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Existence of an Adenosine 5′-Triphosphate Dependent Proton Translocase in Bovine Neurosecretory Granule Membrane†

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ABSTRACT: The addition of ATP to bovine neurohypophysial secretory granules suspended in isotonic sucrose medium induces a positive polarization, $\Delta\psi$, of their interior without affecting their internal pH. In KCl-containing media, ATP failed to generate large $\Delta\psi$ but induced a pH gradient (ΔpH; interior acidic). These observations are consistent with the existence in the neurosecretory granule membrane of an ATP-dependent inward electrogenic H^+ translocase (H^+ pump), capable in KCl-containing media of acidifying the granule matrix by H^+–Cl^- cotransport. The $\Delta\psi$ and ΔpH generated by the H^+ pump, defined as the ATP-induced changes sensitive to the H^+ ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), were blocked by *N*,*N*′-dicyclohexylcarbodiimide, an inhibitor of all H^+ pumps, and were insensitive to oligomycin, a mitochondrial ATPase inhibitor. In sucrose medium, measurements were complicated by a Donnan equilibrium reflecting the presence in the granule of peptide hormones and neurophysins which resulted in a CCCP-resistant resting ΔpH. In KCl-containing media, the Donnan equilibrium was destroyed since the membrane is permeable to cations, but under these conditions a CCCP-resistant K^+-diffusion potential was observed. The ATP-induced $\Delta\psi$ was also monitored by the extrinsic fluorescent probe bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol. The hypothesis of a granule H^+ pump is further supported by the presence of an oligomycin-resistant ATPase in the preparation and the ultrastructural localization of such an activity on the granule membrane. The H^+ pump has been found in both newly formed and aged neurosecretory granules. Its possible physiological function is discussed with reference to that of chromaffin granules, with which it has many similarities.

In the neurohypophysis, the peptide hormones ocytocin and vasopressin are localized in specialized organelles, the neurosecretory granules (NSG). These structures play an important role in the maturation, storage, and liberation of the hormones. It has been demonstrated that the hormones are released from the NSG by exocytosis, but little is known concerning the mechanism of this process [for review, see Morris et al. (1978)]. In vitro, ATP induces the release of vasopressin from isolated NSG (Poisner & Douglas, 1968; Russel & Thorn, 1978), and recently Overgaard et al. (1979) have shown that such release is stimulated by addition of chloride ions. Isolated chromaffin granules of adrenal medulla also release the catecholamines of their matrix by an ATP-dependent process which requires the presence of permeant anions such as chloride (Casey et al., 1976; Hoffman et al., 1976). The latter observation has been explained by the existence in the granule membrane of an inward H^+ pump (Casey et al., 1977; Flatmark & Ingebretsen, 1977; Phillips & Allison, 1978). This pump polarizes the vesicle interior, making it positive with reference to the milieu (Pollard et al., 1976; Scherman & Henry, 1980a) or, in the presence of permeant anions, acidifying it by H^+-anion cotransport, resulting under certain conditions in granule lysis. The possible existence of an H^+ pump in NSG was therefore investigated. The present paper provides pieces of evidence for such a pump in NSG.

Two populations of neurohypophysial NSG have been recently characterized: the newly formed (NF-NSG)[1] and the aged (A-NSG) neurosecretory granules. These two populations differ both in their physiological and in their physicochemical properties (Nordmann et al., 1979; Nordmann & Labouesse, 1981). The present data have been obtained with A-NSG, which are the more abundant material, and have been confirmed with NF-NSG. ATP-induced transmembrane potentials ($\Delta\psi$) and pH gradients (ΔpH) have been measured by the well-documented ion partition technique (Rottenberg et al., 1971; Goldman & Rottenberg, 1973; Casey et al., 1977;

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[1] Abbreviations: A-NSG, aged neurosecretory granules; NF-NSG, newly formed neurosecretory granules; OX-V, bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; Tris, tris(hydroxymethyl)aminomethane.

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Johnson et al., 1978), using [^{14}C]thiocyanate and [^{14}C]-methylamine, respectively. Changes in $\Delta\psi$ were also monitored with the fluorescent probe bis(3-phenyl-5-oxoisoxazol-4-yl)-pentamethine oxonol (OX-V), as previously done for chromaffin granules (Scherman & Henry, 1980a).

Experimental Procedures

Materials. Mes and Hepes were purchased from Sigma (St. Louis, MO). AG-6227 (now commercialized under the trade name of Hexabrix) was a gift from Laboratoires Guerbet (Aulnay-sous-bois, France). [^{14}C]Dextran was obtained from NEN (Dreieich, Germany), thio[^{14}C]cyanate was from the Radiochemical Centre (Amersham, U.K.), and ^3H_2O and [^{14}C]methylamine were from CEA (Saclay, France). The fluorescent probe OX-V was a gift of Dr. B. S. Cooperman. OX-V (0.2 mM), CCCP (2 mM), DCCD (40 mM), and oligomycin (1 mg/mL) were dissolved in ethanol.

Isolation of Neurosecretory Granules. A crude bovine neurohypophysial granule fraction was isolated as described previously (Nordmann & Aunis, 1980) with minor modifications. Sixty to eighty neurohypophyses were obtained from the local slaughter house 1–5 h after the animals were killed. The glands were dissected and put in 0.3 M sucrose buffered with 10 mM Mes (pH 6.6). The neural lobes (separated from the anterior pituitary) were minced and homogenized in sucrose solution ($\eta^{4°C}$ = 1.3480 ; $\rho^{4°C}$ 1.0634), and the homogenate was centrifuged at 800g for 10 min. The resulting pellet was homogenized further with a Teflon homogenizer and centrifuged for 10 min at 800g, and the two supernatants were mixed and centrifuged at 3000g for 10 min. The subsequent supernatant was collected and centrifuged at 5000g for 10 min. The resulting supernatant was centrifuged at 27000g for 20 min, and the pellet, which represents the crude granule preparation, was gently stirred in buffered 0.3 M sucrose and layered onto continuous isoosmotic sucrose–AG-6227 gradients in cellulose nitrate tubes. Gradients were generated from 5.5 mL of 0.36 osm sucrose (0.3 M) and 5.5 mL of 0.36 osm AG-6227 (~33% (v/v); $\eta^{4°C}$ = 1.3870; $\rho^{4°C}$ 1.1877). In some experiments, metrizamide was substituted for AG-6227. The NSG were centrifuged to isopycnic equilibrium for 1.5 h at 129000g_{av}. The two resulting NSG fractions (A- and NF-NSG) were isolated by using a Büchler device with a Gilson (MiniPulse) peristaltic pump and mixed with 0.3 M buffered sucrose before being centrifuged at 27000g for 20 min. The resulting pellets were gently mixed with 1–2 mL of sucrose solution. The protein content of the preparations was determined according to the method of Bradford (1976) using bovine serum albumin as the standard.

