S100β is a target protein of neurocalcin δ, an abundant isoform in glial cells

Katsuo OKAZAKI, Naoko H. OBATA, Shigeo INOUE and Hiroyoshi HIDAKA*
Department of Pharmacology, Nagoya University School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466, Japan

To clarify the function of neurocalcin δ, an isoform found abundantly in glial cells, we attempted to find its target proteins by using neurocalcin δ-affinity chromatography and the ^{125}I-neurocalcin δ gel-overlay method. The 10, 14, 27, 36 and 50 kDa bands found on SDS/PAGE bound to ^{125}I-neurocalcin δ, and 10, 11, 19, 24, 26, 50 and 70 kDa proteins were eluted from a neurocalcin δ-affinity column in a Ca^{2+}-dependent manner. Sequence analysis of proteolytic peptides revealed the following identities: S100β (10 kDa), S100α (11 kDa), myelin basic protein (19 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa) and tubulin β-chain (50 kDa). A zero-length cross-linking study indicated that 1 mol of S100β bound to 1 mol of neurocalcin δ. With the gel-overlay method, purified S100β protein and calcyclin bound to ^{125}I-neurocalcin δ whereas calgizarrin and calvasculin, other members of the S100 family, did not. These findings suggest that S100β is one of the target proteins of neurocalcin δ, and the neurocalcin δ–S100β complex may be involved in Ca^{2+}-signalling in the glial cell.

INTRODUCTION

Neurocalcin is a Ca^{2+}-binding protein found abundantly in the central nervous system [1]. In 1992, it was purified from bovine brain using W-77 affinity chromatography [2]. The 23 kDa (α- and β-isoforms) and 24 kDa (γ1- and γ2-isoforms) isoforms were separated by C18 reversed-phase h.p.l.c. One complete cDNA and an incomplete cDNA for neurocalcin were cloned in the same year [3]. Each of the deduced amino acid sequences from these cDNAs has high homology, but is not identical, with four other native isoforms. Therefore at least six isoforms of neurocalcin from bovine brain have been identified. The cDNA product expressed, named δ-isoform, with a calculated molecular mass of 22284 Da, was detected as a 24 kDa band on SDS/PAGE. It has three putative Ca^{2+}-binding motifs (EF-hand motifs) and binds 2 mol of Ca^{2+}/mol of δ-isoform, thereby suggesting that the C-terminal EF hand may be degenerated.

Our study group has reported the differential distribution of neurocalcin isoforms found using the immunohistochemical approach [7]. Purified neurocalcin-like immunoreactivity was found in the cytosol of bovine cerebrum, cerebellum and spinal cord as well as in the retina. The distribution of neurocalcin is mainly in the nervous system, but it is also found in the zona glomerulosa of the adrenal gland [5,6]. When antibody against expressed neurocalcin δ was applied, nuclei of the neurons in the spinal cord and dorsal root ganglion were stained. The cytosol of glial cells but not axons in white matter expressed neurocalcin δ-like immunoreactivity [7]. These findings suggest that each of the differently located isoforms may play a distinct role in Ca^{2+}-signalling in sensory systems; however, these remain unknown. In the present study, we attempted to identify and characterize target proteins for neurocalcin δ, mainly by using neurocalcin δ-affinity chromatography and the ^{125}I-neurocalcin δ gel-overlay method.

EXPERIMENTAL

Materials

(*S*)-*p*-(2-Aminoethyloxy)-*N*-[2-(4-benzyloxycarbonylpiperazinyl)-1-(*p*-methyloxybenzyl)]-*N*-methylbenzenesulphonamide dihydrochroride (W-77) was synthesized by the method of Hidaka et al. [8]. W-77 was coupled to epoxy-activated Sepharose 6B (Pharmacia LKB Biotechnology, Uppsala, Sweden) as described by Endo et al. [9]. All reagents were of reagent grade or better.

