GENERAL AND COMPARATIVE ENDOCRINOLOGY 84, 155–163 (1991)

Regulation of Secretion of the Teleost Fish Hormone Stanniocalcin: Effects of Extracellular Calcium

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Accepted December 5, 1990

The release *in vivo* and *in vitro* of stanniocalcin (STC) from the corpuscles of Stannius (CS) of the rainbow trout and the European eel was studied. Intraperitoneal injection of $CaCl_2$ (2.45 mmol · kg^{-1} fish) leads to an elevation of both ionic and total calcium in the plasma and results in the release of STC from the CS into the blood. Release of STC *in vitro* is not affected at "physiological" (1.0–1.5 m*M*) or lower Ca^{2+} levels in the incubation medium. High levels of Ca^{2+} (2.5 m*M* and higher), however, stimulate the release of STC, in particular that of stored STC. We hypothesize that variations in extracellular Ca^{2+} in the normocalcaemic range do not *directly* regulate STC release.

The predominant calcitropic hormone in teleost fish is stanniocalcin (STC), previously referred to as hypocalcin (Pang *et al.*, 1974) or teleocalcin (Ma and Copp, 1978). It is produced by the corpuscles of Stannius (CS) (Milet *et al.*, 1979; Fenwick, 1982; Wagner *et al.*, 1986; Wendelaar Bonga and Pang, 1986; Flik *et al.*, 1989). We have shown that in European eels this hypocalcemic hormone exerts its function by controlling plasma ionic calcium (Hanssen *et al.*, 1989).

In mammals the secretion of two major calcitropic hormones is probably regulated directly by the extracellular calcium level. The parathyroid gland parathormone (PTH) secretion is correlated sigmoidally with extracellular ionic calcium (Brown, 1983). Increased calcitonin secretion by C cells is correlated with elevated serum calcium levels (Austin *et al.*, 1979); this has led to the conclusion that external calcium is involved in the regulation of calcitonin secretion.

The role of extracellular Ca^{2+} as a regulator of the secretory activity of the CS is hitherto unclear. Lopez *et al.*, (1984) showed that experimentally induced hypercalcemia in the European eel led to a complete hormone depletion of the cells. $CaCl_2$ infusions in rainbow trout caused a degranulation of CS cells accompanied by acute inhibition of whole body Ca^{2+} influx (Lafeber and Perry, 1988), suggesting a rapid and direct effect of elevated plasma Ca^{2+} on the release of STC. Flik *et al.*, (1989) reported that in goldfish, trout, and eel, $CaCl_2$ injections induced a rise in plasma calcium and a release of immunoreactive STC from the CS. A direct effect of extracellular calcium on the CS cells was suggested before by the results of *in vitro* experiments: incubations of coho salmon CS showed enhanced exocytosis in high Ca^{2+} media but not in low Ca^{2+} media (Aida *et al.*, 1980). STC release by rainbow trout CS-cells in primary culture was dose dependently stimulated by extracellular calcium (Wagner *et al.*, 1989).

In this paper we investigated whether the experimentally induced and acutely stimulating effect of a rise in extracellular calcium on the secretory activity of the CS indeed has relevance for the *in vivo* control of STC secretion. To obtain defined extra-

0016-6480/91 $1.50

cellular Ca^{2+} levels and to exclude factors controlling release of STC *in vivo* (plasma factors, innervation) we studied the effect of extracellular Ca^{2+} on STC-release *in vitro*. The effects of Ca^{2+} concentrations below, above, and in, what is called, the physiological range (1.0–1.5 m*M*) were examined on the release *in vitro* of total immunoreactive and newly synthesized STC. Also the effect of calimycin A23187, which increases intracellular Ca^{2+} (Foreman *et al.*, 1973), was tested on STC release. Levels of immunoreactive STC were assessed with an ELISA technique.

MATERIAL AND METHODS

Animals

European eels (*Anguilla anguilla*) and rainbow trout (*Oncorhynchus mykiss*), weighing around 250 g, were kept in 1000-liter tanks supplied with running Nijmegen tapwater (main ion concentrations in m*M*: Ca^{2+}, 0.7; Na^{+}, 1.9; Cl^{-}, 3.1; Mg^{2+} 0.2; temperature 12°). Rainbow trout were fed daily with Trouvit pellets. The eels were not feeding. The animals had been acclimated to laboratory conditions for at least 14 days. Freshly dissected CS of eel and trout were carefully freed of connective tissue and kidney tissue and were collected in Hanks' balanced salt solution (HBSS; Flow laboratories).

$CaCl_2$ Injections

Rainbow trout were injected intraperitoneally with 300 μl of a $CaCl_2$ solution (2.04 *M* $CaCl_2$ in 0.9% NaCl), a treatment previously shown to produce a significant hypercalcemia (Flik *et al.*, 1989). Control fish were injected with 300 μl 0.9% NaCl. Four hours after injection a blood sample was taken by puncture of the caudal vessels. Fish were killed by spinal transection and the CS were removed. CS were homogenized in 250 μl 0.1 *M* acetic acid.