Internal Water Space Measurement. ^3H_2O (final activity 2 μCi/mL) and the nonpermeant [^{14}C]dextran (1.3 μM, 1 μCi/mL) were added to NSG (0.5–1.5 mg of protein) preincubated in the indicated media (final volume 0.5 mL). After a 2-min incubation at 30 °C for isotope distribution, the suspension was centrifuged for 6 min at 40000g at 30 °C; the supernatant was immediately collected, and the carefully wiped pellet was dissolved in 0.5 mL of 3% Triton X-100 and 1% NaOH. The supernatant (40 μL) added to 160 μL of 3% Triton X-100 and 1% NaOH and the pellet fraction (200 μL) were counted in a Triton–toluene scintillation fluid. The internal water space was derived with a 9825 HP computer from the relative activities of the isotopes in pellet and supernatant as in Casey et al. (1977), with the pellet external dextran volume being about 50% of the pellet total ^3H_2O volume. Dextran excluding volumes (from 3 to 6 μL) were measured in duplicate in each type of medium, with the relative standard error being always less than 10%.

$\Delta\psi$ and ΔpH Measurements. ^3H_2O (2 μCi/mL) and [^{14}C]SCN (25 μM, 1.5 μCi/mL) or [^{14}C]methylamine (36 μM, 1.35 μCi/mL) were added to NSG (0.5–1.5 mg of protein in 0.5 mL). Where indicated the suspension was preincubated for 5 min at 30 °C with the following drugs: CCCP (10 μM), oligomycin (10 μg/mL), or DCCD (75 μM). The amount of ethanol (always less than 0.5%) was adjusted in all experiments. ATP (2.5 mM) and $MgSO_4$ (1.25 mM) were then added to the suspension which was incubated for 5 and 10 min at 30 °C for $\Delta\psi$ and ΔpH measurements, respectively. The suspension was then centrifuged for 6 min at 40000g at 30 °C and processed as above. Internal to external concentration ratios of SCN^- and methylamine were derived as in Casey et al. (1977) from the relative activities of the isotopes in pellets and supernatants. $\Delta\psi$ (at 30 °C) and ΔpH were calculated as

$$\psi_{in} - \psi_{out}\ (mV) = 59 \log \frac{(SCN)_{in}}{(SCN)_{out}}$$

$$pH_{out} - pH_{in} = \log \frac{(methylamine)_{in}}{(methylamine)_{out}}$$

The relative standard error of measurements performed in duplicate (Tables IA, III, V, and VI) was always less than 10%. All experiments were done within 24 h of the cattle being slaughtered. The pH of the supernatant solution was measured immediately after centrifugation.

Mitochondrial and NSG ATPase Activity. Mitochondrial ATPase was measured on submitochondrial particles derived from bovine corticoadrenal mitochondria. The mitochondria were obtained by differential centrifugation at 1000g and 27000g in 0.3 M sucrose. They were then sonicated for 4 min at 20 °C and centrifuged at 45000g for 60 min at 4 °C. The supernatant-containing soluble ATPase activity (F_1) was discarded, and the pellet was resuspended at 4 °C in 0.3 M sucrose and 10 mM Hepes, pH 7.0, and immediately assayed for ATPase activity. The ATPase activity of NSG was also assayed immediately after centrifugation and pellet resuspension.

Mitochondria or NSG (50–100 μg of protein/mL) were incubated at 37 °C with ATP, sodium salt (2 mM), and $MgSO_4$ (2 mM) in 50 mM Tris–succinate buffer at pH 7.5. Oligomycin (generally 10 μg/mL) was added to the incubation mixture 5 min before ATP addition. Ethanol (final concentration 2%) was adjusted in all experiments. Aliquots (50 μL) were withdrawn at various times and assayed for inorganic phosphate (Anner & Moosmayer, 1975).

Ultrastructural Localization of ATPase Activity. A crude granule pellet was fixed as described by Benedeczky and collaborators (Benedeczky et al., 1972; Benedeczky & Carmichael, 1980). Briefly, after gluteraldehyde fixation, the granules were incubated at 37 °C for 1 h in a medium containing 2 mM ATP, 40 mM $MgCl_2$, 50 mM Tris, 50 mM succinic acid, and 2.5 mM lead nitrate, pH 6.5. Controls were incubated without the substrate. The pellets were washed with cacodylate buffer (0.1 M, pH 6.8), postfixed with 2% osmium, dehydrated, and embedded in Epon resin. Ultrathin sections were observed without further staining.

