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Involvement of HPC-1/Syntaxin-1A antigen in transmitter release from PC12h cells

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We examined the effect of antiserum against HPC-1/Syntaxin-1A on the norepinephrine release from digitonin-permeabilized PC12h cells. PC12h cells were permeabilized with digitonin and preincubated with nonimmunized serum or antiserum against HPC-1. The release of norepinephrine was measured in the presence or absence of calcium. The calcium-dependent norepinephrine release was increased in the cells preincubated with anti HPC-1 antiserum. However, with a higher concentration of anti HPC-1 antiserum, the calcium-dependent norepinephrine release was decreased, possibly because of a nonspecific effect. In the case of purified IgG, the same results were obtained. These findings suggested that HPC-1 plays an important role in the exocytosis of transmitter presumably by suppressing the membrane fusion process between the synaptic vesicle and presynaptic membrane.

HPC-1 antigen was originally found as a neuron-specific protein (1). HPC-1 was found on the plasma membrane of axons and presynaptic terminals both in the central and peripheral nervous systems (2,3), and in endocrine cells (4). We have already reported the deduced amino acid sequences of HPC-1 from the rat and bovine brain (1,5). From the database search, we found that HPC-1 was identical to syntaxin 1A (6). This protein is homologous to epimorphine which was an essential factor of epithelial morphogenesis (5). HPC-1 is involved in a protein family of which members are closely related to function in the intracellular membrane transporting mechanisms between either ER, Golgi apparatus, or secretory vesicles (7,8,9). HPC-1 is associated with an N-type calcium channel which localizes on the presynaptic membrane and also is associated with synaptotagmin which is present on the cytosolic surface of synaptic vesicles(10). In this context, although the precise physiologic meaning has not been

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elucidated yet, HPC-1 was implicated in the involvement in the exocytotic process in the presynaptic terminals. It is generally believed that chemical transmitters are stored in the synaptic vesicles and are released through exocytosis. This process is triggered by elevation of intracellular calcium ion. Since digitonin-permeabilized PC12 cells are exocytosis competent and some macromolecules can be introduced into the permeabilized cells, we examined the effect of the antiserum against HPC-1 on the release of norepinephrine from digitonin-permeabilized PC12 cells to examine the role of HPC-1 in the process of neurotransmitter exocytosis.

Materials and Methods

PC12h cells were originally subcloned by Hatanaka et al.(11). In this clone, tyrosine hydroxylase activity was higher than the parent clone PC12. PC12h cells were cultured in DME supplemented with 5% precolostrum newborn calf serum and 5% horse serum. To examine the localization of HPC-1 in PC12h cells, cells were plated onto collagen-coated 35mm dishes. The cells were fixed with 4% paraformaldehyde for 30 min and washed with Ca^{2+}, Mg^{2+}-free PBS. Then, the cells were incubated with antiserum against HPC-1 at 4℃ overnight followed by the incubation with rhodamine-conjugated anti-rabbit IgG (Cappel).

Electrophoresis for Western blotting was performed in 12.5% SDS-PAGE with urea as described previously (12). Tissues from adult rats and PC12h cells were homogenated in 10 volumes (brain) or 2~3 volumes (PC12h) of Ca^{2+},Mg^{2+}-free PBS with 10 mM PMSF at 0℃. Nuclear pellets were removed by centrifugation at 1,000× g for 10 min at 4℃. Membrane fractions were collected by ultracentrifugation at 100,000×g for 1hr at 4℃. Protein concentrations were determined by Bio-Rad protein assay kit. Proteins were transferred to PVDF membrane and visualized by ECL system (Amersham). Antisera were raised in Japanese White Rabbit against the fusion protein expressed in E. coli except for the membrane bound region.

