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Secretion of Newly Taken Up Ascorbic Acid by Adrenomedullary Chromaffin Cells Originates from a Compartment Different from the Catecholamine Storage Vesicle

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SUMMARY

Chromaffin cells in primary culture take up [^{14}C]ascorbic acid from the incubation medium. Cells, stimulated immediately after a short labeling period with [^{14}C]ascorbate, secrete ascorbic acid concomitantly with catecholamines (CA) through a nicotinic receptor-mediated Ca^{2+}-dependent process. A proportional release of CA and [^{14}C]ascorbic acid was observed through a large range of secretion rates obtained by varying the concentration of nicotine or by changing the concentrations of Ca^{2+} and Na^{+} in the external medium. However, under the same conditions of stimulation, different cell preparations secrete 2–10 times more CA than [^{14}C]ascorbate (as percentage of cell content). Furthermore, a different time course of secretion was observed for CA and [^{14}C]ascorbate for each of several secretagogues. In addition, Ba^{2+} is a much more potent stimulus for CA secretion than for secretion of [^{14}C]ascorbate, and Ca^{2+} channel blockers are more potent in inhibiting CA secretion than [^{14}C]ascorbate secretion. These data suggested the possibility that newly taken up ascorbate was being secreted from a compartment altogether distinct from the chromaffin vesicle. This hypothesis was confirmed by subcellular distribution studies, where only a minor fraction of newly taken up [^{14}C]ascorbate was found in the vesicular fraction (P_2) from homogenates of chromaffin cells prepared after a short incubation with [^{14}C]ascorbate. However, the subcellular distribution of [^{14}C]ascorbate follows that of endogenous ascorbate when a short pulse with the label is chased by a prolonged equilibration period in the absence of ascorbate, indicating that a transfer has occurred from the extravesicular compartment(s) to the CA storage organelle. Endogenous ascorbate, which is found both inside and outside the chromaffin vesicle, was also found to be secreted from chromaffin cells, indicating that ascorbic acid could be released simultaneously from two different subcellular compartments.

INTRODUCTION

The biological importance of ascorbic acid is widely accepted. The lack of ascorbate in the diet of mammals that do not synthesize it results in a rapid loss of the vitamin from most tissues; however, in brain the content of ascorbate decreases only after a prolonged deficiency (1, 2). This suggests an important role(s) for ascorbic acid in neural tissue as well as the existence of recycling (3–5) and/or accumulation systems (6–8) to maintain the required levels of the vitamin in its reduced form. Since ascorbic acid is synthesized only in liver (9), even mammals that do not depend on dietary ascorbate should have an efficient uptake and/or recycling system for the cofactor in neural tissue.

In nervous tissue, we find one of the few defined biochemical roles for ascorbic acid, functioning as the electron donor in the hydroxylation of dopamine to norepinephrine (10). The reaction is catalyzed by dopamine β-hydroxylase (EC 1.14.17.1) (10, 11), an enzyme which is enclosed within the chromaffin vesicles of the adrenal medulla (12) and synaptic vesicles of adrenergic neurons (13). Since norepinephrine is synthesized within the chromaffin vesicle and 2 moles of ascorbic acid are required for each mole of dopamine converted to norepinephrine (3, 4), it is essential that the chromaffin vesicles have an efficient way for accumulating the cofactor in order to synthesize the large concentration of CA[2] accumulated within the vesicles (0.5 M; see ref. 14). Indeed, there is a high concentration of fully reduced ascorbate (20 mM) in chromaffin vesicles (15).

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[2] The abbreviations used are: CA, catecholamines; NE, norepinephrine; BSS, balanced salt solution; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-pressure liquid chromatography; ACTH, adrenocorticotropic hormone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; P-286, *N,N*-diisopropyl-*N'*-isoamyl-*N'*-diethylaminoethylurea; D-600, methoxyverapamil.

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We have recently shown that primary cultures of bovine adrenomedullary chromaffin cells do accumulate the vitamin through an active transport system (8) and that, upon stimulation, the newly taken up ascorbate is released together with CA, thereby suggesting vesicular uptake in intact cells (16). By contrast, chromaffin vesicles *in vitro* do not appear to accumulate ascorbic acid (17). In this paper we have further characterized the secretion of newly taken up ascorbic acid from chromaffin cells and studied its subcellular origin.

EXPERIMENTAL PROCEDURES

Cell culture. Chromaffin cells were isolated from bovine adrenal medullae, plated, and maintained in primary culture as previously described by Wilson and Viveros (18). The isolated cells were then plated in multiwell plates (Falcon) at a density of 1×10^6 cells/well and maintained until use at day 3 with fetal calf serum (10%). Cortical cells were isolated using the medullary cell isolation procedure without the Percoll gradient step and were plated at a density of 1×10^6 cells/well and maintained until used on day 3.

