Journal of Supramolecular Structure 7:277–285 (1977)
Molecular Aspects of Membrane Transport 443–451

A Role for Anion Transport in the Regulation of Release From Chromaffin Granules and Exocytosis From Cells

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Release of epinephrine from isolated adrenergic secretory veiscles from the adrenal medulla (chromaffin granules) was found to be inhibited by a number of anion transport blocking agents, including SITS, probenecid, pyridoxal phosphate, and Na-isethionate. High concentrations of permeant anion, such as chloride, are required for granule release and the drugs were found to be competitive inhibitors with respect to chloride. The anion transport blockers were also found to suppress exocytosis of serotonin from human platelets and parathyroid hormone from dissociated bovine parathyroid cells. By contrast, they had no effect on ACTH-activated corticosterone secretion from dissociated rat adrenocortical cells, a process which occurs by diffusion rather than exocytosis. The important anion in the medium for human platelets was hydroxyl ion, rather than chloride, and the most effective drug on platelets was suramin. Isethionate was inactive. In the case of PTH secretion, both chloride and hydroxyl ions were important anions and were both competitively inhibited by anion blocking drugs including Na-isethionate. We conclude from these studies that the chemistry of exocytosis appears to be quite similar to the chemistry of release from isolated secretory vesicles. We suggest that when vesicles are fused to plasma membranes prior to exocytosis they are exposed to higher chloride and hydroxyl ion concentrations of the medium, and that inward anion flux into the vesicle promotes release, possibly by local osmotic lysis. Blockade of exocytosis by anion transport blocking drugs would occur by inhibition of inward anion flux into the fused vesicle, by analogy with previous results from studies on isolated chromaffin granules.

Key words: anion transport, chromaffin granules, exocytosis, platelets, parathyroid hormone

Many neurotransmitters, hormones, and enzymes are stored in intracellular secretory vesicles and, in response to appropriate stimuli, are released into the extracellular compartment by exocytosis (1). The process seems well defined ultrastructurally but the chemical and energetic basis for exocytosis remains obscure.

Received June 17, 1977; accepted June 24, 1977.

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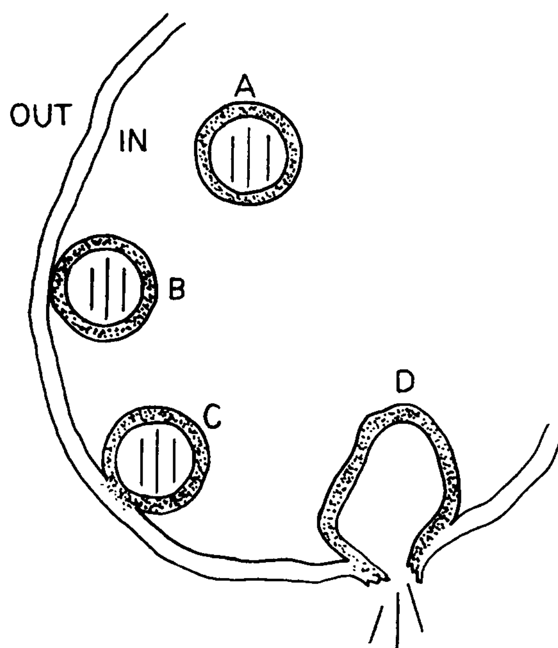

Fig. 1. Schematic representation of possible steps in the process of exocytosis. Isolated secretory granule in the cytoplasm (A) is recruited into juxtaposition (or "fusion") with the plasma membrane (B). The fusion state becomes more intimate, and a trilaminar membrane becomes the only structure separating the intragranular space and the extracellular medium (C). Finally granular contents are released when the fusion complex undergoes breakage or "fission." The nomenclature is based on that suggested by Palade (3).

In secretory systems such as the exocrine pancreas (2, 3), frog neuromuscular junction (4), and mast cells (5, 6), electron microscopy has been intensively applied to visualize exocytosis. As indicated in Fig. 1, the process has appeared to proceed by discrete steps in which isolated secretory vesicles first contact the plasma membrane forming a "pentalaminar" contact or "fusion" complex by an as yet poorly understood process possibly involving calcium. The fusion becomes more intimate, and in some cases it has been shown that the submembrane particles in the plasma membrane portion of the fusion complex move aside. A single bilayer structure then forms dividing vesicle contents and extracellular space. "Fission" of the bilayer finally occurs, resulting in secretion. The mechanism for the latter step is also not known.