To measure the norepinephrine release, PC12h cells were cultured on a collagen-coated 24-well multi-plate. Cells were seeded at a density of about $5\sim8\times10^4$ cells/cm^2 and cultured for at least 4days in an incubator at 37℃ in a humid atmosphere of 5% CO_2. The method of the release measurement was processed as described by Holz et al. (13). Briefly, cells were preloaded with [3]H-norepinephrine (30-50Ci/mmol, 1mCi/ml) at 37℃ for 2~18 hrs in a culture medium supplemented with 0.5mM ascorbate. In these experiments, 23nM norepinephrine was preloaded to the cells/well. Then, the cells were washed twice with culture medium and further cultured for 2 hrs. Before the addition of digitonin, cells were left in buffer K (139mM potassium glutamate, 20mM PIPES, 2mM MgATP, 1mM $MgCl_2$, 5mM EGTA, 5mM glucose) for 10 min at room temperature. Then, cells were permeabilized with 7.5μM digitonin in buffer K containing antiserum or antibody for 6 min at room temperature. Digitonin was washed out, and preincubation with antiserum was continued for a further 18min. Calcium-dependent release was measured by addition of 10μM free-calcium (calculated with EGTA protonation constants from Martell and Smith(14)) in buffer K. After 15min, the buffer was centrifuged at 1,000×g to remove the detached cells, and the radioactivity in the supernatant was counted with a scintillation counter. To solubilize the cells, 1% Triton X-100/buffer K was added to the well. The radioactivity remaining in the cells was also counted. The sum total of released and remained radioactivity was

regarded as the whole amount of NE taken up into the cells. The amount of secretion was calculated as percentage of radioactivity in the supernatant compared to total activity recovered.

Results and Discussion

PC12 cells have been reported to express HPC-1 (15), but there have been no reports about PC12h cells. Figure 1 shows the immunofluorescent labeling of HPC-1 antigen on PC12h cells. The plasma membrane of all PC12h cells were clearly stained. It was obvious that, in the presence of NGF, the plasma membrane of cell soma was labeled as well as the processes. To confirm the expression of HPC-1 in PC12h cells, membrane fraction of the cells were immunoblotted and detected with polyclonal and monoclonal antibody against HPC-1. By using polyclonal antibodies, two main bands were shown in PC12h as well as the brain (Fig. 2, lane 1, 2). The upper one was syntaxin-1B, and the lower one was HPC-1. We raised a monoclonal antibody 14D8 which recognized only 6His-HPC-1 (recombinat rat HPC-1 expressed in E. coli) but not 6His-syntaxin-1B (Fig. 2, lane 7, 8). Both recombinant proteins were recoginized by the polyclonal anti HPC-1 antibodies (Fig. 2, lane 5, 6). The ladder bands of 6His-HPC-1 maight be caused by the stopping of translations before termination. HPC-1 and syntaxin-1B

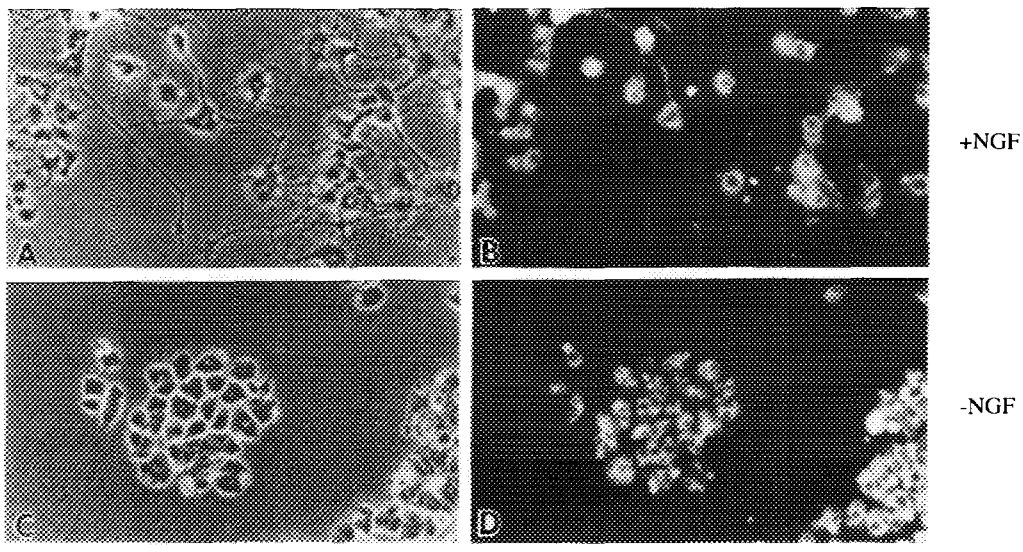

Figure 1. Immunohistochemical staining of PC12h cells.
PC12h cells were cultured in serum-free medium supplemented with NGF (50 ng/ml) (A, B) and serum-containing medium (C,D) for 1 week. They were fixed and stained with anti HPC-1 antiserum using rhodamine-conjugated 2nd antibody.

were very similar, so it was surprising that 14D8 recoginized only 6His-HPC-1. By the 14D8 antibody, only one band was shown in PC12h and brain membrane (Fig. 2, lane 3, 4). Therefore, PC12h cells expressed HPC-1 and the mobility in SDS-PAGE was the same as that in the brain.