Labeling with [^{14}C]ascorbic acid, [^{3}H] NE and [^{3}H] α-aminoisobutyric acid. Plated chromaffin cells after 3 days in culture were washed three times at room temperature with 0.5 ml of BSS containing 150 mM NaCl, 4.2 mM KCl, 1.0 mM NaH_2PO_4, 11.2 mM glucose, 0.7 mM $MgCl_2$, 2.0 mM $CaCl_2$, and 10 mM Hepes (pH 7.4). They were then incubated at 37° for 30 min in BSS (0.5 ml/well) containing 200 μM [^{14}C]ascorbic acid (7.6 mCi/mmole) and 1 mM thiourea to protect ascorbate from oxidation. In some experiments the incubation media also contained 0.5 μM [^{3}H]α-aminoisobutyric acid (10 Ci/mmole) or 0.08 μM [^{3}H]NE (13.4 Ci/mmole). An identical procedure was followed to label cortical cells.

Secretion experiments. After labeling, the chromaffin cells were washed at room temperature by replacing the medium at intervals of 5, 5, and 10 min with 0.5 ml of BSS. This was found to be necessary, as studies on the efflux rate of newly accumulated [^{14}C]ascorbate showed an initial, short-lived, high rate of efflux. Thereafter, the efflux rate was slow and usually less than 4% of the cellular [^{14}C]ascorbate content in a 15-min incubation period (16). Secretion was induced by incubating the washed cells, for 10 min at room temperature, in 0.45 ml of BSS containing any of the following secretagogues: 1–20 μM nicotine, 56 mM KCl, 100 μM veratridine, or 1–3 mM $BaCl_2$. Ouabain (10 μM) was added to the incubation media to prevent ascorbic acid reuptake. This concentration of ouabain did not modify the basal or nicotine-induced secretion of CA. In studies on the effect of *d*-tubocurare or atropine, the drugs were present during the last 10-min wash and during the stimulation period. At the end of the stimulation period, 0.4 ml of medium was withdrawn from each well and pipetted into a chilled tube containing 0.05 ml of 0.5 M perchloric acid to prevent oxidation of catecholamines. The rest of the medium was removed by aspiration and the cells were extracted with ice-cold 50 mM perchloric acid. Aliquots of the cell extract, as well as the acidified secretion media, were analyzed for their content of radioactivity (dual-label program, Packard Tri-Carb 2660) and endogenous CA (19). Because of the rapid oxidation of ascorbic acid in the incubation medium, secretion of endogenous ascorbate was followed by measuring the cell content of ascorbic acid before and after stimulation. Ascorbic acid was determined by HPLC with electrochemical detection as previously described (16). An identical procedure was followed to study the release of [^{14}C]ascorbic acid from cortical cells exposed to nicotine (10 and 100 μM) and ACTH (0.7 and 7 mIU/ml).

Subcellular fractionation. Chromaffin cells were plated in 35-mm plates at the same density used on multiwell plates (5.2×10^5 cells/cm^2). On day 3 after plating, cells from four plates, which were labeled and washed as for the secretion experiments, were suspended in 1.3 ml of 0.3 M sucrose containing 1 mM EDTA and 10 mM Hepes (pH 7.2) and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 800 × *g* for 10 min (Sorvall, SM 24 rotor), and the pellet was rehomogenized in 0.4 ml of buffered sucrose as above and centrifuged again at 800 × *g*. The supernatants were combined and centrifuged at 26,000 × *g* for 10 min to obtain a crude chromaffin granule fraction (P_2). The supernatant (S_2) was centrifuged at 160,000 × *g* for 10 min (Beckman Airfuge, A-100/18 rotor) to obtain a microsomal fraction (P_3) and a postmicrosomal supernatant. Aliquots of the various fractions were treated with perchloric acid containing EDTA (50 mM and 0.01%, respectively, final concentration) and analyzed for their content of ascorbic acid ([^{14}C]ascorbate and total) as indicated above. The CA content was determined by the trihydroxyindole method of Anton and Sayre (19).

Materials. L-[1-^{14}C]ascorbic acid (7.6 mCi/mmole), α-[*methyl*-^{3}H]aminoisobutyric acid (10 Ci/mmole), and DL-[7-^{3}H-(N)]norepinephrine hydrochloride (13.4 Ci/mmole) were all obtained from New England Nuclear Corporation (Boston, Mass.). Nicotine, veratridine, atropine, *d*-tubocurare, bethanecol, and porcine ACTH were obtained from Sigma Chemical Company (St. Louis, Mo.). D-600 was obtained from Kroll Pharmaceuticals Company (Whippany, N. J.), and P-286 from Pittman-Moore (Greensboro, N. C.). All other reagents used were of analytical grade.

RESULTS

Newly taken up ascorbic acid is secreted concomitantly with CA upon stimulation of isolated chromaffin cells. Figure 1 shows that the nicotine and the high potassium-induced secretion of [^{14}C]ascorbic acid is Ca^{2+}-dependent, with no secretion occurring in the absence of the metal ion. Furthermore, the cytoplasmic marker α-aminoisobutyric acid (20, 21) is not released under conditions in which [^{14}C]ascorbate and CA secretion is induced either by nicotine or by K^+ depolarization. We have previously shown that greater than 90% of the ascorbic acid newly taken up remains in the reduced form inside the cultured chromaffin cells (8, 16). When the state of reduction of the ascorbate secreted into the medium was analyzed by HPLC with electrochemical detection, it was found as dehydroascorbate even upon addition of thiourea (1 mM) to the incubation medium. However, inclusion of 200 μM ascorbic acid in the incubation medium allowed for the partial protection of the spontaneously

