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Correlation of the Effects of Bretylium, Guanethidine, and *N,N*-Diisopropyl-*N'*-Isoamyl-*N'*-Diethylaminoethylurea (*P*-286) on the H^+ Electrochemical Gradient across the Chromaffin Granule Membrane and on Chromaffin Granule Function

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SUMMARY

HOLZ, R. W.: Correlation of the effects of bretylium, guanethidine, and *N,N*-diisopropyl-*N'*-isoamyl-*N'*-diethylaminoethylurea (*P*-286) on the H^+ electrochemical gradient across the chromaffin granule membrane and on chromaffin granule function. *Mol. Pharmacol.* **18:** 606–610 (1980).

N,N-Diisopropyl-*N'*-isoamyl-*N'*-diethylaminoethylurea (*P*-286) (0.03–0.3 mM) reduced the ATP, Mg^{2+}-induced electrical potential across the chromaffin granule membrane, inhibited ATP, Mg^{2+}-dependent catecholamine uptake into chromaffin granules, and inhibited carbachol-induced secretion from cultured bovine chromaffin cells. *P*-286 did not alter the intragranular pH. Bretylium and guanethidine did not alter the H^+ electrochemical gradient across the chromaffin granule membrane and did not alter the ATP, Mg^{2+}-induced catecholamine uptake into chromaffin granules. The uptake of bretylium, a quaternary ammonium compound, into chromaffin granules responded to changes in membrane potential in a qualitatively similar manner to that of the lipid-soluble cation triphenylmethylphosphonium cation ($TPMP^+$). This study provides further evidence that catecholamine uptake into chromaffin granules is coupled to the electrical component of the H^+ electrochemical gradient and raises the possibility that chemiosmotic mechanisms of the granules may be involved in catecholamine secretion from chromaffin cells. The study also demonstrates that chemiosmotic processes of chromaffin granules can affect the uptake of drugs into the storage vesicles.

INTRODUCTION

Chromaffin granules, the catecholamine-containing storage vesicles within adrenal medullary chromaffin cells, are intimately involved in the intracellular synthesis and storage of catecholamine and in the secretion of catecholamine from chromaffin cells. Evidence has accumulated that chemiosmotic processes play an important role in the functioning of the granules. *In vitro*, the intragranular H^+ concentration is 15- to 20-fold greater than that of the medium when the medium pH is 7 (1–3). The membrane-bound, Mg^{2+}-dependent ATPase is an electrogenic H^+ pump that can maintain a H^+ electrochemical gradient as large as 120 mV (inside positive) across the granule membrane (4, 5). The H^+ electrochemical gradient is composed of both the H^+ chemical potential {60 mV log $([H^+]_i/[H^+]_o)$, where i and o refer to inside and outside the granule, respectively} and the electrical potential across granule membrane. It is likely that catecholamine uptake into the granules is coupled to both the chemical and the electrical components of the H^+ electrochemical gradient (5, 6). The coupling is responsible for the ATP, Mg^{2+}-dependent stimulation of catecholamine uptake that was first studied by Kirshner (7) and Carlsson *et al.* (8).

Drugs can interfere with chemiosmotic processes in chromaffin granules in a number of different ways. *N*-Ethylmaleimide (9) and dicyclohexylcarbodiimide (DCCD) (10) inhibit the membrane-bound ATPase and inhibit catecholamine uptake into the granules. The H^+ transporter carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) induces a H^+ equilibrium diffusion potential across the granule membrane in the presence of ATP and Mg^{2+} and, thereby, decreases the H^+ electrochemical gradient to zero (3, 5) and inhibits catecholamine uptake (3, 11). Reserpine inhibits the ATP, Mg^{2+}-stimulated catecholamine uptake without altering the ATP, Mg^{2+}-induced increase in the H^+ electrochemical gradient (3). Reserpine probably directly interacts with the catecholamine transport system.