To examine the effect of the antiserum against HPC-1 on transmitter exocytosis, PC12h cells were treated with digitonin as described in materials and methods. Figure 3 shows the effect of the antiserum on the calcium-dependent norepinephrine release. The values are normalized to preimmune serum. The 1/1000 diluted antiserum enhanced the calcium-dependent NE release about 2-fold. When cells were treated with the 1/100 diluted antiserum, the calcium-dependent NE release was not changed in this case, but sometimes decreased (data not shown). Another lot of anti HPC-1 antiserum diluted appropriately also increased the calcium-dependent NE release (data not shown). The two different polyclonal antisera against HPC-1 increased the calcium-dependent NE release, probably because HPC-1 was involved in the inhibitory mechanism on transmitter exocytosis. The decreases of calcium-dependent NE release at a high concentration of the antiserum may have been caused by a non-specific

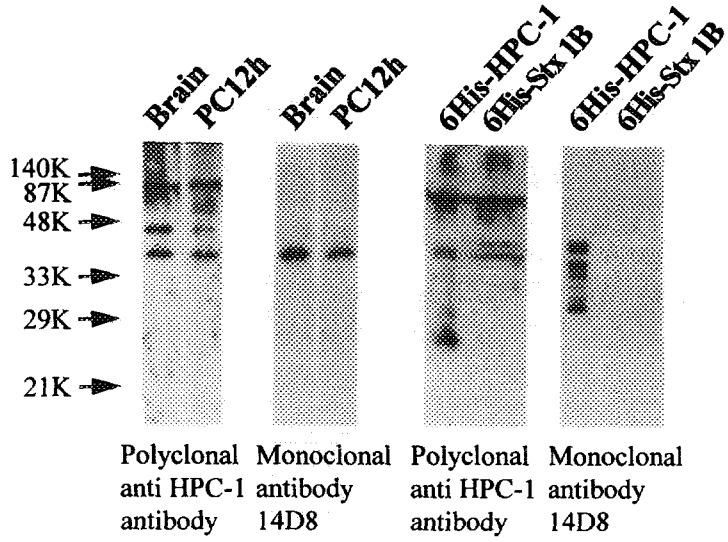

Figure 2. Western blotting of PC12h and brain membrane.
PC12h and brain membrane fractions were analyzed by SDS-PAGE and transferred to PVDF membrane. 6His-HPC-1 and 6-His-Stx 1B are recombinant proteins of HPC-1 and syntaxin-1B expressed in E. coli. The 14D8 antibody recognized only 6His-HPC-1 but not 6-His-Stx 1B. Two main bands were detected with polyclonal anti HPC-1 antiserum, and one band was detected with monoclonal antibody 14D8. Then, HPC-1 must be present in PC12h cells.

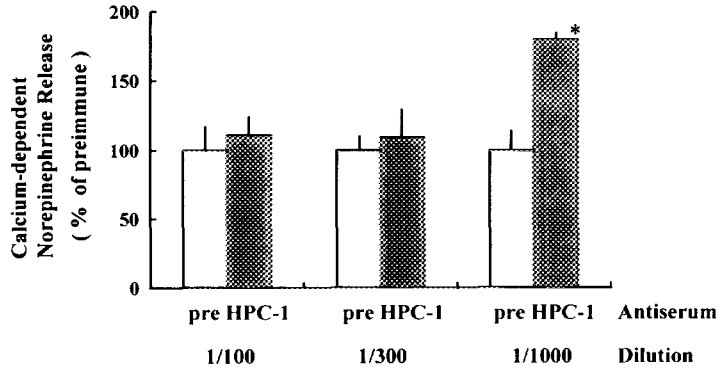

Figure 3. Diluted anti HPC-1 antiserum increased the calcium-dependent NE release. The anti HPC-1 antiserum was diluted as indicated in the figure. Pre was non-immunized rabbit's antiserum. NE release was assayed as described in the materials and methods. Calcium-dependent NE release was represented as % value of the preimmune. Each value is the mean±S.E., n=4; *, P<0.01 in comparison with the preimmune (ANOVA).

interaction of antibodies on the plasma membrane. We tested the effect of protein A purified IgG on the calcium-dependent NE release. As Fig. 4 shows, the calcium dependent NE release was about 2-fold higher at 2.5 or 10.0 μg/ml of anti HPC-1 IgG than preimmune IgG. At 0.625 μg/ml, calcium-dependent NE release was also increased but only slightly. These findings indicate that the increase of calcium-dependent NE release with antiserum against HPC-1 was due to the interaction of antibody of HPC-1 to the antigen.

