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DEPRESSION OF WHOLE-BODY CALCIUM UPTAKE DURING ACUTE HYPERCALCAEMIA IN AMERICAN EEL, *ANGUILLA ROSTRATA*, IS MEDIATED EXCLUSIVELY BY CORPUSCLES OF STANNIUS

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Summary

1. The role of the corpuscles of Stannius (CS) in acute modulation of whole-body calcium influx (J_{in}^{Ca}) in American eel, *Anguilla rostrata*, was investigated by (i) assessing the effects of stanniectomy on J_{in}^{Ca} and plasma total calcium concentration ([Ca_{tot}]), (ii) comparing the abilities of sham-operated and stanniectomized eels to reduce J_{in}^{Ca} during artificially induced hypercalcaemia, and (iii) monitoring the effects of homologous hypocalcin (a 54×10^3 M_r glycoprotein) injection on J_{in}^{Ca}.

2. Stanniectomy (STX) caused a pronounced elevation of J_{in}^{Ca} and hypercalcaemia measured 7 days after surgery.

3. When hypercalcaemia was induced by intra-arterial infusion of $CaCl_2$, a treatment known to cause degranulation of the CS and the specific release of hypocalcin, J_{in}^{Ca} was reduced significantly within 1 h in intact fish. NaCl infusion did not affect plasma [Ca_{tot}] or J_{in}^{Ca} in any group tested.

4. Stanniectomy prevented the reduction of J_{in}^{Ca} associated with the hypercalcaemia induced by $CaCl_2$ infusion.

5. Intra-arterial infusion of $MgCl_2$ caused a significant elevation of plasma total magnesium concentration [Mg_{tot}] but did not alter J_{in}^{Ca}.

6. Intra-arterial infusion of hypocalcin (18.5 nmol kg^{-1} body mass) into intact eels decreased J_{in}^{Ca} to an extent similar to that seen following artificially induced hypercalcaemia.

7. We conclude that the rapid reduction of J_{in}^{Ca} during experimental hypercalcaemia is mediated by hypocalcin released from the corpuscles of Stannius and suggest that calcitonin, another putative hypocalcaemic hormone, is not involved. The results are discussed with respect to the relative importance of hypocalcin and calcitonin as hypocalcaemic hormones in fish.

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Key words: corpuscles of Stannius, calcium, eel, gill, hypocalcin.

Introduction

The corpuscles of Stannius (CS) are small spherical endocrine glands found in teleostean and holostean fishes. In the eel there is a single pair of CS on the ventrocaudal aspect of the kidney. There is overwhelming evidence that type-1 cells of the CS contain a potent hypocalcaemic factor (reviewed by Wendelaar Bonga and Pang, 1986). Removal of the CS causes hypercalcaemia in all teleosts tested (Fontaine, 1964, 1967; Fenwick and Forster, 1972; Fenwick, 1974; Pang *et al.* 1973; Schreibman and Pang, 1975; Kenyon *et al.* 1980; Urasa and Wendelaar Bonga, 1987; Hanssen *et al.* 1989). Further, the hypercalcaemia induced by stanniectomy is prevented by ectopic CS implants (Pang *et al.* 1973; Schreibman and Pang, 1975) or reversed by injections of CS extracts (Kenyon *et al.* 1980; Hanssen *et al.* 1989). Injection of CS extracts also inhibits branchial (So and Fenwick, 1979; Lafeber *et al.* 1988*a*) and whole-body (Lafeber *et al.* 1988*a*; Wagner *et al.* 1986, 1988*a*) calcium uptake (J_{in}^{Ca}) in eel and rainbow trout.

The hypocalcaemic factor within the CS was initially termed hypocalcin by Pang *et al.* (1974) and later teleocalcin (Ma and Copp, 1978; Wagner *et al.* 1986). It is likely that the terms hypocalcin and teleocalcin describe similar proteins (see below). In this paper we advocate the use of the term hypocalcin because of its historical precedence and because it is more descriptive of its function and does not exclude its use with the holosteans.

Hypocalcin is a glycoprotein with excellent homology in the *N*-terminal amino acid sequence in eel, trout and salmon (Wagner *et al.* 1986, 1988*b*; Butkus *et al.* 1987, 1989; Lafeber *et al.* 1988*b*). Estimates of the relative molecular mass of hypocalcin have been somewhat variable and inconsistent owing to methodological problems. Most recent studies, however, report a relative molecular mass of $52 \times 10^3 - 54 \times 10^3$ for the native hypocalcin dimer molecule of trout (Lafeber *et al.* 1988*b*), eel (Butkus *et al.* 1987) and salmon (Wagner *et al.* 1988*a*; Butkus *et al.* 1989).

