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EXPRESSION OF CALCIUM-BINDING PROTEINS, CALBINDIN D28K AND CALRETININ, IN THE FROG TASTE RECEPTOR STRUCTURES

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Considering that information on the expression of calcium-binding proteins (CaBPs) in different cells of the taste receptors is rather limited, we investigated the distribution of such proteins, calbindin D28k (CB) and calretinin (CR), in the taste disc (TD) of the frog *Lithobates catesbeianus*. Western blot analysis revealed that CB and CR are expressed in cells of the fungiform papillae. CB-immunoreactive (ir) and CR-ir cell somata were located in the middle layer of the TD. Most CB-ir and CR-ir cells possessed one rod-shaped apical process and one basal process; in some cells there were several extended basal processes. Apical processes of CR-ir cells were thinner than those of CB-ir units, and CR-ir nerve fibers were ramified in the *lamina propria* directly below the TD. Most CR-ir fiber branches surrounded the TD; however, some penetrated this region, with both types of branches approaching the surface. CB and CR immunoreactivities did not co-occur in TD cells. In the TDs examined, the number of CB-ir cells was significantly greater than that of CR-ir units. Our observations suggest that CB-ir and CR-ir cells in the frog TD correspond to type-II and type-III cells, respectively.

Keywords: calcium binding proteins, calbindin D28k, calretinin, taste cells, frog.

INTRODUCTION

Calcium plays a critical role in intracellular signal transduction in taste cells after taste stimulation [1–3]. Some taste cells have conventional chemical synapses with voltage-gated calcium channels. Other cells respond to taste stimuli without using conventional chemical synapses and calcium release from the internal stores via second messengers. Although calcium-binding proteins (CaBPs) modulate intracellular signals by acting as calcium buffers, transporters, and sensors [4–6], their functions in taste cells have not been fully understood. The CaBP expression has been demonstrated in the taste organs of some animals, including specific types of taste cells in mice [7] and guinea pigs [8].

The frog taste organ, referred to as the taste disc (TD), contains six cell types [9]. Type-I cells are regarded as supporting units; they are divided into three subtypes (Ia, Ib, and Ic). These cells lack neuronal contacts. Type-II and type-III cells are classified as taste receptor cells, with nerve fiber contacts. Type-IV (basal) cells are classified as immature or mechanoreceptive cells. Although CaBP immunoreactivity was shown in the amphibian taste organ [10–13], specific immunoreactive (ir) cell types have not been identified. To clarify the relative distributions and abundance of the CaBPs, namely calbindin D28k (CB) and calretinin (CR), we performed Western blotting and immunohistochemical studies using the TD from the fungiform papillae of the frog *Lithobates catesbeianus*.

METHODS

Ten bullfrogs (*Lithobates catesbeianus*, body mass 350–800 g) were used in the experiments.

Western Blot Analysis. Fresh fungiform papillae and whole brain tissue were collected from frogs, homogenized, and lysed in a 1% radio-immunoprecipitation assay (RIPA) buffer containing 5

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mM EDTA, 150 mM NaCl, 1% NP-40 (nonidet P-40), 0.1% SDS (sodium dodecyl sulfate), 0.5% sodium deoxycholate, 1.0 mM PMSF (phenylmethylsulfonyl fluoride), 10 mg/ml leupeptin, 10 mg/ml aprotinin, 5 mM Na_3VO_4, 10 mM NaF, and 50 mM Tris-HCl (pH 7.5). BCA protein assay kits (Pierce, USA) were used to quantify the samples. A total of 15 μg for each sample was separated on 12.5% SDS-PAGE (SDS-polyacrylamide gel electrophoresis) gels; proteins were then transferred to a polyvinylidene difluoride filter (PVDF). The following primary antibodies were used: mouse monoclonal anti-calretinin (1:1,000, Swant, Switzerland), rabbit polyclonal anti-calbindin D28k (1:10,000, Abcam, USA), and mouse monoclonal β-actin (Santa Cruz, USA) antibodies. The antibodies were diluted in a blocking solution containing 5% skim milk with 0.05% TBST (Tris-buffered saline with Tween), 150 mM NaCl, 0.05% Tween 20, and 20 mM Tris-HCl (pH 7.6). Western blotting was performed as previously described [14, 15].