Plasma Analysis

Blood samples were analyzed as described before (Hanssen *et al.*, 1989). Blood ionic calcium was measured with an ionic calcium analyzer (ICA-1, Radiometer). Plasma total calcium and protein concentration were determined with commercial reagent kits (Sigma and Biorad, respectively). Bovine serum albumin(Biorad) was used as a protein reference.

Incubations

Incubation media were prepared by adding $CaCl_2$ to Ca^{2+}-free HBSS (pH 7.4). In media designated "0" m*M* Ca^{2+} no $CaCl_2$ was added. The addition of EGTA (1 m*M*) to a 0.65 m*M* Ca^{2+} medium resulted in a final Ca^{2+} concentration of 0.1 μ*M* (Sillen and Martell, 1964; Van Heeswijk *et al.*, 1984). Calimycin A23187 was added from a stock solution (5 mg · ml^{-1} in DMSO/methanol 1:9). In control media the solvent was included and it never exceeded 0.1% v/v). Per incubation two corpuscles, each from different eels or from different trout, were transferred to an incubation vessel containing 50 μl incubation medium supplemented with 925 KBq [^{3}H] leucine (Amersham; sp act 5.2 TBq/mmol). Incubation was for 3 hr at 28°. Next the CS were washed in 2 ml incubation medium (three times) and incubated for another 3 hr in 200 μl medium. Since [^{3}H] STC was not only synthesized but also released during the first incubation period it was necessary to carry out a second incubation period without radiolabel in which the release of [^{3}H] STC could be studied. Incubation media were collected and the CS were homogenized in 250 μl acetic acid (0.1 *M*). Part of the incubation media and the CS homogenate was kept separate for ELISA. The remainder of the incubation media was precipitated with TCA (10%, 4° overnight), and the remainder of the CS homogenate was lyophilized. Radioactivity in incubation media and CS homogenate was determined in a LKB Rackbeta LSA with a dpm-program. Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE; Laemmli, 1970) of precipitated proteins from incubation media and CS homogenates indicated that at least 90% of the labeled product (based on optical scans from fluorographs) was a 28 kD (trout) or 30 kD (eel) product that could be identified as trout or eel STC, respectively (Flik *et al.*, 1989).

Enzyme-linked Immunosorbent Assay (ELISA)

A noncompetitive ELISA was carried out according to the method of Kaneko *et al.*, (1988), with some modifications. The STC antiserum used was RADH-1 (Kaneko *et al.*, 1988), raised against trout STC. Eel STC showed a high degree of cross reactivity with the RADH-1 antiserum (Flik *et al.*, 1989). Serial dilutions of the antigen were tested for detection of STC. Trout and eel CS homogenate (prepared as described before), trout and eel CS incubation media, and trout plasma tested produced dose-response curves parallel to the trout STC standard. The wells of microtiter plates (Nunc, immunoplate maxisorp type 1) were precoated for 2 hr at room temperature with 100 μl 1% glutaraldehyde. Wells were washed with distilled water and coated with 100 μl serial dilutions of STC or

unknown samples in coating buffer (0.1 *M* bicarbonate buffer, pH 9.4, with 0.05% Triton X-100; Biorad). Antigen binding was for 1 hr at 37° and subsequently overnight at 4°. Between incubation steps wells were washed with buffer (0.02 *M* phosphate buffered saline; PBS, pH 7.4, with 0.05% Tween 20; Biorad). The remaining binding sites in the wells were blocked with 100 µl block buffer (2% bovine serum albumin in PBS) for 1 hr at 37°. Wells were washed and incubated with 100 µl 1:10,000 RADH-1 in block buffer for 1.5 hr at 37°. Control wells were incubated with block buffer only. Wells were washed and incubated with 100 µl 1:2000 goat anti-rabbit peroxidase immunoconjugate (Nordic) in block buffer for 1 hr at 37°. The amount of peroxidase immunoconjugate bound to the wells was quantified using *o*-phenylene-diamine (OPD; Sigma) as substrate. After washing the wells, 250 µl substrate (0.1% OPD in 0.1 *M* Na_2HPO_4, 0.1 *M* citric acid, 0.05% H_2O_2, pH 5.2) was added at room temperature. The peroxidase reaction was stopped after 2 min with 100 µl 4 *M* H_2SO_4. Absorbance was measured at 492 nm using a EAR-400 microplate reader (SLT Lab Instruments).

Electron Microcopy

Eel CS, incubated as described above, were prefixed for 10 min in 3% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.4, at room temperature. They were then fixed in a solution containing 0.66% osmium tetroxide, 1% glutaraldehyde, and 1.66% potassium dichromate in cacodylate buffer, pH 7.4, for 1 hr at 0°. Then, the CS were block stained in 2% uranyl acetate. The tissues were dehydrated in graded ethanols and embedded in Spurr's resin. Ultrathin sections were poststained with Reynolds's lead citrate and examined in a Philips EM 200 electron microscope.

