0014-4754/95/111040-05$1.50 + 0.20

Research Articles

In vitro development of *Xenopus* skin glands producing 5-hydroxytryptamine and caerulein

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Received 23 January 1995; received after revision 4 April 1995; accepted 13 April 1995

Abstract. The granular glands of amphibian skin synthesize and store a large amount of bioactive amines and peptides which are structurally similar to mammalian brain-gut peptides. To investigate the development of peptide- and amine-producing cells in the granular glands, pieces of dorsal skin taken at various stages from *Xenopus laevis* tadpoles were cultured, and the contents of caerulein and 5-hydroxytryptamine (5-HT) were measured. When pieces of skin from tadpoles at stages 57 to 60 (Nieuwkoop and Faber stages) were cultured in a medium containing 10% fetal calf serum (FCS medium) or one containing FCS treated with charcoal (chFCS medium), the caerulein and 5-HT levels were increased for the six days of the incubation period. The caerulein content was lower in the chFCS medium than in the FCS medium. Addition of thyroxine to the chFCS medium had no significant effect on the caerulein content. These results show that the caerulein- and 5-HT-producing cells of the granular glands can develop in a culture system with FCS- or chFCS-containing media, and suggest that FCS contains substances which are absorbed by charcoal and stimulate development of the amine- and peptide-producing cells of the glands. In a preliminary search for correlation between caerulein and 5-HT synthesis, addition of 5-hydroxytryptophan (5-HTP), a precursor to 5-HT, to the FCS medium increased 5-HT content and, conversely, caused significant decrease in caerulein content, suggesting that accumulation of caerulein in the granular glands is influenced by the amount of 5-HT synthesis. These studies indicate that this culture system is a useful model for investigating the development of peptide- and amine-producing cells.
Key words. *Xenopus laevis*; skin; granular glands; caerulein; 5-hydroxytryptamine; culture.

Amphibian skin has two main types of skin gland: mucous and granular glands. Generally, the latter contain bioactive amines and peptides, some of which share common amino acid sequences with mammalian brain-gut peptides[1]. In *Xenopus*, the granular glands contain a large amount of 5-hydroxytryptamine[2,3], caerulein[3–5], xenopusin[6], thyrotropin releasing hormone (TRH)[7] and magainin[8]. Comparing the amino acid sequences, caerulein is similar to colecystokinin, xenopusin to neurotensin, and TRH is identical to mammalian TRH. The content of amines and peptides in amphibian skin is extremely high when compared with that in the nervous systems and endocrine organs of mammals.

The granular glands develop from epidermal cells during metamorphosis[2,9–12]. In a previous study, we have shown that the *Xenopus* granular glands develop rapidly and accumulate a large amount of 5-HT and caerulein during their metamorphic period[3]. These results suggest that the granular glands may provide a useful model for investigating developmental mechanisms of amine- and peptide-producing cells, including neurons and endocrine cells.

In the present study, we have developed a culture system to investigate the development of amine- and peptide-producing granular gland cells, and have undertaken preliminary experiments to evaluate the usefulness of the culture system for investigating the correlation between peptide and amine synthesis.

Materials and methods

Xenopus laevis tadpoles at various stages (Nieuwkoop and Faber stage[13]) were kept in running tap water for 1–2 h prior to use; they were sterilized by tap water containing 1 million units of penicillin and 1.5 gm of streptomycin per litre. The tadpole skin was taken from the dorsal region of the trunk skin of cold-anesthetized tadpoles. The flayed skin was washed in 67% Hanks solution and attached to a millipore filter (HAWG 02500). Four or six 3 × 3 mm pieces, composed of skin plus millipore filter, were detached as shown in figure 1. The pieces were incubated for up to 8 days in a 24-well dish containing 50% L-15 medium, 10% fetal calf serum (FCS), 40% distilled water, penicillin and streptomycin. In some experiments, FCS was incubated with an excess of charcoal to remove endogenous thyroid hormones