The results of several experimental protocols implicate hypocalcin as a hypocalcaemic hormone in fishes. First, the secretory activity (Wendelaar Bonga *et al.* 1976), volume and density (Urasa and Wendelaar Bonga, 1987) of CS type-1 cells increase as a function of external calcium concentration. Second, elevated calcium levels *in vitro* (Aida *et al.* 1980) or *in vivo* (Lopez *et al.* 1984; Lafeber *et al.* 1988*b*; Lafeber and Perry, 1988) cause degranulation of type-1 cells, specific disappearance of hypocalcin from the CS (Lafeber *et al.* 1988*b*; Lafeber and Perry, 1988) and concomitant inhibition of whole-body J_{in}^{Ca}, similar to that caused by intra-arterial injection of homologous hypocalcin in trout (Lafeber *et al.* 1988*a*). Third, injection of hypocalcin from trout (Lafeber *et al.* 1988*c*) or European eel (Hanssen *et al.* 1989) into stanniectomized eels lowers plasma calcium levels.

It was concluded (Lafeber and Perry, 1988) that hypocalcin is released from CS in response to acute hypercalcaemia to inhibit branchial calcium influx rapidly and thus aid in the regulation of hypercalcaemia. The experimental protocol utilized, however (Lafeber and Perry, 1988), could not exclude the possibility that other putative hypocalcaemic hormones, such as calcitonin (Lopez *et al.* 1976; Milet

et al. 1979), were involved in the acute reduction of J_{in}^{Ca} during experimental hypercalcaemia.

In the present study, we examined the role of hypocalcin in the American eel, *Anguilla rostrata*, and tested specifically the hypothesis that the reduction of calcium uptake during experimentally induced hypercalcaemia is mediated exclusively by corpuscles of Stannius. These objectives were achieved (i) by assessing the effects of stanniectomy on J_{in}^{Ca} and plasma [Ca$_{tot}$], (ii) by monitoring the effects of homologous hypocalcin on J_{in}^{Ca} and (iii) by comparing the responses of intact and stanniectomized eels to intra-arterial CaCl$_2$ injection. In addition, we tested the specificity of the calcium influx inhibition response by comparing J_{in}^{Ca} in CaCl$_2$- and MgCl$_2$-injected eels.

Materials and methods

Experimental animals

Immature American eels, *Anguilla rostrata*, [mean mass=165.2±2.1 g (±S.E.); experimental N=123] were obtained from an eel ladder associated with the Saunders Hydroelectric Dam in Cornwall, Ontario, and were transported on ice to the University of Ottawa. Fish were maintained on a 12h light:12h dark photoperiod in large opaque fibreglass aquaria supplied with flowing, aerated and dechlorinated City of Ottawa tap water ([Na$^+$]=0.12 mmol l^{-1}; [Cl$^-$]= 0.15 mmol l^{-1}; [Ca^{2+}]=0.40–0.45 mmol l^{-1}; [K$^+$]=0.03 mmol l^{-1}; pH 7.5–8.0) for at least 3 weeks prior to experimentation. Water temperature (in holding and experimental facilities) ranged from 9°C (September) to 4°C (January). Fish were not fed throughout the period of this study.

Surgical procedures

Eels were anaesthetized in a solution of ethyl-*m*-aminobenzoate (2 g l^{-1}; MS 222, Sigma) adjusted to pH 7.5–8.0 with Tris buffer (Trizma Base, Sigma). To permit intra-arterial injection and periodic blood sampling, indwelling cannulae were implanted into a pneumogastric artery. An incision was made 3 cm caudal to the heart and right lateroventrally. The pneumogastric artery was isolated caudally to the gall bladder and dorsal to the gas bladder. The artery was ligated caudally and clamped rostrally. A small incision was made and polyethylene tubing (Clay Adams PE 50; i.d.=0.58 mm; o.d.=0.97 mm) was inserted and secured. The cannula was passed through the body wall and sutured to the lateral body wall musculature. The wound was sutured and the fish transferred to an opaque Perspex box (volume=3 l) supplied with flowing water. Fish were allowed to recover from the effects of anaesthesia and surgery for 48 h before experiments commenced. Cannulae were flushed daily with freshwater teleost physiological saline (Wolf, 1963).

