Effects of Changes in Osmolality on the Stability and Function of Cultured Chromaffin Cells and the Possible Role of Osmotic Forces in Exocytosis

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ABSTRACT Recent evidence indicates that osmotic forces may play a role in exocytosis. To examine this possibility and to investigate the osmotic properties of storage granules within cells, we investigated the effects of changes of osmolality on stability and function of cultured bovine chromaffin cells. Cell volume measurements indicated that the cells behaved as osmometers and that the intracellular osmolality rapidly equilibrated with the osmolality of the extracellular medium. Hyperosmotic solutions strongly inhibited nicotinic agonist-stimulated secretion but did not alter nicotinic agonist-stimulated Ca^{2+} uptake. Hyperosmotic solutions also strongly inhibited elevated potassium-stimulated secretion but only weakly inhibited elevated K^{+}-stimulated Ca^{2+} uptake. Thus, hyperosmotic solutions inhibited secretion at a step after calcium entry. Cells exposed to 165 mOs[1] solutions did not lyse and retained their capacity to store and secrete catecholamine upon stimulation. Significant intracellular lysis of chromaffin granules occurred within cells exposed to lower osmolalities. In contrast, 75% of the catecholamine was released from granules from cultured cells or from fresh adrenal medulla incubated in vitro at 210 mOs. The data provide evidence for a role for osmotic forces in exocytosis and suggest that if osmotic stress of the granule occurs during exocytosis, then water influx into chromaffin granules increases granule volume by at least 70%. The results also indicate that the osmotic properties of the granules are altered upon homogenization and subcellular fractionation of the cells.

Exocytosis has been extensively investigated in the perfused adrenal medulla (see reference 34 for review). Calcium entry caused by cholinergic stimulation induces release of catecholamine together with other soluble constituents of the chromaffin granule, the secretory vesicle in chromaffin cells. Cytosolic markers and the chromaffin granule membrane are not released. Although the process has been clearly defined, the biochemical and physiological processes underlying exocytosis are still poorly understood.

There is evidence that osmotic forces play a role in processes involving vesicle membrane fusion. Fusion of artificial vesicles with planar bilayer membranes absolutely requires an osmotic gradient across the vesicle membrane to cause water entry and swelling of the vesicles (5, 35). Mucocyst discharge in *Tetrahymena* is also associated with vesicle swelling (31). Hyperosmotic solutions inhibit exocytosis in dispersed parathyroid cells (2), platelets (30), pheochromocytoma cells (7), and toad urinary bladder (19). However, a systematic study of the effects of hypo-osmotic and hyperosmotic solutions on secretion has not been undertaken and the mechanism of inhibition of secretion by increased osmolality has not been defined.

In this study using monolayer cultures derived from bovine adrenal medulla we have examined the effects of hypo-osmotic and hyperosmotic solutions on the stability and function of chromaffin cells and of chromaffin granules within chromaffin cells. We present evidence that hyperosmotic solutions inhibit secretion by altering cellular function at a step after calcium entry and that the osmotic properties of in situ (intracellular) and in vitro (isolated) storage vesicles differ.

MATERIALS AND METHODS

Cells dissociated from bovine adrenal medulla were prepared and maintained as monolayer cultures in Eagle's minimum essential medium (MEM) containing 10% heat-inactivated fetal calf serum as previously described (10, 11, 15). The culture medium contained 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin, and 1.3 μg/ml Fungizone (Squibb Corp., Princeton, NJ) to

[1] mOs represents milliosmolal.

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prevent bacterial and fungal contamination. The culture medium also contained 10 μM cytosine arabinoside to inhibit fibroblast growth. Cytosine arabinoside-free medium was introduced to the cultures one day before an experiment. Cells were cultured in plastic culture wells or dishes of 16–60 mm diameter at a density of 250,000 cells/cm^2 unless otherwise indicated.

Immediately before an experiment cells were incubated for 30 min with physiological salt solution (PSS) containing 142 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 15 mM HEPES (pH 7.4), and 0.5 mM sodium ascorbate. The osmolality of the solution was 310 mOs. An experiment was initiated by changing the medium to PSS or another solution. Elevated potassium PSS contained 56 mM KCl and 92 mM NaCl. In experiments in which the osmolality was lowered the solutions contained either 40 mM or 70 mM NaCl, instead of 142 mM NaCl, and various amounts of sucrose to vary the osmolality. All experiments were performed at 25°C.

Catecholamine secretion was determined by measuring catecholamine released into the medium and remaining in the cells; data were usually expressed as the percentage of the total catecholamine released. Calcium uptake was measured by incubating cells in PSS containing $CaCl_2$ (0.2 μCi/ml; 2.2 mM). After 2 min the radioactive medium was removed for catecholamine measurement. Cells were rapidly washed three times with 1 ml of 0–4°C nonradioactive PSS. Solution (0.5 ml) containing 1% Triton X-100 and 1 mM EGTA was added to each well. Radioactivity liberated from the cells was counted in 4 ml of ACS (Amersham Corp., Arlington Heights, IL) scintillation counting solution. Data were expressed as nmoles of calcium taken up by the cells. Calcium uptake measured by this method is closely correlated with catecholamine secretion (15).