Immunohistochemical Procedures. After frogs were anesthetized with tricaine (MS-222), they were perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffered solution (pH 7.2). Frog tongues were removed and immersed in the same fixative solution overnight at 4 °C. The samples were washed with PB containing 30% sucrose overnight, embedded in OTC (optimal cutting temperature compound), and frozen in liquid nitrogen. The specimens were sectioned at a thickness of 50 μm using a cryostat. The obtained sections were washed in 0.01 M PBS (phosphate buffered saline, pH 7.4) and blocked for 20 min in 5% normal serum in PBS with 0.3% Triton X-100 (PBT). Rabbit anti-calbindin D28k (1:100,000) and mouse anti-calretinin (1:10,000) primary antibodies were used for staining. Sections were incubated with a mixture of primary antibodies overnight at 4°C and processed with a mixture of secondary antibodies, Alexa Fluor 594 and 488 (Invitrogen, USA), at a dilution of 1:400 overnight at 4°C. The sections were washed with PBT several times after each incubation. Immunostained sections were covered in ProLong Gold mounting medium (Invitrogen, USA) and examined using a fluorescence microscope, BZ-X700 (Keyence, Japan).

Cell Counts. To count CB-ir and CR-ir cells, the ABC method was used. Sections were washed in PBT, treated with 0.3% (w/v) H_2O_2 in methanol for 30 min (to eliminate intrinsic peroxidase activity), and rinsed in PBT. They were then incubated in each of primary antibody at 4°C overnight. Sections were then processed using the Universal Elite ABC Kit (Vector Lab, Inc., USA), and colored using 3,30-diaminobenzidine (Vector Lab, Inc.). Sections were mounted on glass slides, counterstained with methyl green, dehydrated with a graded ethanol series, cleared in xylene, and mounted using Mount-Quick solution (Daido Sangyo, Japan). Immunoreactive cells were counted within 20×100 μm squares using frontal sections of 41 taste discs of four frogs.

Statistical analysis was performed using the Student's *t*-test. The results are presented as means ± ± s.d. Differences were considered significant at $P < 0.05$.

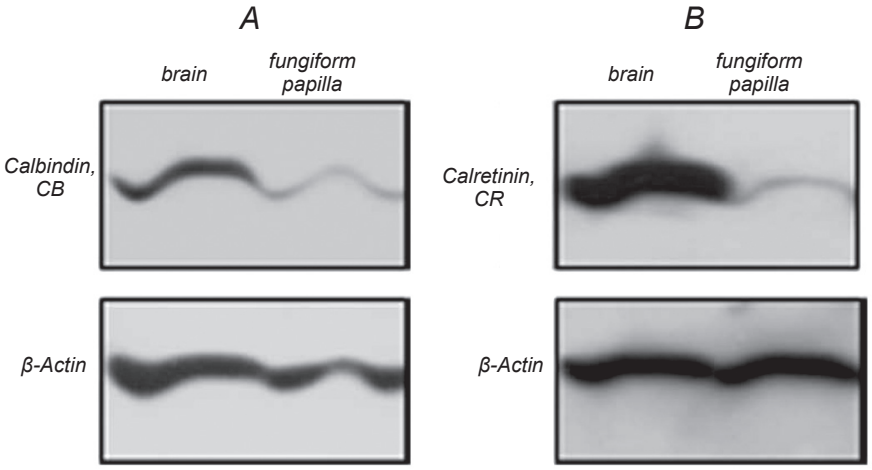
RESULTS

Western Blotting. To examine the cross-reactivities of antibodies to bullfrogs, Western blot analysis was performed. CB and CR antibodies labeled single protein products in the brain and fungiform papillae (Fig. 1), which were of the appropriate molecular masses.

Immunohistochemistry. CB and CR immunoreactivities were readily observed in the frog fungiform papillae (Fig. 2A). CB immunoreactivity was observed in TD cells, and CR immunoreactivity was visualized in TD cells and nerve fibers in the fungiform papillae. CR-ir nerve fibers were ramified in the *lamina propria* located just below the TD. Most branches of the nerve fibers surrounded the TD; some branches were also occasionally observed in the TD. Both branch types approached the TD surface.