Preparation of expressed neurocalcin δ

Neurocalcin δ was expressed in *Escherichia coli* as a maltose-binding protein fusion protein, using pMAL™-c. The fusion protein was expressed and digested according to the manufacturer's (New England BioLabs) instructions. Expressed fusion protein was isolated from bacterial lysate using affinity chromatography with amylose resin, followed by elution with 10 mM maltose. Factor Xa cleavage was carried out at a ratio of factor Xa to fusion protein of 1:800 (w/w) at 4 °C for 48 h. Ion-exchange chromatography using Q-Sepharose was used to isolate the expressed δ-isoform from maltase-binding protein with a linear 0–0.5 M NaCl gradient. The fraction containing semi-purified neurocalcin δ was subjected to W-77-affinity chromatography in the presence of 1 mM $CaCl_2$ and eluted with 30 mM Tris/HCl, pH 7.5, containing 1 mM EGTA (buffer A). The fraction obtained was dialysed against 10 mM NH_4HCO_3, then lyophilized.

Neurocalcin δ-affinity chromatography

All procedures were carried out at 4 °C unless otherwise indicated. The neurocalcin δ-affinity column was prepared by coupling 100 mg of purified neurocalcin to CNBr-activated Sepharose 4B (20 ml gel volume), according to the manufacturer's (Pharmacia) instructions.

Bovine brain (200 g) was homogenized in buffer A, and the homogenate was centrifuged at 100000 *g* for 1 h. After the addition of 1 mM $CaCl_2$, the supernatant was applied to a neurocalcin δ-affinity column equilibrated with buffer B (30 mM

Abbreviations used: PSL, photostimulated luminescence; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; NHS, *N*-hydroxysuccinimidyl esters.
* To whom correspondence should be addressed.

Tris/HCl, pH 7.5, 1 mM $CaCl_2$). After the column had been washed with buffer B, elution was carried out with buffer A.

Amino acid sequence analysis

Purified proteins were digested with either lysyl-endopeptidase at 30 °C for 8 h or with *Staphylococcus aureus* V8 protease at 37 °C for 10 h. Proteolytic fragments were separated on a C18 reversed-phase column (Shimadzu ODS-M; 4 mm × 150 mm) using a Shimadzu h.p.l.c. system (LD-6A) with a linear gradient of 0–80 % acetonitrile in the presence of 0.1 % trifluoroacetic acid, for 70 min. The protein sequence of each fragment was analysed on an Applied Biosystems 473A protein sequencer.

^{125}I-neurocalcin δ gel-overlay method

Purified expressed neurocalcin δ was ^{125}I-labelled with Bolton–Hunter reagent [10]. The gel-overlay technique used was that described by Glenny and Weber [11]. Briefly, after electrophoresis, the gel was fixed in 40 % methanol/7 % acetic acid for 30 min, rinsed several times with distilled water, and incubated for 3 h with 10 % ethanol. It was then incubated with 0.1 M imidazole/HCl, pH 7.0, for 10 min and with 20 mM imidazole/HCl, pH 7.0, containing 0.2 M KCl, 0.1 % BSA, 0.02 % NaN_3 and either 1 mM $CaCl_2$ or 1 mM EGTA (buffer C or buffer D respectively) for 10 min. The gel was incubated with either buffer C or buffer D containing expressed ^{125}I-neurocalcin δ (20 ml; 2×10^6 c.p.m./μg of protein per ml) for 20 min at 4 °C and washed for 24 h with either buffer C or buffer D. The image was visualized for analysis, and the photostimulated luminescence (PSL) value was measured using a bioimage analyser (Model Fujix BAS2000, Fuji Film Corp).

Zero-length cross-linking study of neurocalcin δ

Two-step coupling of neurocalcin δ and S100β was carried out in solution using 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC) and *N*-hydroxysuccinimidyl esters (NHS), by the method of Grabarek and Gergely [12]. Neurocalcin δ (12.5 μg) was incubated at room temperature for 15 min in a buffer containing 20 mM Mes, 0.26 mg/ml EDC and 0.43 mg/ml NHS. Then 20 mM 2-mercaptoethanol (final concentration) and 25 μg of S100β were added and the mixture was incubated for 2 h at room temperature. Samples were electrophoresed and stained with Coomassie Brilliant Blue or by using the Silver Stain Plus kit (Bio-Rad).

