Proc. Natl. Acad. Sci. USA
Vol. 74, No. 10, pp. 4693–4696, October 1977
Physiological Sciences

Calcitonin: Its hormonal action on the gill

(calcium influx/phosphate influx/flow rate/norepinephrine/β-adrenergic receptors)

GÉRARD MILHAUD*, JOHN CLIFFORD RANKIN†, L. BOLIS‡, AND A. A. BENSON§

* Service de Biophysique, Faculté de Médecine St-Antoine, Paris, France; † Department of Zoology, University College of North Wales, Bangor, Gwynnedd, United Kingdom; ‡ Institute of General Physiology, University of Messina, Messina, Italy; and § Scripps Institution of Oceanography, University of California at San Diego, La Jolla, California 92093

Contributed by Andrew A. Benson, July 15, 1977

ABSTRACT **When isolated salmon gills were perfused under simulated *in vivo* conditions, norepinephrine increased and calcitonin decreased the rate of flow of perfusate and the influx of calcium and phosphate through the gill. Only the increased water flow was mediated by β-adrenergic receptors. Differences in sensitivity to both hormones were observed with gills from prespawning and postspawning salmon; gills from freshwater-adapted fish were less sensitive to norepinephrine and more sensitive to calcitonin, suggesting a decreased production of this hormone in the late stage of the life cycle. The antagonistic effects of calcitonin and norepinephrine on the gill strongly implicate their regulatory role for calcium homeostasis *in vivo*; the major process being regulated is the influx of calcium into the gill. In fish, calcitonin performs an important role in regulating gill function.**

It is well known that calcitonin is produced in the ultimobranchial gland of fishes (1). However, the physiological role of this hormone in fish remained unknown. Attempts to lower plasma calcium levels by injection of large doses of hog (2) and salmon calcitonin (3) into elasmobranch fish, *Poroderma africanum* and *Squalus suckleyi*, respectively, failed. Pang and Pickford were unable to elicit hypocalcemia in the teleost fish *Fundulus heteroclitus* using hog thyrocalcitonin (4). We found no significant changes in plasma calcium and phosphate levels during the hours after intravenous administration of 2.5 MRC units/kg of extracted porcine or 25 MRC units/kg of synthetic salmon calcitonin into the teleost fish *Oncorhynchus gorbuscha*. Copp *et al.* observed no effect on plasma and urinary calcium after intravenous infusion of 20 MRC units/kg of salmon calcitonin into *Oncorhynchus nerka* (5). These negative results led investigators to consider calcitonin a vestigial peptide in fishes. Its remarkable activity in the mammalian system, however, engendered our interest in calcitonin as a potential gill hormone.

Isolated salmon gill was perfused under conditions approaching those found *in vivo* in a bath of aerated seawater. The hormone, dissolved in a small volume of saline, was injected into the afferent polythene catheter to the gill. Fishes in prespawning conditions could be studied in their natural environment thanks to the mobility of the research vessel ORV Severiana during the Nimpkish II cruise (Alert Bay Region, British Columbia, September 1976). Seawater prespawning fishes were captured in the neighborhood of Alert Bay, and freshwater fishes in postspawning conditions were obtained in Bond Sound's Ahta River. The external bath solution was that of the natural habitat. The composition of the infused Ringer's solution was varied to mimic the ionic composition of the plasma of fishes adapted to sea or freshwater. The dose-response relationships for norepinephrine and calcitonin were clearly evident. Three rates were directly determined through the gill preparation: (*a*) rate of flow of perfusate, (*b*) calcium influx, and (*c*) phosphate influx. The mediation of these responses by β-adrenergic receptors was investigated using propranolol as β-adrenergic blocking agent. The difference in sensitivity to calcitonin and norepinephrine was assessed between gills from fishes in pre- and postspawning conditions.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

We have adapted the gill preparation of Rankin and Maetz (6). Pacific salmon (*Oncorhynchus gorbuscha, O. keta,* and *O. kisutch*) were captured in purse seines and maintained in large tanks of flowing seawater. Twenty gills obtained from the same number of animals weighing from 2 to 5.4 kg in prespawning conditions and from 0.7 to 1.1 kg in postspawning conditions were perfused by cannulating afferent and efferent arteries with polythene tubing. The Ringer's solution used for seawater salmon differed from that for freshwater salmon (Table 1). All perfusion solutions were filtered through a 0.22-μm Millipore filter to remove small particles and gassed with 95% O_2/5% CO_2 throughout the experiments, resulting in a final pH of 7.3. Heparin (2500 international units) was injected intravenously 10 min before salmon were killed by decapitation posterior to the operculum.