Results

ATP-Induced Transmembrane Potentials in A-NSG. When added to isotonic sucrose-containing medium buffered at pH 6.1, A-NSG were slightly polarized, with their interior being negative with respect to the medium (Table I). The addition of ATP shifted the granule potential toward positive values,

Table I: ATP-Induced Transmembrane Potentials in A-NSG[a]

expt	incubation medium[b]	ATP-$MgSO_4$	drug	$\Delta\psi$ (mV)	$(\Delta\psi)_{CCCP\text{-}sens}$[c] (mV)
A	300 mM sucrose	−		−1	−6
		+		21	16
		+	oligomycin	18	14
		+	CCCP	5	
		+	DCCD	10	5
B	300 mM sucrose	+		19	15
	20 mM KSCN, 260 mM sucrose	+		0	3
	20 mM KCl, 260 mM sucrose	+		16	10
	75 mM KCl, 150 mM sucrose	+		10	3
	75 mM K_2SO_4, 100 mM sucrose	+		25	15
	75 mM CH_3CO_2K, 150 mM sucrose	+		17	14

[a] Experimental conditions are described under Experimental Procedures. The specific dextran excluding volume was 5.4 ± 0.3 μL/mg of protein (±SE; $n = 4$) and did not vary significantly with the external medium. [b] All media were buffered at pH 6.1 with 20 mM Mes-KOH. [c] $(\Delta\psi)_{CCCP\text{-}sens}$ is the CCCP-sensitive fraction of $\Delta\psi$ obtained as the difference between the $\Delta\psi$ observed in the absence and in the presence of CCCP under the same incubation conditions.

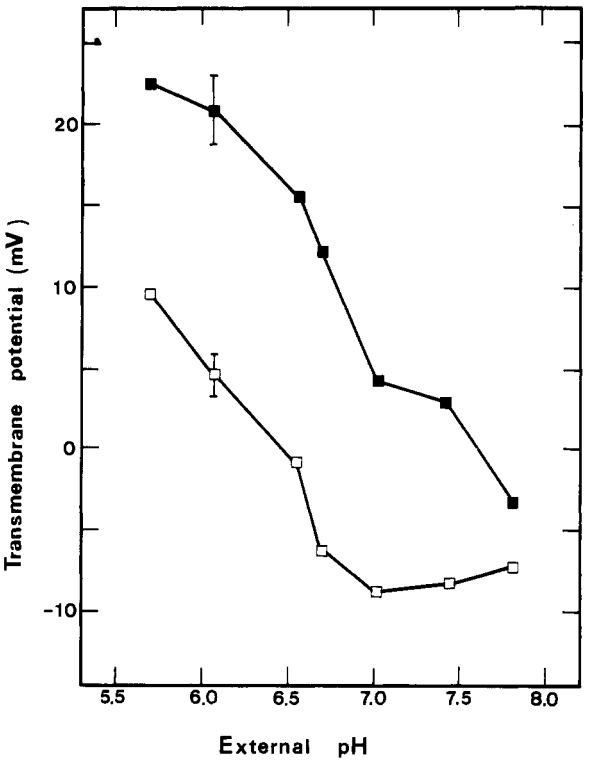

FIGURE 1: pH dependence of the ATP-induced A-NSG transmembrane potential. A-NSG (0.5 mg of protein) were incubated in 300 mM sucrose, 20 mM Mes–KOH from pH 5.5 to pH 6.7 or 20 mM Hepes–KOH from pH 7.0 to pH 7.8, 2.5 mM ATP, and 1.25 mM $MgSO_4$, without (■) or with (□) 10 μM CCCP. The A-NSG preparation used is the same as that used for the experiments described in Table I. Bars are SE ($n = 3$).

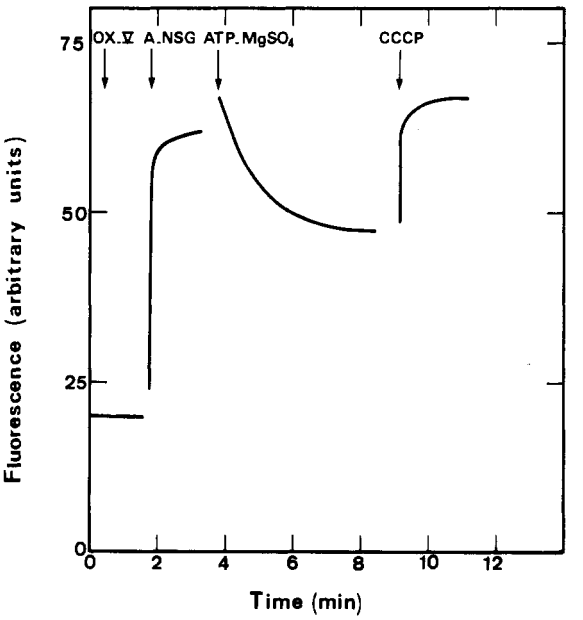

FIGURE 2: OX-V fluorescence change induced by Mg^{2+}–ATP addition. A-NSG (0.1 mg of protein) were added to a 2-mL cuvette containing 300 mM sucrose, 10 mM Mes, pH 6.6, and 0.5 μM OX-V at 25 °C. ATP (2.5 mM) and $MgSO_4$ (1.25 mM) were then added. Fluorescence (excitation and emission wavelengths, 580 and 650 nm) was measured continuously with a Jobin-Yvon JY 3C double monochromator fluorometer as in Scherman & Henry (1980a). CCCP was added at a 5 μM final concentration.

inducing a positive polarization $\Delta\psi$ of 20 mV. This polarization was greatly reduced in the presence of the H^+ ionophore CCCP. The CCCP-sensitive part, $(\Delta\psi)_{CCCP\text{-}sens}$, of the transmembrane potential observed in the presence of ATP was about 15 mV (Table I, experiment A). DCCD (80 μM), which blocks all known H^+ pumps by reacting with their H^+ ionophore, collapsed $(\Delta\psi)_{CCCP\text{-}sens}$, whereas oligomycin (10 μg/mL), which is specific for the mitochondrial H^+ pump, was without effect.

The effect of ionic media was then investigated, and the ATP-induced transmembrane potential ($\Delta\psi$) and its CCCP-sensitive component $[(\Delta\psi)_{CCCP\text{-}sens}]$ were shown to be dependent upon the nature and the concentration of the anion of the medium (Table I, experiment B). SO_4^{2-} (75 mM) and acetate (75 mM) did not alter $(\Delta\psi)_{CCCP\text{-}sens}$ which was collapsed in the presence of Cl^- (33% and 83% inhibition at 20 and 75 mM, respectively) and by SCN^- (82% inhibition at 20 mM). It has to be noted that K_2SO_4 increased $\Delta\psi$ without changing $(\Delta\psi)_{CCCP\text{-}sens}$. This effect might indicate that the CCCP-resistant fraction of $\Delta\psi$ is a K^+-diffusion potential since the membrane has been shown to be permeant to cations such as K^+, Ca^{2+}, and Na^+ (D. Scherman and J. J. Nordmann, unpublished experiment).