Stanniectomy was performed according to Leloup-Hatey (1964). A 3 cm incision was made approximately 1 cm laterally from the mid-line at the level of the vent. The paired CS were revealed on the ventral surface of the kidney after perforation

of the kidney capsule. The corpuscles were removed and the wound sutured. The shams were treated in an identical fashion except for removal of the CS. The eels were allowed to recover for 7 days in 100 l tanks.

Isolation of eel hypocalcin

Corpuscles of Stannius were removed from approximately 1000 American eels and yielded about 200 mg of CS after lyophilization. Hypocalcin was isolated as described previously (Lafeber et al. 1988b) for rainbow trout using concanavalin A affinity chromatography. The material without affinity for concanavalin A (crude CS homogenate minus hypocalcin) is referred to as residue protein and was devoid of hypocalcin, based on SDS–gel electrophoresis.

Determination of whole-body Ca^{2+} fluxes

Fish were placed in individual Perspex flux boxes containing 500 ml of water. 50 μCi of ^{45}Ca (as $CaCl_2$; ICN) was added and allowed to equilibrate for 30 min. ^{45}Ca specific activity of the water was determined on samples removed at 30 min (time=zero) and 4 h after addition of the ^{45}Ca to the water. At 4 h, a blood sample (1.0 ml) was withdrawn either from the arterial cannula or by cardiac puncture (uncannulated eels only) after killing the fish by over-anaesthesia (4 g l^{-1} MS 222). Blood samples were centrifuged (13 000 g for 3 min) and the plasma removed and stored (−20°C) for subsequent ion analyses. The fish was then rinsed in a solution of $CaCl_2$ (10 mmol l^{-1}) and then tap water, to facilitate removal of ^{45}Ca bound to mucus and integumental surfaces (Perry and Wood, 1985). J_{in}^{Ca} was determined by the method of Perry and Wood (1985) utilizing measurements of whole-body ^{45}Ca activity and mean water ^{45}Ca specific activity. To determine whole-body ^{45}Ca activity, fish were cooked in a microwave oven for 1.5 min and then homogenized in a commercial blender with 100 ml of distilled water. Five samples of the tissue homogenate (approximately 0.5 g weighed to the nearest 0.001 g) were dissolved at 60°C in 2 ml of tissue solubilizer (NCS, Amersham). The dissolved samples were neutralized with glacial acetic acid. 5 ml of distilled water and 10 ml of fluor (ACS II, Amersham) were added and the ^{45}Ca activity determined using liquid scintillation counting (LKB Rackbeta). Ca^{2+} net flux (J_{net}^{Ca}) was determined from the change in water Ca^{2+} levels over the 4-h flux period.

Plasma and water analyses

Water and diluted (200×) plasma [Ca_{tot}], [Na^+], [K^+] and [Mg_{tot}] were determined by flame emission or atomic absorption (Mg only) spectrophotometry (Varian model Spectra AA-10). Water and plasma [Cl^-] were determined by amperometric titration (Buchler–Cotlove chloridometer) on undiluted 1 ml and 100 μl samples, respectively. ^{45}Ca activity was determined on 5 ml water samples by liquid scintillation counting. Water pH was measured using a Fisher Accumet pH meter and gel-filled polymer body electrodes.

Experimental protocol

Three series of experiments were performed.

Series I. Effects of stanniectomy on Ca^{2+} fluxes and plasma ion levels

Whole-body J_{in}^{Ca}, J_{out}^{Ca}, J_{net}^{Ca} and plasma ion (Ca_{tot}, Na^+, K^+, Mg_{tot} and Cl^-) concentrations were determined in stanniectomized, sham-operated and intact eels. Fish were transferred to the flux boxes 7 days after surgery and left for 12 h before flux determinations commenced. To control for the effects of possible cyclical or day-to-day changes in fluxes (Wagner *et al.* 1986), experiments were performed simultaneously on the intact, sham-treated and stanniectomized groups. The eels were not cannulated in this series of experiments.