Calculations and Statistics

The STC contect of CS of equivalent weight showed a high degree of variability. Therefore the results of *in vitro* experiments have been presented as percentages STC released to the incubation medium. Data are presented as means ± SEM. For statistical evaluation the Mann–Whitney U test was used. Significance was accepted at $p < 0.05$.

RESULTS

In Vivo

The effects of a rise in blood calcium concentration on the STC release are shown in Figs. 1A and 1B. Following $CaCl_2$ injection both blood, ionic and total calcium levels are elevated from 1.25 to 3.75 m*M* and 2.50 to 5.75 m*M*, respectively (Fig. 1A). Concomitantly the plasma immunoreactive STC level increases from 175 to 800 ng · ml^{-1} and the CS-STC content decreases from 600 to 325 ng · µg protein as determined by ELISA (Fig. 1B).

In Vitro

A comparison between the release of newly synthesized and total immunoreactive STC from trout and eel CS at a medium

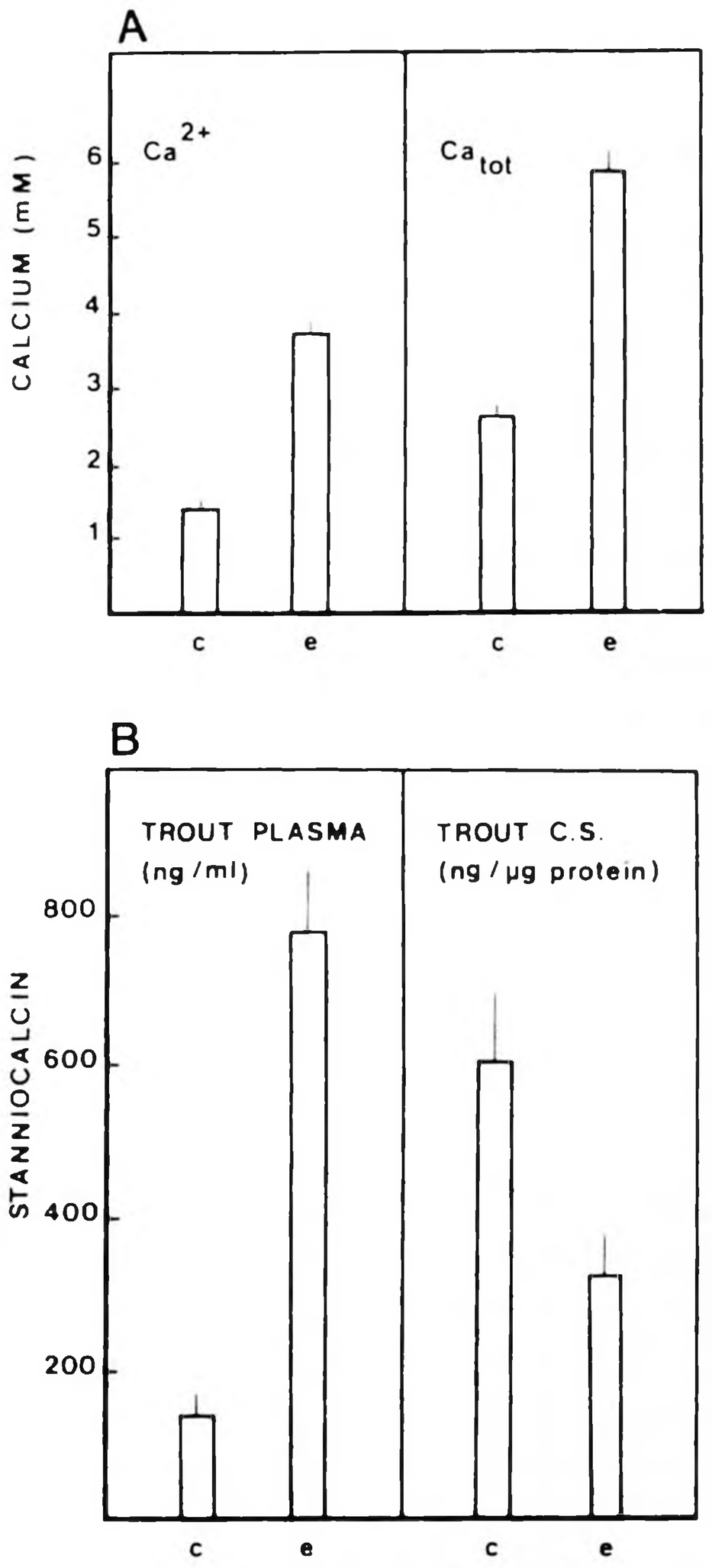

FIG. 1. Effect of $CaCl_2$ injection (e) on ionic and total plasma calcium concentrations (A) and on the STC concentrations in plasma and CS homogenate (B) in rainbow trout. Saline injections served as control (C). ($N = 8$).