The pH dependence of the ATP-induced transmembrane potential is shown in Figure 1. $\Delta\psi$ decreased linearly from 23 mV at pH 5.7 to −3 mV at pH 7.8. The potential observed in the presence of CCCP decreased in a similar way, thus causing $(\Delta\psi)_{CCCP\text{-}sens}$ to be independent of the pH in the 5.6–7.5 pH range. Since potential measurements with the permeant anion SCN^- are accurate only for positive potentials which lead to anion accumulation inside of the granule, our experiments (see Table I) have been conducted at pH 6.1.

The existence of an ATP-induced $\Delta\psi$ in A-NSG was confirmed by experiments with the fluorescent dye OX-V. Ad-

Table II: OX-V Fluorescence Changes in NSG[a]

expt	addn 1	$\Delta I/I$ (%)	addn 2	$\Delta I/I$ (%)	addn 3	$\Delta I/I$ (%)	addn 4	$\Delta I/I$ (%)	addn 5	$\Delta I/I$ (%)
1	ATP	0	$MgSO_4$	30.0	CCCP	0				
2	ATP-$MgSO_4$	24.0	DCCD	3.0	CCCP	2.0				
3	DCCD	0	ATP-$MgSO_4$	0						
4	ATP-$MgSO_4$	30.5	KSCN (2 mM)	21.5	KSCN (5 mM)	13.5	KSCN (10 mM)	3.0	KSCN (20 mM)	0
5	succinate (25 μM)	0	succinate (50 μM)	0	ATP-$MgSO_4$	22.0	CCCP	2.0		
6	NADH	0	ATP-$MgSO_4$	21.0	CCCP	0				
7	ATP-$MgSO_4$	23.0	NADH	23.0	CCCP	−1.0				
8	ATP-$MgSO_4$	17.0	KSCN (2 mM)	10.7	KSCN (5 mM)	6.8	KSCN (10 mM)	2.0	KSCN (20 mM)	0

[a] Experimental conditions are as in the legend to Figure 2. Final concentrations were the following: NSG, 0.1–0.2 mg of protein; DCCD, 80 μM; CCCP, 5 μM; NADH, 50 μM. Experiments 1–7 were performed with A-NSG and experiment 8 with NF-NSG.

Table III: ATP-Induced ΔpH across A-NSG Membrane[a]

expt	incubation medium	ATP-$MgSO_4$	drug	pH_{out}	pH_{in}	ΔpH	$(\Delta pH)_{CCCP\text{-}sens}$[b]
A	300 mM sucrose,	−		6.58	6.12	0.46	
	20 mM Mes, pH 6.6	+		6.58	6.23	0.35	0.07
		+	CCCP	6.60	6.32	0.28	
B	150 mM KCl,	−		6.56	6.50	0.06	
	150 mM sucrose,	+		6.64	6.34	0.30	0.37
	1 mM Mes, pH 6.6	+	oligomycin	6.65	6.37	0.28	0.35
		+	CCCP	6.55	6.62	−0.07	

[a] ΔpH were measured as described under Experimental Procedures. The specific dextran excluding volume was 4.9 ± 0.2 μL/mg of protein (±SE; $n = 3$) and did not vary significantly with the external medium. [b] $(\Delta pH)_{CCCP\text{-}sens}$ is the CCCP-sensitive fraction of ΔpH obtained as the difference between the ΔpH measured in the absence and in the presence of CCCP under the same incubation conditions.

Table IV: Effect of the Incubation Medium on the ATP-Induced ΔpH[a]

incubation medium[b]	drug	pH_{out}	pH_{in}	ΔpH	$(\Delta pH)_{CCCP\text{-}sens}$[d]
300 mM sucrose		6.80	6.41	0.39	0.08
	CCCP	6.67	6.36	0.31	
150 mM sucrose, 75 mM KCl		6.60	6.37	0.23	0.22
	CCCP	6.52	6.51	0.01	
100 mM sucrose, 75 mM K_2SO_4		6.68	6.62	0.06	0.08
	CCCP	6.56	6.58	−0.02	
150 mM sucrose, 75 mM KCH_3COO		6.88	6.82	0.06	0.05
	CCCP	6.89	6.88	0.01	
cytoplasmic-like medium[c]		6.64	6.45	0.19	0.19
	CCCP	6.60	6.60	0.00	

[a] Internal water space was measured under each of the conditions and did not vary with the external medium (4.55 ± 0.22 μL/mg of protein; $n = 6$). [b] All media contained 2.5 mM ATP, 1.25 mM $MgSO_4$, and 1 mM Mes-KOH (pH 6.6). [c] Containing 135 mM potassium glutamate, 5 mM glucose, 1 mM EGTA, 13 mM ATP, 5 mM $MgCl_2$, and 0.1 μM $CaCl_2$. [d] Defined as in Table III.

dition of ATP to a mixture of granules and OX-V induced a large (30%) decrease of fluorescence (Figure 2), similar to that observed with chromaffin granules (Scherman & Henry, 1980a) and submitochondrial particles (Smith et al., 1976). The fluorescence change was completed in 4 min. It was not observed in the absence of the Mg^{2+} cation and was rapidly and totally reversed by addition of CCCP (Figure 2; Table II, experiment 1). It could also be reversed by DCCD (Table II, experiments 2 and 3) and, in a dose-dependent manner, by the permeant anion SCN^- (Table II, experiment 4). Succinate (25 and 50 μM) and NADH (50 μM), which energize submitochondrial particles and induce OX-V fluorescence decrease in these particles (Smith et al., 1976), were without effect on A-NSG (Table II, experiments 5–7).