Series II. Effects of $CaCl_2$ and $MgCl_2$ injections on Ca^{2+} fluxes

Initial experiments were performed to assess the degree and duration of plasma ionic disturbance caused by intra-arterial injections of NaCl (controls), $CaCl_2$ or $MgCl_2$. These experiments were necessary to ensure that hypercalcaemia and hypermagnesaemia were not caused by NaCl injection, but were induced by $CaCl_2$ and $MgCl_2$ injections, respectively, and to determine an appropriate period for measuring Ca^{2+} fluxes after the solute injections. These experiments were also performed simultaneously on intact, sham-treated and stanniectomized fish. Eels were injected *via* the cannula with 1 ml of either 120 mmol l^{-1} NaCl, 70 mmol l^{-1} $CaCl_2$ or 70 mmol l^{-1} $MgCl_2$ immediately followed by 0.5 ml of physiological saline to flush the cannula. A blood sample (200 μl) was withdrawn just prior to injection. Additional blood samples were removed 2, 5, 10, 15, 30, 60, 90, 120, 180, 240 and 300 min after the injection. Physiological saline (200 μl) was re-injected after each blood sample. Plasma was obtained by centrifugation (3 min at 13 000 g), stored and analysed as described above.

These preliminary experiments revealed that flux measurements could be made between 1 and 5 h after injection because $[Ca_{tot}]$ (after $CaCl_2$ injection) and $[Mg_{tot}]$ (after $MgCl_2$ injection) were elevated significantly, but were in a steady-state during this period. Thus, fish were injected with the appropriate solute and after 1 h, a 4 h flux period was initiated. Again, concurrent experiments were conducted on intact, sham-operated and stanniectomized fish. The effects of $MgCl_2$ injection on Ca^{2+} fluxes were determined on intact fish only.

Series III. Effects of hypocalcin injections on Ca^{2+} fluxes

Only intact fish were used in this series of experiments. Fish were injected at time zero of the flux period *via* the cannula with either 200 μl of physiological saline, 16.5 nmol (1 mg) kg^{-1} body mass hypocalcin (in 200 μl saline), or 1.5 mg kg^{-1} protein residue (in 200 μl saline). These doses of hypocalcin and protein residue approximately equal the amount of material obtained from six fish of equal body mass. All injections were followed immediately by 0.5 ml of physiological saline to flush the cannula and ensure delivery of the injected substance to the circulation.

Statistical analysis

Data are presented as means ±1 standard error. Differences between means were evaluated using the Student's unpaired t-test (two-tailed). Significance was accepted at $P \leqslant 0.05$.

Results

Stanniectomy caused a 140 % increase in J_{in}^{Ca} measured 7 days after surgery (Fig. 1). Plasma [Ca_{tot}] in stanniectomized fish was elevated by 65 % (Fig. 1); no other measured plasma ion concentration changed significantly (data not shown). Sham-treatment was without significant effect on either J_{in}^{Ca} or plasma [Ca_{tot}] (Fig. 1).

Intra-arterial injection of $CaCl_2$ caused similar changes in plasma [Ca_{tot}] in the intact, sham-operated and stanniectomized eels (Fig. 2). After the injection there was a pronounced hypercalcaemia (approximately $2 \, mmol \, l^{-1}$ above pre-injection values) followed by a relatively rapid decline of [Ca_{tot}] to reach a new steady state after about 1 h. For the next 4 h, plasma [Ca_{tot}] was elevated above the control levels (NaCl-injected) by approximately $1 \, mmol \, l^{-1}$. No other measured plasma ion concentration changed after injection of $CaCl_2$ (data not shown). Injection of $MgCl_2$ into intact eels resulted in equivalent changes in plasma [Mg_{tot}] (Fig. 3). There was a marked elevation of [Mg_{tot}] after the injection, followed by a rapid decline within the first 60 min, with a new steady-state condition of hypermagnesaemia achieved between 1 and 5 h. Plasma [Ca_{tot}] was unaffected by $MgCl_2$ injection; the occasionally lower plasma [Ca_{tot}] in the $MgCl_2$-injected fish reflected different pre-injection values (Fig. 3). Injection of NaCl did not alter plasma
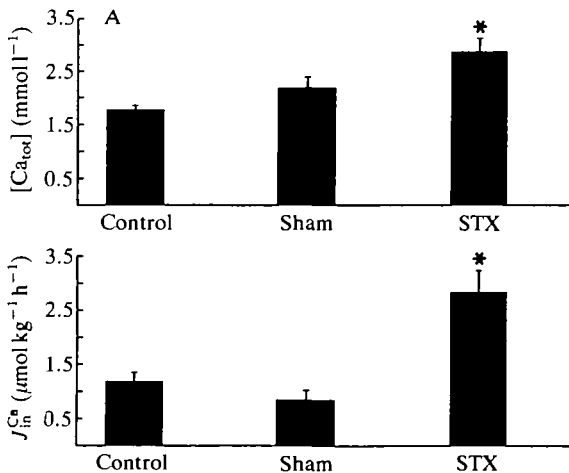

