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Immunocytochemical Observation on Actin in Sensory Epithelium of Rat Macula Utriculi

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= **Abstract** = This study was performed to establish the vestibular preparation technique for immunocytochemical detection of actin in the vestibular hair cells. Frozen sections were chosen for the detection of actin distribution in the macular sensory epithelium. Quick-freezing in the primary embedding followed by secondary embedding was used for the proper orientation of specimens.

Immunohistochemistry by peroxidase-antiperoxidase staining and immunofluorescence demonstrated actin localization in the neck area of the type I hair cells and in the periphery of the perinuclear area as well as in the stereocilia and in the cuticular plate. Two possibilities were suggested: First, immunohistochemically stained area in the neck of the type I hair cells and peripheral perinuclear portion may represent actin filaments of the synaptic area where the hair cells and nerve endings contact. Second, G-actin pool might exist around perinuclear area and react with anti-actin atibody but didn't appear as filamentous area by electron microscopy.

Key words: ***Macula utriculi, Actin, Immunocytochemistry***

INTRODUCTION

The structural similarities between intestinal brush border epithelial cells and inner ear sensory hair cells were noticed by Flock and Cheung in 1977. Since it was shown that microvilli of intestinal epithelial cell contained actin filaments (Mooseker and Tilney 1975), the presence of actin filaments in the inner ear sensory hair cells was speculated. With the aid of decoration with subfragment-1 of myosin(1977), gel electrophoresis and immunofluroescence(1981), the actin filaments in the stereocilia and their rootlets, in the cuticular plate and also in relation to the zonula adherens surrounding the top of the hair cell of the crista ampullaris were demonstrated by Flock and his colleagues.

Following the work of Flock and Cheung(1977), Tilney *et al.* (1980) and Slepecky and Chamberlain(1982) demonstrated actin filaments in the stereocilia and their rootlets. in the cuticular plate of the auditory hair cells of the alligator lizards and chinchillas, respectively.

Recently, Friedmann body in the inner ear receptor cells(Friedmann *et al.* 1965; Slepecky and Chamberlain 1982), which is a structure morphologically similar to the striation of the skeletal muscle cells, was observed under electron microscope. This has called attention to the presence of contractile proteins and their possible organization inside the inner ear receptor cells.

It was thought that more study on any of the contractile proteins which cooperate in performing their functions in the vestibular hair cells would be helpful to understand their sensory excitation mechanism which has not beel clearly explained. To do so, immunohistochemistry has been regarded to be the most useful technque.

Since there has been no report of immunohistochemical approach that examined contractile proteins in the vestibular receptors on the tissue specimen. this study intends to achieve the following

objectives:

1. To establish the vestibular preparation technique which is suitable for the application of immunohistochemistry on tissue sections without damage of vestibular morphological preservation.
2. To observe the presence and localization of actin in the utricular sensory epithelium using immunohistochemistry.

MATERIALS AND METHODS

Adult albino rats of the Sprague-Dawley strain weighing 200-250 gm were sacrificed by decapitation with a small animal head cutter (Harvard Apparatus). From the ventral position, the temporal bone surrounding the external auditory canal was reduced and the remaning bone encompassing the middle ear cavity was removed from the skull. The specimens were immersed in the fixative solution containing 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M Sodium phosphate buffer at pH 7.1. Perilymphatic perfusion was performed for 30-60 minutes at room temperature with fixative. Utricular macular tissues were obtained and processed on ice until the embedding stage. More detailed methods were described in the previous report(Lee 1984).

Frozen sections

Microdissected specimens were washed with several changes of cold phosphate-buffered 30% sucrose solution (0.1 M sodium phosphate, pH 7.1) and kept on ice overnight. The washed tissues were placed flat on a thin aluminum bottle cap for the proper orientation, coated with a thin layer of embedding medium(O.C.T. compound) and partially submerged in a mixture of acetone and dry ice for rapid freezing. The preparation was moved into cryostat chamber set at −20°C, reembedded at the right angle to the original direction for the proper sectioning orientation. Serial sections were cut at 4-5 μm and picked up on cleaned, gel coated slides. After air drying for 10-30 minutes, every fifth slide was observed under light microscope and sections containing sensory epithelium were selected. They were directly processed for immunohistochemical staining or stored in a freezer(−20°C) for up to a week.