Subcellular fractionation was performed with cells maintained in 22-mm, 35-mm, or 60-mm diameter dishes. Ice-cold sucrose medium (310 mOs; 260 mM sucrose, 10 mM Na HEPES pH 7.0, and 0.5 mM ascorbate) was added to the culture (0.5 ml/6 million cells). Cells were scraped from the dishes and homogenized with 12 vigorous strokes of a Dounce homogenizer with a tight-fitting pestle. The crude homogenate was centrifuged at 800 *g* x 10 min. The supernatant (S_1) was then centrifuged at 35,000 *g* x 20 min in a SM 24 rotor in a Sorvall RC-5B centrifuge (Dupont Instruments, Newtown, CT). The amounts of catecholamine in the supernatant (S_2) and in the pellet (P_2) were measured. In experiments in which the osmotic stability of isolated storage vesicles was investigated, the P_2 fraction was resuspended in a small volume of sucrose buffer that was subsequently diluted 20-fold into the indicated solutions.

Intracellular water space of the monolayers was measured by allowing equilibration of 3-O-(methyl-^{3}H)-D-glucose across plasma membranes as described by Kletzien et al. (22). The monolayer was rapidly washed three times with 1 ml of PSS without sugar containing 1 mM phloretin at 0–4°C to inhibit 3-O-methyl-D-glucose efflux from the cells. We found that the efflux of 3-O-methyl-D-glucose during the washing procedure was not measurable. Water space was calculated from the 3-O-methyl-D-glucose trapped in the cells and its concentration in the incubation medium during equilibration.

Osmolalities of solutions were measured using a freezing point depression osmometer. Catecholamine (epinephrine + norepinephrine) was measured by the fluorescence method of Euler and Floding (8). Dopamine-β-hydroxylase (DBH) was measured by the method of Nagatsu and Udenfriend (28) in the presence of 5 μM $CuSO_4$ which gave maximal activity. Soluble DBH was liberated from cells and chromaffin granules by homogenization in 10 mM NaHEPES (pH 7.0), 0.5 mg/ml bovine serum albumin, and 0.1 mg/ml catalase and separated from particulate DBH by centrifugation (35,000 *g* x 30 min). Soluble DBH served as a marker for soluble protein within the storage vesicle. Phenylethanolamine-N-methyl-transferase (PNMT), a specific chromaffin cell cytosolic marker, was measured according to Molinoff et al. (27) (using purified S-adenosylmethionine) in extracts from which catecholamine was removed by Dowex Ag 50-X8 chromatography. Lactate dehydrogenase (LDH) was measured according to Kornberg (25). The ability of cells to exclude trypan blue for at least 10 min in PSS containing 0.2% trypan blue was assessed as a measure of cell viability.

All reagents were readily available commercially. $CaCl_2$ (37.4 Ci/g) and 3-O-(methyl-^{3}H)-D-glucose (80 Ci/mmol) were obtained from New England Nuclear (Boston, MA). S-adenosyl-L-(methyl-^{3}H)methionine (15 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). Data were expressed as mean +/− standard error of the mean. Differences between means of groups were tested for significance with Student's *t* test.

RESULTS

The Effects of Hyperosmotic Solutions on Catecholamine Secretion and Calcium Uptake

The effects of hyperosmotic solutions on DMPP- and elevated potassium-induced catecholamine secretion and calcium uptake were investigated. Solutions made hyperosmotic with sucrose (Fig. 1) strongly inhibited DMPP- and elevated potassium-stimulated catecholamine secretion. Solutions made hyperosmotic with raffinose, a trisaccharide, and NaCl gave similar results. Hyperosmotic solution did not induce the release of the cytoplasmic marker PNMT and did not increase the release of catecholamine from unstimulated cells. The effect of hyperosmotic solution was largely reversible. DMPP-induced secretion in 310 mOs PSS from cells that had been preincubated for 10 min in 640 mOs PSS (containing sucrose) and then allowed to recover for 10 min in 310 mOs PSS was 76% of secretion of cells never exposed to hyperosmotic solution. In the same experiment DMPP-stimulated secretion in 640 mOs PSS was 12% that of cells never exposed to hyperosmotic solution.