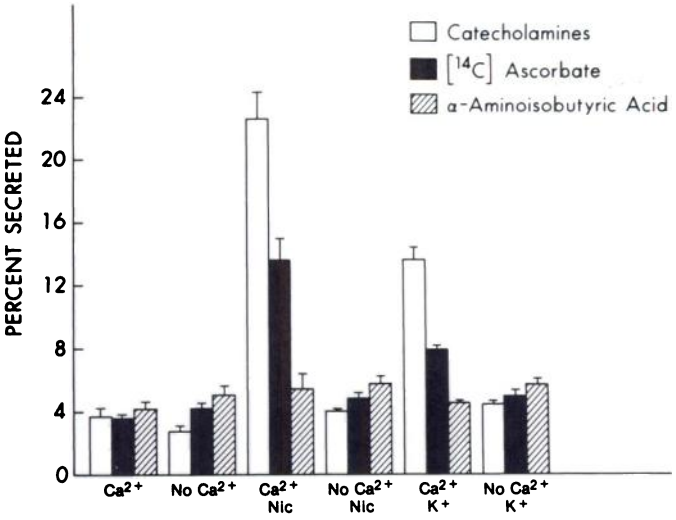

FIG. 1. *Nicotine and K^+-induced secretion of endogenous catecholamines and [^{14}C]ascorbic acid*

Chromaffin cells (1×10^6 cells/well) were incubated in 0.5 ml of BSS containing 200 μM [^{14}C]ascorbic acid and 0.5 μM [^{3}H]α-aminoisobutyric acid for 30 min at 37°. The cells were then washed as described under Experimental Procedures and stimulated for 10 min at room temperature by the addition of 0.45 ml of normal or Ca^{2+}-free BSS containing 10 μM nicotine or 56 mM KCl. The medium and cell extracts were then analyzed for [^{14}C]ascorbate, [^{3}H]α-aminoisobutyric acid, and CA. Results are expressed as the mean ± standard deviation (three wells per treatment). Initial cell radioactivity (radioactivity in the cells and medium at the end of the stimulation period) was 22,876 ± 1,933 dpm of [^{14}C]ascorbate and 44,583 ± 2,968 dpm [^{3}H]α-aminoisobutyric acid, and the endogenous CA content of the cells was 99.8 ± 8.1 nmoles (mean ± standard deviation for the total number of wells in these experiments). Data are shown from a typical experiment repeated at least four times with similar results.

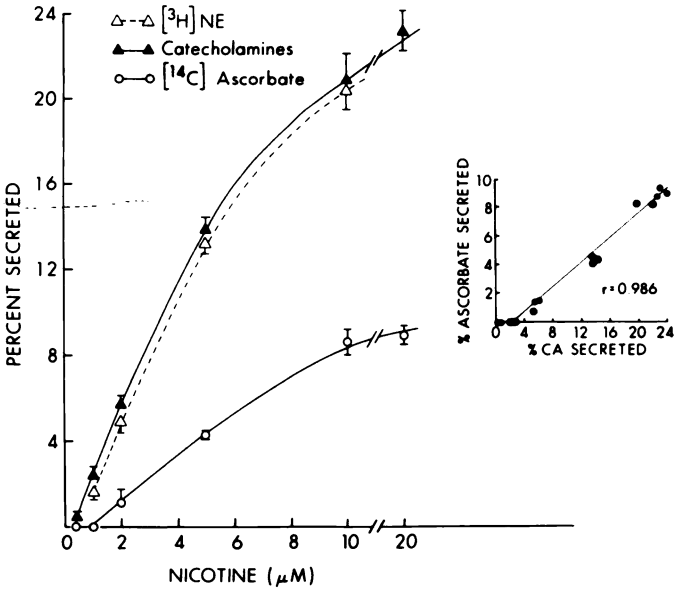

FIG. 2. *Nicotine dose-response relationship for endogenous catecholamine, [^{3}H]NE, and [^{14}C]ascorbate secretion*

Chromaffin cells (1×10^6 cells/well) were labeled with [^{14}C]ascorbate and [^{3}H]NE for 30 min at 37°; the cells were then washed and stimulated with increasing concentrations of nicotine. Results are expressed as percentage secretion of total cell content. The basal (nonstimulated) release has been subtracted. Values for secretion of [^{14}C]ascorbate and CA (endogenous and [^{3}H]NE) are all significantly different. Values are given as the mean ± standard deviation (slope = 0.44). Initial cell radioactivity was 20,197 ± 2,315 dpm of [^{14}C]ascorbic acid and 401,673 ± 21,230 dpm of [^{3}H]NE, and the endogenous CA content was 81.5 ± 3.6 nmoles (mean ± standard deviation).

released (basal) and secreted [^{14}C]ascorbate. The percentage degradation (30%) was equal for both cold and radioactive ascorbate, indicating that ascorbic acid is secreted in the fully reduced form.