Peroxidase-Anti-Peroxidase(PAP) staining

DAKO PAP KIT SYSTEM(K548) was used along with rabbit antiactin IgG as the primary antibody which was purchased from Biomedical Technologies Inc.(Cat. No.: BT-560). The PAP staining was performed at room temperature in the following procedure: (0.05M Tris Buffer, pH 7.6, was used throughout this procedure unless otherwise indicated.).

Sectons were covered with 3% hydrogen peroxidase for 5 minutes. After rinsing with distilled water and washing with Tris Buffer for 5 minutes, normal swine serum was applied for 20 minutes followed by tapping off excess serum. Incubation of the sections overnight at 4°C with primary natibody diluted 1:100 with Trif buffer, was followed by buffer wash for 20 minutes. Then, swine anti-rabbit immunoglobulin was layered on sections for secondary antibody for 30 minutes and ashed with Tris buffer for 20 minutes. The end product was revealed with a freshly made solution of 3-amino-9-ethyl-carbozole in N, N dimethyl formamide mixed with 0.1 M acetate buffer, pH 5.2, and 0.3% hydrogen peroxidase in water for 15-30 minutes. Finally, sections were sahsed in tap water and DAKO glycergel was used as the mounting medium.

The method specificity of this immunocytochemical reaction was tested with specimens in which preimmune rabbit sera was substituted for the anti-actin IgG. Control also included application of primary antibody preincubated with chicken gizzard actin(2.7 mg of actin/1 ml of IgG) purchased from Sigma Chemical Company.

Immunofluorescence

Sections were treated with normal swine serum in 0.05M Tris buffer, pH 7.6, for 20 minutes at room temperature, incubated overnight at 4°C in anti-actin IgG diluted 1:20 in Tris buffer, and washed three times with 0.01 M phosphate buffered saline(PBS), pH 7.2. The second stage included incubation in fluorescein-labelled swine anti-rabbit immunoglobulin(DAKO, F205) diluted 1:20 in 0.01M PBS, pH 7.2, for 30 minutes. After the final washing with PBS, ethanol cleaned coverslips were mounted in a 9:1 dilution of glycerol:PBS. The specimens were viewed using A.O. microscope fitted with a fluorescence vertical illuminator for incident light excitation.

RESULTS

In this study, primary embedding in O.C.T. compound before reembedding for frozen sections was employed in order to obtain in proper sectioning direction. Freezing artifact which caused intracellular ice crystal formation decreased after quick freezing in aceton-dry ice bath. Light micrograph of frozen sections stained by PAP method are shown

gs. 1, 2, 4-6. When sections were not proces-
through rapid freezing as shown in Fig. 1,
ation of the intracellular ice crystal-filled area
ered with the observation of actin localization.
sucrose-rapid freezing treatment, improved
hological preservation was acquired(Fig. 2).
P staining revealed actin of hair cells with
nish granules in the hairs(Figs. 2, 5) and in
pical area which was thought to correspond to
lar plate(Figs. 1, 4). Some of the hair cells
d actin positive areas surrounding perinuc-
area(Figs. 5, 6). In some of the type I hair
neck areas with positive staining was also
(Fig. 6). Supporting cells showed few stained
. Above findings were also demonstrated in
n the immunofluorescent stained sections-
3, 7). Controls which used adjacent sections
reimmune rabbit serum in place of the prim-
ibody were shown in Fig. 5a and showed no
d area.