Ca^{2+} concentration of 1.25 m*M* is shown in Fig. 2. The release of total immunoreactive STC from trout and eel CS during the labeling incubation period and the nonradioactive incubation period thereafter was comparable. In trout CS 70% of the newly synthesized STC was released during the 3-hr labeling period and only 4% during the 3-hr incubation period thereafter. For eel CS these numbers were 40 and 16%, respectively. Because of the relatively high basal release of newly synthesized STC during the nonradioactive incubation period we selected eel CS for investigation of the effects of medium Ca^{2+} levels on CS secretory activity. *In vitro* incubation of eel CS showed that the release of newly synthesized and total immunoreactive STC was not significantly affected by medium Ca^{2+} levels ranging from 0 to 2 m*M* (Fig. 3). EGTA added to obtain incubation media with a final Ca^{2+} concentration of 0.1 μ*M* did not diminish the secretory response. Increased release of newly synthesized and total immunoreactive STC was observed at 2.5 and 3.75 m*M* Ca^{2+}. Examination of electron micrographs of eel CS following incubation under various conditions (Figs. 4A–4D) showed marked degranulation at 3.75 m*M* Ca^{2+}; at 0.1 μ*M*, O m*M*, and at 1.25 m*M* Ca^{2+} no visible degranulation occurred. The effects of calimycin (A23187) have been summarized in Fig. 5. Calimycin at a concentration of 1.3×10^{-5} *M* increases release of both newly synthesized and total immunoreactive STC.

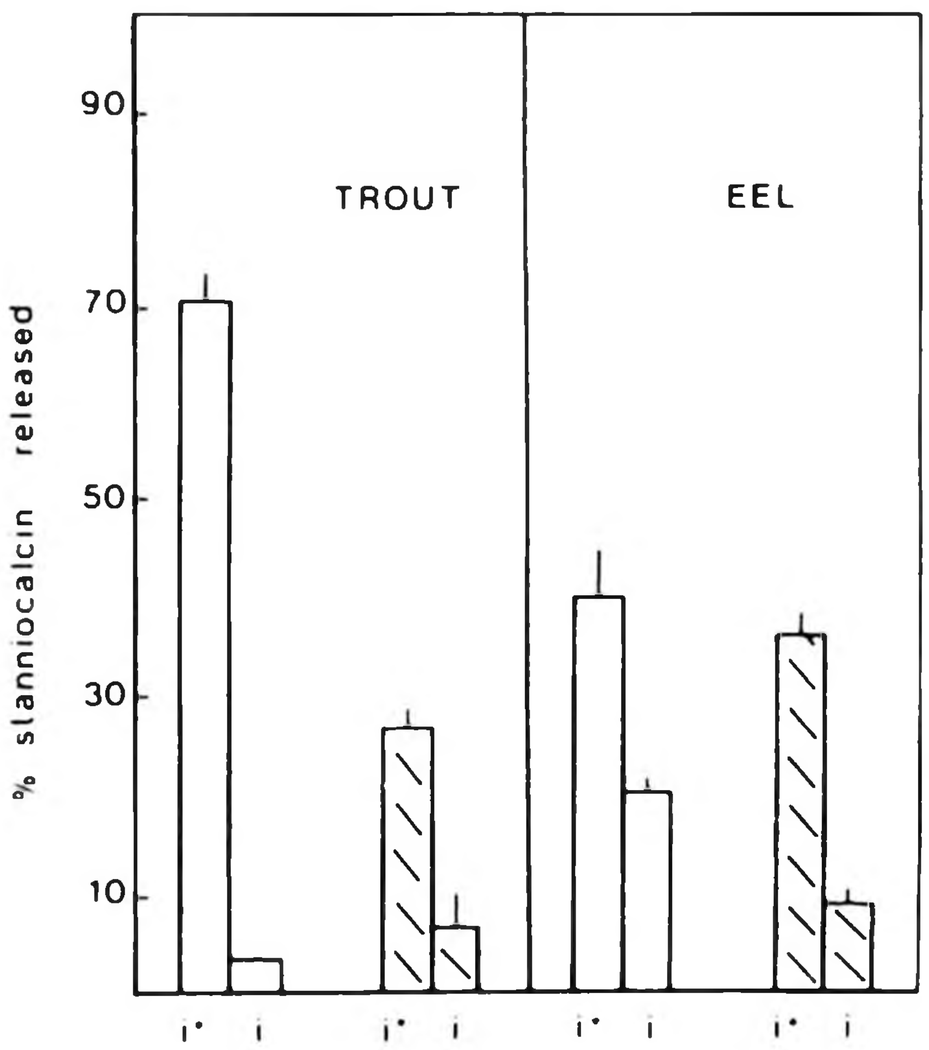

FIG. 2. Release of newly synthesized (□) and total immunoreactive (▨) STC from trout and eel CS in 1.25 m*M* Ca^{2+} medium. STC in labeling incubation medium (i*), nonradioactive incubation medium (i), and CS homogenate together is 100%. ($N = 4$).

DISCUSSION

The noncompetitive ELISA described here appears suitable for the quantitation of STC. Both trout and eel STC could be easily detected with a sensitivity of approximately 18.5 p*M* (the M_r for trout STC is 54 kDa; Flik *et al.*, 1989). The STC-ELISA and the STC-RIA used by Kaneko *et al.* (1988) have a sensitivity of approximately 74 p*M* and 18.5 p*M*, respectively. The STC-RIA developed by Wagner *et al.* (1989) has a detection limit of approximately 74 p*M*.