ATP-Induced pH Gradient across the A-NSG Membrane. When A-NSG were incubated in 300 mM sucrose buffered at pH 6.6, a ΔpH of 0.46 (interior acidic) was observed (Table III, experiment A). This resting pH gradient was slightly decreased by addition of ATP, and ΔpH measured in the presence of ATP was quite unaffected by addition of CCCP, with the CCCP-sensitive ΔpH being 0.07 pH unit.

In contrast, in hypertonic KCl-containing media (Table III, experiment B), the resting ΔpH was small (0.06), but the ATP addition induced a marked increase of the ΔpH (0.3 pH unit). ATP addition resulted in an acidification of the granule interior. With the low buffering capacity of the incubation mixture used in these experiments, a concomitant alkalinization of the medium (0.1 pH unit) was also observed. Similar results were obtained with two NSG preparations in more than 15 experiments. The ΔpH observed in the presence of ATP was collapsed by the ionophore CCCP, which affected both the alkalinization of the external medium and the acidification of the granule matrix. Oligomycin did not modify the CCCP-sensitive ATP-induced ΔpH.

The CCCP-sensitve ΔpH induced by ATP addition, $(\Delta pH)_{CCCP\text{-}sens}$, was stable for more than 1 h (Figure 3). During the same period of time, the intragranular water space did not vary much (see legend to Figure 3), indicating that the granules did not lyse significantly. Nevertheless, it has to be pointed out that the experiment was conducted in hypertonic solution (osmolarity adjusted to 450 mosm with sucrose) to prevent the granule lysis which might have resulted from the osmolarity increase caused by H^+–Cl^- cotransport.

The origin of the difference in what is observed in sucrose and ionic media was then investigated. The magnitude of $(\Delta pH)_{CCCP\text{-}sens}$ was shown to depend on the nature of the anion

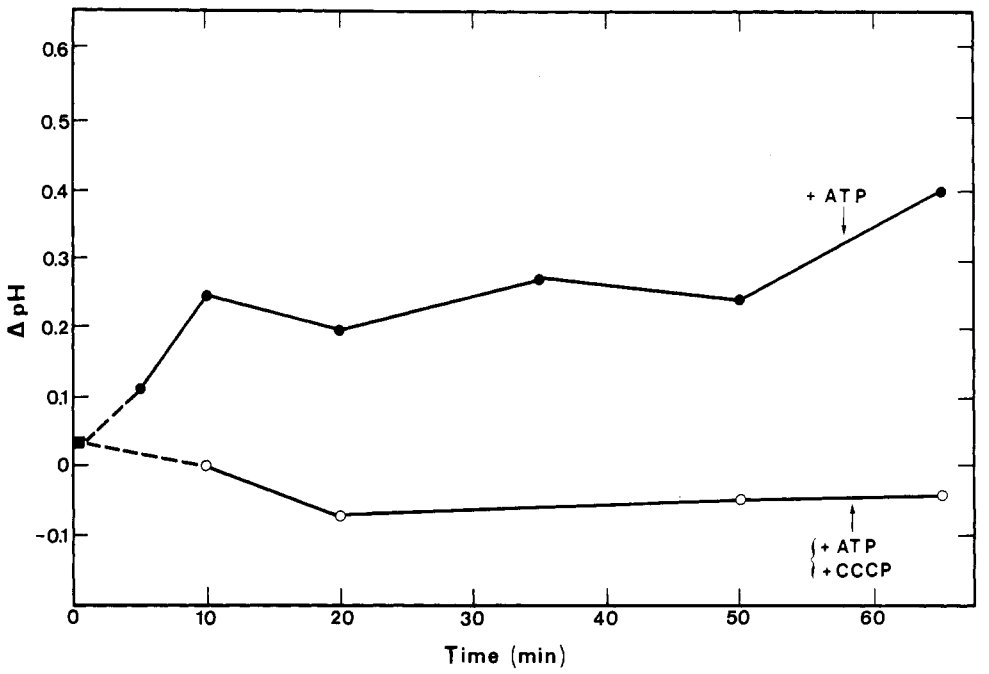

FIGURE 3: Time course of the ATP-induced ΔpH. A-NSG (1.6 mg of protein/mL) were incubated for various times at 35 °C in a 1 mM Mes buffer, pH 6.6, containing 150 mM sucrose, 150 mM KCl, 2.5 mM ATP, and 1.25 mM $MgSO_4$ in the absence (●) or in the presence (○) of 10 μM CCCP. The pH gradient was measured as described under Experimental Procedures. The specific internal water space was 4.7, 4.4, and 4.3 μL/mg of protein at 0, 15, and 60 min, respectively. On the same figure is indicated an experiment without ATP (■) which is a more precise zero time value since the technique involves a 6-min centrifugation and is thus not rapidly terminated.

Table V: ATP-Induced Transmembrane Potential in NF-NSG[a]

incubation medium	ATP-$MgSO_4$	drug	Δψ (mV)	$(\Delta\psi)_{CCCP\text{-}sens}$[b] (mV)
300 mM sucrose,	−		2.65	
20 mM Mes-KOH, pH 6.1	+		9.6	7.4
	+	oligomycin	10.3	8.1
	+	CCCP	2.2	

[a] NF-NSG were derived from the same preparation as that used for the experiments described in Table I. The specific internal water space was 5.0 ± 0.3 μL/mg of protein (mean ± SE; $n = 3$). [b] $(\Delta\psi)_{CCCP\text{-}sens}$ is defined as in Table I.

