Processing and secretion in the neurohypophysis

Stability of isolated secretory vesicles and role of internal pH

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A possible role of low pH in secretory vesicles for processing and secretion in the neurohypophysis was investigated. Subcellular fractionation of guinea-pig neural lobes revealed that a proton pump present in the membranes from this tissue could not be ascribed to secretory vesicles. However, a proton pump was found in coated microvesicles. Secretory vesicles isolated from rats and guinea pigs were stable under conditions known to lyse secretory vesicles from the adrenal medulla owing to the generation of a proton gradient. These results suggest that the internal pH of secretory vesicles from the neurohypophysis is closer to neutral than is the pH in chromaffin secretory vesicles. Processing of a neurophysin–glycopeptide intermediate from the biosynthesis of vasopressin in intact secretory vesicles incubated *in vitro* was activated by the addition of NH_4Cl , known to increase the intravesicular pH. This activation of neurohormone processing was also apparent in isolated nerve endings incubated in the presence of NH_4Cl , suggesting that NH_4Cl can also be used to increase the intravesicular pH in intact nerve endings. However, NH_4Cl did not affect the secretion of neurohormones, indicating that a low intravesicular pH is not important for exocytosis in the neurohypophysis. Our results indicate that a low pH generated during processing by mechanisms other than ATP-dependent proton transport may inhibit the processing enzymes, thereby preventing extensive breakdown of neurohormone precursors.

INTRODUCTION

Secretory vesicles from the neurohypophysis have been demonstrated to have an acidic interior (Scherman *et al.*, 1982; Russell & Holz, 1981). The presence of a proton-pumping ATPase in these vesicles has been suggested to explain their acidification (Russell, 1984; Scherman *et al.*, 1982).

In the adrenal medulla a proton-pumping ATPase has been localized in the secretory-vesicle membrane (Johnson *et al.*, 1982; Apps & Reid, 1977). In this tissue such an enzyme might play an important role in the uptake of the secretory material (Bashford *et al.*, 1976). This is not the case in the peptidergic neurons of the neurohypophysis, where the secretory material is packed into the vesicles in the Golgi zone of the cell body located in the hypothalamus (Brownstein, 1982). However, it has been suggested that the enzyme may be important for other functions of the secretory vesicle, such as proteolytic processing of the neurohormones that they contain (Brownstein, 1982; Russell & Holz, 1981; Scherman *et al.*, 1982). The idea that a low intravesicular pH is necessary for normal processing of neuropeptides in the neurohypophysis has been supported by the finding that at least certain processing enzymes appear to have an acidic pH optimum (Turner, 1984). In the pituitary such acidic enzymes have been found only in the anterior lobe (Gainer *et al.*, 1984). A role in exocytosis has also been suggested for this proton-pumping ATPase, creating gradients believed to be essential for the fusion of the secretory vesicles with the plasma membrane (Grinstein *et al.*, 1982; Pollard *et al.*, 1977). H^+ -transporting ATPases would then be expected to be present in most types of secretory vesicles.

There are, however, certain pieces of evidence that suggest that some types of secretory vesicles might not have an internal pH much lower than 7. Certain authors have found an internal pH in secretory vesicles from the neurohypophysis as high as 6.5 (Scherman *et al.*, 1982), and in secretory vesicles from the parotid gland an internal pH of 6.8 has been demonstrated (Arvan *et al.*, 1984). Furthermore, the demonstration that the amidating enzyme involved in the normal processing of hormones in the pituitary has a pH optimum above 7.0 (Eipper *et al.*, 1983) does not support the idea of a low pH in all types of secretory vesicles.

Using a newly developed method for the isolation of secretory vesicles from the guinea-pig neurohypophysis (Saermark *et al.*, 1984), we have now investigated the possible co-purification of ATPase activity with the hormone-containing vesicles, and studied the pH-dependency of the processing in intact secretory vesicles of an intermediate of vasopressin biosynthesis.

EXPERIMENTAL

Materials

All reagents were of analytical grade. Reagents for enzyme assays were from Sigma except Malachite Green (Fluka). [^{35}S]Cysteine (~ 10000 Ci/mmol) was obtained from Amersham.