Depending on the pre- and postspawning condition of the fish, the gill was suspended by the cannulae in a bath of stirred and aerated seawater or freshwater (250–350 ml) containing, respectively, 200–350 μCi of $^{45}CaCl_2$ or [^{32}P]phosphate. The two cannulae were fitted tightly into cannulae of larger diameter filled with Ringer's solution. The gill was perfused under hydrostatic pressure from a reservoir at a variable height (40 cm above the bath water level). The outlet cannulae terminated in an electronic drop counter connected to a potentiometric chart recorder. To test for leakage in some experiments, bovine serum albumin labeled with ^{125}I was added to the Ringer's solution used for perfusion. Fractions of the perfusate were collected at regular time intervals, usually every 2 min. The radioactivity of the samples (0.5 ml) was measured in a Beckman LS-100 liquid scintillation counter after addition of 7 ml of scintillation fluid (toluene/Triton X-100; 2/1, and 5.5 g of 2,5-diphenyloxazole per liter). Norepinephrine and synthetic salmon calcitonin (Sandoz) were injected as 10-ml pulses into the inlet cannula at increasing concentrations; the pH of calcitonin/Ringer's solution was 7.2. Propranolol was added to the Ringer's solution used for perfusion. Samples of seawater and freshwater were collected and analyzed by the following techniques: atomic absorption for calcium and magnesium, flame photometry for sodium, and spectrophotometry for chloride and phosphate.

Table 1. Composition of seawater and freshwater Ringer's solutions

	Seawater, mM	Freshwater, mM
NaCl	143.1	114.4
$NaHCO_3$	26.2	26.2
$Na_2HPO_4 \cdot 12H_2O$	1.6	1.6
KCl	3.4	2.0
KH_2PO_4	0.3	0.3
$CaCl_2$	1.3	1.3
$MgSO_4 \cdot 7H_2O$	4.1	2.0
$(NH_4)_2SO_4$	0.4	0.4

Polyvinylpyrrolidone (20 g) and 1 g of glucose were added per liter of Ringer's solution (6).

RESULTS

Ionic composition of seawater and freshwater

The large differences in the ionic composition are reported in Table 2. Compared to that for freshwater, the seawater content of calcium was very high and that of phosphate was quite low.

Hormonal effects on gills from fishes in prespawning condition

Effects of Norepinephrine. *Flow rate.* A typical action of 10^{-7}–10^{-6} M norepinephrine is shown in Figs. 1 and 2. This catecholamine increased the rate of flow of perfusate. The effect was barely detectable at a concentration of 10^{-9} M. The dose-response curve (Fig. 2) indicated a mean effective dose (ED_{50}) of 10^{-8} M. *Calcium influx through the gill.* The influx of calcium from seawater was increased by a factor of 8, as shown in Fig. 3. The dose-response curve (Fig. 2) indicated that this effect is discernible at concentration 10^{-9} M with an ED_{50} of 5×10^{-8} M. *Phosphate influx through the gill.* Similarly, the influx of phosphate from seawater is increased as shown in Fig. 1. The dose-response curve (Fig. 2) indicates that this effect is barely detectable at concentration 10^{-9} M with an ED_{50} of 10^{-8} M. The maximal increase is 2.7-fold (10^{-7} M). *Propranolol* (5×10^{-5} M) reversed or blocked the action of norepinephrine on the flow rate but did not prevent the increase of calcium influx through the gill (Fig. 4). Two or more replicate experiments were always performed.

Effects of Calcitonin. *Flow rate.* Calcitonin decreased the rate of flow of perfusate (Fig. 5). The dose-response curve had an ED_{50} of 2 μg (2.9×10^{-7} M) (Fig. 6); the threshold for detection was less than 1 μg (1.4×10^{-7} M). *Calcium influx.* The influx of calcium from seawater was decreased by a factor of 5.6. The ED_{50} was of the order of 3 μg (4.3×10^{-7} M) (Fig. 6). *Phosphate influx.* The phosphate influx from seawater decreased by a factor of 3.8 for 20 μg (2.9×10^{-6} M). This effect was discernible at 2 μg (2.9×10^{-7} M). *Propranolol* (5×10^{-5} M) did not affect the decrease in the rate of flow or calcium influx (Fig. 4).