The relation between nicotine concentration and the secretion of CA (endogenous or newly taken up [^{3}H]NE) and [^{14}C]ascorbic acid is shown in Fig. 2. Endogenous and newly taken up CA followed an identical pattern of secretion in agreement with previous reports (22). Therefore, in the following experiments, secretion of CA is referred indistinctly to endogenous or newly taken up CA. An excellent correlation between secretion of CA and [^{14}C]ascorbate was observed at nicotine concentrations of 2 μM and above, but at low concentrations of agonist (0.5 and 1 μM) there was no detectable stimulated release of [^{14}C]ascorbate whereas CA levels in the medium were significantly above basal release. Also, consistently there was a lower percentage secretion of [^{14}C]ascorbate as compared with CA, and the same was observed during K^+-induced secretion (Fig. 1). Similarly, an analysis of the Ca^{2+} dependency of the nicotine-induced secretion of CA and [^{14}C]ascorbate (Fig. 3) showed a good correlation between the secretion of both molecules, but the percentage secretion of [^{14}C]ascorbic acid was always lower than the percentage secretion of CA. The same was found when studying the Na^+ dependency for the nicotine-induced secretion of CA and ascorbate (results not shown).

The nicotine-induced secretion of [^{14}C]ascorbic acid from cultured bovine chromaffin cells was completely blocked by *d*-tubocurare and not modified by the presence of atropine. Furthermore, muscarinic agonists such as bethanecol were unable to induce the release of [^{14}C]ascorbate and/or CA from isolated chromaffin cells. These results are shown in Fig. 4.

The time course for the secretion of [^{14}C]ascorbic acid and CA induced by several secretagogues was studied and is expressed in Fig. 5 as secretory rates at different time intervals after stimulation. With all stimuli the rates of secretion of [^{14}C]ascorbic acid and CA at each time studied were different. With the three secretagogues the rate of CA secretion reached a maximum after 2 min of stimulation, and then decreased except for that induced by veratridine, which remained constant throughout the duration of the experiment. In contrast, upon stimulation with nicotine or K^+, the rate of [^{14}C]ascorbate secretion reached its maximum by 5 min, and with veratridine increased linearly with time. The rate of secretion of [^{14}C]ascorbate, induced by nicotine or K^+, did not decrease but remained at the maximal rate by 10 min of continued exposure to the secretagogues.

The above results were suggestive that catecholamines and newly taken up ascorbic acid could be contained and released from different compartments. To study the possibility that the nicotine-induced release of newly taken up [^{14}C]ascorbate might originate from contaminant cortical cells, we isolated, cultured, and labeled adrenocortical cells with [^{14}C]ascorbate. Cortical cells, cultured

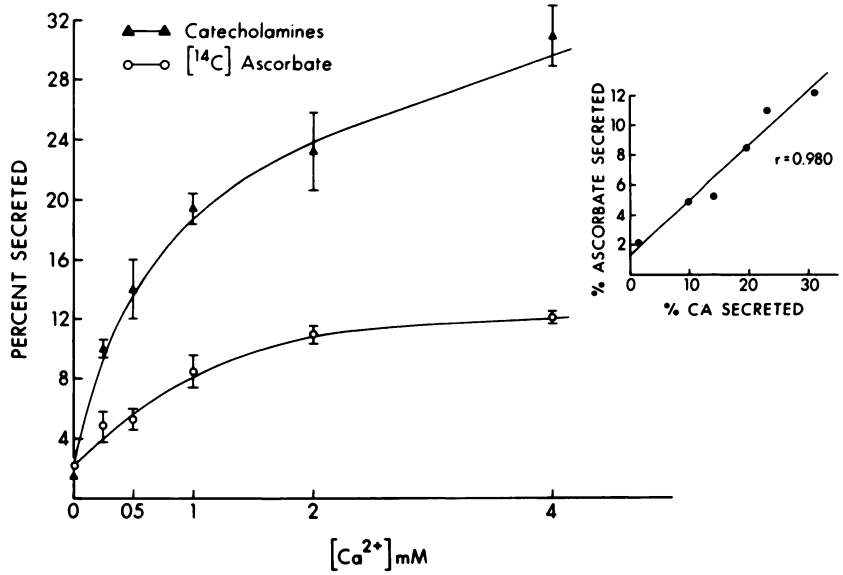

FIG. 3. *Ca^{2+} dependence for the nicotine-induced release of [^{14}C]ascorbate and catecholamines*

Cells (10^6/well) were stimulated with 10 μM nicotine at different Ca^{2+} concentrations. Results are expressed as percentage secretion of total cell content. Basal (nonstimulated) release was measured for each condition and subtracted out. Results are expressed as the mean ± standard deviation (slope = 0.36). Initial cell radioactivity was 22,214 ± 3,152 dpm of [^{14}C]ascorbic acid, and the endogenous CA content was 84.7 ± 5.1 nmoles (mean ± standard deviation).

under identical conditions as those for chromaffin cells, accumulated [^{14}C]ascorbic acid by a saturable process (results not shown); however, no release of the newly taken up ascorbic acid was observed when these cells were exposed to ACTH or high concentrations of nicotine. In addition, ACTH did not modify the basal or nicotine-induced secretion of [^{14}C]ascorbate and [^{3}H]NE from cultured adrenomedullary cells (Table 1).