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N,N-Diisopropyl-*N'*-isoamyl-*N'*-diethylaminoethylurea (*P*-286), bretylium, and guanethidine all interfere with chromaffin granule or sympathetic nerve vesicle function. *P*-286 inhibits ATP, Mg^{2+}-stimulated catecholamine uptake into isolated chromaffin granules (12) and also inhibits acetylcholine-induced secretion from adrenal medulla (12, 13). Bretylium at high concentrations causes release of norepinephrine from sympathetic nerves and at lower concentrations inhibits physiological release of norepinephrine (14). Guanethidine profoundly depletes norepinephrine stores in sympathetic nerves (15). Both bretylium and guanethidine are taken up into sympathetic nerves and are released upon physiological stimulation (15, 17). In this report the interaction of *P*-286, bretylium, and guanethidine with chemiosmotic processes of chromaffin granules is investigated and correlated with catecholamine transport across the granule membrane and, in the case of *P*-286, with catecholamine secretion from dissociated bovine chromaffin cells maintained in tissue culture.

MATERIALS AND METHODS

Chromaffin granules were purified in isotonic medium using a sucrose, Ficoll, D_2O discontinuous gradient according to Trifaro and Dworkind (18). Chromaffin granule studies were performed in 285 mM sucrose, 10 mM K_2SO_4, and 10 mM potassium phosphate, pH 6.8–7.0, at 31°C. [^{3}H]Triphenylmethylphosphonium cation ($TPMP^+$), [^{14}C]SCN^-, [^{3}H]methylamine, and [^{14}C]bretylium concentration ratios across the granule membrane were determined in pelleted granules (1, 3). Generally, 0.05–0.1 μCi/ml of isotope was used. Intravesicular volumes were determined with ^3H_2O and [^{14}C]dextran under conditions identical to those used to measure concentration ratios (1, 3). At the concentrations used, these compounds did not alter catecholamine uptake into chromaffin granules. Granules were pelleted by centrifugation at 12,000*g* for 3 min in an Eppendorf 5412 centrifuge. Whole pellets were analyzed for radioactivity. One and one-half-milliliter volumes were sampled of a chromaffin granule suspension containing 0.7 to 1.4 mg protein/ml. Incubations and centrifugations were performed at 30–31°C. There were three samples per group. Processing and centrifugation of samples took 4–5 min after incubation. These delays are not included in figures where time courses are shown. Membrane potential was calculated from the Nernst equation, $V_i - V_o = -z60$ mV log (C_i/C_o), where z is the charge of the univalent ion, C is the concentration, and i and o refer to inside and outside the granule, respectively. The intragranular pH was determined from the methylamine ratio across the granule membrane and from the pH of the medium (1, 3).

Catecholamine transport across the granule membrane was determined according to Hasselbach and Taugner in the presence and absence of ATP (5 mM) and Mg^{2+} (5 mM) (9). Absolute transport rates were determined. The sum of epinephrine and norepinephrine was measured by the fluorometric method of Euler and Floding (19). Proteins were measured according to Lowry *et al.* (20) with bovine serum albumin as a standard.

Cells, disaggregated from bovine adrenal medullae according to the method of Fenwick *et al.* (21), were added to 16-mm-diameter uncoated culture wells (Costar, Cambridge, Mass.) (250,000 cells/well) containing 1 ml Eagles minimum essential medium (MEM) (GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated, fetal calf serum (GIBCO), 10 μM 5-fluorodeoxyuridine (an inhibitor of cell division and fibroblast proliferation), 50 μg/ml gentamycin, and 2.5 μg/ml Fungizone (Squibb, Princeton, N.J.). After 4 days at 34°C in 5% CO_2, 95% air, chromaffin cells formed monolayers which contained 6–15 nmol catecholamine/well. Cells which had been maintained for 4 days were incubated at 25°C in Ca^{2+}-containing Locke's solution (142 nM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.2 mM $CaCl_2$, 5.6 mM glucose, and 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4) supplemented with 0.1 mg/ml sodium ascorbate. Drugs were present where indicated. After 12 min the incubation medium was added to a test tube containing 0.056 ml 50% trichloroacetic acid (TCA). One milliliter of 5% TCA was added to the well and the floor of the well was scraped to liberate catecholamine remaining in the cells. Catecholamine in the TCA extracts was measured by the method of Euler and Floding (19). Data are expressed as the fraction of the total catecholamine present in the medium at the end of the 12-min incubation.