Fig. 1. The effects of sham-treatment ($N=6$) or 7-day stanniectomy (STX; $N=5$) on (A) plasma total calcium concentration ([Ca_{tot}]) and (B) whole-body calcium uptake (J_{in}^{Ca}) in American eel, *Anguilla rostrata*. * Significantly different ($P \leqslant 0.05$; Student's t-test) from control (intact) fish ($N=5$).
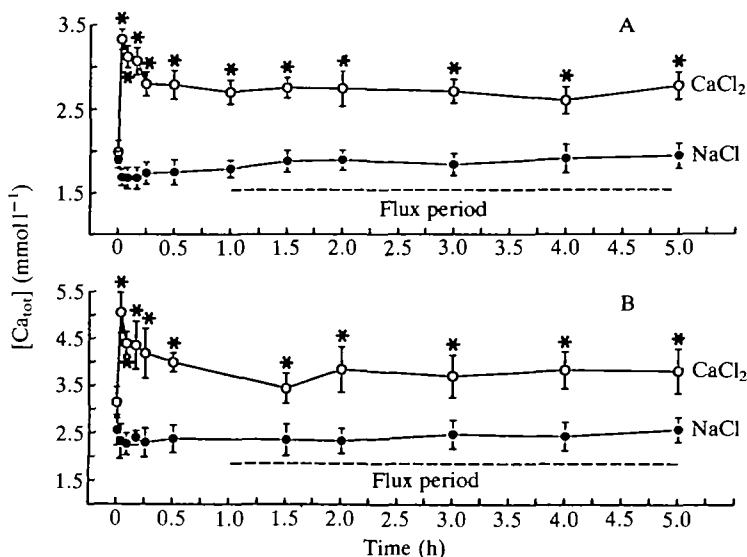
Fig. 2. The effects of intra-arterial injections of $120 \, \text{mmol} \, l^{-1}$ NaCl (●——●; $N=5$ in each group) or $65 \, \text{mmol} \, l^{-1}$ CaCl$_2$ (○——○; $N=6$ in each group) on plasma calcium concentration ($[\text{Ca}_{\text{tot}}]$) in (A) intact ($N=11$) and sham-treated ($N=11$) eels [the values from the shams and intact fish are combined ($N=22$) since there were no significant differences between the two groups] and (B) 7-day stanniectomized eels ($N=11$). *Significantly different from value in the NaCl-injected fish at corresponding time ($P \leqslant 0.05$). Note that a new steady-state plasma $[\text{Ca}_{\text{tot}}]$ was achieved 1 h after the injection. The horizontal dashed line indicates the period when calcium influx was determined.

$[\text{Ca}_{\text{tot}}]$ (Fig. 2), $[\text{Mg}_{\text{tot}}]$ (Fig. 3) or any other measured plasma ion concentration (data not shown).

The hypercalcaemia induced by injection of CaCl$_2$ caused reductions of $J_{\text{in}}^{\text{Ca}}$ in the intact (control) and sham-operated eels of 63 % and 57 %, respectively, compared to the NaCl-injected eels (Fig. 4). $J_{\text{in}}^{\text{Ca}}$ was not affected by CaCl$_2$ loading in the stanniectomized fish (Fig. 4). The induction of hypermagnesaemia in intact eels did not significantly affect $J_{\text{in}}^{\text{Ca}}$ (Fig. 5); once again, the potent inhibitory effect of elevated plasma $[\text{Ca}_{\text{tot}}]$ on $J_{\text{in}}^{\text{Ca}}$ was observed.

Intra-arterial injection of hypocalcin in intact eels caused a decrease in $J_{\text{in}}^{\text{Ca}}$ of 46 % compared to eels injected with physiological saline (Fig. 6). Injection of CS protein residue did not affect $J_{\text{in}}^{\text{Ca}}$ (Fig. 6).