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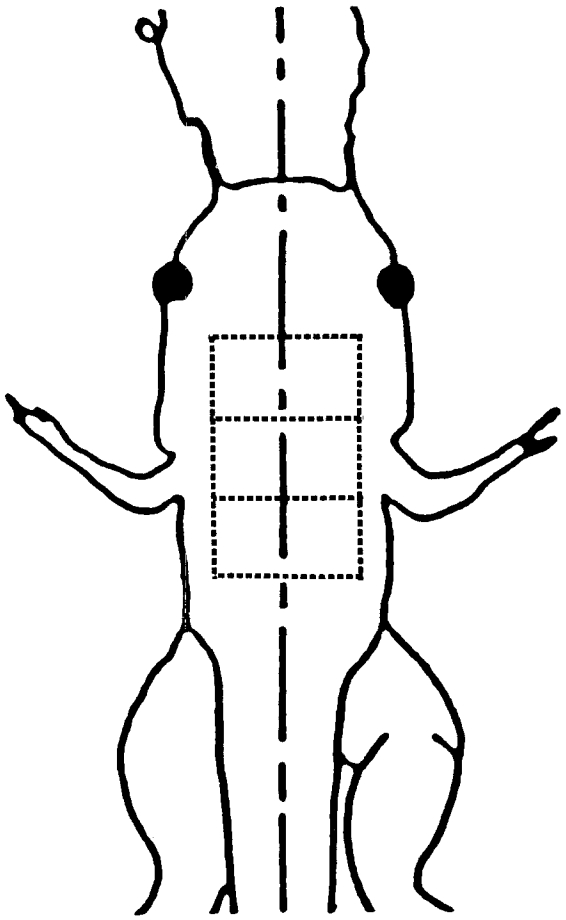

Figure 1. The area of skin used in culture experiments is shown by the dashed line. Each side of the midline (dot-dashed line) is used in culture experiments as follows: Day 0 vs Day 6, FCS medium vs chFCS medium, chFCS medium vs chFCS medium plus T_4, control vs 5-HTP.

from FCS, and tyroxine (T_4) (Sigma) or 5-hydroxytryptophan (5-HTP) (Sigma) added to medium containing FCS (FCS medium) or medium containing charcoal-treated FCS (chFCS medium). Caerulein and 5-HT content of cultured skin was measured by radioimmunoassay using anti-CCK 10 antibody (R5911)[14], which has been demonstrated to detect caerulein[3], and an HPLC-fluorometric system, respectively. Immunohistochemistry for 5-HT and caerulein was performed using anti-5-HT antibody (Immunonuclear corporation) and anti-CCK 10 antibody (R5911). The methods for the HPLC-fluorometric system, radioimmunoassay, and immunohistochemistry have been described elsewhere[3]. To obtain skin secretions from the skin grands, noradrenaline (10^{-3} M) in a volume of 200 µl was injected directly into the dorsal lymph sac of stage 65–66 tadpoles. The secretions released on the skin surface were collected in H_2O. Caerulein and 5-HT in the secretions were measured by HPLC system using synthetic caerulein (Calbiochem) or 5-HT (sigma) as standard[3]. The data were analyzed by Student's *t* test.

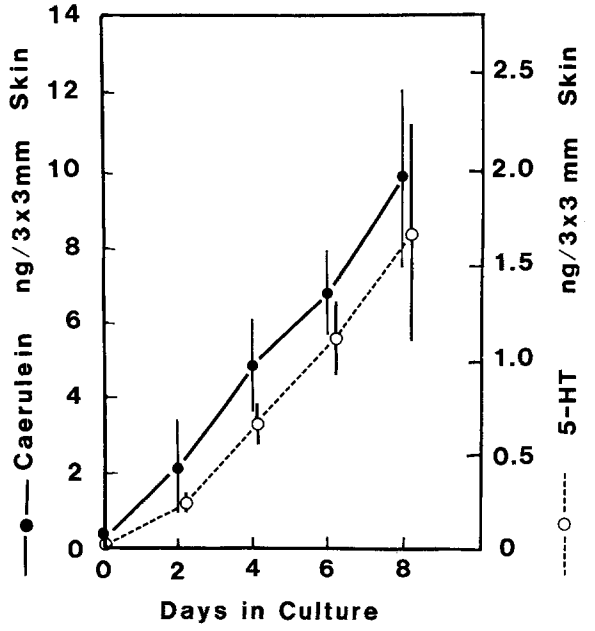

Figure 2. Time course of 5-HT and caerulein accumulation in piece of cultured skin from stage 58 tadpoles.

Results

In time-course experiments, pieces of skin prepared from stage 58 tadpoles were cultured in an FCS medium. The content of caerulein and 5-HT in cultured skin increased linearly during the eight days of the culture period (fig. 2). Next, pieces of skin from tadpoles at various stages were cultured in FCS medium, and the content of 5-HT and caerulein in the uncultured and cultured skin was examined (table 1). Although caerulein in stage 57 skin and 5-HT in stage 57 and 58 skin were undetectable on Day 0 of culture, both substances were detectable at Day 6. After 6 days of culture, the caerulein content in stage 58, 59 and 60 skin increased 75-, 21- and 17-fold, respectively, and the 5-HT level in stage 59 and 60 skin was elevated 54- and 3-fold, respectively.