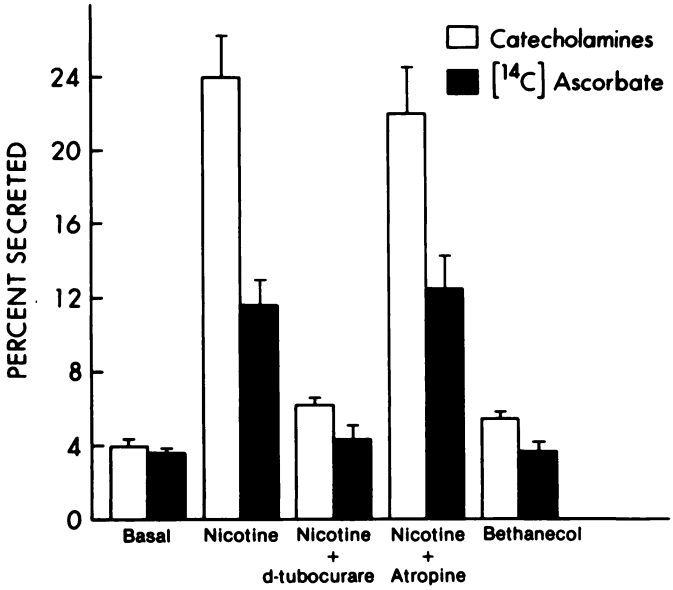

FIG. 4. *Effect of nicotinic and muscarinic blockers on the nicotine-induced release of catecholamines and [^{14}C]ascorbic acid from chromaffin cells*

Labeled and washed cells were stimulated for 10 min with 10 μM nicotine or 300 μM bethanecol. Atropine (1 μM) and *d*-tubocurare (10 μM) were present in the medium in the last 10-min wash and during stimulation. Results are expressed as percentage of the total cell content; mean ± standard deviation of a typical experiment (three wells per treatment) repeated three or more times. Initial cell content was 21,635 ± 1,220 dpm of [^{14}C]ascorbate and 97.3 ± 12.3 nmoles of endogenous CA (mean ± standard deviation).

In order to assess further the existence of different releasable compartments in chromaffin cells, we studied the effect of a number of agonists and antagonists of adrenomedullary CA secretion on [^{14}C]ascorbate secretion. As has been indicated before and shown in Figs. 1–4, nicotine and K^+ induced a 1.5- to 3-fold higher percentage secretion of CA than ascorbic acid in different cells preparations, but this ratio was constant for each cell preparation. However, Table 2 shows that stimulation of cells with Ba^{2+} in regular BSS markedly increased the ratio of percentage CA secreted/ascorbate secreted; furthermore, Ba^{2+} in Ca^{2+}-free BSS induced the almost exclusive secretion of CA. P-286, a compound known to inhibit CA secretion from perfused bovine adrenals (23), was found to be a very potent inhibitor of the nicotine-induced secretion of both CA and [^{14}C]ascorbate acid from isolated chromaffin cells. However, the nicotine-induced secretion of catecholamines was found to be more sensitive than the secretion of [^{14}C]ascorbate to the action of Ca^{2+} channel blockers, particularly Ni^{2+}. The preferential stimulation and inhibition of CA secretion as compared with ascorbate provides further support to the hypothesis of different subcellular origin for the secreted chatecholamines and newly taken up ascorbic acid.

Additional evidence for the existence of separate compartments originating CA and ascorbate secretion was obtained by studying the subcellular distribution of endogenous and newly taken up [^{14}C]ascorbic acid in cultured chromaffin cells. Figure 6 shows the distribution of endogenous catecholamines and ascorbic acid in homogenates prepared from isolated and washed adrenal chromaffin cells that had been for 2 days in culture. Of

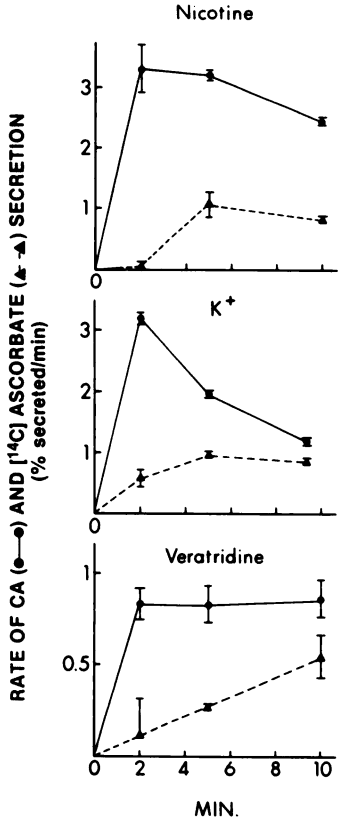

FIG. 5. *Changes in the secretion rate of catecholamines and [^{14}C] ascorbate in response to various secretagogues*

Labeled and washed cells were stimulated with nicotine (10 μM), K^+ (56 mM), or veratridine (100 μM) for different periods of time, and rates were calculated by dividing the amount secreted during each successive period and the computed time. Basal release rate at each time point has been subtracted. Results are expressed as the mean ± standard deviation (five wells per treatment). Initial cell content was 22,401 ± 2,071 dpm of [^{14}C]ascorbic acid and 78.2 ± 4.3 nmoles of endogenous CA (mean ± standard deviation).

the total cell ascorbate, 32% was found in the crude granular fraction (P_2). This value is very close to a value of 34% previously reported by Ingebretsen *et al.* (15)