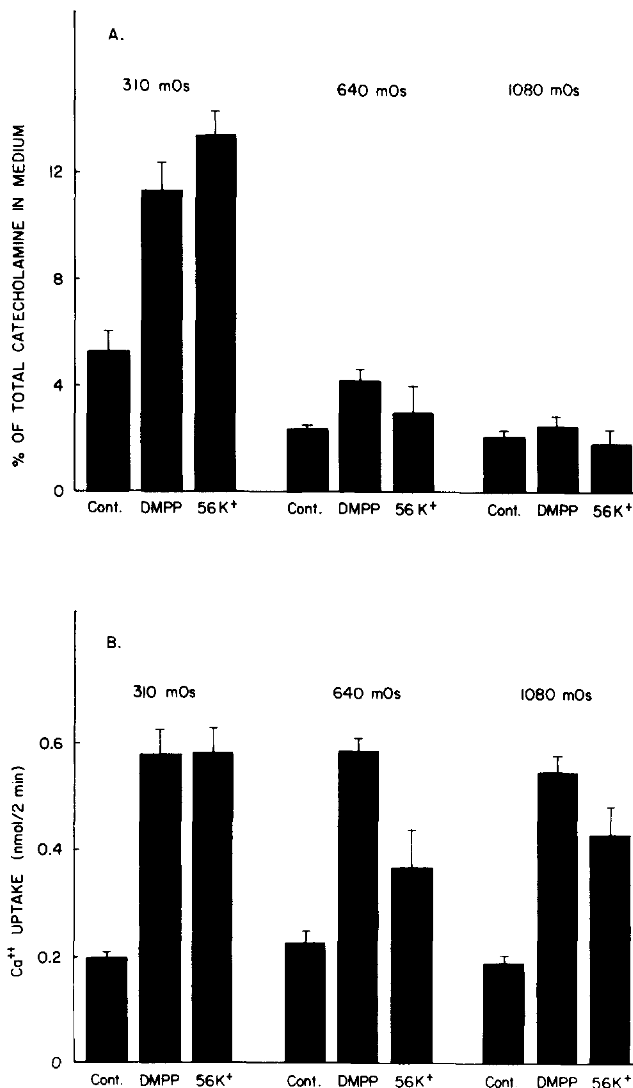

FIGURE 1 The effect of hyperosmotic solutions on catecholamine secretion (*A*) and Ca^{++} uptake (*B*). Cells (in 16-mm diameter wells) were incubated in PSS (310 mOs) or in PSS made hypertonic by additions of sucrose. After 7 min the solutions were replaced by $^{45}Ca^{++}$-containing solutions of the same osmolality without secretagoque (*Cont.*) or with DMPP (10 μM) or with 56 mM K^+. Catecholamine secretion and $^{45}Ca^{++}$ uptake were determined at 2 min. There were 4 wells/group.

The virtually complete inhibition of DMPP-stimulated secretion by hyperosmotic solutions is in marked contrast to the virtual absence of inhibition of DMPP-stimulated radioactive calcium uptake into the cells (Fig. 1 *B*). The inhibition of elevated potassium-induced secretion by solutions made hyper-

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osmotic with sucrose was associated with a variable and a relatively small degree of inhibition of calcium uptake. In a previous study correlating calcium uptake with catecholamine secretion (15) inhibition of secretion by calcium antagonists was associated with a significantly larger relative inhibition of calcium uptake than of catecholamine secretion. In the present experiments solutions made hyperosmotic with sucrose inhibited elevated potassium-induced calcium uptake significantly less than elevated potassium-induced catecholamine secretion. This is apparent in 1,080 mOs solution, which inhibited elevated potasium-induced calcium uptake 36% and inhibited elevated potassium-induced secretion 100% (Fig. 1 *B*). Thus, increased osmolality inhibited both DMPP- and elevated potassium-induced secretion at a step after calcium entry into the cell.

The effect of different osmolalities on DMPP-stimulated secretion is presented in Fig. 2. One-half maximal inhibition of secretion occurred at ~420 mOs.

Stability of Cells in Hypotonic Media

A likely site for osmotic effects in chromaffin cells is the chromaffin granule, which in vitro is exquisitely sensitive to changes in the osmolality (14, 17, 26). However, no data are available concerning the osmotic characteristics of the granule in situ. The effects of hypo-osmotic solutions on the stability and function of chromaffin cells and intracellular chromaffin granules were examined in the following experiments.

Reduction of the osmolality from 310 mOs (iso-osmotic) to 165 mOs did not release the cytoplasmic markers phenylethanolamine-N-methyltransferase (PNMT) (Table I) or lactate dehydrogenase (data not shown) and did not reduce the ability of cells to exclude trypan blue. In other experiments solutions of 114 mOs and 10 mOs (5 mM Na HEPES) released 40 ± 2% and 80 ± 2%, respectively, of the total PNMT into the medium. Thus, chromaffin cells in monolayer culture can withstand a substantial reduction of osmolality without lysis.

Although the cells did not lyse at 165 mOs, they released 17% of their catecholamine (Table I). The percentage of soluble dopamine-β-hydroxylase (DBH) released, which is a measure

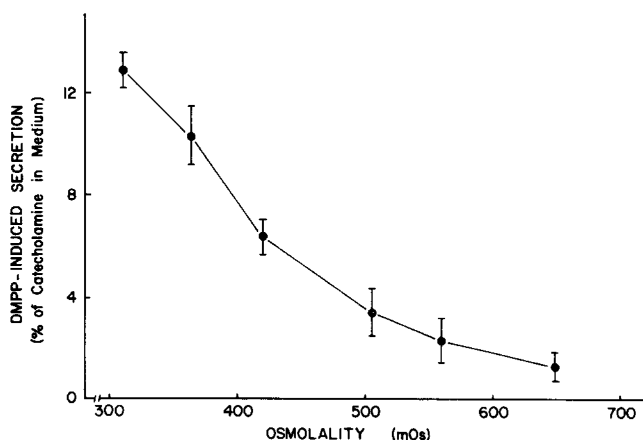

FIGURE 2 Inhibition of DMPP-stimulated catecholamine secretion at elevated osmolalities. Cells were incubated in PSS containing various amounts of sucrose to increase the osmolality. After 12 min, solutions were replaced with solutions at the same osmolality with or without 10 μM DMPP. The amount of catecholamine released into the medium was determined after 12 min. Data expressed as DMPP-induced catecholamine release. There were four 16-mm diameter wells/group.