Our results clearly show that artificially produced high extracellular Ca^{2+} levels induce a release of STC *in vivo* as well as *in vitro*. Assuming that such high levels of Ca^{2+} do not occur in normocalcemic eels we conclude that plasma Ca^{2+} is normally not a determining factor in the direct control of STC release.

In Vivo

Hypercalcemia, induced by $CaCl_2$ injection, resulted in the release of immunoreactive STC from the CS. This observation confirms reports showing that hypercalcemia induced by $CaCl_2$ injection causes depletion of the hormonal content of CS cells (Lopez *et al.*, 1984; Lafeber and Perry, 1988; Flik *et al.*, 1989). This is consistent with the hypocalcemic function of CS in fish (Pang *et al.*, 1974; Milet *et al.*, 1979; Fenwick, 1982; Wagner *et al.*, 1986; Wendelaar Bonga and Pang, 1986). With an average CS weight of 2.5 mg per fish we calculated that, following this $CaCl_2$ challenge, about 86 μg STC was released into the blood (1 mg CS contained about 125 μg extractable protein). Our ELISA data show a

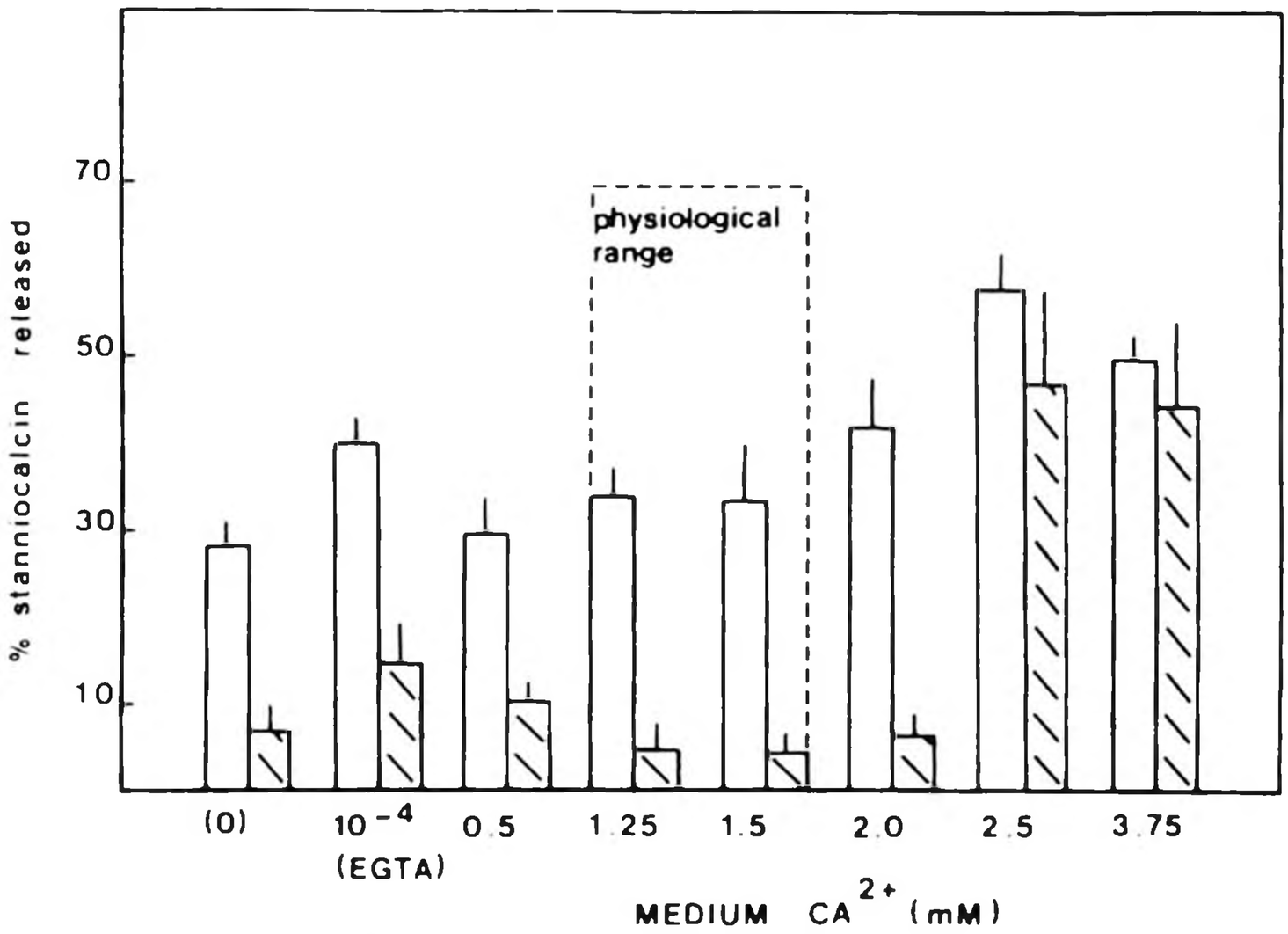

FIG. 3. Effect of the medium Ca^{2+} concentration on the release of newly synthesized (□) and total immunoreactive (▨) STC from eel CS. STC in nonradioactive incubation medium and CS homogenate together is 100%. ($N = 8$).

rise in plasma immunoreactive STC after $CaCl_2$ injection from 175 to 800 ng · ml^{-1}, equivalent to a rise from 3.2 to 14.8 n*M*, respectively. Assuming a plasma volume of 7.5 ml (3% of the body weight) the total increase in irSTC will amount to 4.7 μg 4 hr after injection, indicating that 95% of the irSTC initially released has been cleared from the plasma.