Isolation of membrane fractions

Secretory vesicles were isolated from guinea-pig neurohypophyses (one or two) on 400 μl Percoll gradients centrifuged in a standard bench-top micro-centrifuge (O. Dich, Copenhagen, Denmark) as described

previously (Saermark *et al.*, 1984). Briefly, neural lobes were homogenized in a buffer (190 mM-sucrose/20 mM-Tes/25 mM-EGTA, pH 7.0) and centrifuged at 3000 $g_{av.}$ for 5 min. The supernatant was centrifuged at 22000 $g_{av.}$ for 10 min, and the pellet (P_2) was used for the final isolation of the secretory vesicles on Percoll gradients (50% Percoll, unless otherwise stated). In some experiments, a buffer with a lower ionic strength (250 mM-sucrose/0.1 mM-EGTA/1 mM-Tes, pH 7.0) was used throughout.

In some experiments, sucrose gradients were used for the isolation of secretory vesicles. The pellet (P_2) prepared in the low-ionic-strength buffer was placed on a sucrose gradient made up in 800 μ l inserts for the SW 50 rotor (Beckman) and centrifuged at 100000 $g_{av.}$ for time periods as indicated. The gradients were made up of 1.75 M-, 1.4 M-, 1.35 M- and 1.3 M-sucrose in low-ionic-strength buffer in layers of 100 μ l, except for the 1.35 M-sucrose (200 μ l).

Coated microvesicles were isolated as described previously (Saermark *et al.*, 1984). Isolated nerve endings (secretosomes) were prepared as described by Gratzl *et al.* (1977).

Assays

Acid phosphatase (EC 3.1.3.2) was measured in a medium containing 100 mM-glycerophosphate and 50 mM-acetate, pH 5.0 and Malachite Green was used for the measurement of phosphate liberation (Saermark

et al., 1983). Mg^{2+} -ATPase (EC 3.6.1.3) was assayed by using Malachite Green for the measurement of phosphate liberation (Saermark *et al.*, 1983). The assay could detect 0.2 nmol of P_i . The medium contained 10 mM-Tes, 0.5 mM-EGTA, 1 mM- Mg^{2+} (free) and 2 mM-ATP (hypo-osmotic medium). Activation of the Mg^{2+} -ATPase activity by NH_4Cl was measured by addition of 50 mM- NH_4Cl to the incubation medium and subtracting the ATPase activity in the absence of NH_4Cl . Lysis of the vesicles was prevented in these experiments by addition of sucrose (320 mosM). The measurements were carried out at 1 mM- Mg^{2+} (free) (Saermark & Vilhardt, 1979), and the medium included 10 mM-Tes, 2 mM-ATP and 0.5 mM-EGTA. Effects of NH_4Cl on internal pH were determined as described by Saermark *et al.* (1985a), by using 9-aminoacridine. The incubation conditions are given in the Results section.

Release of neurohormones from secretory vesicles and the conversion of neurohormone precursors were measured by reverse-phase h.p.l.c. This was carried out on a Nucleosil 5 μ C_8 column (150 mm $\times$ 5 mm; LKB, Sweden) and a pre-column (10 mm $\times$ 5 mm) packed with the same material. The gradients of acetonitrile were made up in 0.1% trifluoroacetic acid, and the column was eluted at 1 ml/min. All reagents used for h.p.l.c. were from Rathburn (U.K.) and were of h.p.l.c. grade. Percoll was removed from samples of secretory vesicles before h.p.l.c. by diluting the samples 5-fold and centrifuging them at 22000 $g_{av.}$ for 10 min. Pellets were extracted in 0.1 M-HCl, frozen and thawed before the h.p.l.c. analysis.

Fig. 1. Secretory vesicles isolated from the neurohypophysis

(a) Electron micrograph of secretory vesicles obtained from five rats by isolation on two Percoll micro-gradients by the method of Saermark *et al.* (1984). The Percoll concentration was 50% (b) Material (P_2) applied to the Percoll gradients. Magnification $\times$ 7400 for both micrographs.

Fig. 2. H.p.l.c. analysis of acid extracts of secretory vesicles and their osmotically released contents

Secretory vesicles were prepared from one guinea-pig neurohypophysis 24 h after hypothalamic injection of [^{35}S]cysteine. Half of the material was extracted in 0.1 M-HCl immediately. The rest was diluted in water to 100 mosM, incubated for 10 min at 37 °C and centrifuged at 100000 g_{av} for 5 min (Beckman Airfuge). Half of this supernatant was removed and extracted in 0.1 M-HCl. Both samples were subjected to reverse-phase h.p.l.c. as described in the Experimental section. (a) Radioactivity in the fractions obtained from h.p.l.c. of the secretory vesicles; (b) fractionation of the material obtained by osmotic lysis of the vesicles. The gradient of acetonitrile is indicated (●) in (a) and the arrows 1–5 indicate the retention times of vasopressin, oxytocin, their related neurophysins and a neurophysin-related biosynthetic intermediate respectively (Jones *et al.*, 1984). Buffer A is 10% acetonitrile in 0.1% trifluoroacetic acid, and buffer B 80% acetonitrile in 0.1% trifluoroacetic acid.