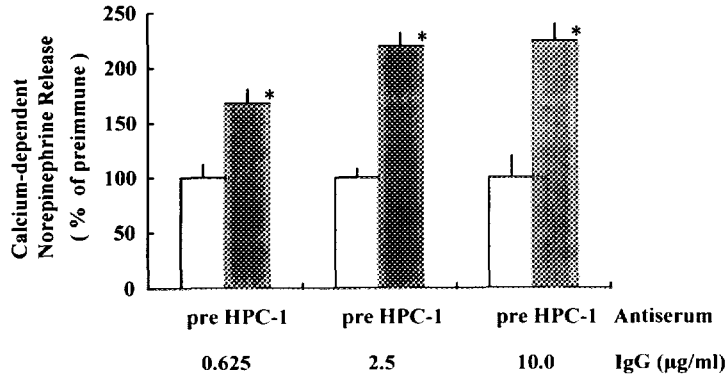

Figure 4. Purified anti HPC-1 IgG also increased the calcium-dependent NE release. Anti HPC-1 IgG was purified by protein A and dialyzed to water and dried. Before use, it was added to buffer K at the concentration in the figure. Data were represented as in figure 3. Each value is the mean±S.E., n=4; *, P<0.01 in comparison with the preimmune (ANOVA).

By contrast, there is a report that antibodies against HPC-1 inhibit calcium-dependent catecholamine secretion from digitonin-permeabilized chromaffin cells (16). The possible reason for this discrepancy is the differences of antibodies and cells. They used monoclonal antibodies and chromaffin cells. Antibodies, especially monoclonal antibodies, do not usually inhibit the functions depending on the antigen. We used two different polyclonal antibodies and obtained the same results. Therefore, HPC-1 was inhibited and resulted in the increase of calcium dependent NE release. In addition, they indicated that the secretion was decreased at more than 25 μg/ml of IgG. As Fig.4 shows, the increase of NE release were demonstrated at less than 10 μg/ml of IgG. This difference might be for the different titers of the IgG's, and/or the different recognition site of the antibodies. In other strategy of the inhibition of HPC-1 in living PC12 cells, Bennet et al. microinjected soluble fragments of syntaxin, which resulted in a decrease in the percentage of cells with high levels of surface dopamine β-hydroxylase, DβH, and a corresponding increase in the percentage of cells with low levels of surface DβH (15). In addition, Blasi et al. reported that botulinum neurotoxin type C1 (BoNT/C1) inhibited glutamate release from isolated nerve terminals, and that the inhibition was associated with the selective breakdown of HPC-1(17). The BoNT/C1-induced cleavage caused a soluble fragment of HPC-1, so it might result in the generation of soluble HPC-1 fragment in the synaptosome. In both studies, the soluble HPC-1 fragment was increased in the cell or synaptosome. If the soluble fragments gave rise to the competitive inhibition with membrane-anchored HPC-1, HPC-1 would function as an accelerator on exocytosis. On the other hand, if the soluble HPC-1 fragments had functional activity on transmitter exocytosis as membrane-bound HPC-1, the decrease of exocytosis suggested that HPC-1 had a function as a suppressor on exocytosis. We now have evidence that soluble HPC-1 lacking membrane-bound domain possesses functional activity on the secretory process (manuscript in preparation). Also, the overexpression of HPC-1 in the β-TC cell, an insulinoma cell line, provided a decrease of secretion of glucose-induced insulin (Nagamatsu et al., submitted). In addition, the intracellularly applied anti HPC-1 antibody in the rat hippocampal neurons cultured on the glial micro-islands increased the epsc amplitude transiently (5 to 20 min) up to 60% (data not shown). These findings also suggested that HPC-1 plays a role in some inhibitory mechanism in secretion and regulatory exocytosis.

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