Table 2. Chemical composition of seawater (Alert Bay) and freshwater (Ahta River, Bond Sound)

	Seawater, mM	Freshwater, mM	Ratio
Calcium	8.4	0.65	12.9
Magnesium	41.4	2.51	16.5
Sodium	400	25	16
Chloride	500	32	15.6
Phosphate	0.01	0.05	0.2

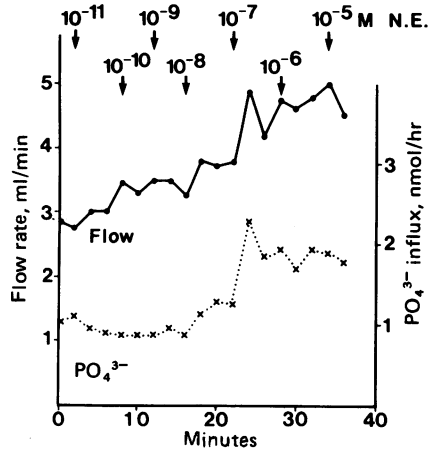

FIG. 1. Effect of norepinephrine (N.E.) on the rate of flow and phosphate influx through the gill. Flow rate is measured directly; phosphate influx is expressed per kg of fish/hr multiplied by 8 (number of gill arches). Female *Oncorhynchus keta* in prespawning condition (4.05 kg).

Hormonal effects on gills from fish in postspawning condition

Effects of Norepinephrine. *Flow rate.* An increase occurred but required higher concentrations of catecholamine, as shown by the ED_{50} (3×10^{-7} M). *Calcium influx.* An increase occurred at elevated concentrations, ED_{50} at 10^{-6}–10^{-5} M.

Effects of Calcitonin. *Flow rate.* The flow rate was decreased and the ED_{50} was approximately 2 μg (2.9×10^{-7} M). *Calcium influx.* The decrease was evident at a much lower concentration than in prespawning condition with an ED_{50} of 2 μg (2.9×10^{-7} M).

DISCUSSION

In fish the gill represents the major organ for ionic, water, and respiratory exchange with the environment. Norepinephrine increases the branchial flow of water. This effect can be prevented by β-blocking agents such as propranolol, suggesting a mediation by β-adrenergic receptors. Calcium influx through the gill is also enhanced by norepinephrine. Large amounts of calcium are involved since the calcium content of seawater is high (8.4 mM) compared to the plasma calcium level of fish (2 mM). This effect is not sensitive to propranolol and appears not to involve β-receptors. The dissociation of the two responses by propranolol suggests that they result from a direct effect on gill epithelium rather than from a hemodynamic effect. Phosphate influx is also increased by catecholamine but the actual amounts

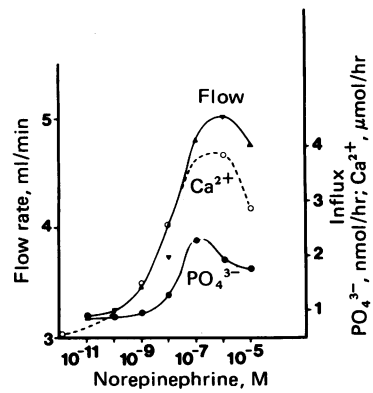

FIG. 2. Norepinephrine: dose-response relationship on the rate of flow and calcium and phosphate influx through the gill.

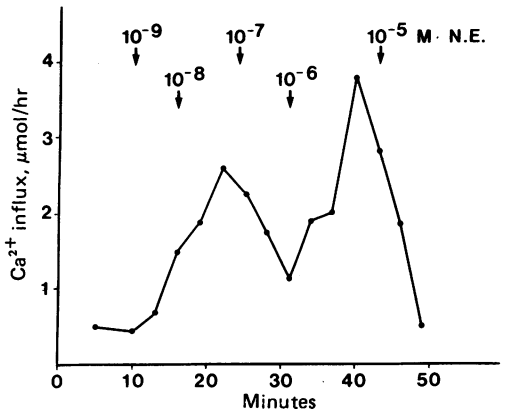

FIG. 3. Effect of norepinephrine (N.E.) on calcium influx through the gill expressed per kg of fish/hr multiplied by the number of gills. Female *Oncorhynchus keta* in prespawning condition (4.05 kg).

are very small due to the low concentration of phosphate in seawater, $^1/_{1000}$ that of calcium.