Conversion of neurophysin–glycopeptide in intact secretory vesicles was analysed by incubation *in vitro* of secretory vesicles radiolabelled by hypothalamic injection of [^{35}S]cysteine 4 h before their isolation (Robinson & Jones, 1983). The incubation was carried out in a buffer identical with the homogenization buffer, except for the use of Mes and Tris for buffering at pH below and above 7 respectively. After incubation, the secretory vesicles were centrifuged (200000 g_{av} -min), the pellet was extracted in 0.1 M-HCl and the neurohormone components were isolated by h.p.l.c. The radioactivity in the h.p.l.c. fractions was determined by scintillation counting.

Radiolabel incorporation *in vivo*

Anaesthetized (Hypnorm; D.A.K., Denmark) guinea pigs and rats were given bilateral injections of [^{35}S]cysteine (2 μl ; 20 μCi) into each of the paraventricular nuclei. Animals were killed 4 h or 24 h after injection, and the neural lobe of the pituitary was removed and immediately homogenized in the appropriate homogenization buffer at 0 °C. In some experiments, radiolabelled neurohypophyses were used for the preparation of secretosomes.

Secretion experiments

Radiolabelled secretosomes were incubated as described by Jones *et al.* (1984). Verapamil was dissolved in ethanol (0.5% final concn., including the controls). KCl concentrations as indicated were used to stimulate secretion.

Other procedures

Electron microscopy was done on samples fixed in 1% glutaraldehyde and embedded as described by Gratzl & Dahl (1978). Percoll was removed before this procedure by centrifugation as described above for the h.p.l.c. analysis. The pellet was resuspended in the buffer, and a pellet for fixation was obtained by centrifugation in an Airfuge (Beckman) at 100000 g_{av} for 5 min.

RESULTS

Isolation of membrane fractions

A method developed for the isolation of secretory vesicles from the neurohypophysis of a single guinea pig (Saermark *et al.*, 1984) can also be used to purify secretory vesicles from rat neural lobes. Fig. 1 shows an electron micrograph of these vesicles purified on 60% Percoll gradients, and also a picture of the fraction (P_2) from which the vesicles are finally purified (see the Experimental section).

Radiolabelling of secretory vesicles

When secretory vesicles are isolated from animals injected with [^{35}S]cysteine into the hypothalamus 24 h before the experiment, their content of neurohormones can be demonstrated to be labelled with [^{35}S]cysteine (Robinson & Jones, 1983). During subcellular fractionation this incorporated radioactivity co-purifies with the vesicles, as shown by the parallel enrichment of the vesicle fraction in the content of immunoreactivity and ^{35}S radioactivity (Saermark *et al.*, 1984).

Fig. 2(a) shows the h.p.l.c. analysis of an acid extract of secretory vesicles isolated from the guinea-pig neural lobe 24 h after injection of [^{35}S]cysteine into the hypothalamus of a guinea pig. More than 90% of the ^{35}S radioactivity was recovered in the acid extract from the secretory vesicles. The peaks correspond to the neurohormones in the neurohypophysis, as indicated in Fig. 2(a). Since this h.p.l.c. chromatogram is identical with the results obtained from an acid extract of a whole pituitary (Jones *et al.*, 1984), it is concluded that the content of [^{35}S]cysteine in sedimentable subcellular fractions can be used as a marker for the secretory vesicles. This assumption is further supported by analysis of ^{35}S -containing material released from purified secretory vesicles by osmotic lysis. Fig. 2(b) shows an h.p.l.c. analysis of the ^{35}S -containing neuropeptides released by osmotic lysis, which is essentially the same as

Fig. 3. ATPase and acid phosphatase activity in secretory-vesicle fractions as a function of the Percoll concentration at high and low ionic strength

The fraction P_2 obtained from four guinea-pig neural lobes by differential centrifugation was placed on various Percoll gradients made up in buffers with either high (a) or low (b) ionic strength. In (a) is shown the yield of neurohormones (●), Mg^{2+} -ATPase activity (○) and acid phosphatase activity (▲) in the secretory-vesicle fraction as a function of the Percoll concentration. The results are expressed as percentages of the amount applied on the gradient ($\pm$ S.D., $n = 3$). The amount of ATPase activity and acid phosphatase activity in the fraction P_2 applied on the gradients was 134 and 20 nmol of P_i /h per mg respectively. In (b) is shown the neurohormone content and ATPase activities in the secretory-vesicle fraction as a function of the Percoll concentration at low ionic strength. The yield of Mg^{2+} -ATPase activity (○) and neurohormones (●) was determined and expressed as percentage of the amount applied on the gradient, as in (a). Also shown in (b) is the distribution of ATPase activity activated by 25 mM- NH_4Cl (▲), calculated as the difference between Mg^{2+} -ATPase activity with and without NH_4Cl (means of two expts.).

an acid extract of the secretory vesicles (Fig. 2a), and as previously published data on acid extracts of neurohypophyses (Jones *et al.*, 1984; Robinson & Jones, 1983).