Table VI: ATP-Induced ΔpH across NF-NSG[a]

expt	incubation medium	ATP-$MgSO_4$	drug	pH_{out}	pH_{in}	ΔpH	$(\Delta pH)_{CCCP\text{-}sens}$[b]
A	300 mM sucrose,	−		6.60	6.32	0.28	
	20 mM Mes-KOH, pH 6.6	+		6.58	6.46	0.12	−0.04
		+	CCCP	6.58	6.42	0.16	
B	150 mM KCl,	−		6.78	6.76	0.02	
	150 mM sucrose,	+		6.70	6.57	0.13	0.16
	1 mM Mes-KOH, pH 6.6	+	oligomycin	6.70	6.63	0.07	0.10
		+	CCCP	6.70	6.73	−0.03	

[a] NF-NSG were derived from the same preparation as that used for the experiments described in Table III. Specific dextran excluding volume was 5.3 ± 0.4 μL/mg of protein (±SE; $n = 4$) in sucrose medium and did not vary significantly in KCl-containing medium. [b] Defined as in Table III.

of the medium (Table IV). $(\Delta pH)_{CCCP\text{-}sens}$ was large in chloride-containing media and small in the presence of sulfate or acetate. It has to be pointed out that anions had opposite effects on the $(\Delta\psi)_{CCCP\text{-}sens}$ (Table I) and the $(\Delta pH)_{CCCP\text{-}sens}$ (Table IV). In addition, a cytoplasmic-like medium containing potassium glutamate (135 mM) was found to induce a ΔpH as large as that obtained in the presence of KCl.

Energization of NF-NSG. NF-NSG were also energized by addition of ATP, and the results were qualitatively similar to those described on A-NSG. When NF-NSG were suspended in isoosmotic sucrose, a CCCP-sensitive ATP-induced transmembrane potential $(\Delta\psi)_{CCCP\text{-}sens}$ was observed (Table V), which was resistant to oligomycin. As for A-NSG, this potential could be monitored with the fluorescent probe OX-V (Table II, experiment 8). The ATP-induced fluorescence decrease was reversed by CCCP and DCCD (data not shown) or by the anion thiocyanate (Table II, experiment 8).

The addition of ATP also induced a CCCP-sensitive acidification of the matrix of NF-NSG suspended in KCl but not in sucrose (Table VI). The CCCP-sensitive ΔpH was partially resistant to oligomycin.

It has to be noted that the A-NSG and the NF-NSG used for similar experiments (Tables I and V or III and VI) were derived from the same preparation and therefore that the effects, though qualtiatively similar, were less marked with NF-NSG.

ATPase Activity of A-NSG and NF-NSG Preparations. A particulate-bound ATPase was detected in the two types of preparations. The enzyme was Mg^{2+} dependent, and its activity, measured after centrifugation to eliminate any soluble

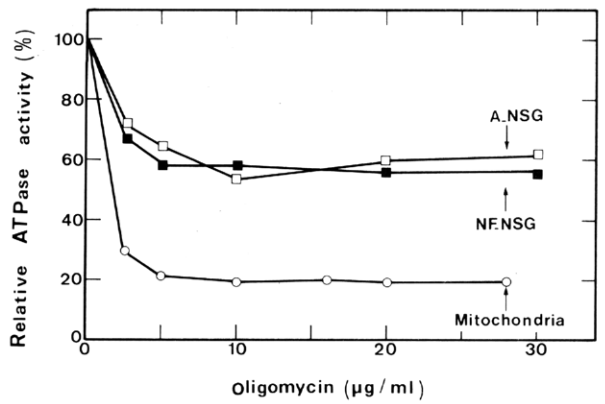

FIGURE 4: Inhibition by oligomycin of NSG and mitochondrial ATPase activity.

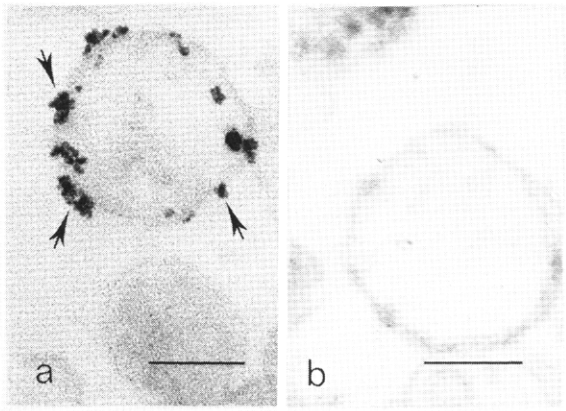

FIGURE 5: Ultrastructural localization of ATPase. Part a shows a clear precipitate (arrows) around the membrane of a granule incubated in the presence of ATP. No precipitate can be observed in controls (b). Bar: 0.1 μm.

form of the enzyme, was 90 ± 4.5 (n = 4) and 60 ● 5 (n = 2) nmol of ATP hydrolyzed (mg of protein)$^{-1}$ min^{-1} for A-NSG and NF-NSG, respectively. Both A-NSG and NF-NSG ATPase activities were more resistant to oligomycin than mitochondrial ATPase activity (Figure 4). In some experiments, the ATPase activity of NF-NSG was found to be less resistant to oligomycin than that of A-NSG (data not shown).

The existence of an ATPase on NSG was further supported by ultrastructural localization experiments similar to those performed on chromaffin granules (Benedeczky & Carmichael, 1980). A lead salt precipitate was observed on the membrane of granules incubated in the presence of substrate (Figure 5a). This precipitate was absent when the substrate was omitted (Figure 5b). Although not all granules were as reactive as the NSG shown in Figure 5a, the majority of them had a much darker membrane than the controls.

Discussion

Existence of an ATP-Dependent Electrogenic Proton Pump. The major conclusion of this work is that there exists an electrogenic H^+ pump in NSG. It is based on the observation that addition of ATP to NSG results in a positive polarization and/or an acidification of the granule interior, depending upon the anion composition of the medium. That ATP exerts its effect by energizing an H^+ pump is shown by the fact that the uncoupler CCCP, which increases the H^+ conductance of biological membranes and thus prevents electrogenic H^+ accumulation, decreased dramatically the ATP-dependent $\Delta\psi$ or ΔpH. In sucrose medium, $(\Delta\psi)_{CCCP\text{-}sens}$ was large and $(\Delta pH)_{CCCP\text{-}sens}$ was low since the H^+ pump only polarized the granule interior; under these conditions the observed pH gradient originated mainly from a Donnan equilibrium (see below). The converse was true in KCl-containing media where neutral H^+–Cl^- cotransport resulted in a limited $(\Delta\psi)_{CCCP\text{-}sens}$ and a large $(\Delta pH)_{CCCP\text{-}sens}$, with the CCCP-resistant $\Delta\psi$ being due to a K^+-diffusion potential (see below).