DISCUSSION

ently, the launching of space shuttles height-
he interest in the vestibular apparatus which
cally has been less studied than the auditory
However, the difficulty in obtaining and pro-
g the vestibular sensory area, which is mi-
nd surrunded by temporal bone, has made a
progress in the immunocytochemical
ch. It appears that the immunohistochemi-
roach to the contractile protein of the ves-
hair cells was reported only by Flock and
ner(1982) who demonstrated actin in the
ilia of the crushed macular epithelium. The
osmium as a fixative in the study would
e the visibility of the specimens and faciliate
ue processing. There has been no report on
ect of osmium fixation on antigenicity of
but Maupin-Szamier and Pollard(1978)
that actin filament was damaged by fixation
O_4.

-like filaments in nonmuscle cells have a
to interact with heavy meromyosin(HMM)
MM decoration technique would be more
for the detection of actin than use of anti-
ntobody. It was not chosen in this study,
, since HMM was reported to induce actin
nerize, which might cause displacement of
urthermore, it requires a detergent treat-
r cell membrane penetration because the
the exogenous HMM should be performed
xation for the binding of HMM to intracellu-
lar actin.

New observation in this study after the applica-
tion of the immunohistochemical staining on tissue
sections of the utricular epithelium is that the pre-
sence of actin did not appeared to be limited to the
apex of the hair cells and stereocilia. Actin was also
revealed around the neck area of type I hair cells
and in the periphery of the perinuclear area.
Although there have been reports on the presence
of actin beneath the cuticular plate of the cochlear
hair cells(Zenner 1981; Flock and Bretscher 1982),
no demonstration of actin in such a location has
been made in case of vestibular hair cells. Ever
electron microscopic studies after S-1 decoratior
showed actin filaments only in the apical portion of
vestibular hair cells(Flock and Cheung 1977). A
possible explanation for this finding is that actin
positive areas surrounding the neck area of type I
hair cells and perinuclear area correspond to the
synaptic areas where hair cells and nerve endings
make contact. This speculation is supported by the
finding of Lebeux and Willemot(1975) who demon-
strated actin-like filaments in the synaptic areas by
means of HMM labeling. Also, maillet-stained
nerve calyces(Lindeman 1969) showed appearance
similar to the immunohistochemically stained neck
and basal area. Difference in the immunohistoche-
mical staining between type I and type II hair cells
was hardly able to be known under the light micro-
scope unless the neck area was stained.

Althouth it appeared likely that a few stained
areas in the basal portion of the supporting cells
corresponded to parts of unmyelinated fibers ex-
tending to the nerve ending, we cannot exclude the
possibility of presence of actin in the supporting
cells.

When the results of the present study was com-
pared with that of electron microscopic studies
obtained in our laboratory(unpublished), the former
method showed positively stained peripheral peri-
nuclear area in addition to the filamentous areas
shown in the electron micrographs. From the fact
that electron microscopy shows only filamentous
actin and primary antibody used in this study can
bind both G- and F-actins, the possibility of G-ac-
tin existing in the peripheral perinuclear area was
suggested. This suggestion was supported by the
presence of unpolymerized actin in the cytosol(Pol-
lack *et al.* 1975). Thus pool of G-actin may be
present within cells and utilized to form the F-actin
when it is necessary to perform some cellular func-
tions.

More extensive investigation into the use of the purified anti-actin for immunoelectron-microscopic approach will be the next step to provide correct answer to the specific location of actin of the vestibular hair cells.

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=국문초록=

免疫組織化學的 觀察을 通한 흰쥐 內耳 卵形囊의 「액틴」 分布像의 硏究

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이병란 · 성기준 · 조사선 · 이광호 · 백상호 · 장가용

內耳 前庭器官에 있는 感覺上皮에 의한 감각의 受容 및 전달기전에 대한 연구는 극히 미진한 실정으로 최근에는 內耳感覺有毛細胞와 구조적 유사성을 지닌 小腸上皮細胞內에 존재하는 것으로 알려진 收縮性 蛋白質이 이러한 역할을 하리라고 추정되고 있다. 본 연구는 조직표본상에서 免疫組織化學的인 方法을 이용하여 그 관찰을 가능케하는 조직처리기법을 확립한 후 광학현미경하에서 수축성 단백질의 일종인 액틴의 內耳感覺神經上皮內의 분포를 관찰하여 다음과 같은 결론을 얻었다.