Osmotic lysis released most of the [^{35}S]cysteine-labelled material from the secretory vesicles. At 100 mosm, $87 \pm 9\%$ ($n = 8$) of the total amount of ^{35}S was released as labelled neuropeptides. An acid extract of the material bound to the lysed membranes showed that most of the radioactivity remaining membrane-bound was found in the neuropeptides, although SDS/polyacrylamide-gel electrophoresis showed that certain membrane proteins were radiolabelled (Saermark *et al.*, 1985b), but such proteins accounted for less than 10% of the total secretory-vesicle ^{35}S radioactivity.

ATPase activity in isolated secretory vesicles

The use of different Percoll gradients at high and low ionic strength to purify the secretory vesicles resulted in different amounts of Mg^{2+} -ATPase activity in the resulting fractions of secretory vesicles. This change was obvious when both the absolute activity and the activity relative to neurohormone content were compared. For example, vesicles purified at 30% Percoll and low ionic strength (see the Experimental section) contained an ATPase activity of 1.5 mmol/h per mg of protein, whereas vesicles isolated in the high-ionic-strength buffer at 60% Percoll has an activity of 0.2 μ mol/h per mg.

Fig. 3 shows that the content of Mg^{2+} -ATPase activity and [^{35}S]cysteine-labelled neurohormones in the fraction of secretory vesicles was strongly dependent on the Percoll concentration used for purification. Increasing the Percoll concentration from 40 to 50% caused no significant decrease in the yield of secretory vesicles, as judged from the recovery of ^{35}S -labelled neurohormones in the vesicle fraction, whereas the ATPase activity was markedly decreased. At higher Percoll concentrations a gradual decrease in the yield of secretory vesicles was seen. However, at these Percoll concentrations no ATPase activity was recovered in the secretory-vesicle fraction (Fig. 3). On these gradients acid phosphatase activity showed a distribution similar to that of ATPase activity rather than to the neurohormones when the Percoll concentration was increased (Fig. 3).

A decrease in ionic strength influenced the yield of secretory vesicles at different Percoll concentrations. When a buffer with low ionic strength was used for the Percoll gradients, the yield of secretory vesicles started to decrease at Percoll concentrations about 45% (Fig. 3). However, the decrease in ATPase activity with increasing Percoll concentration was much more pronounced than the decrease in neurohormones, and this decrease was prominent at Percoll concentrations that did not affect the yield of neurohormones (Fig. 3).

The ATPase activity measured in the fraction P_2 used for isolation of the secretory vesicles could be activated by NH_4Cl (see below). When the increase in ATPase activity caused by NH_4Cl was measured as a function of the Percoll concentration, it was found to decrease in a similar way to the basal Mg^{2+} -ATPase activity, but not in parallel with the decrease in the yield of the neurohormones (Fig. 3). These data suggest that the ATPase activity activated by NH_4Cl is not located in the same structure as the neurohormones.

Rate zonal centrifugation in sucrose gradients has been used to analyse the content of ATPase activity in bovine secretory vesicles (Vilhardt & Hope, 1974). Using a

Fig. 4. Rate zonal centrifugation of secretory vesicles on sucrose gradients

The fraction P₂ from four guinea pigs prepared in low-ionic-strength buffer was centrifuged on sucrose gradients for various lengths of time (see the Experimental section). The yield of Mg²⁺-ATPase activity (○) and neurohormones (●) obtained at the 1.4 M-/1.75 M-sucrose interface was determined and expressed as % of the activity in P₂ (Fig. 3) (means of two expts.).

similar approach, we measured the time-dependent sedimentation of ATPase activity and secretory vesicles on a 800 μ l sucrose gradient (see the Experimental section, and Saermark *et al.*, 1985b). More than 40% of the neurohormones in the starting material (P₂) could be sedimented at 1.4 M-sucrose on these gradients after 3 h centrifugation at 100000 g_{av} . (Fig. 4). Mg²⁺-ATPase activity was also found on these gradients, but the sedimentation rates of the ATPase activity and the neurohormones were not parallel (Fig. 4), and the ratio of ATPase activity to neurohormone content was decreased compared with that in P₂ (Fig. 4).

