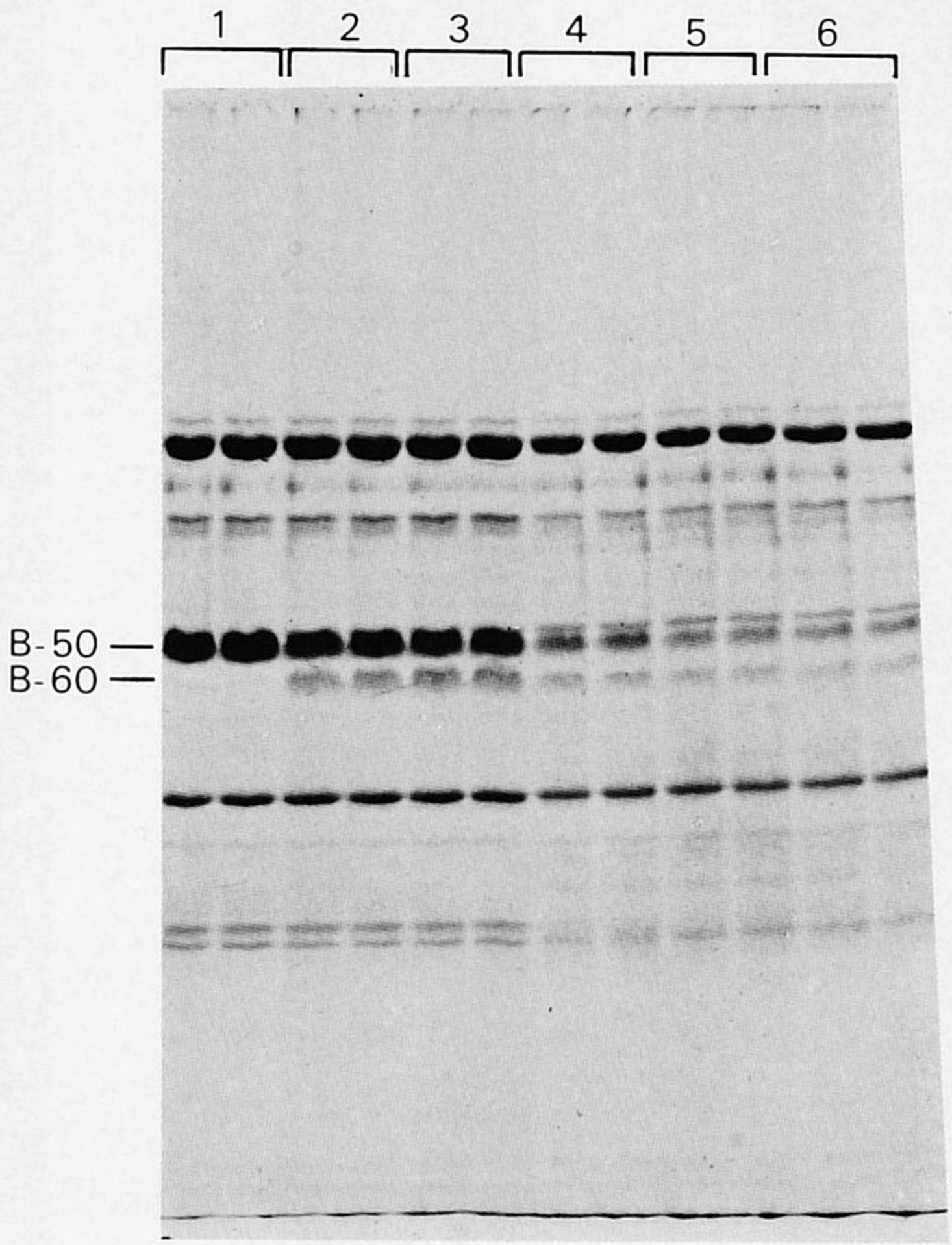

FIG. 4A. Protein staining pattern of the proteolysis of ASP 57–82% proteins in the presence of different concentrations of Ca^{2+}. Duplicate samples of ASP 57–82% proteins (3 μg, volume 30 μl) were incubated for 8 hrs at 30°C in the presence of 1 mM EGTA. Numbers on top refer to: 1, control, non-incubated; 2, incubated; 3, incubated with 0.1 mM Ca^{2+}; 4, with 1 mM Ca^{2+}; 5, with 2 mM Ca^{2+}; 6, with 5 mM Ca^{2+}.

calmodulin. Samples of freshly prepared ASP 57–82% were incubated for 8 hrs at 30°C in buffer A containing 1 mM EGTA and various concentrations of $CaCl_2$ (0; 0.1; 1; 2; 5 mM).