Calcitonin elicits an opposite effect with catecholamine on the three parameters. It decreases the influx of water, calcium, and phosphate from seawater. The hormone can prevent excess calcium entry from the calcium-rich seawater and thereby contributes to the homeostasis of the "milieu interieur" of the fish. It is remarkable that calcitonin plays an analogous role in fish and in mammals, protecting the "milieu interieur" from an increase in calcium. In mammals the primary nondietary source of calcium is bone. Calcitonin, by inhibiting bone resorption, affects the bone-blood equilibrium. In fish the source of calcium is seawater, and calcitonin decreases calcium influx through the gill.

The difference in sensitivity between gills from fish in prespawning and postspawning condition is striking. The ED_{50} for norepinephrine was higher by almost two orders of magnitude in postspawning conditions, both for water and calcium influx. This observation is in agreement with the physiological catecholamine concentration found in teleost blood by Fontaine *et al.* (7). Under postspawning conditions, the decreased response could be due to the saturation of receptors. After spawning the gills are most sensitive to calcitonin, corresponding

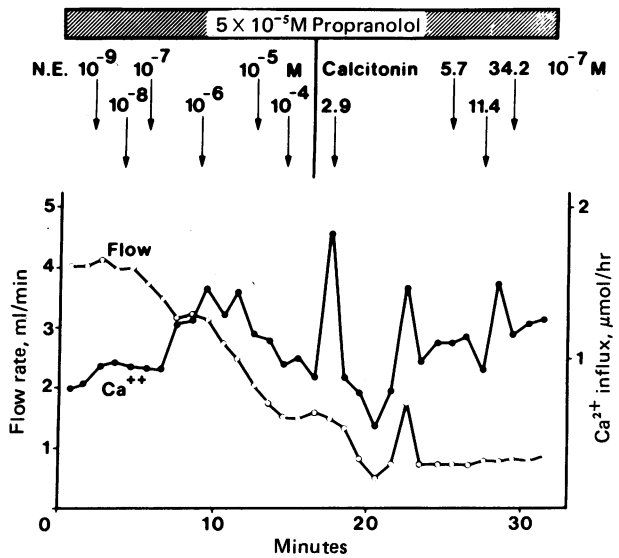

FIG. 4. Effect of propranolol added to the Ringer's solution used for perfusion on the rate of flow and calcium influx through the gill. Male *Oncorhynchus keta* in prespawning condition (4.00 kg).

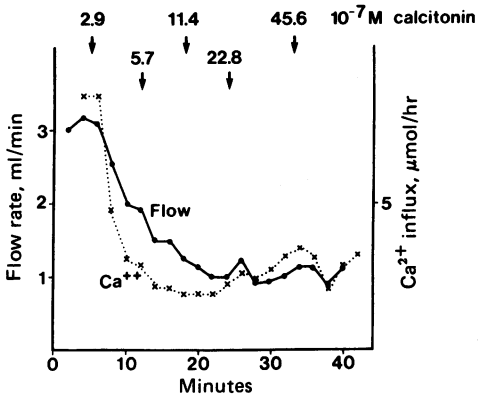

FIG. 5. Effect of calcitonin on the rate of flow and calcium influx through the gill. Male *Oncorhynchus keta* in prespawning condition (3.6 kg).

with a decreased production or secretion of calcitonin or an increased receptor sensitivity at this stage of the life cycle or both. This hypothesis is being tested by assessing the circulating levels of calcitonin and the hormonal content of the ultimobranchial bodies. The death of fish experimentally returned from freshwater to seawater could be explained if calcitonin production could not be raised rapidly enough to control the increased calcium influx. The imbalance would actually be exaggerated by the excess of catecholamine output, which could not be matched by an adequate calcitonin production. After spawning, the condition of the fish deteriorates rapidly. The gills appeared very healthy, as might be expected for a critical organ situated at the interface between the environment and the "milieu interieur," enabling the adaptation from seawater to freshwater, with relatively minor changes in the composition of the "milieu interieur" itself. The decreased sensitivity to norepinephrine and the increased sensitivity to calcitonin suggests function of quite different receptors for each hormone.