Effect of NH₄Cl on ATPase activities in fractions from the neurohypophysis

Coated microvesicles purified from the guinea-pig neurohypophysis contain high Mg²⁺-dependent ATPase activity (14 ± 3 μ mol/h per mg; $n = 5$). This is at least 5 times the ATPase activity found in the secretory-vesicle fractions (see above). When the coated microvesicles were incubated in the presence of NH₄Cl (50 mM), this activity was increased to $210 \pm 23\%$ of controls (s.d.; $n = 5$). A similar NH₄Cl-dependent activation is also found for the ATPase activity in the fraction (P₂) used for the purification of the secretory vesicles. The activity in this fraction is increased to $142 \pm 15\%$ of controls when NH₄Cl is included in the medium. However, although the activation of the ATPase activity by NH₄Cl is pronounced in fraction P₂ and the coated-microvesicle fraction, the effect of NH₄Cl is much smaller on the ATPase activity in the secretory vesicles. In three experiments this activation was less than 20% of the Mg²⁺-ATPase activity in vesicles purified at high ionic strength in 50% Percoll, suggesting that the Mg²⁺-ATPase activity in the fraction of secretory vesicles may represent a contamination.

Effect of NH₄Cl on internal pH in the secretory vesicles

NH₄Cl is able to change the internal pH in secretory vesicles isolated from bovine neurohypophyses (Scherman *et al.*, 1982; Russell, 1984). To test whether this is also the case for the secretory vesicles used in the present study, we incubated secretory vesicles in the presence of 1 μ M-9-aminoacridine in a medium containing 10 mM-Tes, 10 mM-Mes, 0.5 mM-EGTA and 130 mM-KCl (pH 7.3) (Shuldiner *et al.*, 1972). When the vesicles were added to the medium a decrease in fluorescence of the 9-aminoacridine was found ($\Delta F/F = 0.35$). When NH₄Cl (25 mM) was included in the medium (containing 105 mM-KCl), no significant change in 9-aminoacridine fluorescence was seen ($\Delta F/F < 0.03$). Addition of monensin (1 μ g/ml) or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (1 μ g/ml) to the medium also completely abolished the change in 9-aminoacridine fluorescence. These data support the previous reports showing that NH₄Cl changes the internal pH in secretory vesicles (Scherman *et al.*, 1982; Russell & Holtz, 1981; Russell, 1984). When the external pH was lowered to 6.4, no change in 9-aminoacridine fluorescence was found on addition of secretory vesicles. This supports the data of

Table 1. Release of hormone content from secretory vesicles

Secretory vesicles were incubated at 37 °C in a medium containing 130 mM-KCl, 0.5 mM-EGTA and 20 mM-Tes, pH 7.0, and the additions indicated in the Table. After 30 min the release of hormone content was determined by centrifugation of the samples as described in Fig. 3. The results are given as release (% of total), as means $\pm$ s.d. for the numbers of experiments in parentheses. Abbreviation: n.d., not determined.

Addition	Secretory vesicles from . . .	Release (%)	
		Guinea pigs	Rats
None		8 $\pm$ 2 (10)	16 $\pm$ 3 (5)
ATP (1 mM)		8 $\pm$ 3 (10)	19 $\pm$ 3 (5)
ATP (10 mM)		5 $\pm$ 2 (5)	n.d.
NH ₄ Cl (50 mM)		8 $\pm$ 1 (5)	15 $\pm$ 4 (5)
Adenosine 5'-[β - γ -imido]triphosphate (1 mM)		9 $\pm$ 2 (8)	14 $\pm$ 3 (3)

Table 2. Conversion in intact secretory vesicles of neurophysin-glycopeptide intermediate (NPGP) from the synthesis of vasopressin and its related neurophysin (NP)

Secretory vesicles labelled *in vivo* purified 4 h after injection of [³⁵S]cysteine were incubated for 4 h in iso-osmotic buffer followed by centrifugation at 22000 *g*_{av.} as described in the Experimental section. The incubation conditions are given below (NH₄Cl = 50 mM). The conversion of NPGP into NP was monitored by h.p.l.c. analysis (Jones *et al.*, 1984). Results are means $\pm$ s.d. (*n* = 3); n.d., not determined; * and ** indicate values significantly different (*P* < 0.05) from each other and from that at 0 °C respectively. The osmolality was kept constant by a decrease in KCl and NH₄Cl was added. Replacing KCl by sucrose did not change the results.