In Vitro

We found that variations in extracellular Ca^{2+} *in vitro* between 0 and 2.0 m*M* did not significantly affect the basal release of newly synthesized or total immunoreactive STC. Application of the Ca^{2+}-chelator EGTA, which in our experimental design reduced external Ca^{2+} to 0.1 μ*M* (Sillen and Martell, 1964; Van Heeswijk *et al.*, 1984), did not decrease basal STC release. Electron micrographs of CS incubated in 0.1 μ*M*, 0 m*M*, and 1.25 m*M* Ca^{2+} medium all showed a large number of secretory granules and no apparent signs of altered secretion. However incubation of CS in 2.5 or 3.75 m*M* Ca^{2+} media stimulated release of both newly synthesized and total immunoreactive STC, and electron micrographs of CS incubated in 3.75 m*M* Ca^{2+} medium showed marked degranulation of the cells.

A stimulatory effect on the newly synthesized and total immunoreactive STC release was also obtained with calimycin (A23187). The equipotent stimulatory effect of high external Ca^{2+} (2.5–3.75 m*M*) and calimycin (1.3×10^{-5} *M*) on STC release indicates that STC release may be triggered by a surge of internal Ca^{2+}. Since addition of $CoCl_2$ to the medium inhibits high external Ca^{2+} stimulated STC secretion (Wagner *et al.*, 1989), STC release is probably triggered by a Ca^{2+}-influx through voltage-independent Ca^{2+} channels in the plasma membrane. External Ca^{2+} concentrations in the physiological range (1.0–1.5 m*M*), or lower, are apparently unable to open these channels, since they do not induce release. With respect to high external Ca^{2+} levels our results are in line with histological observations of Aida *et al.* (1980), who found degranulated cells in salmon CS tissue blocks after incubation in media containing 3 and 6 m*M* Ca^{2+} and after treatment with calimycin (A23187) in the presence of 1.5 m*M* Ca^{2+}. These authors also found that media containing 1.5 m*M* Ca^{2+} or lower did not induce degranulation. After addition of