As a departure point for our own studies, we assumed that the chemistry of release as defined by isolated secretory vesicles might also apply to the fission step in exocytosis (see Fig. 1D). We therefore devoted some of our subsequent efforts to the study of release of epinephrine from adrenal medullary secretory vesicles, or chromaffin granules. We have recently found that anion transport blocking drugs such as SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid), probenecid, pyridoxal phosphate, and others are able to block release of epinephrine from isolated chromaffin granules. We have also found that the blocking drugs inhibit secretion from several types of cells that release by exocytosis, but not by one cell type that secretes by diffusion. In this paper we suggest that permeant anions in the external medium, such as Cl^- and OH^-, may play an important role in regulating exocytosis.

MATERIALS AND METHODS

Chromaffin Granule Release

Chromaffin granules were prepared by differential centrifugation as previously described (7, 8) from 10–12 pairs of fresh bovine adrenal glands. For analysis of release, granules were incubated in an isotonic medium (335 mosmol) containing 500 μg granule protein, 1 mM $MgSO_4$, 1 mM ATP, 50 mM Hepes-NaOH buffer, pH 6.0, and 90 mM KCl in a total volume of 1.75 ml. After incubation for 10 min at 37°C, samples were mixed with 1 ml cold 0.33 M sucrose and centrifuged at 20,000 × g for 20 min. Aliquots of supernatant solution were then assayed for released endogenous epinephrine using the trihydroxy indole reaction (9). In experiments with isethionate, chloride was kept constant and 0.3 M sucrose was varied reciprocally with 0.15 M isethionate to generate different isethionate concentrations. SITS was obtained from British Drug House, and pyridoxal phosphate and probenecid were obtained from Sigma Chemical Company (St. Louis, Missouri). Suramin was obtained from Imperial Chemical Industries (London, England).

Human Platelets

Platelet-rich plasma in 0.1% EDTA was isolated from fresh human blood by differential centrifugation and platelets were labeled with [^{3}H]serotonin (final concentration = 0.2 μM) by incubation at 18°C for 45 min (10). The platelets were then chilled, centrifuged, washed twice with cold Rossi's medium (11), and resuspended in ice cold 0.3 M sucrose containing 1 mg/ml human serum albumin. Platelets in a 50-μl volume were then mixed with 450 μl of a prewarmed medium containing 130 mM NaCl, 25 mM Mes-NaOH buffer, pH 7.32, and thrombin (0.16 units/ml). After 1 min at 37°C, 10 μl of 10% glutaraldehyde were added to terminate the reaction, and the platelets were sedimented at 1,030 × g for 10 min. Samples of supernatant were subjected to liquid scintillation counting to determine released [^{3}H]5HT, and the percent release was determined from the relation, % R = 100 (released cpm – blank)/(maximum released cpm – blank). The maximum release was determined by incubation of platelets in 100-fold excess thrombin or A23187 for 1 min, and the blank was determined by incubating platelets in the absence of thrombin. [^{3}H] Serotonin was obtained from New England Nuclear Corporation (Boston, Massachusetts). Suramin was obtained from Imperial Chemical Industries (United Kingdom).

Isolated Parathyroid Cells

Dispersed parathyroid cells were prepared from fresh bovine parathyroid glands by mincing and digestion with 0.2% collagenase and 50 μg/ml DNase (12). Washed cells (150,000–200,000/0.5 cm^3) were incubated for 30–60 min at 37°C with 0.5 mM $CaCl_2$, 0.5 mM $MgSO_4$, and 0.2% bovine serum albumin in either a) Eagle's medium number 2 (bicarbonate deleted) with 20 mM Hepes, pH 7.5, or b) 150 mM NaCl, 1 g/liter dextrose, 0.25 g/liter K_2HPO_4, 0.3 g/liter KCl, and 20 mM Hepes, pH 7.5. The drugs indicated above were added at the beginning of the incubation and the reaction was terminated by sedimenting the cells for 30 sec in a microfuge (Beckman). Parathyroid hormone (PTH) in supernatant samples was determined by radioimmunoassay (12). Results are expressed as percent of release occurring at 0.5 mM calcium without added drugs.