Conditions	Ratio NPGP to NP in:	
	Secretory vesicles	Secretosomes
pH 7.3; 0 °C	0.85 $\pm$ 0.10	0.71 $\pm$ 0.07
pH 7.3; 37 °C	0.45 $\pm$ 0.04*	0.47 $\pm$ 0.09**
pH 6.0; 37 °C; + NH ₄ Cl	0.56 $\pm$ 0.06	n.d.
pH 7.0; 37 °C; + NH ₄ Cl	0.58 $\pm$ 0.04	n.d.
pH 7.3; 37 °C; + NH ₄ Cl	0.32 $\pm$ 0.06*	0.24 $\pm$ 0.03**
pH 8.0; 37 °C; + NH ₄ Cl	0.28 $\pm$ 0.03	n.d.

Scherman *et al.* (1982), suggesting an internal pH above 6 in these secretory vesicles.

Stability of neurosecretory vesicles

Secretory vesicles incubated in a buffer containing 0.5 mM-EGTA, 10 mM-Tes and 260 mM-sucrose for 30 min at 37 °C release $8 \pm 2\%$ (*n* = 8) of their content. The vesicles were also stable when 130 mM-KCl was included in the medium instead of sucrose (Table 1). When incubated in a similar medium, bovine secretory vesicles were found to lyse on addition of ATP to the medium (Russell & Thorn, 1978; Russell, 1984). This effect was not observed when guinea-pig secretory vesicles were incubated in the presence of ATP (2 mM) and KCl (130 mM) at pH 7.0. Addition of MgCl₂ (1 mM free) or 10 mM-ATP and MgCl₂ (1 mM free) did not change this result (Table 1).

No time-dependent change in stability was observed when these secretory vesicles were isolated 6 or 17 days after injection of the [³⁵S]cysteine used to label their content. Secretory vesicles isolated from guinea pigs 6 days after injection of the label released $8 \pm 1\%$ (*n* = 3) of their content in the presence of ATP (1 mM), Mg²⁺ (1 mM free) and KCl (130 mM), compared with $9 \pm 1\%$ in the presence of Mg²⁺ and KCl alone.

Secretory vesicles isolated 17 days after injection of the animals released 6 and 9% of their content in presence of ATP and MgCl₂ (*n* = 2), compared with 8 and 9% in the presence of MgCl₂ alone.

Similar results were obtained with secretory vesicles prepared from rat neural lobes (Table 1). Although the percentage of content released from rat vesicles was somewhat higher (16–19%), ATP had no effect on vesicle stability.

The secretory vesicles from both rats and guinea pigs became unstable when the external pH was increased to pH 8, whereas a decrease in pH to 6.0 did not affect their

Fig. 5. Secretion of neurohormones from secretosomes in the presence of NH₄Cl

Secretosomes were isolated from one guinea-pig neurohypophysis radiolabelled by [³⁵S]cysteine. The secretosomes were incubated in the presence of increasing concentrations of KCl, and secretion of neurohormones was measured as release of radioactivity (Jones *et al.*, 1984) (○). In a parallel series of incubations 50 mM-NH₄Cl was included in the medium (●). The inhibition by verapamil under both conditions was tested (△, ▲ respectively). The total amount of radioactivity was 30 000–50 000 c.p.m. in the secretosome preparations. Results are means $\pm$ s.d. (*n* = 3).

stability. Secretory vesicles from rats incubated at pH 8.0 in a buffer containing 0.5 mM-EGTA, 10 mM-Tris and 250 mM-sucrose released $28 \pm 5\%$ (*n* = 3) of their content when ATP (1 mM) and Mg²⁺ (1 mM free) were included in the medium, compared with $33 \pm 6\%$ in the presence of Mg²⁺ alone. Guinea-pig secretory vesicles released similar amounts of neurohormones under these conditions. Although the release of neurohormones is significantly higher at pH 8.0 than at pH 7.0, ATP failed to have any effect on the stability of the secretory vesicles at either pH. It was observed that NH₄Cl increased the release of neurohormones at pH 8.0 in both the presence and the absence of ATP (but not at pH 7.0; Table 1). In guinea-pig secretory vesicles this release was $52 \pm 7\%$ (*n* = 3) in the presence of 25 mM-NH₄Cl alone at pH 8.0.