TABLE I
Release of Catecholamine, DBH, and PNMT from Cells

Osmolality	Percent of total in medium		
	Catecholamine	Soluble DBH	PNMT
Experiment A			
310 mOs	2.4 ± 0.4	0.9 ± 0.2	3.5 ± 1.1
164 mOs	17.2 ± 0.9*	4.3 ± 0.3*	3.6 ± 0.3
Experiment B			
310 mOs, PSS 0 divalent ions	1.3 ± 0.3	2.4 ± 0.8	ND
310 mOs, PSS 2.2 mM Ba^{++}	36.2 ± 0.8‡	36.0 ± 0.4‡	ND

In experiment A, cells were incubated for 15 min in 40 mM NaCl, 5.6 mM KCl, 5.5 mM glucose, 5 mg/ml BSA, 0.5 mM ascorbic acid, and various amounts of sucrose to obtain the indicated osmolalities. There were 6 dishes/group (35-mm diameter). In experiment B, cells were preincubated for 15 min in PSS with either 0 divalent ions or 2.2 mM Ba^{++}. There were 4 wells/group (16-mm diameter). *ND*, not done.
* $p < 0.001$ vs. 310 mOs in Experiment A.
‡ $p < 0.001$ vs. PSS with 0 divalent ions in Experiment B.

of the release of soluble protein constituents of chromaffin granules, was much less (4%) and was approximately equal to the percentage of PNMT released. In contrast barium, a known secretagogue (34), stimulated proportional release of catecholamine and soluble DBH as a consequence of exocytosis (Table I, *Experiment B*). Thus, the catecholamine release in hypo-osmotic medium was not exocytotic. The release may have been caused by a small amount of intracellular lysis of chromaffin granules and subsequent leakage of catecholamine into the extracellular medium (see below).

Cellular Water Space and Medium Osmolality

To evaluate the ability of cells to alter volume and intracellular osmolality when exposed to different medium osmolalities, cellular water space was determined by measuring the cellular uptake of 3-O-methyl-D-glucose (22). Steady state uptake of the sugar was attained by 5 min. Uptake was probably limited by sugar transport, and not by changes in cellular water space since the time course of the uptake was independent of the final water space attained. Fig. 3 demonstrates that cellular water space increased in hypo-osmotic media and decreased in hyperosmotic medium. When the data are graphed as a Van't Hoff plot, a straight line is obtained which indicates that the cells were responding as osmometers. The non-zero Y intercept may have resulted from a small amount of binding of 3-O-methyl-D-glucose to the cells. Thus, water entry rapidly reduced the intracellular osmolality of cells in hypo-osmotic solutions, thereby exposing the intracellular granules to an osmolality equal to that of the extracellular solution.

Ability of Chromaffin Cells to Secrete After Exposure to Hypo-osmotic Medium

To investigate the ability of chromaffin cells to secrete after incubation in hypo-osmotic medium, cells (total catecholamine 10.5 nmol) were incubated in hypo-osmotic medium for 20 min (Fig. 4*A*), allowed to recover in normal medium (PSS, 310 mOs) (Fig. 4*B*), and finally incubated in the presence or absence of carbachol (0.3 mM) in PSS (Fig. 4*C*). Incubation at 220 mOs released little catecholamine but incubation at 114

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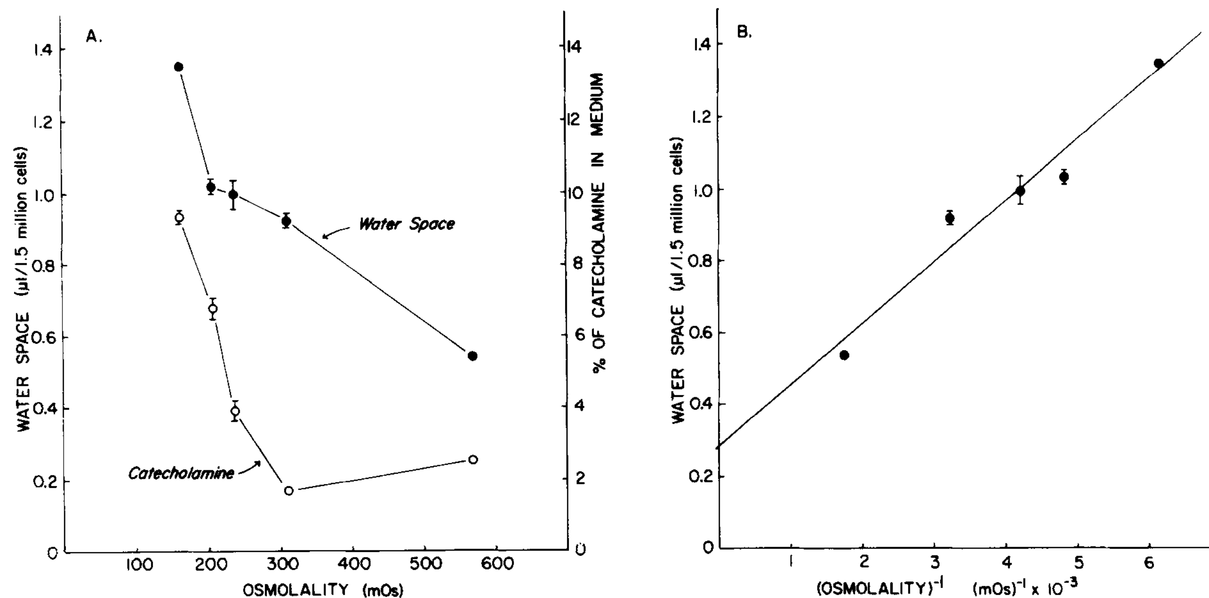