P-286 was obtained from Dr. Franklin Marshall, Dow Chemical USA, Indianapolis, Ind. FCCP was a gift from Dr. P. G. Heytler, E. I. Dupont de Nemours Co., Wilmington Del. *l*-[7-^{3}H]Norepinephrine (3.8 Ci/mmol), [^{3}H]triphenylmethylphosphonium bromide (3.59 Ci/mmol), [^{3}H]methylamine (1.4 Ci/mmol), [^{14}C]dextran (2.76 mCi/g), and ^3H_2O (1 mCi/g) were obtained from New England Nuclear Co. (Boston, Mass.). [^{14}C]KSCN (59 mCi/mmol) was obtained from Amersham–Searle (Arlington Heights, Ill.). [*N*-$^{14}CH_3$]Bretylium I (0.48 mCi/mmol) was synthesized and kindly provided by Dr. Raymond E. Counsell (Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Mich.). Drugs were added to media by making a 1:200 dilution from concentrated stock solutions in water or ethanol. Controls contained an appropriate amount of solvent.

Data are expressed as the mean ± standard error of the mean. Significance was determined by Student's *t* test.

RESULTS

P-286 (0.1 mM) inhibited ATP, Mg^{2+}-stimulated catecholamine uptake into granules by 45–60% (Figs. 1A and 2A). Guanethidine sulfate (0.1 mM) and bretylium iodide (0.1 mM) did not affect catecholamine uptake. Neither *P*-286, guanethidine, nor bretylium significantly altered catecholamine efflux from the granules. To examine the effects of these agents on the H^+ electrochemical gradient across the chromaffin granule membrane, granule membrane potential and the intragranular H^+ concentration were measured. *P*-286 significantly reduced the SCN^- concentration ratio across the membrane (Fig. 1B).[1] From the Nernst equation one calculates that *P*-286 reduced the potential from 51 to 25 mV (inside positive). *P*-286 did not alter the intragranular H^+ concentration

[1] The apparent concentration ratio of SCN^- in the absence of ATP and Mg^{2+} is less than one.

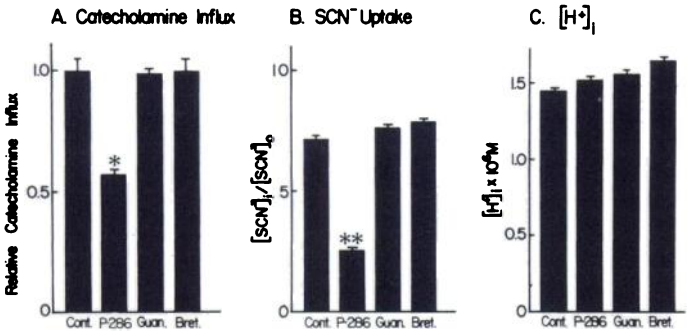

FIG. 1. *Effects of P-286, guanethidine, and bretylium on catecholamine influx (A), thiocyanate uptake (B), and intragranular H^+ concentration (C) in chromaffin granules*

Chromaffin granules were incubated for 15 min in the absence of drug (Cont.) or in a solution containing *P*-286 (0.1 mM), guanethidine (Guan.; 0.1 mM), or bretylium iodide (Bret.; 0.1 mM). * $P < 0.01$ vs control. ** $P < 0.001$ vs control.

(Fig. 1C). Neither guanethidine nor bretylium altered the electrical potential or the intragranular H^+ concentration (or the H^+ concentration gradient) across the granule membrane.

The effects of various concentrations of *P*-286 on granule membrane potential (measured by the SCN^- concentration ratio), on catecholamine influx into isolated granules, and on carbachol-induced secretion from cultured bovine chromaffin cells was determined (Fig. 2). *P*-286 reduced the granule membrane potential from +47 to +18 mV (inside positive) over approximately the same concentration range (3×10^{-5}–1×10^{-4}) that the drug inhibited catecholamine uptake by 65% and inhibited carbachol-induced secretion from chromaffin cells by 90%.