TABLE 1

Secretion of [^{14}C]ascorbic acid and [^{3}H]NE from adrenocortical and adrenomedullary cells in culture

Cortical and medullary cells were labeled with [^{14}C]ascorbic acid (200 μM) for 30 min at 37°. The incubation medium for medullary cells also contained [^{3}H]NE (0.1 μM). The cells were then washed as described under Experimental Procedures and exposed for different periods of time with ACTH, nicotine, or a combination of both. In all cases, ouabain (10 μM) was present during the stimulation period. Secretion is expressed as percentage of total cell content. Results are presented as the mean ± standard deviation of quadruple samples from two different cell preparations. Initial cell radioactivity in cortical cells was 30,642 ± 2,172 dpm of [^{14}C]ascorbate/10^6 cells and for medullary cells 33,829 ± 924 dpm of [^{14}C]ascorbate and 414,935 ± 32,662 dpm of [^{3}H]NE/10^6 cells.

Condition	Time	% Secretion	
		[^{14}C]ascorbate	[^{3}H]NE
Cortical cells			
Basal	60 min	16.2 ± 2.9	—
ACTH, 0.7 mU/ml	60 min	15.6 ± 1.1	—
ACTH, 7 mU/ml	60 min	20.5 ± 1.5	—
Basal	15 min	10.9 ± 1.6	—
Nicotine, 10 μM	15 min	11.4 ± 302	—
Nicotine, 100 μM	15 min	10.3 ± 2.5	—
Medullary cells			
Basal	10 min	2.8 ± 0.3	4.8 ± 0.3
Nicotine, 10 μM	10 min	10.9 ± 0.8	22.9 ± 1.0
ACTH, 7 mU/ml	10 min	2.1 ± 0.4	4.0 ± 0.6
Nicotine, 10 μM + ACTH, 7 mU/ml	10 min	10.8 ± 0.6	22.5 ± 1.2

upon subcellular fractionation of the bovine adrenal medulla. The catecholamine content of that same fraction was over 60% of total cell CA. Figure 7A shows that, although the distribution of newly taken up [^{3}H]NE was identical with that of endogenous catecholamines, the [^{14}C]ascorbate taken up after a 1-hr pulse was found mainly in the 26,000 × *g* supernatant (94%), indicating that after this short labeling period, only a minimal amount of ascorbic acid had accumulated in the chromaffin vesicles. However, when chromaffin cells were

TABLE 2

Differential secretion of CA and [^{14}C]ascorbate from chromaffin cells stimulated by secretagogues in the presence or absence of inhibitors

Chromaffin cells were labeled with [^{14}C]ascorbate acid (200 μM) and [^{3}H]NE (0.1 μM) for 30 min at 37°; the cells were then washed and stimulated either for 10 min (B and C) or 15 min (A) at room temperature. The inhibitors were present in the medium during the last 10-min wash and during stimulation. Secretion is expressed as percentage of total cell content. The basal (nonstimulated) release for each condition has been subtracted. Stimulation with $BaCl_2$ was done in the absence of ouabain. Results are presented as the mean ± standard deviation of triplicates from one experiment repeated at least two times with similar results. Initial cell content was 31,431 ± 1,933 dpm of [^{14}C]ascorbate and 435,221 ± 28,652 dpm of [^{3}H]NE per 10^6 cells, respectively.

Secretagogues	Inhibitors	Catecholamines	[^{14}C]Ascorbate	Ratio CA/[^{14}C]ascorbate
		% secretion		
A. Nicotine, 10 μM		26.4 ± 1.0	15.2 ± 0.4	1.7
Ba^{2+}, 2 mM		33.1 ± 0.2	5.9 ± 0.8	5.6
Ba^{2+}, 3 mM		41.1 ± 1.0	6.9 ± 0.2	6.0
B. Ba^{2+}, 1 mM[a]		32.2 ± 0.9	3.0 ± 0.4	10.7
		41.0 ± 0.4	4.2 ± 0.5	9.8
		% inhibition		
C. Nicotine, 10 μM	P-286, 10 μM	97.0 ± 1.0	96.2 ± 3.3	1.0
Nicotine, 10 μM	D-600, 5 μM	59.3 ± 3.9	40.1 ± 4.6	1.5
Nicotine, 10 μM	NI^{2+}, 1 mM	83.1 ± 0.9	29.8 ± 3.6	2.8

[a] In Ca^{2+}-free BSS.

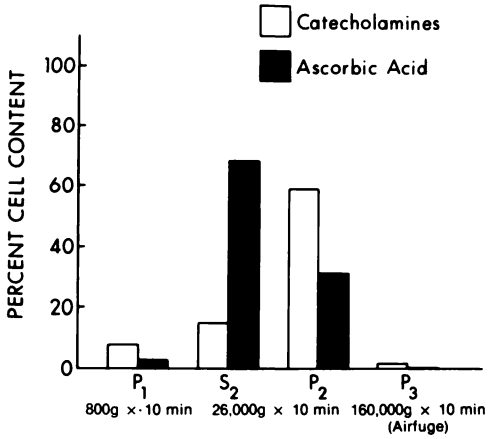

FIG. 6. *Subcellular distribution of endogenous CA and ascorbic acid in isolated chromaffin cells*

Day 2 cells were washed following the procedure for secretion experiments and then were homogenized in 0.3 M sucrose/1 mM EGTA/10 mM Hepes (pH 7.2) (15×10^6 cells/ml) with a Dounce homogenizer and fractioned as described under Experimental Procedures. The total content of CA and ascorbate was 1.39 μmole and 26.3 nmoles/15×10^6 cells, respectively.