Isolated Rat Adrenocortical Cells

Adrenals were obtained from male Holtzman rats (260–300 g). The medulla-reticularis-fasciculata tissue was expressed and the remaining tissue minced with a razor

TABLE I. Influence of Isethionate on Kinetics of Release of Epinephrine From Isolated Chromaffin Granules

Isethionate, mM	Cl^- $K_{1/2}$ mM	ISETH K_i mM	V_{max}[a]
0	69.2	–	7.1
10	81.2	57.3	7.1
20	105.9	37.7	7.1
30	131.8	33.1	7.1

[a]% total catecholamine released/min.

blade. Adrenocortical cells were mechanically dispersed following enzymatic digestion with collagenase at room temperature. Isolated cells ($1-3 \times 10^5$/ml) were incubated in Ham's F-10 medium containing 2.5 mM calcium and 10% fetal calf serum at 37°C for 2 h. Following the incubations the cells were removed by centrifugation and the corticosterone in the medium was assayed directly by radioimmunoassay. The steroid assay was accurate (90–110%) and precise (intraassay cultivariant = 7%; interassay cultivariant = 10%). A medium blank determined in each assay was invariably zero.

RESULTS

Chemistry of Release From Isolated Chromaffin Granules

Chromaffin granules are 2,000 Å diameter secretory vesicles from adrenal medulla which contain large amounts of epinephrine, ATP, and specific proteins (13). The isolated granules are stable in isotonic sucrose, but release their total contents when exposed to Mg-ATP and high concentrations of permeant anion such as chloride at pH 6, and 37°C (see Ref. 7 for details and complete references). The mechanism of release in this process is osmotic lysis since granule release is suppressed by increased extracellular osmotic strength (8, 14, 15). The anion requirement atracted our attention since most cells have anion gradients across their plasma membranes (120 mM external Cl^- versus 5–30 mM internal Cl^-; $pH_{out} > pH_{in}$), and secretory vesicles in the "fusion" state (Fig. 1B, C) would naturally be exposed to these gradients.

In the case of release from isolated chromaffin granules, we found that release was a saturable function of $[Cl^-]$. By contrast, impermeant anions would not support release but rather would competitively inhibit release with respect to $[Cl^-]$. Isethionate ($HO\text{-}C_2H_4\text{-}SO_3^-$), a typical impermeant anion, was a competitive inhibitor of release with respect to Cl, in that it changed the K_m but not the V_{max} of Cl^--induced release (Table I).

We had previously found that Cl^- (as ^{36}Cl) actually entered the granule in the presence of Mg-ATP (15), and that the role of Mg-ATP was to provide a positive electric potential within the granule to attract the anion (8). This suggested that permeant anion entry was competitively blocked by impermeant anions, and we therefore concluded that a specific anion transport site might exist on the granule membrane.

Anion transport across red cell membranes occurs by exchange and is sensitive to specific, impermeant drugs (16–18). Examples are shown in Fig. 2, and we decided to test these compounds on Cl^--dependent ATP-mediated release from granules. As shown in Table II, these drugs blocked granule release in a dose-dependent fashion and kinetic analysis revealed that they also competed with Cl^-.