Purification of S100β

S100β was purified from bovine brain by the modified method of Isobe et al. [13]. Briefly, bovine brain was chopped and homogenized in a cold-room (4 °C) in buffer A and centrifuged at 100000 *g* for 60 min. To the supernatant was added solid $(NH_4)_2SO_4$ [about 85 %-satd. $(NH_4)_2SO_4$] and the pH was adjusted to 4.7 with NaOH. The precipitate was collected by centrifugation, dissolved in and dialysed extensively against buffer A. The extract was chromatographed on DEAE-Sephadex A-50 with a gradient of 0–0.5 M NaCl. Fractions containing 9 and 10 kDa proteins were collected separately. $CaCl_2$ was added to each fraction (final 2 mM). The sample was applied to a phenyl-Sepharose CL-4B column and eluted with buffer A. A 10 kDa band was isolated as a single band on SDS/PAGE. With the fraction containing 9 kDa protein, 9 and 30 kDa proteins were isolated in a Ca^{2+}-dependent manner.

Other methods

Protein concentration was determined by the method of Bradford [14] with BSA as standard. Tricine SDS/PAGE was performed as described by Schagger and von Jagow [15]. Analysis of homology and hydrophobicity was carried out with a Macintoch computer using GENETYX software.

RESULTS

^{125}I-neurocalcin δ gel overlay

Bovine brain homogenate (20 μg) was subjected to SDS–PAGE and the gels were incubated in buffer containing ^{125}I-neurocalcin δ. Several bands were detected as neurocalcin δ-binding proteins in the presence of 1 mM $CaCl_2$. The molecular masses of the major bands were 10, 14, 27, 36 and 50 kDa (Figure 1a). In the presence of 1 mM EGTA, no bands were detected. When the fraction eluted from the neurocalcin δ-affinity column was applied, 10 and 36 kDa bands were detected in the presence of 1 mM $CaCl_2$.

Neurocalcin δ-affinity chromatography

Seven major proteins were eluted from a neurocalcin δ-affinity column in a Ca^{2+}-dependent manner; the molecular masses were 10, 11, 19, 24, 36, 50 and 70 kDa (Figure 1b). Fractions were collected and separated on a C18 reversed-phase column with a linear gradient of 0–80 % acetonitrile in the presence of 0.1 % trifluoroacetic acid (Figure 2): six major peaks were isolated. Each protein obtained was lyophilized, digested, separated on a C18 reversed-phase column and sequenced. Table 1 shows the amino acid sequence of the proteolytic peptides from each peak. Tentative identification of each peptide sequence was carried out by a computer-aided homology search. The partial amino acid sequences of the 19 kDa protein (peak 1) were AQHGRPQDEDPVVHF and GXGLXLSRFSXGAEG, which were identified as myelin basic protein. The partial amino acid sequences of the 36 kDa protein (KVIELN and LWRDGRG from peak 2) were tentatively identified as glyceraldehyde-3-phosphate dehydrogenase. KDVNAAIATIK from the 50 kDa

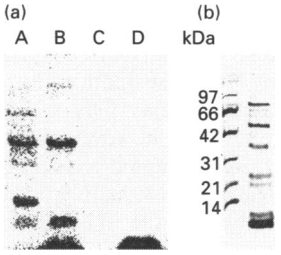

Figure 1 (a) ^{125}I-neurocalcin δ gel overlay and (b) ^{125}I-neurocalcin δ-affinity chromatography

(**a**) Bovine brain homogenate (lanes A and C) and the fraction eluted from a ^{125}I-neurocalcin δ-affinity column (lanes B and D) (20 μg each) were subjected to SDS/PAGE. After the treatment as described in the Experimental section, the gel was incubated with ^{125}I-neurocalcin δ in the presence of 1 mM $CaCl_2$ (lanes A and B) or EGTA (lanes C and D). (**b**) Bovine brain homogenate was applied to a neurocalcin δ-affinity column in the presence of 1 mM $CaCl_2$ and eluted in the presence of EGTA. Seven major bands were detected on SDS/PAGE.