Effect of NH₄Cl on processing and secretion

NH₄Cl has been shown to change the internal pH in secretory vesicles from the neurohypophysis (Russell, 1984). Using this method we studied the role of internal pH on the conversion of a neurophysin-glycopeptide intermediate from the biosynthesis of vasopressin, known to be metabolized in isolated secretory vesicles (Jones *et al.*, 1984). Table 2 shows the effect of NH₄Cl on the processing of the intermediate in intact secretory vesicles. The final centrifugation step after the incubation (see the Experimental section) ensured that only the conversion of the intermediate into free neurophysin and glycopeptide inside intact secretory vesicles was measured. The

addition of NH_4Cl increased rather than decreased the conversion of the intermediate into free neurophysin at pH 7.3. An increase in external pH accentuated the effect of NH_4Cl , whereas the opposite was seen with a decrease in external pH.

The effect of NH_4Cl on the conversion of neurophysin-glycopeptide makes it possible to demonstrate that NH_4Cl also affects the internal pH in secretory vesicles, even inside intact nerve endings. As shown in Table 2, the effect of NH_4Cl is the same whether the secretory vesicles are isolated or inside intact nerve endings.

Since NH_4Cl affects the processing of the intermediate in much the same way in isolated secretory vesicles and nerve endings, NH_4Cl can be used to investigate the role of the internal pH in exocytosis. The addition of NH_4Cl to isolated secretosomes did not affect the secretion of the ^{35}S -labelled neurohormones in response to K^+ depolarization (Fig. 5), although such a release was completely blocked by verapamil (Fig. 5).

DISCUSSION

Using a newly developed method for the rapid purification of secretory vesicles from the neurohypophysis of a single laboratory animal, we have investigated the stability and ATPase activity of these vesicles. Secretory vesicles from the guinea pig as well as the rat appear to be stable in the presence of ATP, in contrast with previous data obtained from bovine material (Russell & Thorn, 1978; Russell, 1984), where it was found that the addition of ATP to bovine secretory vesicles leads to lysis of the vesicles.

Preparations of secretory vesicles from bovine material have been found to contain ATPase activity (Scherman *et al.*, 1982; Russell & Holz, 1981). However, all subcellular-fractionation studies have failed to show an enrichment of ATPase activity in the fraction of secretory vesicles (Vilhardt & Hope, 1974; Gratzl *et al.*, 1980; Saermark *et al.*, 1983, 1984). These studies were based on bovine material, where the long preparation time may represent a problem. In the present study we have been able to isolate secretory vesicles from a single guinea pig within a very short time. Using this method, we have investigated the influence of increasing Percoll concentrations on the content of ATPase activity in the secretory-vesicle fraction. Increasing Percoll concentrations decrease the ATPase activity without a similar effect on the yield of neurohormones. At both high and low ionic strength a decrease in the ATPase activity is apparent without a parallel decline in neurohormone yield. The ionic strength affects the density of the vesicles, as would be expected according to theoretical considerations (Steck *et al.*, 1970).

NH_4Cl increased the ATPase activity in the material used for the final isolation of the secretory vesicles. Such an effect would be expected if the ATPase activity was coupled to proton transport, as has been demonstrated for the enzyme in coated microvesicles (Stone *et al.*, 1983; Forgac *et al.*, 1983). However, this activity was decreased in the fraction of secretory vesicles, in parallel with the decrease in basal ATPase activity when the Percoll concentration was increased.

The isolation of secretory vesicles on Percoll gradients is an isopycnic technique. However, rate zonal centrifugation also shows that the ATPase activity and neurohormones in the neurohypophysis are present in different

structures (Vilhardt & Hope, 1974). Using a small-scale approach, we have investigated the time-dependent sedimentation of ATPase activity and neurohormones on sucrose gradients. Our results confirm the previous report by Vilhardt & Hope (1974) that the sedimentation rates for ATPase activity and neurohormones are different, suggesting that they are located in different structures.

The present results, and those obtained from the parotid gland (Arvan *et al.*, 1984), suggest that a low internal pH in secretory vesicles may not be necessary for normal functioning of the vesicles in processing and secretion in all types of cells. This is further supported by the finding that the amidating enzyme involved in the normal processing of hormones in the pituitary has a pH optimum at 7.8 (Eipper *et al.*, 1983). The results obtained on processing of an intermediate from the degradation of the vasopressin precursor (see the Results section) show that increasing the internal pH in secretory vesicles from the guinea pig increases the rate of conversion of this intermediate. Although several basic as well as acidic proteinases appear to be present in subcellular fractions from the neurohypophysis (Pelaprat *et al.*, 1984), we can exclude that enzymes not present in the secretory vesicles may be involved, since the method used ensures that only processing in intact granules is measured (see the Experimental and Results sections). Although certain processing enzymes have an acidic pH optimum (Gainer *et al.*, 1984), this appears not to be a prerequisite for all kinds of proteolytic processing.