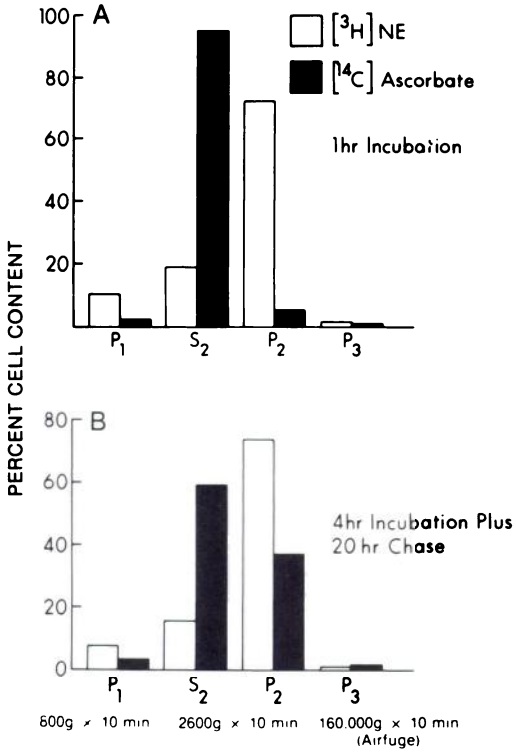

FIG. 7. *Subcellular distributions of [^{14}C]ascorbic acid and [^{3}H]NE in isolated chromaffin cells*

Cells were incubated in the presence of 200 μM [^{14}C]ascorbic acid and 0.37 μM [^{3}H]NE at 37°. A, Cells were incubated for 1 hr (day 3), washed, homogenized, and fractioned as for Fig. 6. B, Cells were incubated for 4 hr (day 2), washed, and incubated for a further 20 hr at 37° in BSS; the cells were then homogenized and fractioned as above. The washing procedure in both experiments was identical with that used for the secretion experiments. The amount of the radioactive material present in the homogenates, as calculated from their specific activities, was 20.6 and 14.6 nmoles of [^{14}C]ascorbic acid, and 0.096 and 0.467 nmole [^{3}H]NE for A and B, respectively.

incubated for 4 hr with [^{14}C]ascorbate followed by a prolonged equilibration period (Fig. 7B), marked changes were observed in the subcellular distribution of the [^{14}C] ascorbic acid. By 24 hr, the label was found in the chromaffin vesicles in a percentage close to that of endogenous ascorbate. Four hours of incubation with [^{14}C] ascorbate without further equilibration showed only a 1.5-fold increase of accumulation in the P_2 fraction with respect to cells incubated for 1 hr (data not shown).

DISCUSSION

Adrenomedullary chromaffin cells have been shown to secrete their products from more than one compartment (24, 25). The discussion of the results will be centered around two main aspects: first, we will be dealing with the evidence for the multicompartmental secretion of ascorbic acid and later with the possible physiological function of the secreted ascorbate from these multiple compartments.

We have confirmed that newly taken up [^{14}C]ascorbic acid is secreted along with catecholamines upon stimulation of isolated chromaffin cells (16). The secretion of both [^{14}C]ascorbate and CA is prevented in the absence of Ca^{2+} and is markedly inhibited in the presence of Ca^{2+} channel blockers (Table 2), indicating an absolute Ca^{2+} dependency for the process. The secretion of newly taken up [^{14}C]ascorbic acid from bovine chromaffin cells, like that of CA, is mediated through the activation of nicotinic receptors, as it is induced by nicotine and not by bethanecol and it is blocked by *d*-tubocurare but not by atropine (Fig. 4). The above observations have been considered characteristic of secretion by exocytosis of chromaffin vesicles (26, 27). Furthermore, the proportional release of CA and [^{14}C]ascorbic acid observed when analyzing the nicotine concentration-relationship (Fig. 2) and the Ca^{2+} dependence of the nicotine-induced secretion (Fig. 3) led us to conclude that the chromaffin cell had a mechanism to accumulate ascorbic acid into the CA storage vesicle, the site of intracellular dopamine-β-hydroxylation (16).

However, upon further analysis of the data, we noticed that, even though the release of both molecules was proportional, the amounts being released (expressed as percentage of cell content) were different, usually 2 times more CA than [^{14}C]ascorbic acid being secreted. This difference in the proportion of [^{14}C]ascorbate secreted could be explained if only a fraction of the newly taken up ascorbate accumulated inside the chromaffin vesicles, and if only this fraction was being secreted. An alternative explanation is that the secreted [^{14}C]ascorbate could come from a compartment altogether distinct from the CA storage vesicles. The latter is strongly supported by the differential effects of Ba^{2+}, D-600, and Ni^{2+} (Table 2), and the different time courses of secretion observed for CA and [^{14}C]ascorbate as induced by various secretagogues (Fig. 5). Two molecules of similar molecular weight, secreted by exocytosis from the same storage particle, must be released with identical time courses, in a fixed ratio, identical with the ratio of products stored

in the vesicles, and this ratio should be constant in spite of inhibition of secretion by Ca^{2+} channel blockers. The possibility that the secreted [^{14}C]ascorbic acid might originate from contaminant adrenocortical cells was ruled out, since these cells do not release newly taken up [^{14}C]ascorbic acid when exposed to ACTH or high concentrations of nicotine (Table 1), indicating that cultured cortical cells may be less responsive than freshly isolated cells (28).