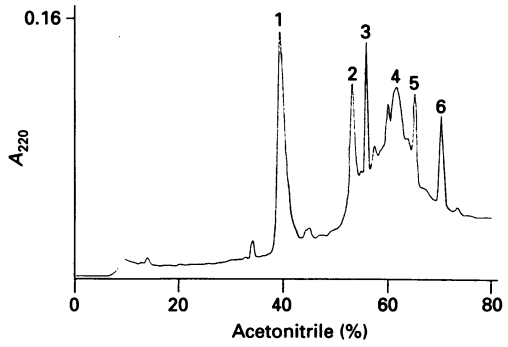

Figure 2 Isolation of neurocalcin δ-binding proteins by h.p.l.c.

The fractions obtained from neurocalcin δ-affinity chromatography were separated on a C18 reversed-phase h.p.l.c. column. Six major peaks (1–6) were collected, digested with proteases and analysed on a protein sequencer.

Table 1 Partial amino acid sequences of neurocalcin δ-binding proteins

	Peak sequence of proteolytic fragment	Identified protein
1	AQHGRPQDEDPVVHF GXGLXLSRFSXGAEG	Myelin basic protein
2	KVIELN and LWRDGRG	Glyceraldehyde-3-phosphate dehydrogenase
3	DTDSEEEIREAFRV	Calmodulin
4	GDKYKLSKKE	S100α
5	KDVNAAIATIK	Tubulin β-chain
6	VMETLDNDGDGECDFQEFMAFVAMV	S100β

protein (peak 5) appeared to be tubulin β-chain, and GDKYKLSKKE from the 11 kDa protein (peak 4) was tentatively identified as S100α. The amino acid sequence of the peak-3 peptide was DTDSEEEIREAFRV which was tentatively identified as calmodulin, and that of the peptide of the 10 kDa protein (peak 6) was VMETLDNDGDGECDFQEFMAFVAMV, identified as the sequence of S100β protein. The 70 kDa protein was not sequenced because it was lost during the h.p.l.c. procedure. The 23 kDa and 46 kDa bands reacted with the antibody against neurocalcin δ on immunoblot analysis.

^{125}I-neurocalcin δ binding of S100β and other S100 family proteins

To confirm the specific binding of S100β to neurocalcin, we used a procedure of ^{125}I-neurocalcin δ gel overlay with purified S100β. The 9 and 20 kDa proteins obtained in the purification of S100 were digested and sequenced. LSHFLE and EFEHE from the 9 kDa protein was identified as S100β and AVVALIDVFHQY from the 10 kDa protein was also identified as S100β. Both 9 and 10 kDa proteins identified as S100 β bound to ^{125}I-neurocalcin δ in a Ca^{2+}-dependent manner (Figure 3). We also examined the Ca^{2+}-dependent binding of calmodulin and other members of the S100 family to ^{125}I-neurocalcin δ to determine whether the binding of S100β to neurocalcin δ is specific. We subjected 1 μg of S100β, calcyclin, calgizzarin, calvasculin and calmodulin to SDS/PAGE and incubated the gel in buffer containing ^{125}I-neurocalcin δ in the presence of 1 mM $CaCl_2$ or EGTA. S100β and calcyclin bound to ^{125}I-neurocalcin δ in the presence of Ca^{2+}

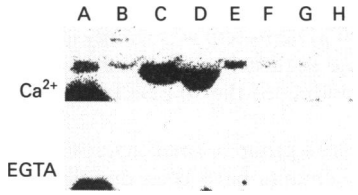

Figure 3 Binding of Ca^{2+}-binding proteins to ^{125}I-neurocalcin δ

Lane B, semipurified neurocalcin δ; Lane C, S100β (10 kDa); lane D, S100β (7 kDa); lane E, calcyclin; lane F, calgizzarin; lane G, calvasculin; lane H, calmodulin; 1 μg was applied to each lane. Lane A, 10 kDa bands homogenate from bovine brain homogenate.