The difficulties in demonstrating the ATPase proposed to generate an acidic interior do not imply that secretory vesicles from the neural lobe may not have an acidic interior. Substantial evidence indicates a pH lower than 7.0 (Castel *et al.*, 1984). This acidification may come from processing of vasopressin and its binding to the neurophysins. An interesting possibility indicated by our results is that the acidification may stop processing and thereby prevent extensive breakdown of the secretory products.

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REFERENCES

- Apps, D. K. & Reid, A. G. (1977) *Biochem. J.* **167**, 297–300
- Arvan, P., Rudnick, G. & Castle, J. D. (1984) *J. Biol. Chem.* **259**, 13567–13572
- Bashford, C. L., Casey, R. P., Radda, G. & Ritchie, G. A. (1976) *Neuroscience (Oxford)* **1**, 399–412
- Brownstein, M. J. (1982) *Trends Neurosci.* **5**, 318–320
- Castel, M., Gainer, H. & Dellmann, H.-D. (1984) *Int. Rev. Cytol.* **88**, 304–384
- Eipper, B. A., Mains, R. E. & Glembofski, C. C. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5144–5148
- Forgac, M., Cantley, L., Weidenmann, B., Altsteil, L. & Branton, D. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1300–1303
- Gainer, H., Russell, J. T. & Peng Loh, Y. (1984) *FEBS Lett.* **175**, 135–139
- Gratzl, M. & Dahl, G. (1978) *J. Membr. Biol.* **40**, 343–364
- Gratzl, M., Dahl, G., Russell, J. T. & Thorn, N. A. (1977) *Biochim. Biophys. Acta* **470**, 45–57
- Gratzl, M., Torp-Pedersen, C., Dartt, D., Treiman, M. & Thorn, N. A. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1615–1628

- Grinstein, S., Vander Meulen, J. & Furuga, W. (1982) *FEBS Lett.* **148**, 1–4
- Johnson, R. G., Beers, M. F. & Scarpa, A. (1982) *J. Biol. Chem.* **257**, 10701–10707
- Jones, P. M., Saermark, T. & Robinson, I. C. A. F. (1984) *J. Endocrinol.* **103**, 347–354
- Pelaprat, D., Seidah, N. G., Sikstrom, R. A., Lambelin, P., Hamelin, J., Lazure, C., Cromlish, J. A. & Chretien, M. (1984) *Endocrinology (Baltimore)* **115**, 581–590
- Pollard, H. B., Tack-Goldman, K., Pazoles, C. J., Creutz, C. E. & Schulman, N. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5295–5299
- Robinson, I. C. A. F. & Jones, P. M. (1983) *Neurosci. Lett.* **39**, 273–278
- Russell, J. T. (1984) *J. Biol. Chem.* **259**, 9496–9507
- Russell, J. T. & Holz, R. W. (1981) *J. Biol. Chem.* **256**, 5950–5953
- Russell, J. T. & Thorn, N. A. (1978) *Acta Endocrinol. (Copenhagen)* **87**, 495–506
- Saermark, T. & Vilhardt, H. (1979) *Biochem. J.* **181**, 321–330
- Saermark, T., Thorn, N. A. & Gratzl, M. (1983) *Cell Calcium* **4**, 151–171
- Saermark, T., Jones, P. M. & Robinson, I. C. A. F. (1984) *Biochem. J.* **218**, 591–599
- Saermark, T., Flint, N. & Evans, W. H. (1985a) *Biochem. J.* **225**, 51–58
- Saermark, T., Jones, P. M. & Robinson, I. C. A. F. (1985b) *Biochim. Biophys. Acta* **819**, 114–118
- Scherman, D., Nordmann, J. J. & Henry, J. P. (1982) *Biochemistry* **21**, 687–694
- Schuldiner, S., Rottenberg, H. & Avron, M. (1972) *Eur. J. Biochem.* **25**, 64–70
- Steck, T., Strans, J. H. & Wallach, D. F. H. (1970) *Biochim. Biophys. Acta* **203**, 385–395
- Stone, D. K., Xie, X.-S. & Racker, E. (1983) *J. Biol. Chem.* **258**, 4059–4063
- Turner, A. J. (1984) *Trends NeuroSci.* **7**, 258–260
- Vilhardt, H. & Hope, D. B. (1974) *Biochem. J.* **143**, 181–189

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