Calcitonin is produced in the fish by the ultimobranchial body, which is almost embedded in the gill. Thus, a much higher concentration of the hormone could be supplied to the gill if a special circulating system were collecting the secretion and directly irrigating the gills. Consequently the hormone concentration would be much higher than in systemic blood. The effects reported *in vitro* involve calcitonin concentrations of the order of 10^{-7} M. In *Oncorhynchus tshawytscha* circulating calcitonin levels were 2 μg/liter (0.58×10^{-9} M) in male and 13 μg/liter (3.8×10^{-9} M) in female prespawning fishes (8). This difference between *in vitro* and *in vivo* may seem rather large but could well be explained by dilution effects of the gland effluent blood by the systemic blood. An alternative

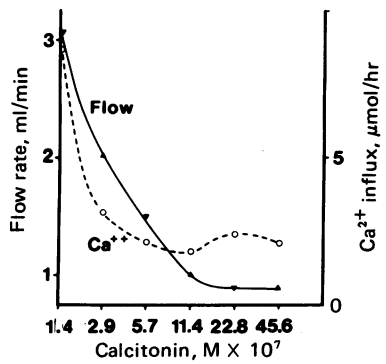

FIG. 6. Calcitonin: dose-response relationship on the rate of flow and calcium influx through the gill.

explanation might be a response to pulsed flow compared to continuous perfusion. This was confirmed using the same concentrations as those found *in vivo* (personal communication from C. Milet, J. Peignoux, and E. Martelly).

The composition of the "milieu interieur" is affected by net fluxes (f_{net}), which are the balance between influx (f_{in}) and outflux (f_{out}) in gills, according to ref 1.

$$f_{net} = f_{in} - f_{out}$$

The calcium influx through the gills of seawater fishes was thought to be a passive process, the calcium moving down its concentration gradient. This is not the case in freshwater-adapted fish.

Seawater fish excrete calcium to the environment through the gills in order to maintain homeostatic conditions. Efflux is an active process since calcium has to move against a concentration gradient. In freshwater-adapted fish, the calcium efflux can be considered a passive process. Any complete study of a dynamic equilibrium in fish should involve the measurements of two parameters. The present investigation was primarily concerned with influxes and is therefore not in a position to give quantitative indications of net fluxes. The isolated gill preparation is well suited for the study of influx but is less reliable for efflux measurement, which may be affected by undetected leaks in the perfusion system, leading to erroneously high outflux values. Nevertheless, in a previous investigation we observed outfluxes for calcium and phosphate. The result suggested that calcitonin enhances the outfluxes of both. If calcium homeostasis is controlled by the influx rather than by the outflux through the gill, it is likely that the influx is the hormonally controlled process, with norepinephrine and calcitonin influencing this process antagonistically.

We sincerely appreciate the generous cooperation and advice of Mr. Ray Scheck, Fishery Officer, Alert Bay, Department of Fisheries, Environment Canada, Mr. George Jolliffe, Jr., Alert Bay, Mr. D. W. Wilkie, Director, Scripps' Aquarium Museum, and Mr. Lewis Sisson and his crew of ORV Severiana. Active participation of Chancellor W. D. McElroy and Mr. Robert O. Peterson made the Nimpkish II cruise possible. This work was supported by the Foundation for Ocean Research and by National Science Foundation Grant BMS 75-18036 and Grant BMS 74-21615 in support of R/V Alpha Helix, on which preliminary studies were carried out.

1. Copp, D. H., Cockroft, D. W. & Kueh, Y. (1967) *Science* **158**, 924.
2. Louw, G. N., Stahl, P. D. & Kenny, A. D. (1969) *Zool. Afr.* **4**, 95–98.
3. Copp, D. H., Brooks, C. E., Low, B. S., Newsome, F., O'Dor, R. K., Parkes, C. O., Walker, V. & Watts, E. G. (1970) in *Calcitonin 1969*, eds. Taylor, S. & Foster, G. (Heinemann Medical Books, London), pp. 281–294.
4. Pang, P. K. T. & Pickford, G. E. (1967) *Comp. Biochem. Physiol.* **21**, 573–578.
5. Copp, D. H., Byfield, P. G. H., Kerr, C. R., Newsome, F., Walker, V. & Watts, E. G. (1972) in *Calcium, Parathyroid Hormone and the Calcitonins*, eds. Talmage, R. V. & Munson, P. L. (Excerpta Medica, Amsterdam), pp. 12–20.
6. Rankin, J. C. & Maetz, J. (1971) *J. Endocrinol.* **51**, 621–635.
7. Fontaine, M., Mazeaud, M. & Mazeaud, F. (1963) *C. R. Hebd. Seances Acad. Sci.*, **256**, 4562–4565.
8. Deftos, L. J., Murray, T. M., Powell, D., Habener, J. F., Singer, F. R., Mayer, G. P. & Potts, J. T. (1972) in *Calcium, Parathyroid Hormone and the Calcitonins*, eds. Talmage, R. V. & Munson, P. L. (Excerpta Medica, Amsterdam), pp. 140–151.