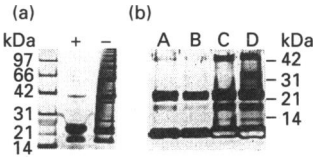

Figure 4 (a) Effect of a reducing agent on polymerization of neurocalcin δ and (b) zero-length cross-linking study of S100β and neurocalcin δ

(**a**) Semipurified neurocalcin δ (3 μg) was electrophoresed in the presence (+) or absence (−) of 2-mercaptoethanol. The gel was stained with Coomassie Brilliant Blue. (**b**) Neurocalcin δ and S100β were incubated in the presence of 1 mM EGTA (lanes A and C) or $CaCl_2$ (lanes B and D) as described in the Experimental section. The assay mixture was electrophoresed and stained with Coomassie Brilliant Blue (lanes A and B) or by a silver stain kit (lanes C and D).

but not in its absence. The PSL value of calcyclin was smaller than that of S100β [651 compared with 5590 (lane D) PSL/70 mm^2]. The PSL values of calgizzarin (lane F) and calvasculin (lane G) were 53 and 20 PSL/70 mm^2 respectively in the presence of Ca^{2+}. The PSL value of calmodulin, a 4-EF-hand Ca^{2+}-binding protein, was not measurable. We prepared a calcyclin-affinity column coupled with CNBr-activated Sepharose 4B and examined the binding of neurocalcin δ to calcyclin: neurocalcin δ was trapped in the calcyclin-affinity column and eluted in the presence of EGTA (results not shown).

Stoichiometry of S100β binding to neurocalcin δ

When semipurified neurocalcin δ was subjected to SDS/PAGE in the presence of the reducing agent 2-mercaptoethanol, there appeared a 24 kDa band, which was neurocalcin δ, a 46 kDa band, which was its dimer, and a 17 kDa band, which was an incomplete form of neurocalcin δ generated during the purification procedure. On SDS/PAGE in the absence of 2-mercaptoethanol, multipolymer bands were detected (Figure 4a).

Stoichiometry of S100β to neurocalcin δ was analysed by the zero-length cross-linking method using EDC in the presence of NHS. In the presence of Ca^{2+}/neurocalcin δ, the molecular mass of the cross-linking product was found to be 33 kDa on SDS/PAGE (Figure 4b). In the presence of EGTA, cross-linking products were not observed. Complete (24 kDa) and incomplete (17 kDa) forms of neurocalcin δ and their polymers (46 kDa and 70 kDa) were found in both lanes.

DISCUSSION

In this study, we searched for target proteins of neurocalcin δ using both ^{125}I-neurocalcin gel overlay and neurocalcin δ-affinity chromatography. In each experiment, several bands were

detected as candidates. Both methods detected 10 and 36 kDa bands, and the 10 kDa protein was readily identified as S100β by partial amino acid sequencing. Therefore we initially attempted to confirm the binding of the 10 kDa protein, S100β, to neurocalcin δ.

S100 proteins are a group of small acidic Ca^{2+}-binding proteins [16]. Two S100 subunits have been described, α and β. Three S100 dimeric isoforms have been isolated and characterized: S100αo, S100α and S100β, which differ in their subunit compositions ($\alpha\alpha$, $\alpha\beta$ and $\beta\beta$ dimers respectively). In the nervous system, expression of S100β is restricted to glial cells, whereas S100α is expressed, at a much lower level, in some neuronal cells as well as in non-nervous tissue. We isolated 9 and 10 kDa protein from bovine brain using Ca^{2+}-dependent phenyl-Sepharose chromatography. Partial amino acid sequences of these proteins were identified as S100β. The 9 kDa protein may be a heterogeneous or degraded form.

Neurocalcin δ has a high affinity for S100β, a glia-specific protein. Among other members of the S100 family, calcyclin [17], calvasculin [18] and calgizzarin [19], which are not expressed in the nervous system, had lower affinities than S100β as assessed by the gel-overlay method. Calcyclin is composed of 90 amino acids and, like S100β, has a hydrophilic structure at the C-terminus. Calvasculin and calgizzarin have additional structures at the C-terminus, which is rich in hydrophobic amino acids in both cases (Figure 5). The differences in binding affinities to neurocalcin δ among the members of the S100 family may be due to the differences in hydrophobicity at the C-terminus.