FIGURE 3 The effects of osmolality on cellular water space and catecholamine release. Cells that had been plated at 1.5 million cells/16 mm diameter well (three times normal density) were incubated in solutions containing 70 mM NaCl, 5.6 mM KCl, 10 mM HEPES (pH 7.4), 2.0 mM glucose, 0.5 mM ascorbate, and various concentrations of sucrose to vary the osmolality. Solutions also contained 3-O-(methyl-^{3}H)-D-glucose (1.5 µCi/ml). After 20 min the solutions were removed and catecholamine in the medium and water space of cells were determined as described in Materials and Methods. There were 5 wells/group. The water space at normal osmolality (310 mOs) was 0.92 ± 0.01 µl/1.5 million cells plated. (*A*) Cellular water space (●) catecholamine release (○) vs. osmolality. (*B*) Van't Hoff plot. Water space vs. 1/osmolality.

and 29 mOs released 51% and 82%, respectively, of the total catecholamine (Fig. 4*A*). Cells that had not been exposed to hypo-osmotic medium secreted 25% of the catecholamine in response to carbachol (Fig. 4*C*). Carbachol-induced secretion from cells that had been incubated in 220 mOs was only partially inhibited (Fig. 4*C*). Carbachol also stimulated secretion, in cells exposed to 114 mOs and 29 mOs solutions, which was 13% and 26%, respectively, of the total catecholamine remaining in the cells. Because isolated chromaffin granules lose >80% of their catecholamine when exposed to 220 mOs (14, 17, 26; see below), the results suggest that chromaffin granules in situ are more resistant to osmotic lysis than isolated chromaffin granules. They also indicate that chromaffin cells retain a remarkable amount of function after exposure to low osmolality medium.

Subcellular Distribution of Catecholamine and Dopamine-β-hydroxylase (DBH) in Cells that Had Been Exposed to Hypo-osmotic Solutions

To determine whether exposure of cells to hypo-osmotic medium caused intracellular lysis of storage granules, cells were exposed to various osmolalities for 10 min, homogenized in 300 mOs buffered sucrose and the homogenate subjected to differential centrifugation (Fig. 5). Between 70% and 80% of the catecholamine and soluble DBH (after removal of unhomogenized cells) was sedimented in the P_2 (chromaffin granule) fraction of cells which had been incubated at 310 mOs and 210 mOs. In cells incubated at 114 mOs 29% of the catecholamine and 59% of the soluble DBH was sedimented in the P_2 fraction. Thus, there was no detectable intracellular lysis of granules at 210 mOs and only partial intracellular lysis of granules at 114 mOs.

In Vitro Osmotic Fragility of Storage Vesicles Isolated from Cultured Chromaffin Cells

To determine whether chromaffin granules from cultured chromaffin cells lyse in hypo-osmotic solutions, a P_2 fraction containing chromaffin granules was prepared from cultured chromaffin cells and incubated in potassium methylsulfate solutions at various osmolalities. Chromaffin granules from fresh adrenal medulla were also investigated. Chromaffin granules from cultured cells released 70–80% of their content by 200 mOs and those from fresh adrenal medulla released a somewhat larger fraction (Fig. 6). The osmotic stability of chromaffin granules from cultured cells (and from fresh adrenal medulla) was the same in salt and sucrose solutions (data not shown). The retention of catecholamine by chromaffin granules within cells (data from Fig. 5, and other experiments) is graphed together with data from isolated chromaffin granules (Fig. 6). The data demonstrate that in situ chromaffin granules are more resistant to osmotic lysis than in vitro chromaffin granules.

Osmotic Stability of In Situ Chromaffin Granules in Cells Stimulated to Secrete

If a decrease in the osmotic stability occurs in a large proportion of chromaffin granules in cells undergoing secretion, more granule lysis should occur in secreting cells than in nonsecreting cells, especially in hypo-osmotic solution. Cells were equilibrated at 310 mOs or 165 mOs then stimulated to secrete by the addition of barium to medium at the same osmolality. After 20 min, by which time the cells had been induced to secrete 27% of the total catecholamine in 310 mOs or 18% of the total catecholamine in 165 mOs, the medium was changed to 310 mOs sucrose solution. Cells were homogenized

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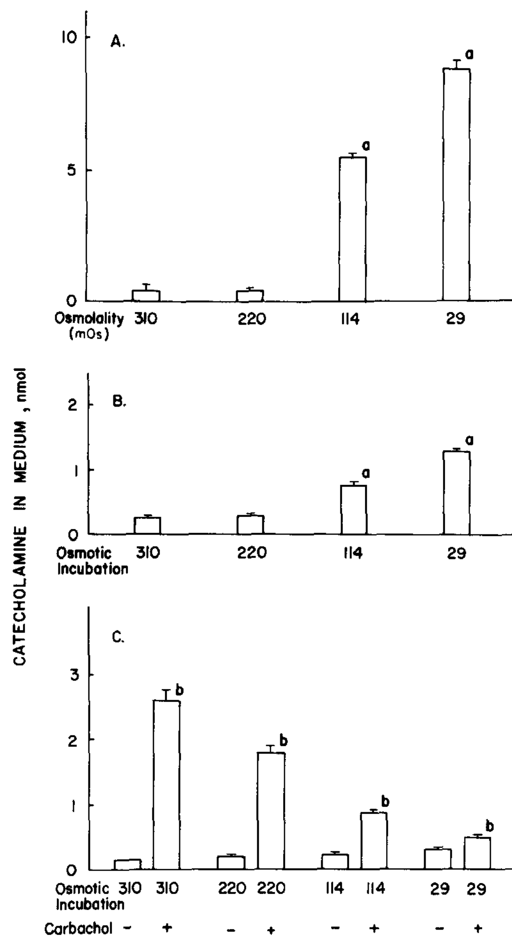