As can be seen in Figure 4A, in the absence of exogenous calcium there was a specific conversion of B-50 to B-60. If, however, 2 mM calcium was added, the recovery of all protein bands was markedly reduced suggesting a calcium stimulated, non-specific proteolysis. This effect of calcium was dose-dependent with the order of effectiveness increasing with increasing concentration (Figure 4A).

From Figure 4B it can be concluded that calmodulin (1.8 μg calmodulin/30 μl incubation) mimicked the effect of calcium alone (Figure 4A) and that the combination of calmodulin and calcium led to a total disappearance of ASP 57–82% proteins. The data therefore suggest that the specific conversion of B-50 to B-60 has no calcium/calmodulin requirement. In

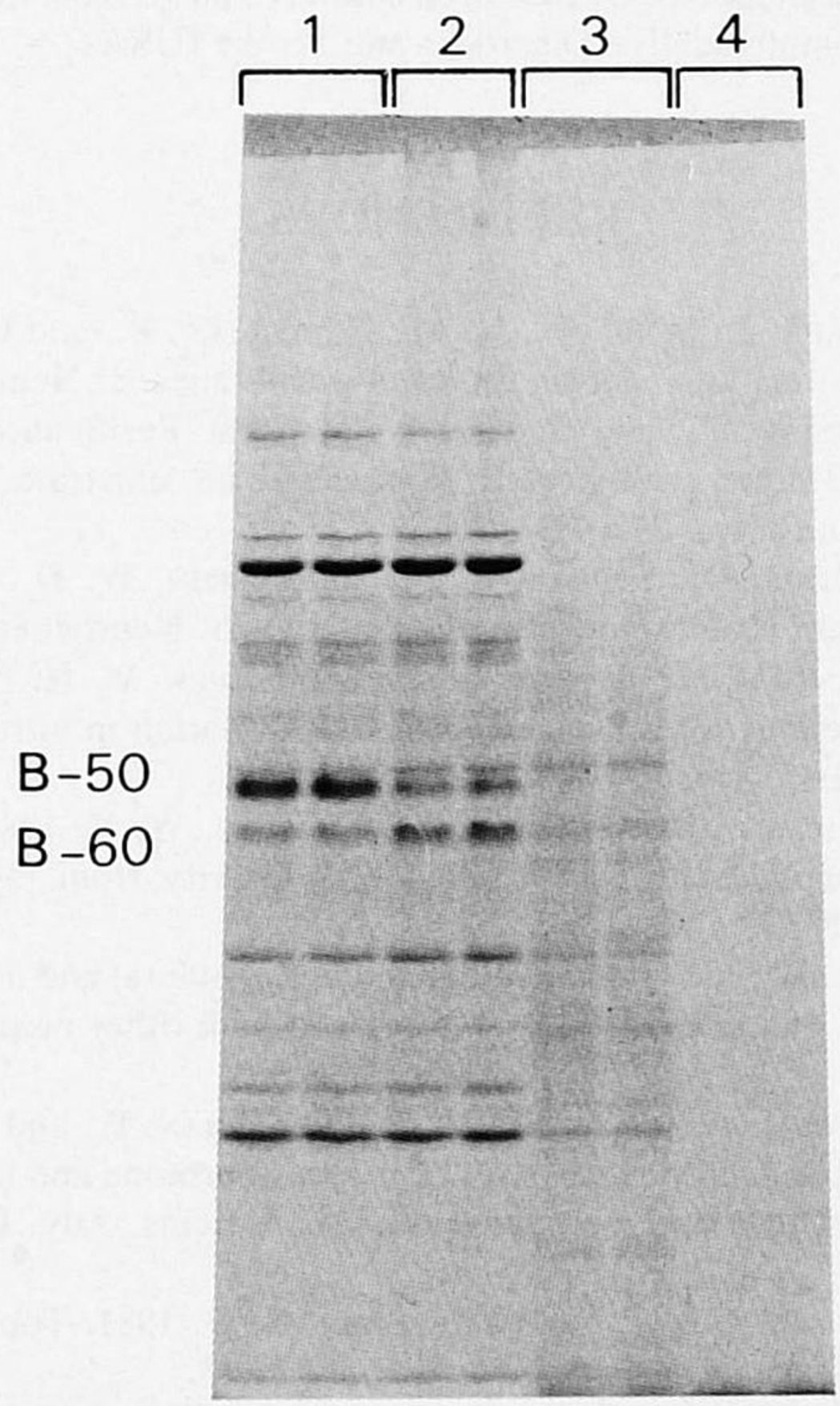

FIG. 4B. Effect of calmodulin on proteolysis. 1, control, non-incubated; 2, incubation for 8 hrs at 30°C; 3, incubated with 1.8 μg calmodulin; 4, with 1.8 μg calmodulin and 2 mM Ca^{2+}. In all conditions 1 mM EGTA was present.