In a study of stoichiometry by the method of zero-length cross-linking, a 33 kDa band was formed in the presence of Ca^{2+}. It suggested that 1 mol of S100β bound to 1 mol of neurocalcin δ in a Ca^{2+}-dependent manner. On SDS/PAGE, neurocalcin δ was detected as multipolymer bands in the absence of 2-mercaptoethanol. The 24 kDa band, which was eluted from the neurocalcin δ-affinity column and coexisted with myelin basic protein in a fraction of peak 1 after h.p.l.c., reacted with antibody against neurocalcin δ. The binding of S100β to the polymer of neurocalcin δ cannot be discussed using results from a zero-length cross-linking study only, although its binding to neurocalcin δ may regulate the polymerization of neurocalcin δ in the cells.

(a)

S100β	DSDGDGECDFQEFMAFVAMITTACHEFFEHE
S100α	DEDGDGEVDFQEYVVLVAALTVACNNFFWENS
Calcyclin	DRNKDQEVNFQEYVTFLGALALIYNEALKG
Calgizzarin	DSNRDNEVDFQEYCVELSCIAMMCNEFFEGCPDKEPRKK
Calvasculin	DLNSDGQLDFQEFLNLIGGLAVACHESFVKAAPPQKRF

(b)

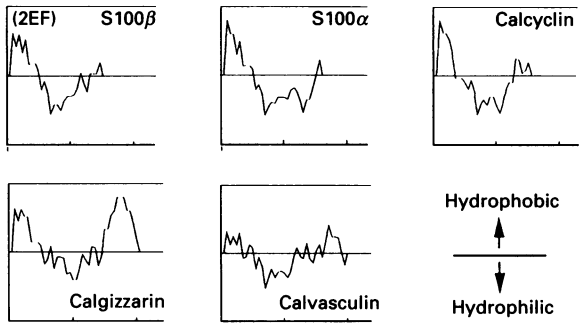

Figure 5 (a) Comparison of amino acid sequences of members of the S100 family and (b) hydropathy plots of the S100 family members

(**a**) The amino acid sequence from the second EF-hand motif (2EF) to the C-terminus. (**b**) Hydrophobicities of the amino acid sequences demonstrated in (**a**) were analysed.

We have reported that neurocalcin δ is expressed mainly in glial cells. On the basis of these findings, we speculate that neurocalcin δ and S100β may form a complex at high Ca^{2+} concentrations and be involved in Ca^{2+}-signalling in glial cells or in the protection against high-Ca^{2+} toxicity.

A 36 kDa protein from neurocalcin δ-affinity chromatography was sequenced and identified as glyceraldehyde-3-phosphate dehydrogenase. This is a glycolytic enzyme that has also been reported to be a target protein of calcyclin [20]. In ^{125}I-neurocalcin δ gel overlay, 36 and 50 kDa bands from bovine homogenate were positively stained. However, these bands from neurocalcin δ-affinity chromatography did not bind to ^{125}I-neurocalcin δ. This discrepancy in results may be the result of denaturation of these proteins during the neurocalcin δ-affinity chromatography procedure. This possibility appears to be corroborated by the evidence that neurocalcin δ formed polymers during neurocalcin δ-affinity chromatography and zero-length cross-linking studies, but bound only weakly to ^{125}I-neurocalcin δ. However, we may have to search for other 36 or 50 kDa proteins as target proteins for neurocalcin δ. In the ^{125}I-neurocalcin δ gel-overlay study, 14 and 27 kDa bands were positively stained. Further investigations of this point are in progress.

During an immunohistochemical study now in progress, we have found neurocalcin immunoreactivity in microtubules (K. Okazaki, S. Iino, S. Kobayashi and H. Hidaka, unpublished work), and association with microtubuli has been reported for other Ca^{2+}-binding proteins such as calbindin-28K [21]. Further examinations need to be carried out, although we have obtained negative data in the ^{125}I-neurocalcin gel-overlay experiment as mentioned above. S100β, S100α, tubulin and calmodulin, which were eluted from a neurocalcin δ-affinity column, are Ca^{2+}-binding proteins. These findings suggest the importance of interactions among Ca^{2+}-binding proteins.