In immunohistochemical observations, uncultured skin taken from stage 57 tadpoles contained a small number of granular gland rudiments that rarely possessed caerulein or 5-HT-positive granules (fig. 3A). After six days in culture, developed granular glands containing caerulein- and 5-HT-positive granules were observed (fig. 3B). In uncultured skin from stage 58 tadpoles, rudiments of the granular glands with or without immunoreactive materials, and developing granular glands with small cavities filled with immunoreactive granules, were observed (fig. 3C). After six days in culture, the glands developed further and had a lumen filled with a large number of immunoreactive granules (fig. 3D). Occasionally, pieces of cultured skin were removed from millipore filters during the culture period. In such cases, the granular glands also developed well and accu-

Table 1. Accumulation of caerulein and 5-HT in skin from tadpoles at various stages cultured in FCS medium for six days.

Stage	N	Caerulein (ng/piece)[a]		N	5-HT (ng/piece)[a]	
		Day 0	Day 6		Day 0	Day 6
57	8	<0.01	1.57 ± 0.50	6	<0.05	0.11 ± 0.04
58	8	0.29 ± 0.05	21.7 ± 6.70	6	<0.05	1.30 ± 0.48
59	9	2.84 ± 0.51	59.7 ± 9.96	6	0.27 ± 0.02	14.6 ± 1.17
60	6	4.09 ± 1.67	69.9 ± 18.6	6	3.21 ± 1.02	10.3 ± 2.89

[a]Mean ± SE.

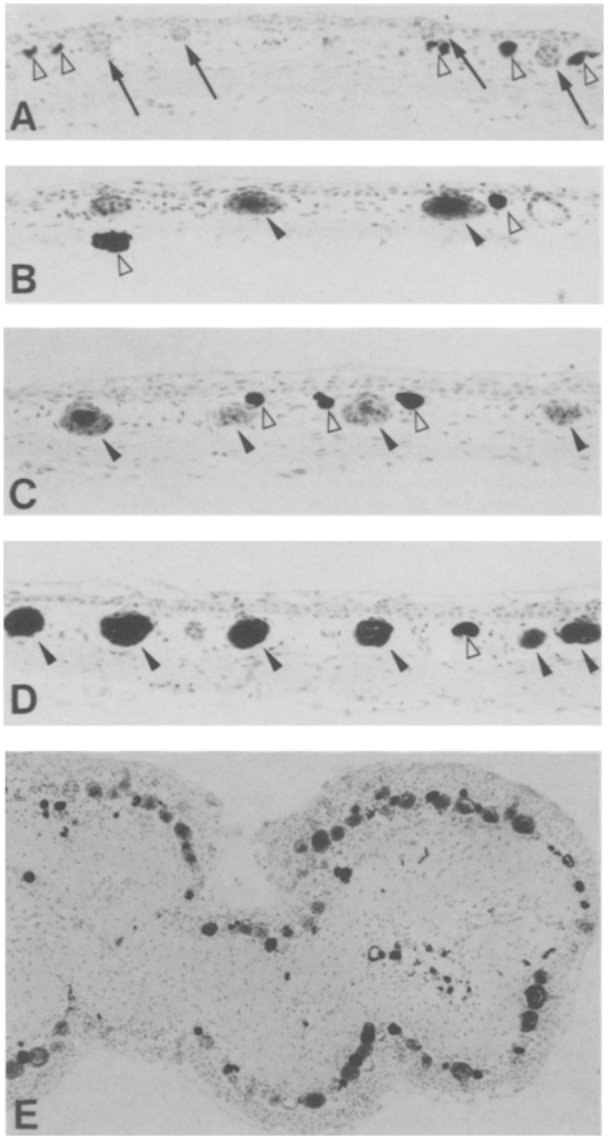

Figure 3. Immunohistochemistry of skin from tadpoles at stage 57 (*A*, *B*) and 58 (*C*, *D*, *E*) on Day 0 (*A*, *C*) and Day 6 (*B*, *D*, *E*) of culture, showing caerulein (*A*, *B*, *E*) and 5-HT (*C*, *D*) immunoreactivity. No immunoreactivity could be detected in the gland rudiments (arrow) of uncultured skin from stage 57 tadpole (*A*). The granular glands containing immunoreactive materials are shown by arrowheads. Open arrowheads indicate melanophores. (*E*) shows the skin which was removed from millipore filter during culture period. In such cases, well-developed granular glands filled with caerulein-positive granules could be found. *A*–*D*, ×110; *E*, ×55.

mulated a large number of immunoreactive granules within the lumen (fig. 3E).