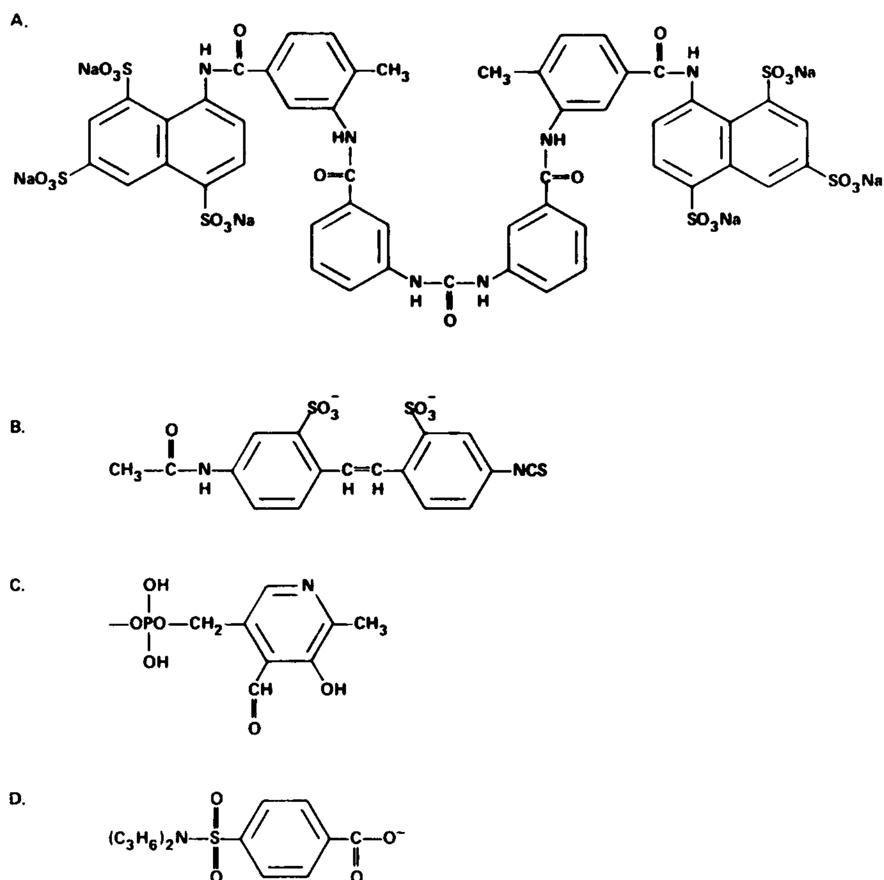

Fig. 2. Chemical structures of anion transport blocking drugs. A) Suramin, B) SITS, C) pyridoxal phosphate, D) probenecid.

TABLE II. Influence of Anion Transport Blockers on ATP, Cl-Induced Release of Epinephrine From Isolated Chromaffin Granules

Additions	Concentration	% Release in 10 min	% Inhibition
None	–	67 ± 2	–
SITS	50 μM	48 ± 1	28
	100 μM	31 ± 2	54
	500 μM	10 ± 4	85
Probenecid	50 μM	58 ± 2	13
	100 μM	48 ± 2	28
	500 μM	18 ± 3	73
Pyridoxal Phosphate	2 mM	53 ± 2	21
	5 mM	35 ± 2	48

[a]The reaction mixture was as described in Methods, except that the chloride concentration in all cases was 90 mM.

TABLE III. Influence of Anion Transport Blocking Drugs on Serotonin Secretion From Human Platelets

Additions	Concentration	% Release in 1 min	% Inhibition
None	–	58	–
Suramin	1 μM	46 ± 2	20
	5 μM	19 ± 2	66
Pyridoxal Phosphate	10 μM	48 ± 3	18
	100 μM	8 ± 2	86
SITS	10 μM	52 ± 3	10
	100 μM	16 ± 2	72
Probenecid	1 mM	41 ± 3	30
	5 mM	6 ± 2	90

TABLE IV. Influence of Anion Transport Blocking Drugs on PTH Secretion From Isolated Bovine Parathyroid Cells

Additions	Concentration	% Release[a]	% Inhibition
None	–	100	–
SITS	100 μM	87 ± 4	13
	1 mM	52 ± 2	48
	10 mM	10 ± 4	90
Probenecid	100 μM	88 ± 4	12
	1 mM	66 ± 4	34
	10 mM	15 ± 3	85
Na Isethionate (as an	100 mM	55 ± 5	45
isotonic replacement for NaCl)	150 mM	30 ± 4	70
Sucrose (as an isotonic replacement for NaCl)	300 mM	28 ± 2	72

[a]Reactions were carried out at pH 7.5.

Influence of Anion Transport Blocking Drugs on Secretion From Platelets

Human platelets secrete serotonin from storage sites in secretory vesicles by exocytosis (19–21) and this process was also found to be blocked by the anion transport blocking drugs. As shown in Table III, the effect was dose-dependent. Suramin (bis(m-amino-m-aminobenzoyl-p-methylbenzyl)-1-naphthylamine-7,6,8-trisulfonate carbamide) was found to be the most potent blocker of platelet release, and also suppressed chromaffin granule release though at somewhat higher concentrations. The data in Table III were collected using thrombin as the stimulus, and similar drug effects were obtained when A23187, a calcium ionophore, was used instead.

Platelets proved to be insensitive to removal of NaCl from the medium or substitution of Na-isethionate for NaCl, though they were quite sensitive to small reductions in the pH. Detailed kinetic analysis revealed that the anion transport blocking drugs were in fact competitive inhibitors with respect to OH^- ions.