Myelin basic protein is a 13–21 kDa protein and is specific to glial cells. Our two antibodies against native and expressed neurocalcin δ did not react with myelin, whereas axon has immunoreactivity to the antibody against native neurocalcin [7]. Moreover, this binding may reflect a tendency for the basic protein to bind to the acidic protein. Therefore evidence supporting the binding of myelin basic protein to neurocalcin δ is still lacking.

The present study has demonstrated that neurocalcin δ has a high affinity for members of the S100 family, especially S100β protein. Several Ca^{2+}-binding proteins and an enzyme are candidates as neurocalcin δ-binding proteins. We propose that each neurocalcin isoform has a distinct function or multifunctions and is involved in Ca^{2+}-signalling in neural cells.

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REFERENCES

1 Hidaka, H. and Okazaki, K. (1993) Neurosci, Res. **16**, 73–77
2 Terasawa, M., Nakano, A., Kobayashi, R. and Hidaka, H. (1992) J. Biol. Chem. **267**, 19596–19599
3 Okazaki, K., Watanabe, M., Ando, Y. Hagiwara, M., Terasawa, M. and Hidaka, H. (1992) Biochem. Biophys. Res. Commun. **185**, 147–153
4 Nakano, A., Terasawa, M., Usuda, N., Morita, T. and Hidaka, H. (1992) Biochem. Biophys. Res. Commun. **186**, 1207–1211
5 Nakano, A., Watanabe, M., Okazaki, K., inoue, S., Kato, M., Nimura, Y., Usuda, N., Morita, T. and Hidaka, H. (1992) J. Endocrinol. **138**, 283–290
6 Bastianelli, E., Okazaki, K., Hidaka, H. and Pochet, R. (1993) Neurosci. Lett. **161**, 165–168
7 Okazaki, K., Iino, S., Kobayashi, S. and Hidaka, H. (1994) Biochim. Biophys. Acta, **1223**, 311–317

8 Hidaka, H., Inagaki, M., Kawamoto, S. and Sasaki, Y. (1984) Biochemistry **23**, 5036–5041
9 Endo, T., Tanaka, T., Isobe, T., Kasai, H., Okuyama, T. and Hidaka, H. (1981) J. Biol. Chem. **256**, 12486–12489
10 Bolton, A. E. and Hunter, W. M. (1973) Biochem. J. **133**, 529–539
11 Glenny, J. R. and Weber, K. (1980) J. Biol. Chem. **255**, 10551–10554
12 Grabarek, Z. and Gergely, J. (1990) Anal. Biochem. **185**, 131–135
13 Isobe, T., Nakajima, T. and Okuyama, T. (1977) Biochim. Biophys. Acta **494**, 222–232
14 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
15 Schagger, H. and von Jagow, G. (1987) Anal. Biochem. **166**, 368–379
16 Hilt, D. C. and Kligman, D. (1991) in Novel Calcium-Binding Proteins (Heizmann, C. W., ed), pp. 65–103, Springer-Verlag, Berlin
17 Tokumitsu, H., Kobayashi, R. and Hidaka, H. (1991) Arch. Biochem. Biophys. **291**, 202–207
18 Watanabe, Y., Kobayashi, R., Ishikawa, T. and Hidaka, H. (1992) Arch. Biochem. Biophys. **292**, 563–569
19 Watanabe, M., Ando, Y., Todoroki, H., Minami, H. and Hidaka, H. (1991) Biochem. Biophys. Res. Commun. **181**, 644–649
20 Filipek, A., Gerke, V., Weber, K. and Kuznicki, J. (1991) Eur. J. Biochem. **195**, 795–800
21 Legrand, C., Clos, J., Legrand, J., Langley, O. K., Ghandour, M. S., Labourdette, G. and Gombos, D. (1981) Neuropathol. Appl. Neurobiol. **7**, 299–306

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