To exclude the effect of endogenous thyroid hormones in FCS, pieces of skin from tadpoles at stages 57 to 59 were cultured in chFCS medium. This charcoal treatment reduced the T_4 level of FCS from 188 ng/ml to less than 1 ng/ml. When pieces of skin obtained from tadpoles at stages 58, 58$^-$ and 59 were cultured in chFCS medium, the caerulein content in the cultured skin was much higher than in the uncultured skin of tadpoles at 58 (0.29 ± 0.05 ng/piece) and 59 (2.84 ± 0.51 ng/piece) (fig. 4, left figures), whereas in stage 57 skin cultured in chFCS medium, caerulein was undetectable. The caerulein content in cultured skin was lower in the chFCS medium than in the FCS medium in tadpoles at stages 58$^-$ (20%), 58 (37%) and 59 (67%). The difference was larger in skin from tadpoles at earlier stages. Addition of T_4 (10^{-7} M and 10^{-6} M) to the chFCS medium had no significant effect on caerulein accumulation in skin from tadpoles at stages 57 to 59 (fig. 4, middle and right figures).

Next, we examined the correlation between caerulein and 5-HT synthesis in cultured skin. Prior to culture experiments, the ratio of 5-HT to caerulein in secretions released from skin glands of stage 65–66 tadpoles was examined. HPLC analysis showed that the ratio of 5-HT to caerulein was constant (8.43 ± 0.56) in each individual specimen (table 2), suggesting the presence of a mechanism to regulate the rate of 5-HT and caerulein synthesis in the granular glands. When pieces of skin from stage 58 tadpoles were cultured in FCS medium containing 5-HTP (10^{-3} M), 5-HT content in cultured skin was increased, whereas caerulein content decreased (table 3).

Discussion

The presnet study has shown that the granular glands can develop rapidly and accumulate a large amount of 5-HT and caerulein in tadpole skin cultured in FCS medium or in chFCS medium, although accumulated caerulein in cultured skin was less in the chFCS than the FCS medium. The difference in caerulein accumulation between the chFCS and FCS medium suggests that FSC contains factor(s), absorbed by charcoal, which stimulate development of the peptide- and amine-producing

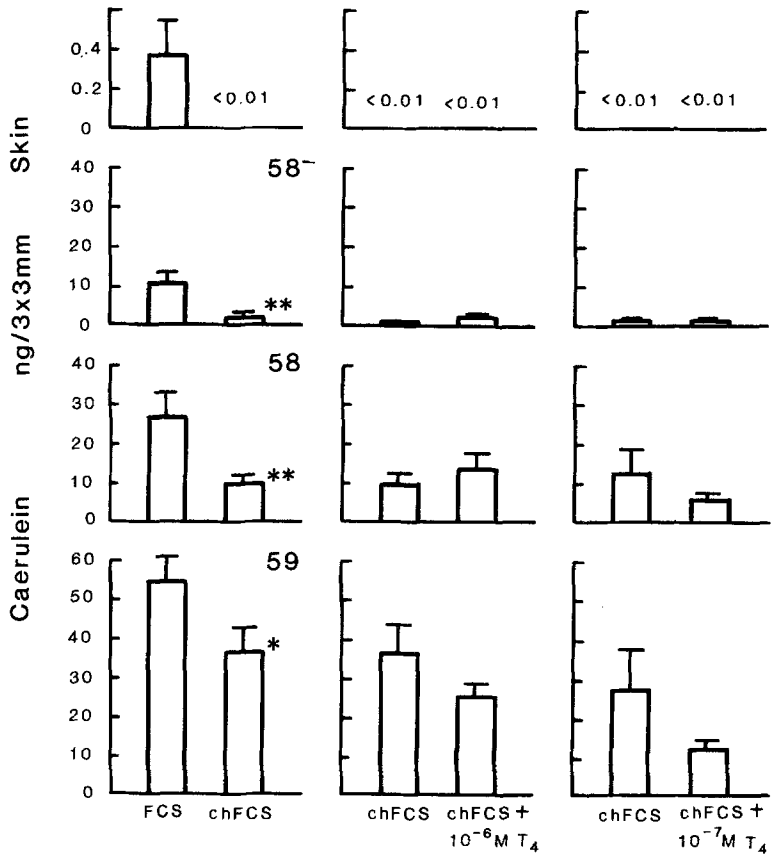

Figure 4. Comparison of effect of FCS, chFCS and T_4-containing chFCS media on caerulein accumulation in cultured skin from tadpoles at various stages. In each group, 7–12 skin pieces were used. Bars represent mean values plus SE. **Differs from FCS medium at $p < 0.01$; *differs from FCS medium at $p < 0.05$.