contrast, when these modulators are added an overall non-specific breakdown of proteins was obtained. At present it is unclear whether calcium activates an endogenous protease other than that responsible for the B-50/B-60 conversion or whether it affects the substrate specificity or acceptability of the B-50 protease.

If the specific proteolytic breakdown of B-50 in SPM is to have a physiological role, it is tempting to conclude that the enzyme by splitting B-50 into B-60 and PIP provides a local supply of a peptide (PIP) which can modulate the degree of phosphorylation of B-50 (5), a factor supposed to be involved in membrane polyphosphoinositide metabolism (12, 15).

ACKNOWLEDGMENTS

The authors wish to acknowledge Mrs. Greet Hoekstra for skillful technical assistance. Part of this research was supported by Research Grant NS 08309 from the National Institutes of Neurological and Communicative Disorders and Stroke (USA).

REFERENCES

1. Zwiers, H., Tonnaer, J., Wiegant, V. M., Schotman, P., and Gispen, W. H. 1979. ACTH-sensitive protein kinase from rat brain membranes. J. Neurochem. 33:247–256.
2. Zwiers, H., Schotman, P., and Gispen, W. H. 1980. Purification and some characteristics of an ACTH-sensitive protein kinase and its substrate protein in rat brain membranes. J. Neurochem. 34:1689–1700.
3. Zwiers, H., Veldhuis, D., Schotman, P., and Gispen, W. H. 1976. ACTH, cyclic nucleotides and brain protein phosphorylation in vitro. Neurochem. Res. 1:669–677.
4. Zwiers, H., Wiegant, V. M., Schotman, P., and Gispen, W. H. 1978. ACTH-induced inhibition of endogenous rat brain protein phosphorylation in vitro: structure-activity. Neurochem. Res. 3:455–463.
5. Zwiers, H., Verhoef, J., Schotman, P., and Gispen, W. H. 1980. A new phosphorylation-inhibiting peptide (PIP) with behavioral activity from rat brain membranes. FEBS Lett. 112:168–172.
6. Zwiers, H., Aloyo, V., and Gispen, W. H. 1981. Behavioral and neurochemical effects of the new peptide dynorphin (1–13): comparison with other neuropeptides. Life Sci. 28:2545–2553.
7. Gispen, W. H., Zwiers, H., Wiegant, V. M., Schotman, P., and Wilson, J. E. 1979. The behavioral active neuropeptide ACTH as neurohormone and neuromodulator: The role of cyclic nucleotides and membrane phosphoproteins. Adv. Exp. Med. and Biol. 116:199–224.
8. Mahler, H. R., Kleine, L. P., and Sorensen, R. G. 1981. Topography of synaptic phosphoproteins. Trans. Am. Soc. Neurochem. 12:213.
9. Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulphate and analysis by gel electrophoresis. J. Biol. Chem. 252:1102–1106.

10. WALLACE, R. W., and CHEUNG, W. Y. 1979. Calmodulin: Production of an antibody in rabbit and development of a radioimmunoassay. J. Biol. Chem. 254:6564–6571.
11. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
12. JOLLES, J., ZWIERS, H., VAN DONGEN, C., SCHOTMAN, P., WIRTZ, K. W. A., and GISPEN, W. H. 1980. Modulation of brain polyphosphoinositide metabolism by ACTH-sensitive protein phosphorylation. Nature (Lond.) 286:623–625.
13. WEBER, W., and HILZ, H. 1979. Stoichiometry of cAMP binding and limited proteolysis of protein kinase regulatory subunits R I and R II. Biochem. Biophys. Res. Commun. 90:1073–1081.
14. TAKI, Y., KISHIMOTO, A., INOUE, M., and NISHIZUKA, Y. 1977. Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissue I. J. Biol. Chem. 252:7603–7609.
15. JOLLES, J., ALOYO, V., and GISPEN, W. H. 1981. Molecular correlates between pituitary hormones and behavior. Pages 285–316, *in* BROWN, I. R. (ed.), Molecular approaches to neurobiology, Academic Press, New York.