Discussion

In these experiments whole-body $J_{\text{in}}^{\text{Ca}}$ was measured. Although the relative contributions of the gills and skin to whole-body $J_{\text{in}}^{\text{Ca}}$ are still debated (e.g. Perry d Wood, 1985), it seems probable that the effects of hypocalcin on $J_{\text{in}}^{\text{Ca}}$ observed in this study were mediated at the gills (Lafeber *et al.* 1988*b*). Thus, we are
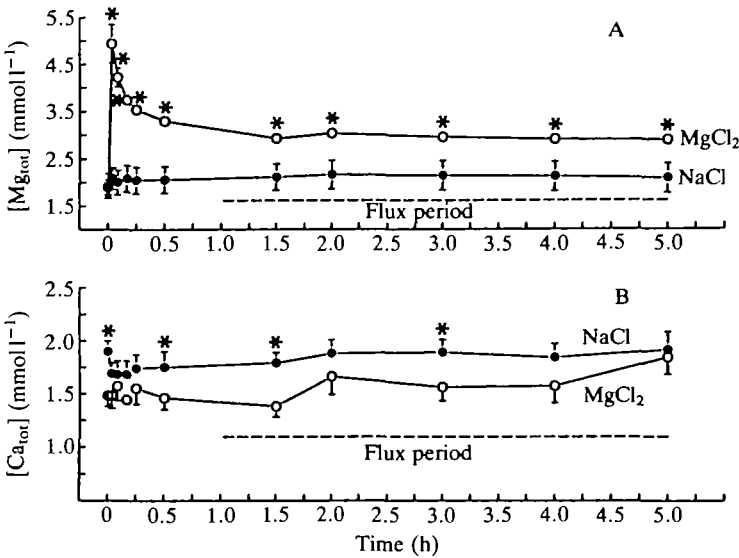
Fig. 3. The effects of intra-arterial injections of 120 mmol l^{-1} NaCl (●————●; $N=5$) or 65 mmol l^{-1} MgCl$_2$ (○————○; $N=6$) on (A) plasma magnesium concentration ([Mg$_{tot}$]) and (B) plasma calcium concentration ([Ca$_{tot}$]) in intact eels. All other details as in Fig. 2.

confident that the changes in whole-body J_{in}^{Ca} reported here were due primarily to modification of inward transbranchial calcium movement.

The results of a previous study using an identical protocol to elevate plasma [Ca$_{tot}$] (Lafeber and Perry, 1988) demonstrated that hypercalcaemia caused degranulation of CS cells and a depletion of CS hypocalcin. Consequently, it was assumed that hypocalcin was released into the circulation and inhibited branchial
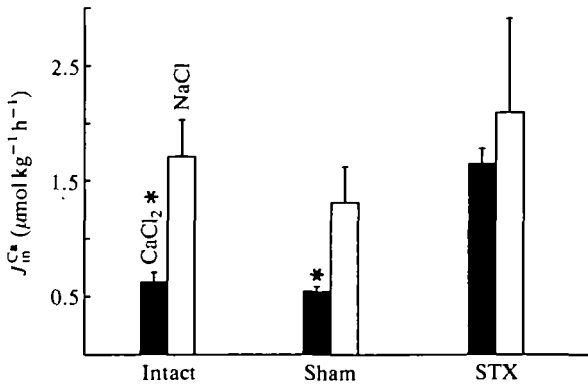

Fig. 4. The effect of intra-arterial injection of CaCl$_2$ (filled bars; $N=5$ in each group) on whole-body calcium uptake (J_{in}^{Ca}) in control (intact), sham-treated and stanniecto-mized (STX) eels. * Significantly different ($P \leqslant 0.05$) from J_{in}^{Ca} in NaCl-injected fish (open bars; $N=6$ in each group).
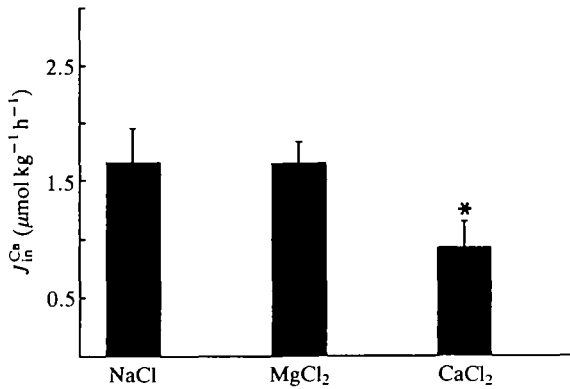
Fig. 5. The effect of intra-arterial injection of $MgCl_2$ ($N=6$) or $CaCl_2$ ($N=6$) on whole-body calcium uptake (J_{in}^{Ca}) in intact eels. * Significantly different ($P\leqslant0.05$) from J_{in}^{Ca} in the NaCl-injected ($N=6$) fish.