Table 2. Ratio of 5-HT to caerulein in secretion released from skin gland after noradrenaline injection.

Individual animal	Content in secretion (10^{-5} mole)		
	caerulein	5-HT	5-HT/caerulein
1	7.09	50.8	7.17
2	29.8	195	6.56
3	7.09	70.3	9.92
4	10.6	105	9.92
5	5.67	46.9	8.27
6	4.25	37.2	8.74
Mean			8.43
±SE			0.56

Table 3. Effect of 5-HTP in accumulation of 5-HT and caerulein in cultured skin from stage 58 tadpoles.

Treatment	N	5-HT[a] (ng/piece)	N	Caerulein[a] (ng/piece)
Control	11	1.40 ± 0.23	8	13.3 ± 2.57
5-HTP (10^{-3} M)	10	8.12 ± 1.82*	8	0.10 ± 0.02*

[a]Mean ± SE. *Differs from control at $p < 0.001$.

cells of the granular glands. Further, the fact that the difference between chFCS and FCS was greater in the skin from tadpoles at an earlier stage implies that these factors are more critical in the earlier process of development.

In spite of the fact that thyroid hormones stimulate development of skin glands in vivo[9,10,15], addition of T_4 to the chFCS medium failed to increase caerulein content. This suggests that stimulating factor(s) other than thyroid hormones, although probably acting in collaboration with them, exist in FCS. In this respect, it is notable that in the small intestine of *Xenopus* tadpoles, in vitro changes from larval to adult form are caused by the addition of triiodothyronine, cortisol, and insulin to chFCS medium[16]. In contrast to the present results, conventional histological studies have shown that T4 stimulates development of gland rudiments in skin cultures from tadpoles before forelimb eruption[17,18] which occurs at stage 58[13]; there are, however, no data concerning peptide- and amine-producing cells. Since FCS-containing medium was used in these cultures, the difference in results may be due to the different culture media. It also remains possible, however, that there is a different mechanism for the formation of the cell mass of the gland rudiments and the differentiation of peptide- and amine-producing cells.

The present data show that an increase of 5-HT in the granular glands caused a decrease in caerulein content.

These results suggest that, in the granular glands, 5-HT synthesis affects caerulein synthesis, and that this culture system is useful for investigating the correlation between peptide and amine synthesis. In this respect, it is of note that in cultured bovine chromaffin cells, a decrease in adrenal catecholamines following treatment with reserpine and catecholamine-depleting agents produces an increase in opioid peptide content[19–21]. By contrast, it has been reported that in cultured sympathetic ganglia, noradrenaline, somatostatin and substance P are independently regulated[22]. It should be noted that neural tissues contain several types of neuron which synthesise different neurotransmitters and neuropeptides. In contrast with neural tissues, each granular gland possesses the same peptides and amines in the lumen[3,23]. Further studies using the skin culture system may provide useful information about the correlation between peptide and amine synthesis.

However, some problems remain to be solved. For example, in the present experiment, we could not use tadpoles before stage 57, since preliminary experiments have shown that when skin taken from tadpoles before this stage was cultured for eight days, 5-HT and caerulein could not be detected, and cultured skin was damaged by culture for more than eight days. However, in stage 57 skin, gland rudiments are already present. It has also been reported that a low level of thyroid hormones can be detected in the plasma of *Xenopus* tadpoles at stage 57[24], and that expression of thyroid hormone receptor genes can be found in the skin of premetamorphic *Xenopus* tadpoles[25]. Therefore, to investigate the early developmental processes of caerulein and 5-HT producing cells, a long-term culture system needs to be developed.

Acknowledgments. We are grateful to colleagues at the University of Shizuoka and at the Institute for Physiological Sciences, Okazaki, for help with the caerulein RIA, and also to Hideo Takasu (Sumitomo Pharmaceutical Co., Ltd.) for help with the T_4 RIA.

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