FIGURE 4 Effect of hypoosmotic solutions on subsequent carbachol-stimulated catecholamine secretion. (*A*) Osmotic incubation. Cells were incubated in 0.25 ml of solution containing 40 mM NaCl, 5.6 mM KCl, 0.5 mM $MgCl_2$, 5.6 mM glucose, 5 mg/ml bovine serum albumin, 0.5 mM ascorbic acid, 10 mM HEPES (pH 7.4) and either 185 mM sucrose (310 mOs solution), 100 mM sucrose (220 mOs solution) or 0 mM sucrose (114 mOs solution). One incubation was performed in 10 mM HEPES, 5.6 mM glucose, 5 mg/ml bovine serum albumin, 0.5 mM ascorbic acid (pH 7.4, 29 mOs solution). (*B*) Isotonic recovery. After 20 min the solutions in *A* were removed and replaced by PSS (310 mOs) to allow recovery from the previous incubation. (*C*) Carbachol stimulation. After 20 min the solution in *B* was removed and PSS with or without carbachol was added; secretion was assessed after 20 min. Milliosmolalities of the solutions in which cells had been incubated in *A* are indicated in the abscissa in *B* and *C*. Catecholamine content per well was 10.5 nmol. There were 8 wells per group in *A* and *B* and 4 wells/group in *C*. *a*, $p < 0.001$ vs. catecholamine release from cells incubated continuously in 310 mOs solutions. *b*, $p < 0.01$ vs. unstimulated catecholamine release from cells treated with the same osmolality solutions in *A*.

and subjected to subcellular fractionation in 310 mOs sucrose solution. The percentages of intracelluar catecholamine in the chromaffin granule fraction in nonsecreting and secreting cells incubated at 310 mOs were 56% and 67%, respectively. The percentages of intracellular catecholamine in the chromaffin granule fraction in nonsecreting and secreting cells incubated at 165 mOs were both 60%. Similar results were obtained when secretion was induced by carbachol. Thus, there is probably not a large change in the osmotic state of a large proportion of intracellular chromaffin granules coincident with secretion. It is possible, however, that only those few storage vesicles about to release their contents by exocytosis undergo a decrease in osmotic stability. The change would not be apparent in the above experiment in which the osmotic stability of the entire vesicle population was investigated.