1. 고농도의 서당으로 처리후 급속동결하는 방법으로 항원성의 손실이 없이 형태를 보존할 수 있었다.

2. Peroxidase-anti-peroxidase(PAP)염색을 한 표본에서 액틴은 不動毛(stereo cilia), 小皮板(cuticular plate)과 같은 感覺細胞의 上部이외에도 제1형 有毛細胞頸部 및 核周圍부분의 주변部 그리고 상피세포의 基低部位에 分布되어 있었다.

3. 免疫螢光法을 이용한 조직에서의 액틴은 PAP염색의 것과 같은 분포양상이었다.

이상과 같은 관찰성적을 전자현미경 소견과 비교해 보면 有毛細胞의 頸部 및 그 세포 核周圍部에서 관찰된 액틴은 有毛세포와 신경 말단부간의 接合(synapse)부위에 나타나는 細絲型 액틴(F-actin)으로 추측되나 有毛상피세포내 핵주위세포질의 末梢部에는 顆粒性액틴(G-actin)이 actin-pool의 形態로 存在하고 있을 가능성도 배제할 수 없었다.

LEGENDS FOR FIGURES

Fig. 1. A frozen sectioned and PAP stained section of the macula utriculi. Areas filled with intracellular ice crystals (arrow heads) are seen. EL, Endolymphatic space; CP, Cuticular plate. (x400).

Fig. 2. Sucrose-rapid freezing treatment prior to frozen sectioning and PAP staining improved morphological preservation of macula utriculi. (x400).

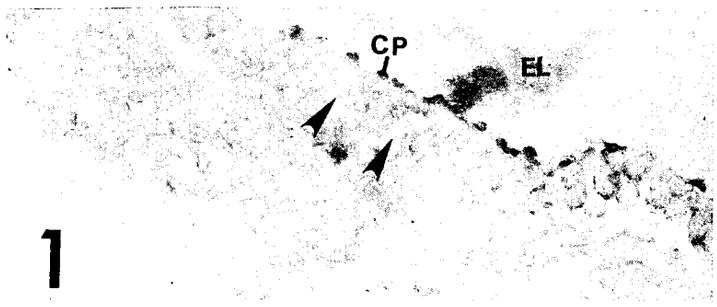
Fig. 3. Immunofluorescent micrograph of the macula utriculi after frozen sectioning stereocilia(ST) and cuticular plate(CP) are seen stained with anti-actin antibody. EL, Endolymphatic space. (x400).

Fig. 4. Cuticular plates(CP) of hair cells are showing brownish granules. PAP staining. (x1,000).

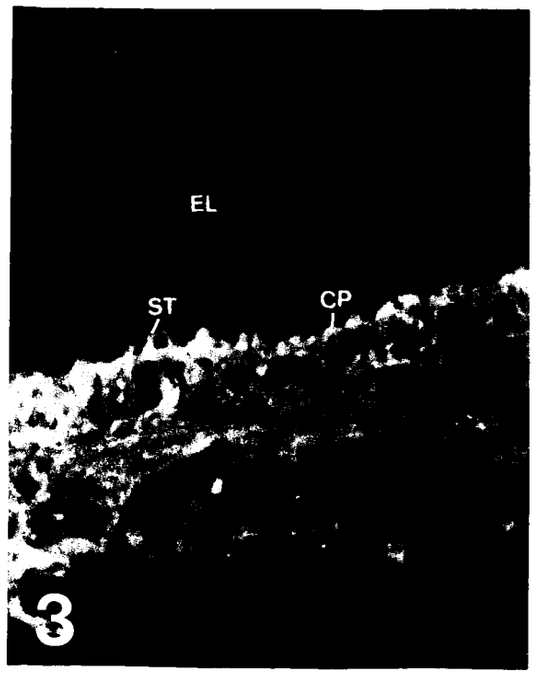
Fig. 5. PAP stained areas are seen in the stereocilia(ST) of hair cells and also in a few areas in the basal protion of the supporting cells (arrow heads)

Fig. 6. Perinuclear areas of the hair cells and neck area (arrow head) of type I hair cells are stained brown by PAP. (x1,000).