Influence of Anion Transport Blocking Drugs on Secretion of Parathyroid Hormone

Parathyroid hormone (PTH) is also stored in secretory vesicles and is released from dispersed bovine parathyroid cells when stimulated either with low calcium (0.5 mM) or

TABLE V. Influence of SITS on ACTH-Activated Corticosterone Release From Isolated Rat Adrenal Cortical Cells

Condition	Corticosterone released in 2 h (ng/ml) Experiment 1	Experiment 2
Control	104 ± 4	195 ± 47
ACTH (1 mU/ml)	830 ± 125	2,240 ± 464
SITS (0.1 mM)	200 ± 43	282 ± 67
ACTH + SITS	1,050 ± 173	1,831 ± 243

β-adrenergic agonists (12). We decided to test the anion transport blocking drugs on exocytosis in this second cell type, and found that they also inhibited PTH secretion (see Table IV). Inhibition was found regardless of whether stimulation was evoked with low calcium or isoproterenol. Probenecid and SITS were not as potent in the case of the PTH cells as in the chromaffin granules or platelet systems, but they did prove to be competitive inhibitors with respect to $[Cl^-]$. As expected from the similar chloride sensitivity of PTH cells and chromaffin granules, isethionate also proved to be an effective inhibitor of PTH secretion.

As indicated in Table IV, replacement of NaCl by sucrose, or replacement of chloride by isethionate, decreased PTH release at pH 7.4 to only 30% of the original release level. However, nearly complete inhibition was obtained by, in addition, lowering the pH down to 6.5. This result suggested that hydroxyl ions might also be important in PTH release, and probenecid also proved to be a competitive inhibitor of PTH release with respect to $[OH^-]$, just as it did with serotonin release from the platelets.

Influence of Anion Transport Blocking Drugs on Corticosterone Secretion

Steroids are presumed to be released by diffusion rather than by exocytosis. We therefore studied the influence of SITS on corticosterone release from isolated rat adrenal cortical cells. As shown in Table V, SITS did not inhibit ACTH-activated corticosterone release. In fact, a small increment in cortisol secretion was noted in one experiment.

DISCUSSION

These data have led us to consider anion transport as a key regulatory event in secretion by exocytosis. Both hydroxyl ions and chloride ions appear to be implicated as anionic substrates for the transport sites. The fact that isolated chromaffin granules are also sensitive to these agents suggests that the site of action of the impermeant blocking drugs in secreting cells may be at regions of the external cell surface that contain secretory vesicle anion transport sites. These regions might be the "fusion" complexes described in Fig. 1 (B and C).

By analogy with the results from studies on isolated chromaffin granules, anion transport in secreting cells may ultimately be directed into the fused vesicle interior (14). Chromaffin granules have relatively low internal concentrations of chloride [approximately 30 mM (7)] and have relatively acidic interior compartments (pH 5.5–6.25) (8, 22, 23), but little is known about the interior environment of other secretory vesicles. It is perhaps not surprising that the concentrations of OH^- and Cl^- are higher outside

cells than inside. In this regard it is possible that other secretory vesicle interiors are similar to the chromaffin granule interior and that they would also support local $[OH^-]$ or $[Cl^-]$ gradients when fused to the plasma membrane of the secreting cell. These anion chemical potentials may be related to electric potentials, and anion transport may be accompanied by a counterion, but we have no definitive information on this point. Based on chromaffin granule studies, it is likely that anion transport in exocytosis represents flux rather than simply exchange.

It is evident that the anion transport blocking drugs inhibit release with different potencies in different secreting systems. This may be due either to structural heterogeneity of anion transport sites in different cell types, or to variation in the accessibility of the drugs to transport sites in different cells. That anion transport is specifically involved in exocytotic secretion, but not in other mechanisms of secretion, is indicated by the failure of SITS to inhibit steroid secretion. The latter is believed to occur by immediate diffusion of newly synthesized hormone.

One of the most important conclusions from these studies is that the chemistry of exocytosis appears to be quite similar to the chemistry of release from isolated secretory vesicles. Both processes are blocked by the same ion transport blocking drugs and depend on specific permeant anions. In the paradigm for the exocytotic process represented in Fig. 1, the "fission" step (D) involves breakage of the trilaminar membrane separating the granule interior from the extracellular medium. This may be the anion-dependent step equivalent to release in the chromaffin granule system. The actual motive force for release from chromaffin granules is osmotic lysis (8, 14, 15, 24), and a similar force may operate in secretory cells as well.

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