CB-ir cells were abundantly distributed in the TD (Fig. 2B), with their cell somata located in the middle TD layer. Single thick rod-shaped apical processes that reached the TD free surface were observed in most cells. CB-ir cells also had single basal processes or none at all. CB-ir cells tended to form groups in the TD and extended their apical processes closely to the TD surface. CR-ir cells were also abundantly observed in the TD (Fig. 2B), with their cell somata also located in the middle TD layer. Single thin rod-shaped apical processes were generally observed in these cells; these processes reached the free surface of the TD. Apical processes of CR-ir cells were notably thinner than those of CB-ir cells and were occasionally forked. CR-ir cells also possessed either single or several basal processes.

The Keyence 3D-analysis software results are shown in Fig. 2C. XY, YZ, and XZ section images



F i g. 1. Western blot analysis of calcium binging proteins, calbindin D28k (CB) and calretinin (CR). CB (A) and CR (B) were detected in homogenates of the frog brain and fungiform papilla tissues. *β*-Actin was used as a loading control.

Р и с. 1. Результати Вестерн-блотингу кальційзв'язуючих протеїнів калбіндину D28k (CB) та калретиніну (CR).

were created using a 50-µm-thick specimen. In every section image examined, no CB or CR co-immunoreactivities were observed in TD cells.

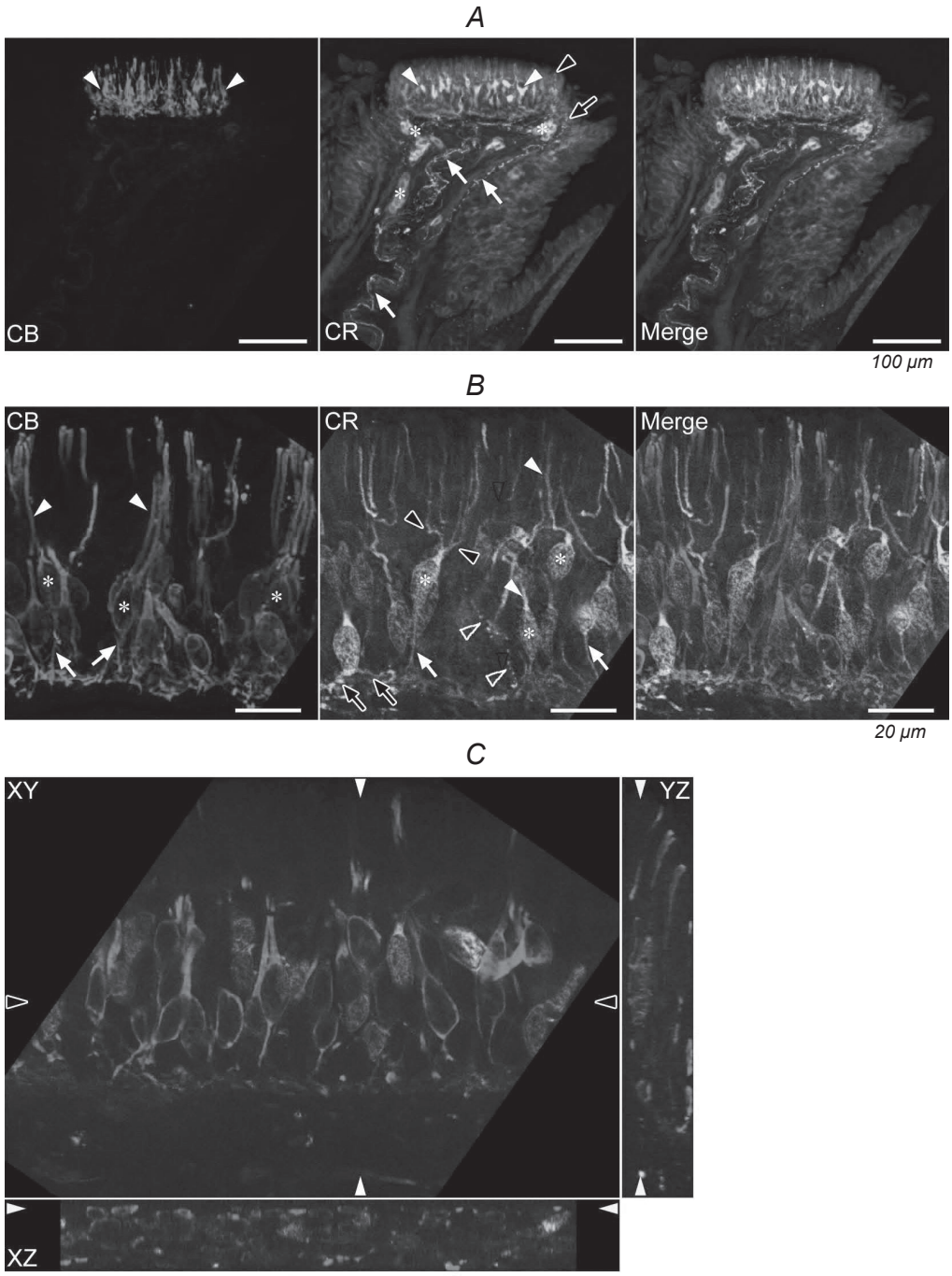
Immunoreactive cell numbers were counted in 20×100 µm squares; 41 taste discs from four frogs were used (Fig. 3). The minimum and maximum observed CB-ir cell numbers were 16 and 36, respectively. Among CR-ir cells, the respective minimum and maximum counts were 7 and 24 (Fig. 3A). The average numbers of CB-ir and CR-ir cells (± s.d.) were 26 ± 5.30 and 16.3 ± 4.50 (n = 41), respectively (Fig. 3B). In the TDs examined, CB-ir cell counts were significantly greater than those of CR-ir cells ($P < 0.001$).