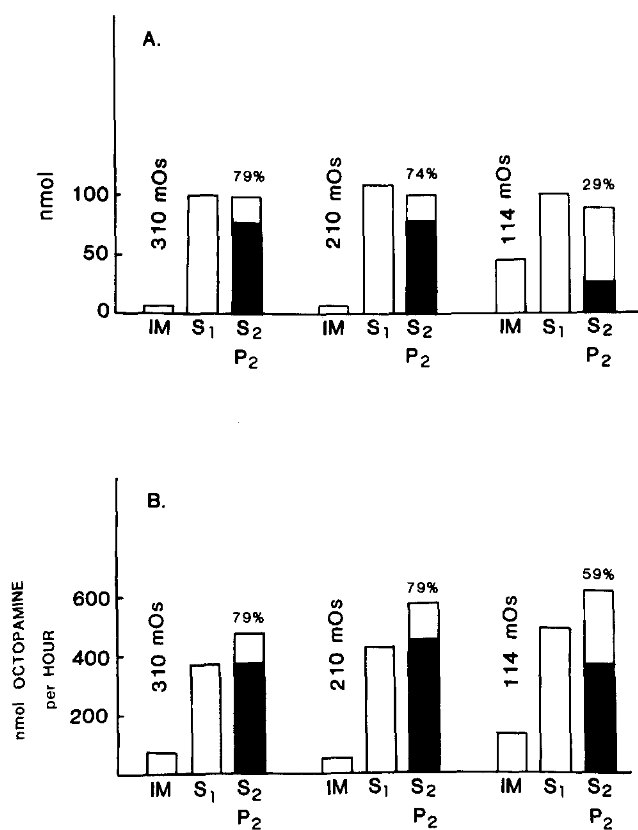

FIGURE 5 Effects of incubation of cells in media of differing osmolalities on the subcellular distributions of catecholamine and dopamine-β-hydroxylase. (*A*) Catecholamines. (*B*) Soluble DBH. Dishes (35-mm diameter) of chromaffin cells were washed briefly with 0 Ca^{++} PSS with 10 μM pargyline and then incubated at 25°C in solutions of differing osmolalities of the following composition: 40 mM NaCl, 5.6 mM KCl, 10 mM HEPES (pH 7.4), 5.6 mM glucose, 5 mg/ml BSA, 0.5 mM ascorbate, 10 μM pargyline and 0 mM, 100 mM, or 185 mM sucrose. The solutions were 114 mOs, 210 mOs and 310 mOs, respectively. After 10 min the solutions were removed, aliquots were saved for both DBH and catecholamine determinations. Solution (0-4°C, 1.25 ml) containing 260 mM sucrose, 10 mM HEPES (pH 7.1), 5 mg/ml BSA and 0.1% ascorbate solution was then added to each dish. Dishes were scraped and subjected to differential centrifugation as described in Materials and Methods. *IM* represents contents of incubation medium. S_1 contains the total catecholamine or DBH in the supernatant of a low speed centrifugation. P_2 is the pellet of a high speed centrifugation and contains unlysed chromaffin granules. It is represented by the filled column. S_2 is the supernatant from the high speed centrifugation and contains soluble constituents of lysed chromaffin granules. It is represented by the length of the open column above the closed column. The contents of $P_2 + S_2$ should equal the contents of S_1. One dish of chromaffin cells was fractionated at each osmolality. Assays were performed in triplicate. Percentages indicate the fraction $P_2(100)/(P_2 + S_2)$. The 20% deficit of soluble DBH in S_1 compared to soluble DBH in the sum of $S_2 + P_2$ (fractions that are derived from S_1) may have resulted from incomplete recovery of soluble DBH from S_1. Soluble DBH in S_1 was liberated by diluting the fraction into one volume of H_2O (with 5 mg/ml BSA and 0.1 mg/ml catalase) which may not have been sufficient to release completely the soluble DBH (33). DBH was released from P_2 by resuspension of the pellet directly in 20 mOs medium (with 5 mg/ml BSA and 0.1 mg/ml catalase).

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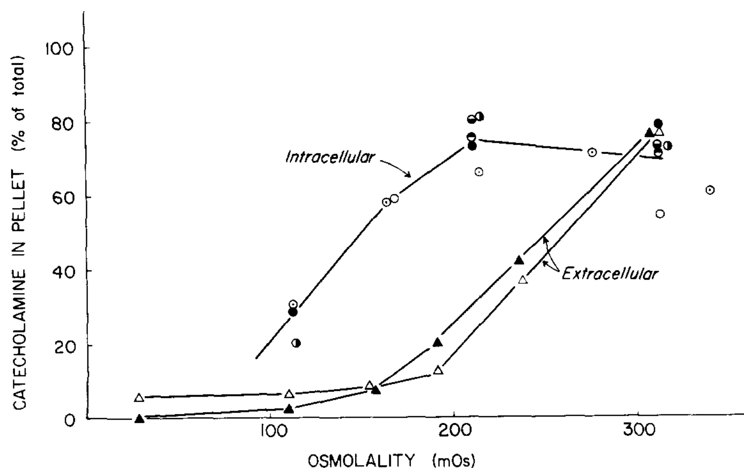

FIGURE 6 Comparison of the extracellular and intracellular osmotic stability of chromaffin granules. To determine the extracellular osmotic stability of chromaffin granules, chromaffin granules isolated from cultured chromaffin cells (▲) or from fresh bovine adrenal medulla (△) in 260 mM sucrose, 1 mM EGTA and 10 mM HEPES (pH 7.0) and 0.5 mM ascorbic acid (300 mOs), were diluted into solutions containing various concentrations of potassium methylsulfate, 0.5 mM ascorbate, 1 mM EGTA, and 10 mM HEPES (pH 7.0) at 25°C. After 10 min the solutions were cooled to 0–4°C and centrifuged at 35,000 $g \times 20$ min. Catecholamine was determined in supernatant and pellet. There were 3 samples/group. Standard errors of the mean were <1% and error bars were omitted from the figure. Intracellular osmotic stability was determined by incubating cells in solutions of differing osmolalities and performing subcellular distributions at 300 mOs as described in Materials and Methods and Fig. 5. The percentage of catecholamine from S_1 that sedimented at 35,000 $g \times 20$ min is plotted (circles). Data from six experiments including those from Fig. 5 are presented and indicated by differently filled circles. The line is drawn through the average value of the percentages at each osmolality.

DISCUSSION

Possible Role of Osmotic Forces in Exocytosis

An important finding in this study was that hyperosmotic solutions inhibited exocytosis stimulated by nicotinic agonists or by elevated K^+ in chromaffin cells at a step after Ca^{2+} entry into the cells. A possible osmotically sensitive compartment is the chromaffin granule. Hyperosmotic solutions in vitro stabilize chromaffin granules if they are stressed osmotically by a variety of different mechanisms (3, 14). Within the cell, granule swelling during exocytosis could stress and destabilize the granule membrane, and, thereby, promote fusion of the granule membrane with the plasma membrane. Hyperosmotic solution, by causing water to leave the granule, would maintain the granule membrane in a less reactive state and prevent membrane fusion.

It is difficult to evaluate possible direct inhibitory effects on secretion of increased intracellular ionic strength or of increased concentration of intracellular components. Recently Knight and Baker (23), using suspended chromaffin cells with leaky plasma membranes, demonstrated a similar inhibition of exocytosis by solutions made hyperosmotic with sucrose. In this preparation low molecular weight species in the medium including ATP, Ca^{2+}, and sucrose probably equilibrate with the cell interior. Thus, in this system, changes in intracellular ATP or ionic strength are not responsible for the inhibition caused by hyperosmotic solutions. It is possible, however, that in both intact and leaky cells hyperosmotic solutions inhibit exocytosis by altering the spatial relationships between the releaseable pool of chromaffin granules and the plasma membrane.