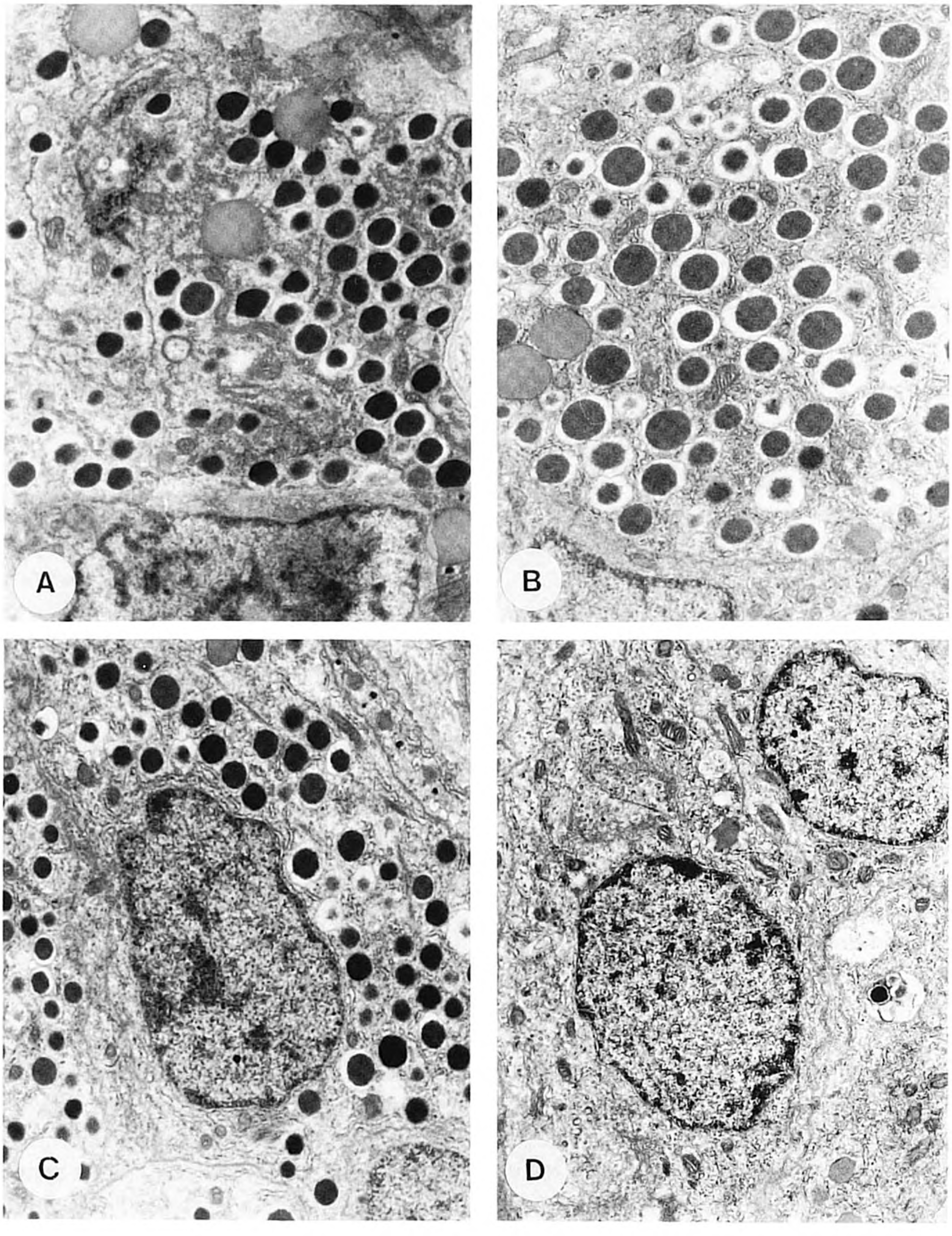

FIG. 4. Electron micrographs of eel-CS after incubation in media containing 0.1 μM (A), 0 mM (B), and 1.25 mM (C) Ca^{2+} show CS cells with many large secretory granula. Incubation in 3.75 mM Ca^{2+} (D) shows degranulated cells. Magnification, × 15,000.

EDTA resulting in a Ca^{2+} concentration of 0.16–0.21 mM (values that we calculated from their data) no stimulatory effect on degranulation occurred. However, Wagner *et al.* (1989) showed a dose-related stimulation of the STC release from trout CS cells in primary culture between 1 and 2.5 mM external Ca^{2+} that plateaued beyond 2.5 mM. In accordance with our results, these authors found that calimycin mimicked the stimulatory effect of 1.8 mM external Ca^{2+} on STC release. In accordance with the re-