DISCUSSION

In our study, CB-ir and CR-ir cells and nerve fibers were examined in the frog fungiform papillae. The frog TD contains six types of cells [9]. Among these six types, type-Ib cells are referred to as wing cells; they have apical processes with three sheet-shaped plates. Both type-II and type-III cells have rod-shaped apical processes and are thought to be taste receptor cells. The cell somata of these cells are located in the middle layer of the TD. Type-II cell apical processes are 2–3 µm in diameter, while those of type-III cells are thinner (0.6–0.8 µm) [9]. Type-III cells also extend one or more basal processes toward the basal membrane [9]. Although type-II cells were reported to be bipolar units, numbers and shapes of the basal processes were not clarified earlier [9]. We found that the CB-ir cell somata are located in the middle layer of the TD, and these cells possess thick apical processes. Most of CB-ir cells also possessed single basal processes oriented toward the basal membrane. However, basal processes were not clearly observable in some of these cell types. Based on the features described, the cells identified in our study correspond most closely to type-II cells. Moreover, we found that CB-ir cell apical processes tend to form a cluster from several cells, a feature also similar to that of type-II cells [9, 16, 17].

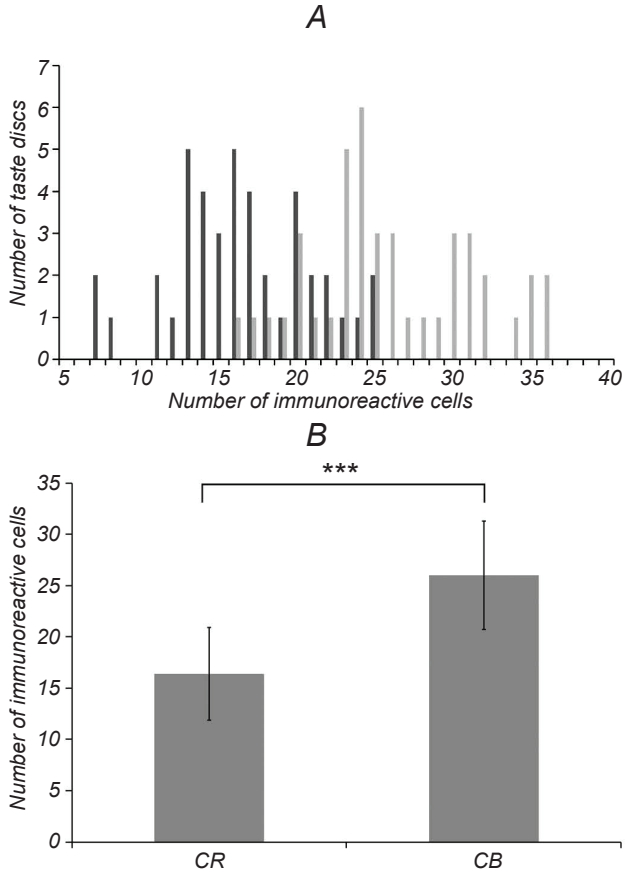
Our results indicate that the CR-ir cell somata were also located in the middle layer of the TD. Extended thin apical processes were present in CR-ir cells, and most of these cells possess only one process each. Several basal processes were observed in some CR-ir cells, and these features correspond to those of type-III cells. Moreover, branching apical processes were also observed in CR-ir cells. It has been reported that type-III cells have branching thin apical processes [16, 18]; such branching apical processes were also reported for type-Ib (wing) cells [9]. However, as CR-ir sheet-shaped processes were not observed, it is highly possible that the CR-ir cells identified in our study are type-III cells.