J_{in}^{Ca}. These data were consistent with a hypocalcaemic hormonal role for CS hypocalcin, but could not exclude the possibility that other hypocalcaemic hormones were modulating (in whole or in part) the branchial regulatory response. The results of the present study, however, demonstrate unequivocally that hypocalcin promotes the depression of J_{in}^{Ca} during acute hypercalcaemia. The absence of any reduction in J_{in}^{Ca} following $CaCl_2$ infusion in the stanniectomized eels indicates that this response is controlled specifically by hypocalcin and that other hormones are not involved.

Calcitonin is frequently named as a putative hypocalcaemic hormone in fish, yet the results of experiments designed to test the role of calcitonin in fish are often either inconclusive or contradictory (for recent review, see Fenwick, 1989). For example, injections of calcitonin in fish have been reported to cause hypocalcaemia (Chan *et al.* 1968; Wendelaar Bonga, 1981), to have no effect on plasma [Ca_{tot}]
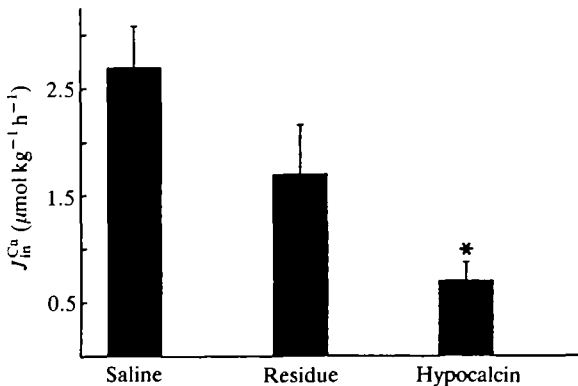

Fig. 6. The effect of intra-arterial injection of CS hypocalcin ($N=6$) or protein residue ($N=6$) on whole-body calcium uptake (J_{in}^{Ca}) in intact eels. *Significantly different ($P\leqslant0.05$) from J_{in}^{Ca} in eels injected with physiological saline ($N=6$).

(Pang, 1971; Hirano *et al.* 1981) or even to induce hypercalcaemia (Fouchereau-Peron *et al.* 1987). Calcitonin receptors have been localized in fish gill (Fouchereau-Peron *et al.* 1981) and addition of calcitonin to perfused gill preparations of eel (Milet *et al.* 1979) or salmon (Milhaud *et al.* 1977) was shown to inhibit J_{in}^{Ca}. Further, partial ultimobranchialectomy of goldfish acutely transferred from fresh water to 30 % sea water resulted in a substantial increase in plasma $[Ca_{tot}]$ (Fenwick, 1975). In contrast, investigations of calcitonin *in vivo* in freshwater-adapted eel have demonstrated little, if any, effect on J_{in}^{Ca} (D. L. Kingsbury and J. C. Fenwick, in preparation). The conflicting results of experiments with calcitonin are in sharp contrast to the consistent hypocalcaemic effects of hypocalcin and the hypercalcaemic effects of stanniectomy. Since acute regulation of internal calcium balance in fishes is mediated primarily by adjustment of branchial Ca^{2+} fluxes (see Fenwick, 1989), it seems likely that hypocalcin, rather than calcitonin, is the dominant hypocalcaemic hormone in fishes. Of course, it is conceivable that hypocalcin is, in part, permissive and required for calcitonin to elicit effects. This possibility has not been tested.