According to Mitchell (1961), an H^+ pump is characterized by its protonmotive force, defined as the steady-state H^+ electrochemical gradient $\Delta\mu_{H^+} = \Delta\psi + 59\Delta pH$ (at 30 °C) observed in the presence of ATP and independent of the experimental conditions. In sucrose medium, $\Delta\psi$ and ΔpH (expressed in mV) were, at pH 6.6 and in the presence of ATP, 14 (Figure 1) and 21 mV (Table III, experiment A), respectively, thus leading to a figure of 35 mV for the $\Delta\mu_{H^+}$ of A-NSG. In KCl-containing media, $\Delta\psi$ was about 10 mV (Table I, experiment B) and ΔpH was 18 mV (Table III, experiment B), resulting in a $\Delta\mu_{H^+}$ of 28 mV. These figures are confirmed by those observed in a separate set of experiments performed at pH 6.6 on granules purified only by differential centrifugation: in the presence of ATP, values of 19 mV for $\Delta\psi$ and 28 mV for ΔpH in sucrose medium and 6 mV for $\Delta\psi$ and 32 mV for ΔpH in KCl-containing medium were observed, thus leading to 47 and 38 mV for the $\Delta\mu_{H^+}$ in sucrose and KCl media, respectively. The consistency of the figures obtained under the two sets of conditions supports the contention of the existence of an H^+ pump in NSG. This conclusion is also supported by the pH dependency of the ATP-induced $\Delta\psi$ observed in 0.3 M sucrose (Figure 1). Increasing the external pH results in a decrease of $\Delta\psi$, which can be accounted for by the increase of ΔpH and the constancy of the $\Delta\mu_{H^+}$ generated by the H^+ pump. A similar result has been reported for chromaffin granules (Scherman & Henry, 1980a).

The granule preparations were contaminated by mitochondria (Nordmann et al., 1979), but the observed ATP-dependent $\Delta\mu_{H^+}$ did not originate from contaminating submitochondrial particles for the following reasons: (i) The ATP-induced $(\Delta\psi)_{CCCP\text{-}sens}$ (Tables I and V) and $(\Delta pH)_{CCCP\text{-}sens}$ (Tables III and VI) in A- and NF-NSG were largely resistant to the mitochondrial inhibitor oligomycin. (ii) Succinate and NADH which energize submitochondrial particles did not polarize NSG. Moreover the hypothesis of the existence of a granular ATPase is supported by the ultrastructural localization of this activity on the NSG membrane (Figure 5) and by the resistance of oligomycin of the ATPase activity of the preparation. The resistance to this antibiotic was higher in fractions derived from NSG by suspension in hypoosmotic buffer and separated from intact granules by centrifugation on a metrizamide–sucrose gradient (Table VII). Fractions 5 and 6, which had a low density and a high ATPase to cytochrome c oxidase ratio, were likely to be NSG membranes separated from mitochondria; 66–70% of their ATPase activity was resistant to oligomycin vs. 25–30% for intact NSG.

The NSG H^+ pump can thus be compared with that of chromaffin granules. The two preparations, although they have membranes differing in their permeability properties (see below), have similar H^+ pumps. Both are resistant to oligomycin (Bashford et al., 1975b); DCCD, which blocks the H^+ ionophore of all known H^+ pumps, also inhibited both preparations. The kinetics of the polarization of NSG, monitored by the dye OX-V, resembles that of chromaffin granules (Scherman & Henry, 1980a). It has nevertheless to be pointed out that the protonmotive force of NSG (about 40 mV) is lower than that reported for chromaffin granules (120–150 mV) (Phillips & Allison, 1978; Johnson & Scarpa, 1979;

Table VII: Sucrose-Metrizamide Density Gradient Centrifugation of Osmotically Shocked NSG[a]

	fraction no.							
	1	2	3	4	5	6	7	8
density	1.17	1.14	1.13	1.12	1.11	1.09	1.08	1.07
phospholipids (mM)	0.18	0.81	1.16	0.72	0.30	0.17	0.10	0.22
ATPase (nmol of ATP hydrolyzed min^{-1} mL^{-1})	ND[b]	111	63	30	24	10	3	2
ATPase resistant[c] to oligomycin (%)		30 ± 8	25 ± 2	31 ± 8	48 ± 2	66 ± 4	70 ± 13	81 ± 15
cytochrome *c* oxidase[d] (A_{550} min^{-1} mL^{-1})	4.5	85.0	41.5	7.6	1.7	0.3	1.4	ND[b]
ATPase activity/cytochrome *c* oxidase activity		1.3	1.5	4.0	14.2	34.0	2.4	

[a] NSG (ρ 1.11–1.14) purified by linear sucrose-metrizamide gradient centrifugation were diluted 10 times in 10 mM Hepes, pH 6.8, and incubated at 4 °C for 6 h. After centrifugation at 100000*g* for 10 min, they were resuspended in a 0.4-mL buffer containing 0.3 M sucrose, layered onto a sucrose-metrizamide gradient identical with the previous one, and centrifuged at 129000g_{av} for 1 h. The fraction volume was about 1 mL. Fractions 2 and 3, NSG; fractions 5 and 6, NSG membranes separated from mitochondria. [b] ND, not determined. [c] Derived from four and two measurements of ATPase activity, in the absence and in the presence of oligomycin, respectively. [d] Measured spectrophotometrically.

Scherman & Henry, 1980b). However, methodological problems in the measurement of the water exchangeable space might account for this discrepancy since dextran, which has been used in the present study, also gave low figures (15–30 mV) with chromaffin granule ghosts (Johnson et al., 1979; Scherman & Henry, 1981). Another possibility might be the presence of other organelles which would contribute significantly to the measured water space, but this hypothesis is not supported by electron microscopy of the preparation (Nordmann et al., 1979).