In the taste buds of transgenic mice, which express green fluorescent protein (GFP) driven by the IP_3R_3 (inositol 1,4,5-trisphosphate receptor type 3-guanosine 5'-diphosphate) promoter [19], a marker



F i g. 2. Immunostaining in the fungiform papillae of the frog taste disc (TD) with antibodies to CB and CR. A) Low-magnification photomicrographs of the TD. CB-immunoreactive (ir) or CR-ir TD cells are shown with white arrowheads; CR-ir nerve fibers are shown with white arrows; branches of the CR-ir nerve fibers in the TD and around the TD are shown with black arrowheads and black arrows, respectively. Red blood cells show non-specific staining (asterisks). B) High-magnification photomicrographs of the TD. CB-ir or CR-ir cell somata in the TD are indicated with asterisks. CB-ir or CR-ir cells usually possess an apical process (white arrowheads) and a basal process (white arrows). CR-ir cells sometimes extend branched apical processes (black arrowheads) and several basal processes (black arrows). CR-ir nerve fibers in the TD are shown with white outlined arrowheads. C) 3D-analysis of CB-ir and CR-RI cells in the TD; XY, XZ, and YZ section images were created using Keyence 3D-analysis software. Black and white arrowheads on the XY images show the points where XZ and YZ images were created, respectively. Yellow arrowheads on XZ and YZ images show the points where an XY image was created. In every section image examined, no CB and CR co-immunoreactivities were observed in TD cells.

Р и с. 2. Результати імуномічення в грибоподібних папілах смакового диску (TD) жаби антитілами щодо CB та CR.



F i g. 3. Number of CB-ir and CR-ir cells in the TD. A) Diagram showing the number of the TDs containing CB-ir and CR-ir cells (gray and black bars, respectively). B) Diagram showing the average numbers of CB-ir and CR-ir cells. The average numbers of CB-ir and CR-ir cells were 26 ± 5.30 and 16.3 ± 4.50 (mean ± s.d., $n = 41$), respectively. In the TDs examined, CB-ir cell counts were significantly greater than those of CR-ir cells (Student's t-test, three asterisks show the difference with $P < 0.001$).

Р и с. 3. Кількість CB- та CR-імунореактивних клітин у смаковому диску.

for type-II taste cells [20], CB and CR were found to be expressed in approximately half of type-II cells, in addition to those, CB and CR were expressed in cells not showing the type-II cell marker [7]. The differences between CB-ir and CR-ir cell types observed in frogs in the present study and those reported in mice may be the manifestations of species-related specificity. As it is difficult to precisely measure the thicknesses of cytoplasmic processes, observations by electron microscopy are needed to better identify these cell types.

CB (i.e., spot-35 protein)-ir cells have been reported to be expressed in the frog taste organ, with immunoreactive cell bodies located in the middle layer of the TD [10]. Spot 35 protein is synonymous with CB [8]. However, the immunoreactivities were also observed in the supporting cells located near the surface of the taste organ in *Xenopus* [11]. In our study, CB immunoreactivities were not observed in type-Ia (mucus) cells adjacent to the TD surface. In the middle layer, CB-ir cells showed features resembling those of type-II cells.

Cells in the kidney nephron and CNS neurons were found to express specific CaBPs [4]. Thus, the expression of CaBPs depends on the cell type. CB-ir and CR-ir neurons were observed in the *Xenopus* spinal cord, with only a small percentage of neurons double-labeled in the dorsal and ventrolateral fields of the grey substance [21]. In our study, no double-labeled TD cells were observed.