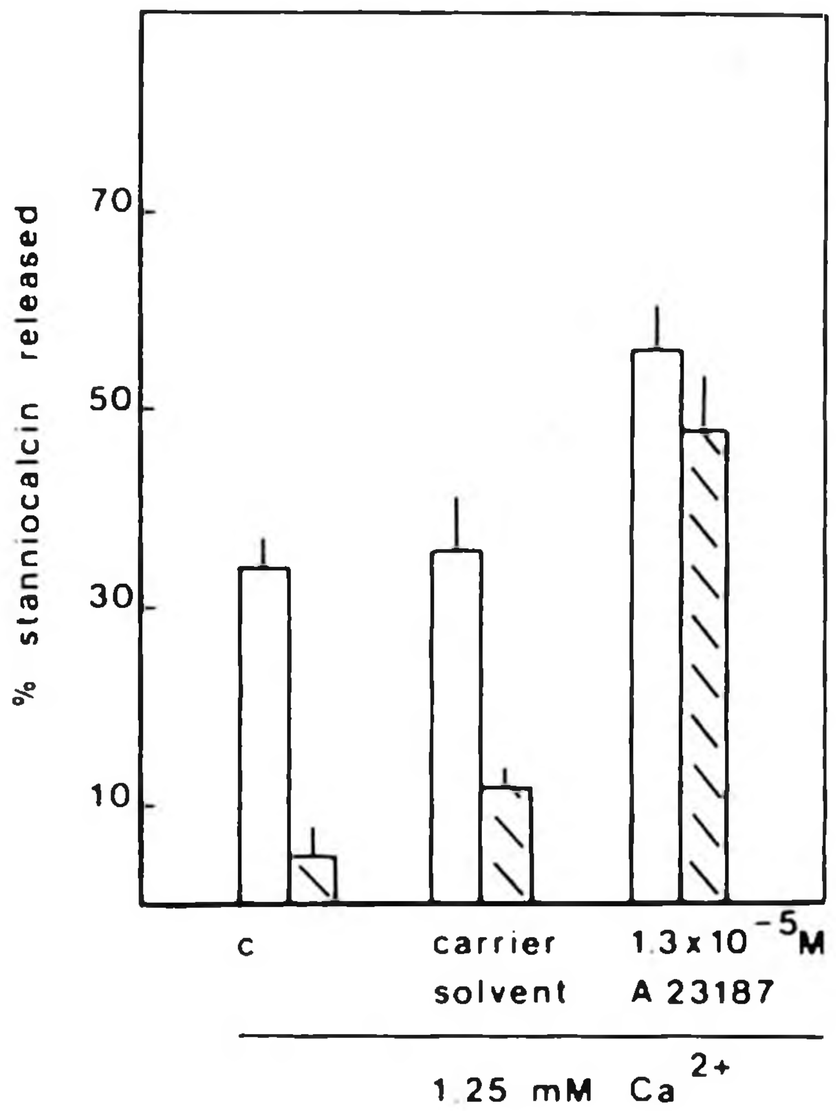

FIG. 5. Effect of calimycin (A23187; 1.3×10^{-5} *M*) added to 1.25 m*M* Ca^{2+} medium on the release of newly synthesized (□) and total immunoreactive (▨) STC from eel CS. Media containing 1.25 m*M* Ca^{2+} and 1.25 m*M* Ca^{2+} with an equivalent amount of carrier solvent were used as controls. STC in nonradioactive incubation medium and CS homogenate together is 100%. ($N = 8$).

sults of Aida *et al.* (1980), Wagner *et al.* (1989) found no stimulation of the STC-release after reduction of the medium Ca^{2+} concentration to 0.8 m*M* or 1 μ*M* (values that we calculated from their data), by addition of 1 or 2 m*M* EGTA to 1.8 m*M* Ca^{2+} medium, respectively. The enhanced STC release at increasing Ca^{2+} concentrations between 1 and 2.5 m*M* found by Wagner *et al.* (1989) is at variance with our results. It may be the result of species specific differences or of the preparation of the primary CS-cell suspension, which inevitably affects the cellular membrane and possibly cellular Ca^{2+} homeostasis.

In our experiments we find a basal STC release at external Ca^{2+} concentrations around and below the physiological range that is not accompanied by visible degranulation of the CS cells. Even at an external Ca^{2+} concentration of 0.1 μ*M* the basal release has not changed. This indicates that at these external Ca^{2+} concentrations a Ca^{2+} influx from the medium does not occur or is insufficient to initiate STC release. The basal STC release may have been initiated by a Ca^{2+} release from internal stores. However, one may not exclude the possibility of a low rate of secretion at basal or even reduced levels of intracellular Ca^{2+} (Penner and Neher, 1989).

Secretory Pools

When CS are incubated in 2.5 or 3.75 m*M* external Ca^{2+} medium the release of total irSTC has increases 7 times, that of newly synthesized STC 1.7 times. This suggests that upon stimulation by high external Ca^{2+}, stored STC is preferentially released. This is in accordance with the electron micrographs of cells from CS incubated in 3.75 m*M* external Ca^{2+} medium.

We conclude that there are more STC pools in the CS cells that are controlled separately. One pool is characterized predominantly by newly synthesized STC that appears to be secreted independently of an external stimulus; another pool contains stored STC that is released upon stimulation by factors that induce increased Ca^{2+} influx in the CS cells. Separate intracellular hormone pools, one consisting of newly synthesized hormone, the other consisting of stored product, have been described in a variety of endocrine cells (e.g., Morrisey and Cohn, 1979). Experiments by Walker and Farquhar (1980) have shown the existence of two individually recruitable secretory pools in prolactin cells of the rat. Newly synthesized prolactin was preferentially released in unstimulated cells whereas TRH-stimulated prolactin cells preferentially release stored prolactin. These observations parallel our results on STC release.

Physiological Significance

Several authors have suggested that the secretory activity of the CS is directly controlled by variations in external (plasma) Ca^{2+} levels (Aida *et al.*, 1980; Flik *et al.*, 1989). However, the high Ca^{2+} stimulated

STC release *in vivo* does not allow the conclusion that *in vivo* the secretory activity of the CS is directly controlled by extracellular Ca^{2+}: the hypercalcemia induced by $CaCl_2$ injection in our studies as well as in the studies cited above (3.75 m*M* or higher; Lopez *et al.*, 1984; Lafeber and Perry, 1988; Flik *et al.*, 1989) is above what we call the physiological range (1.0–1.5 m*M*). Our *in vitro* data show that differences in Ca^{2+} concentrations in a range corresponding with physiologically relevant plasma Ca^{2+} concentrations (1.0–1.5 m*M*) have no effect on STC secretion. We conclude that the high Ca^{2+} stimulus for the *in vitro* release of stored STC most likely has no physiological relevance since it is far beyond these physiological plasma Ca^{2+} concentrations. We suggest that, at least in eels, effects of plasma Ca^{2+} fluctuations on STC secretion *in vivo* are indirect and possibly mediated by the nervous system. The CS are richly innervated (Krishnamurthy and Bern, 1971; Wendelaar Bonga *et al.*, 1977) and observations of Unsicker *et al.* (1977) demonstrated the presence of nerve fibers containing noradrenalin, adrenalin, and 5-hydroxytryptamine in the CS of *Salmo irideus*. Preliminary results show a stimulatory effect of the acetylcholine agonist carbachol on the irSTC release. This indicates that a nervous factor should be considered to be involved in the regulation of STC secretion.

ACKNOWLEDGMENTS

We thank Mr. Coen van der Meij for preparing the electron micrographs. We also thank Dr. Mayer-Gostan for her interest in and stimulating comments on this study.

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