That the secreted [^{14}C]ascorbate and CA originate from different subcellular compartments was confirmed when analyzing the subcellular distribution of newly taken up [^{14}C]ascorbic acid and [^{3}H]NE in cells treated in a way identical with that for the secretion experiments. After a 1-hr pulse, a very small amount (4.6%) of [^{14}C] ascorbic acid is found in the crude granular fraction. If one normalizes this value to 100% recovery of CA in the granular fraction (29), the percentage of [^{14}C]ascorbate in that same fraction would increase to 6.6%. Therefore, with a nicotine-induced CA secretion averaging about 25% of the cell content, the maximal amount of [^{14}C] ascorbic acid secreted from the granules under these conditions would be at most 1.5% of the cell [^{14}C]ascorbate content. Our results show that the nicotine-induced secretion of [^{14}C]ascorbic acid ranges from 12% to 15% of the cell content, thus the bulk of the [^{14}C]ascorbate secreted cannot originate from the chromaffin granules. Nevertheless, by chasing a 4-hr pulse for a further 20 hr, a marked change in the distribution of [^{14}C]ascorbate occurs, indicating that [^{14}C]ascorbate had slowly accumulated into the CA storage organelles.

The possibility that the released [^{14}C]ascorbate could originate from a free-cytosolic pool was ruled out by following the release of a cytosolic marker ([^{3}H]α-aminoisobutyric acid) of a similar molecular weight (Fig. 1). Previous investigations of secretion of macromolecular cytosolic components during stimulation of the adrenal medulla have given negative results (26). α-Aminoisobutyric acid and NE are actively transported into central nervous system cells by a Na^+-dependent process, but the amino acid is not taken up by the storage vesicles and is not co-released with NE upon electrical stimulation (20, 21). The results presented here corroborate our earlier finding (16) and demonstrate that newly accumulated [^{14}C]ascorbic acid is not secreted from a free pool in the cell cytoplasm, as no release of [^{3}H]α-aminoisobutyric acid can be detected under conditions in which CA and [^{14}C]ascorbate are co-secreted. Thus, newly taken up ascorbic acid appears to be secreted exocytotically via stimulation of nicotinic receptors from a compartment different from the CA storage vesicle. Endogenous ascorbic acid, which is partly contained in the chromaffin vesicles (35% of ascorbate cell content), is also secreted from isolated chromaffin cells upon stimulation with nicotine.[3] Because during exocytotic secretion, the entire water soluble content of the chromaffin vesicle is extruded to the extracellular space (26) secretion of endogenous ascorbate must originate, at least in part, from the chromaffin vesicle. Thus, ascorbic acid would be secreted from more than one compartment at the same time.

Chromaffin cell secretion from organelles other than chromaffin vesicles has not been much explored; however, there is good evidence for the release of products from lysosomes and endoplasmic reticulum. Nicotine induces a Ca^{2+}-dependent secretion of lysosomal enzymes from decorticated perfused adrenal glands (24). In addition, stimulation of CA secretion from the adrenal medulla by K^+ depolarization and other agents produces a Ca^{2+}-dependent release of a particular acetylcholinoesterase (ACHE-5) (25). This isoenzyme was found to be nonsedimentable upon subcellular fractionation of the adrenal medulla, although it was localized, using electron microscopic histochemistry, exclusively in the cisternae of the endoplasmic reticulum (25). The possibility that the secreted [^{14}C]ascorbate originates from lysosomes is unlikely, since we find no radioactive label in the P_2 fraction (containing lysosomes) from homogenates of cells incubated for a short time with [^{14}C]ascorbate (Fig. 7A); however, we cannot disregard the possibility that newly accumulated ascorbic acid would be released from endoplasmic reticulum cisternae, a mechanically fragile compartment which we cannot isolate intact by subcellular fractionation.

Finally, the role of ascorbic acid in neuronal function does not appear to be restricted to dopamine-β-hydroxylation. Indeed, the evidence above presented for the existence of more than one compartment for the release of ascorbic acid, of which at least one is not associated with the chromaffin vesicle and CA synthesis, suggests extracellular functions for ascorbic acid such as a hormone or a neuromodulator. Recent studies have indicated a possible modulatory role for ascorbate in neurotransmission, i.e., inhibition of dopamine-sensitive adenylate cyclase (30), Na^+,K^+-ATPase (31) neurotransmitter receptor binding (32) and blockade of the amphetamine-induced stereotypy *in vivo* (33). In addition, a depolarization-induced release of ascorbic acid has been demonstrated *in situ* by electrical stimulation of the rat brain thalamo-cortical pathway (34). These reports and our data suggest that the multicompartmental secretion of ascorbic acid from neural tissues might play a novel neuromodulatory role.

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