Previous studies have demonstrated gradual development of hypercalcaemia after stanniectomy or lowering of plasma $[Ca_{tot}]$ after homologous hypocalcin injection in stanniectomized eels (e.g. Hanssen *et al.* 1989). It was speculated that these effects resulted from stimulation or inhibition, respectively, of J_{in}^{Ca}. The present results provide the first *in vivo* evidence that the changes in plasma $[Ca_{tot}]$ after stanniectomy or hypocalcin injection are caused (at least in part) by hypocalcin-mediated control of whole-body calcium uptake with the presumed site of action being the gill. Removal of the CS, and hence the source of hypocalcin, must cause plasma levels of hypocalcin to decline, resulting in stimulation of J_{in}^{Ca}. Interestingly, this indicates that J_{in}^{Ca} is tonically depressed by basal levels of hypocalcin. This source of tonic inhibition can either be removed (e.g. after stanniectomy), reduced (e.g. in fish acclimated to low environmental $[Ca^{2+}]$; Wendelaar Bonga *et al.* 1976; Urasa and Wendelaar Bonga, 1987) or increased (e.g. after hypocalcin injection or in fish acclimated to Ca^{2+}-enriched water; Wendelaar Bonga *et al.* 1976). Such a tonic action of hypocalcin on J_{in}^{Ca} would provide very flexible control of plasma calcium balance, whereby J_{in}^{Ca} is either inhibited or stimulated depending on calcium requirements or environmental conditions.

The $1.0 \, mmol \, l^{-1}$ elevation of plasma $[Ca_{tot}]$ 7 days after stanniectomy can be accounted for fully by the observed stimulation of J_{in}^{Ca} ($+1.65 \, \mu mol \, kg^{-1} \, h^{-1}$) if one assumes this stimulation is representative of the entire 7-day period and there was little change in the rate of internal calcium turnover. It is more difficult to assess whether the similar degree of hypocalcaemia within only 24 h after injection of hypocalcin in stanniectomized eels (Hanssen *et al.* 1989) could be attributable solely to inhibition of J_{in}^{Ca}. It is clear, however, that the magnitude of the hypocalcin-mediated inhibition of J_{in}^{Ca} would need to be much greater than reported in the present study using intact eels. This is a distinct possibility hypercalcaemia potentiates the inhibitory effect of hypocalcin on J_{in}^{Ca}. Evidence

for such a phenomenon is that experimentally induced hypercalcaemia caused a greater reduction in J_{in}^{Ca} than did injection of hypocalcin in normocalcaemic fish (compare Figs 4 and 5). In the study of Hanssen *et al.* (1989), hypocalcin was injected 15 days after stanniectomy, at which time plasma total calcium levels had risen to approximately $6\,mmol\,l^{-1}$. In addition, it is conceivable that chronic reduction of endogenous hypocalcin levels after stanniectomy enhanced the responsiveness of the gill to exogenous hypocalcin.

The mechanism whereby hypocalcin reduces branchial calcium uptake has not been elucidated. Our current understanding of the mechanisms involved in gill transepithelial uptake of Ca^{2+} (Perry and Flik, 1988; Fenwick, 1989) suggests two possible modes of action. The most likely is that hypocalcin decreases the permeability of apical Ca^{2+} channels in branchial chloride cells, which are thought to be the cells involved in calcium influx (Perry and Wood, 1985; Ishihara and Mugiya, 1987; Perry and Flik, 1988). In this way, rapid adjustments of J_{in}^{Ca} could be achieved. A second possibility is that the affinity of the basolateral Ca^{2+}-ATPase to Ca^{2+} is decreased. Preliminary data obtained using gill basolateral membrane vesicles, however, reveal no effect of hypocalcin on vesicular Ca^{2+} transport (G. Flik, unpublished observations). The latter possibility, therefore, seems unlikely. Calcium uptake was not depressed during hypermagnesaemia. This suggests that the control of hypocalcin secretion from the corpuscles of Stannius is specific to adjustments of plasma calcium, and not a general response to divalent cations.

In summary, the criteria required to identify hypocalcin as a hypocalcaemic hormone in the eel have been met and its mode of action established. First, injection of homologous hypocalcin causes hypocalcaemia (Hanssen *et al.* 1989) and reduction of calcium uptake (this study). Second, removal of the source of hypocalcin (stanniectomy) causes hypercalcaemia and stimulation of calcium influx (this study). Third, hypocalcin is released from the CS during hypercalcaemia (Lafeber and Perry, 1988) and calcium uptake is depressed concurrently (this study). Fourth, stanniectomy prevents the depression of calcium uptake during hypercalcaemia (this study). We conclude that hypocalcin is the dominant hypocalcaemic hormone in the eel and regulates acute hypercalcaemia by inhibiting branchial calcium uptake.

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