Hyperosmotic solutions inhibited stimulated hormone secretion from dispersed parathyroid cells (2) and from platelets (30) but also increased hormone release from unstimulated cells, thus indicating that increased osmolality could have multiple effects. In those experiments calcium uptake, reversibility of the effects of hyperosmotic solutions, and release of cytosolic markers were not determined to evaluate changes in calcium metabolism, toxicity, and cell lysis that may have accompanied treatment with hyperosmotic solutions. Increased osmolality also increased spontaneous quantal release of acetylcholine from the neuromuscular junction, an effect that may have been caused by increased cytosolic calcium concentrations (9). In the present experiments reversibility of the effects on secretion, absence of release of catecholamine from unstimulated cells, and absence of release of cytosolic markers indicate that increased osmolality did not grossly disturb chromaffin cell function. It is unlikely that inhibition of secretion from chromaffin cells by hyperosmotic solutions was caused by depletion of intracellular energy stores. Calcium sequestration, which is a necessary component of the calcium uptake measurement (15), is an energy-requiring process. Calcium uptake and, therefore, calcium sequestration were unaltered in hyperosmotic solution in which catecholamine secretion was strongly inhibited.

Granules in cells exposed to hyperosmotic medium (165 mOs) should take up water and be sensitive to any further osmotic stress. Because of the inability of a secretory stimulus to cause intracellular lysis of a detectable fraction of the intracellular granules in cells exposed to 165 mOs medium, it is possible that only those granules near the plasma membrane about to undergo exocytosis were subjected to osmotic stress. We estimate below that the water space of intracellular granules approximately doubled in size before intracellular granules lysed. If osmotic lysis of granules occurs during exocytosis, then at normal osmolality (310 mOs) the amount of osmotically active solutes within a granule undergoing exocytosis must double.

Osmotically active solutes could be increased within the granule to induce water influx by: (*a*) a permeability increase of the granule membrane to cytosolic constituents, (*b*) dissolution of the storage complex within the chromaffin granule or (*c*) activation of a pump in the granule membrane that can transport solutes into the granule. Any of these mechanisms could be activated by calcium. One possibility that has been suggested is that osmotic stress is caused by chloride influx into the granule from the extracellular medium after association of the chromaffin granule membrane with the plasma membrane (29). However, other laboratories have found that exocytosis from cultured bovine adrenal medullary cells (21), from perfused adrenal medulla (6) from chromaffin cells with leaky plasma membranes (23) and from pheochromocytoma cells (7) occurs in the absence of chloride or other anions in the medium. We have not further investigated anion dependency.

Osmotic Stability of Intracellular Chromaffin Granules

Chromaffin granules within cells were significantly more stable to osmotic stress than were isolated chromaffin granules.

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Reduction of the osmolality from 310 mOs to 210 mOs resulted in release from isolated granules of >75% of the catecholamine which is in agreement with other studies (14, 17, 26). In contrast, chromaffin granules within cells were stable at 200–220 mOs which was demonstrated by the absence of release of catecholamine from cells, by subcellular distributions of catecholamine and DBH and by the ability of cells to secrete after exposure of cells to these low osmolalities.

The osmotic stability of in situ chromaffin granules may be explained by the shape of the granules. Pleomorphic granules with oval or elongated shapes are common in thin section and freeze-fracture electron micrographs of adrenal medulla and of cultured bovine chromaffin cells (1, 20, 31). These nonspherical shapes (spheres have minimal surface to volume ratios) suggest that the granule volume can probably increase without increasing granule surface area. Because reduction of the osmolality to below 165 mOs was required for substantial intracellular lysis of granules, intracellular granules must be able to approximately double the volume of their water space without lysis. If the water space of intracellular granules is 67% of the total granule volume as in isolated granules (17), this would result in a 70% increase in total granule volume. The ~100 mOs decrease in osmotic stability of isolated granules compared with in situ granules (Fig. 6) indicates that upon isolation, the granules became less stable to hypo-osmotic solutions, perhaps because of damage or because of loss of cytoplasmic components that maintain the integrity of the granule membrane or the granule interior.

The Relationship of the Stability of In Situ Chromaffin Granules to Chemiosmotic Mechanisms of Chromaffin Granules

The intragranular pH of isolated chromaffin granules is 5.7 in the absence of extragranular ATP (4, 12, 16, 17). The granule membrane contains a H^+ pump ATPase (4) that in the presence of MgATP increases the H^+ electrochemical gradient by shifting the granule membrane potential from inside negative to inside positive values (13) and, under some conditions, by further acidifying the granule interior (4). Catecholamine transport into the granules is coupled to the H^+ electrochemical gradient (12, 18, 24). One consequence of the chemiosmotic processes of the granules is that extragranular chloride is cotransported with hydrogen ion (which is partially buffered) in the presence of MgATP and causes osmotic lysis of the granule in vitro (3, 29). Only if osmotic lysis is inhibited by hyperosmotic solutions, will MgATP further acidify the granule interior as a consequence of cotransport of hydrogen ion and chloride (4, 29). These data with isolated granules suggest that for granules to be stable within cells in the presence of millimolar ATP and Mg^{++} and 10–20 mM chloride, there can be no net acidification of the granule interior due to the hydrogen ion pump and cotransport of chloride (or another permeant ion). However, because granules in situ are osmotically more stable than isolated granules, it should be possible for the granule hydrogen ion pump to acidify the granule interior and cause net uptake of chloride without compromising the integrity of the granule.

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