Fig. 7. Note the neck area (arrow head) of a type I hair cell stained by immunofluorescence. (x1,000).


CP
EL
1


EL
2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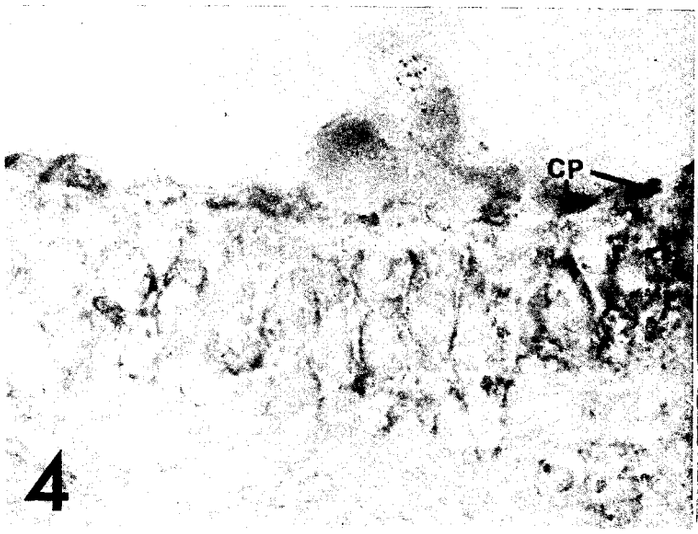
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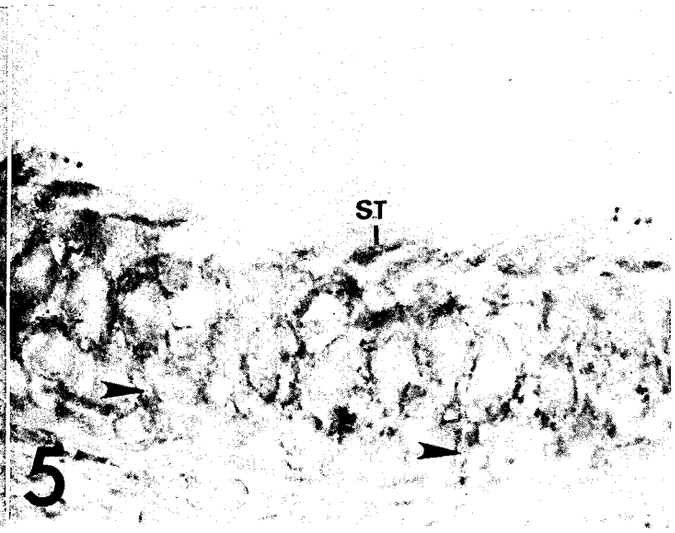
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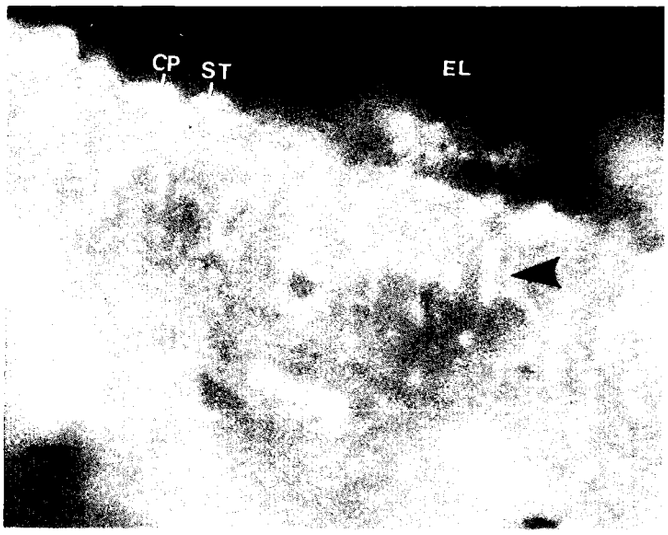
6


CP
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