It has been reported that type-II cells are fewer in their number than type-III cells [16, 17]. According to our estimations, the number of CR-ir cells is significantly smaller than that of CB-ir cells; thus, it is possible that some type-III cells do not express CR.

The precise functions of CaBPs in taste cells have not been elucidated. In general, the dissociation constant (K_D) values of most CaBPs are well above the intracellular free calcium concentrations (determining the Ca^{2+} current). Thus, most CaBP binding sites are not occupied by calcium under resting conditions, such as CaBPs limit increases in Ca^{2+} currents from the resting levels rather than when clamping such a current at a given level [6]. There is growing evidence that CB does not function only as a calcium buffer, but also binds to and regulates target proteins, including membrane ATPases, IMPase (5'-nucleotidase), RanBPM (Ran-binding protein M), procaspase-3, caspase-3, and TRPV5 (transient receptor potential channel vanilloid subtype 5), in a variety of cells [6]. IMPase is a key enzyme in the IP_3 (inositol trisphosphate) second messenger pathway. Interestingly, interaction between CB and IMPase was demonstrated *in situ* in Purkinje neurons (in acute mouse cerebellar brain slices) [22]. Mammalian type-II cells respond to sweet, bitter, or umami taste stimuli; these cells lack voltage-gated calcium channels and depend on calcium release from the internal stores via second-messenger pathways to generate stimuli-appropriate output signals [3, 23, 20]. In contrast, mammalian type-III cells have conventional chemical synapses and exhibit voltage-gated calcium currents in response to taste stimuli [1, 2]. Type-Ib (wing) cells and type-II and type-III cells have been shown

to produce action potentials in the frog TD with depolarizing current pulses [18, 24]. Frog type-Ib (wing) and type-II cells responded to quinine stimuli, whereas type-III cells did not [18]. Interestingly, frog type-III cells express voltage-gated calcium channels, while type-Ib (wing) and type-II cells do not [24]. Okada et al. [25] showed that injection of the second messenger IP_3 induced an increase in the internal calcium concentration in isolated frog taste disc cells.

According to the combined results, CB and CR may act as calcium buffers to terminate internal signal transduction cascades or as calcium sensors to modulate signal transduction enzymes in frog type-II and type-III cells.

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All experiments were carried out in agreement with the existing international ethical norms and according to the guidelines specified for animal experimentation in the Matsumoto Dental University.

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ЕКСПРЕСІЯ КАЛЬЦІЙЗВ'ЯЗУЮЧИХ ПРОТЕЇНІВ КАЛБІНДИНУ D28k ТА КАЛРЕТИНІНУ В СТРУКТУРАХ СМАКОВОГО РЕЦЕПТОРА ЖАБИ

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Р е з ю м е

Беручи до уваги те, що інформація про експресію кальцій-зв'язуючих протеїнів (CaBP) у різних клітинах смакових рецепторів обмежена, ми досліджували розподіл таких протеїнів – калбіндину D28k (CB) та калретиніну (CR) у смаковому диску (TD) жаби *Lithobates catesbeianus*. Аналіз за допомогою Вестерн-блотингу показав, що CB і CR експресуються в клітинах грибоподібних папіл. CB-імунореактивні (ir) та CR-ir соми клітин локалізовані в середньому шарі TD. Більшість CB-ir- та CR-ir-клітин мали один паличкоподібний апікальний відросток та один базальний відросток; у декотрих клітин було по декілька довгих базальних відростків. Апікальні відростки CR-ir-клітин були тоншими, ніж такі в CB-ir-клітин; нервові волокна із CR-реактивністю розгалужувались у межах *lamina propria* безпосередньо під TD. Більшість CR-вмісних гілочок волокон оточували TD, проте деякі гілочки проникали в цю ділянку і гілочки обох типів досягали поверхні. Комбінації імунореактивності щодо CB і CR у клітинах TD не спостерігалися. В обстежених TD кількість CB-ir-клітин була істотно більшою, ніж така CR-ir-клітин. Наші спостереження дають підстави думати, що CB-ir- та CR-ir-клітини в TD жаби відповідають клітинам II та III типів.

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