There is a difference between the protonmotive force of A- and NF-NSG. This point is now under investigation, and at the present time, it is not known whether this difference reflects different properties of the two granule types or some technical problem due to the small amount of NF-NSG available.

Passive Properties of NSG Membrane. It has already been observed (D. Scherman and J. J. Nordmann, unpublished experiment) that the internal pH of NSG is governed by a Donnan equilibrium resulting from the presence of impermeant anions (hormones and neurophysins) inside the granule. The variation of the Donnan equilibrium with pH (D. Scherman and J. J. Nordmann, unpublished experiment) is also likely to be responsible for the variation with pH of the polarization of NSG suspended in isotonic sucrose in the presence of ATP and CCCP (Figure 1). As previously observed (D. Scherman and J. J. Nordmann, unpublished experiment), the fact that the Donnan equilibrium is destroyed in ionic media indicates that the NSG membrane is permeable to cations (Na^+, K^+, and Ca^{2+}). Such a salting out phenomenon is obvious in Table III where the resting ΔpH was larger in the absence than in the presence of the permeant K^+ ions. Moreover, the addition of Mg^{2+}–ATP to A-NSG in 0.3 M sucrose slightly decreased the resting ΔpH, an effect which indicates permeation of the membrane by the cations present in the ATP–Mg^{2+} solution. The permeability of NSG membrane to cations is further substantiated by the existence, when K^+ ions were associated with moderately permeant anions such as chloride or sulfate, of a K^+-diffusion potential responsible for the CCCP-resistant fraction of $\Delta\psi$ (Table I, experiment B). On the other hand, it should be pointed out that chromaffin granule membrane is impermeable to cations (Johnson & Scarpa, 1976).

The effects of anions on $(\Delta\psi)_{CCCP\text{-}sens}$ and $(\Delta pH)_{CCCP\text{-}sens}$ (Tables I and IV) indicate a permeability increasing in the series acetate < SO_4^{2-} < Cl^- < SCN^- qualitatively comparable to that reported for chromaffin granule membrane (Dolais-Kitabgi & Perlman, 1975; Casey et al., 1976; Phillips & Allison, 1978). The two membranes thus differ with respect to their permeability to cations but not to anions.

Function of the ATP-Dependent Proton Pump of NSG. The H^+ pump of NSG bears large resemblance to that of chromaffin granules. The latter has been convincingly involved in the uptake of the monoamines stored in the granules (Bashford et al., 1975a; Johnson & Scarpa, 1979; Phillips, 1978; Scherman & Henry, 1980b) whereas no known transport function can be attributed to the former. In vitro, the H^+ pump described in the present study is likely to be responsible for the reported ATP-evoked liberation of neurohormones observed in KCl-containing media (Overgaard et al., 1979) since H^+–Cl^- cotransport should induce an osmotic lysis. Such a phenomenon has been proposed to be physiologically relevant to the mechanism of exocytosis (Pazoles & Pollard, 1978). It is striking to note that NSG and chromaffin granules have the same sophisticated bioenergetic equipment. A possible role of proton influx and granule lysis during exocytosis remains to be elucidated.

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Isolation, Purification, and Properties of Respiratory Mucus Glycoproteins†

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ABSTRACT: The major glycoprotein from human tracheobronchial secretions and from primary explant cultures of human tracheal epithelium has been purified to apparent homogeneity. Mucin was solubilized in buffer and fractionated on Sepharose CL-4B, followed by CsBr density gradient centrifugation of the void volume fraction. High- and low-density fractions were obtained in ratios ranging from 2:1 to 5:1. The high-density (1.46) fraction appeared homogeneous by exclusion chromatography and recentrifugation in CsBr and had an amino acid composition characteristic of a mucin-type structure (threonine, serine, proline, glycine, and alanine comprise two-thirds of the amino acid residues). The carbohydrate, which is nearly 80% by weight, was O-glycosidically linked via GalNAc, sulfated (5.4% by weight), and contained fucose, galactose, glucosamine, galactosamine, and sialic acid. The low-density fraction had an amino acid composition distinct from that of the high-density fraction (threonine, serine, proline, glycine, and alanine comprise 51% of the amino acid residues) and a lower sulfate content. The size distribution of the saccharides in the low-density fraction was similar to that of the high-density fraction; the same sugars were present although the ratios were different. The low-density fraction contained 3 times more noncovalently associated lipid than did the high-density fraction. Several distinct classes of lipids were identified. Neutral lipids (mono-, di-, and triglycerides, cholesterol, and cholesteryl esters) comprised 56% by weight of the total lipid. Glycolipids and phospholipids were also identified. Palmitate (16:0), stearate (18:0), and oleate (18:1) were the major fatty acids in all classes of lipids.

Human tracheobronchial secretions consist of a heterogeneous population of macromolecules, salts, and water. Respiratory mucus, as well as mucus from other sources (e.g., gastric, salivary, and cervical mucus), serves as a lubricative and protective barrier and, in addition, provides a clearance mechanism for particulate matter from the respiratory tract.

Human tracheobronchial mucus has been characterized in terms of total protein, carbohydrate, lipid, and inorganic salts (Basch et al., 1941; Masson et al., 1965; Chernick & Barbero, 1959). Rheological properties have also been studied (Mitchell-Heggs, 1977; Charman & Reid, 1972, 1973).

The major glycoprotein present in tracheobronchial secretions is a large, carbohydrate-rich glycoprotein whose macromolecular structure regulates the viscoelastic properties of mucus. Isolation and characterization of the individual components from mucus are required to understand structure–function relationships.

Mucus contains several components in addition to the major mucin glycoprotein, most of which are lower molecular weight proteins and serum-type glycoproteins (Havez et al., 1968; Roussel et al., 1975; Boat et al., 1976; Feldhoff et al., 1976, 1979; Rose et al., 1979). Resolution of these components often involves the use of exclusion chromatography (Boat et al.,

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