Bretylium is taken up into sympathetic nerve terminals and secreted together with norepinephrine upon nerve stimulation (16). These data suggest that bretylium is taken up into sympathetic nerves and then into intraterminal storage vesicles. The ability of chromaffin granules, which are homologous to sympathetic nerve storage vesicles, to take up [^{14}C]bretylium was, therefore, investigated. Because bretylium, a quaternary ammonium compound, is positively charged, its rate of uptake could be influenced by the granule membrane potential. The combination of 5 mM ATP and 5 mM Mg^{2+} shifts the granule membrane potential from −70 mV (inside negative) to +50 mV (inside positive) (5). Bretylium was taken up at a constant rate for 2 h in the absence of ATP and Mg^{2+} (Fig. 3A). ATP and Mg^{2+} inhibited bretylium uptake by 60% at 2 h. When added after some bretylium had accumulated in the granules, ATP and Mg^{2+} caused a significant decline of the bretylium content of the granules. The effects of ATP and Mg^{2+} on bretylium uptake were qualitatively similar to the effects of ATP and Mg^{2+} on membrane potential-sensitive $TPMP^+$ uptake (Fig. 3B). However, $TPMP^+$ uptake was greater than that of bretylium and reached a steady state in the presence and absence of ATP and Mg^{2+}. The effects of ATP and Mg^{2+} on $TPMP^+$ concentration ratios across the granule membrane were virtually identical to those previously reported (5). The apparent uptake of $TPMP^+$ in the presence of ATP and Mg^{2+} has been previously investigated (5) and is caused by binding to granules and not to the accumulation of unbound $TPMP^+$ within granules.

These data suggest that, as with the transport of $TPMP^+$, the transport of the positively charged bretylium is affected by membrane potential. When the granule membrane potential is negative inside, bretylium influx is enhanced. When the potential is positive inside, bretylium influx is retarded. Binding may contribute to the apparent uptake of bretylium and may account for a significant fraction of the apparent uptake in the presence of ATP and Mg^{2+}.

To further examine the effects of granule membrane potential on bretylium uptake into the granules, the effect of changing the granule potential in the presence of ATP and Mg^{2+} with FCCP was investigated (Fig. 4). FCCP, a H^+ carrier, shifts the granule membrane potential in the presence of ATP and Mg^{2+} from +50 mV (inside positive) to the H^+ equilibrium potential, approximately −70 mV (inside negative). The inhibition of bretylium uptake by ATP and Mg^{2+} should be reversed by FCCP. Indeed, FCCP (2 μM) restored bretylium up-

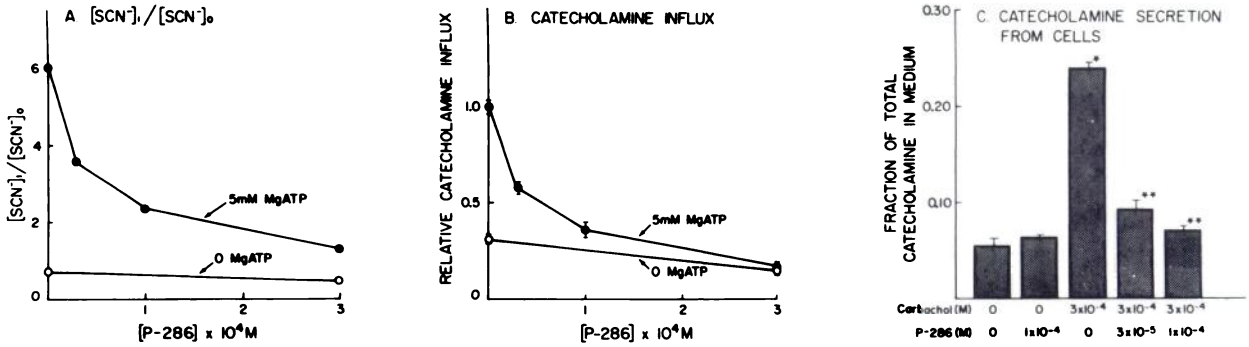

FIG. 2. *Effects of various concentrations of P-286 on thiocyanate uptake (A) and catecholamine influx (B) into chromaffin granules and on catecholamine secretion from chromaffin cells (C)*

Chromaffin granules were incubated in various concentrations of *P*-286 for 15 min in A and for 10 min in B. In C, the amount of catecholamine secreted from chromaffin cells after 12 min was determined in the various drug-containing media. In A and B, the standard error of the mean bars of some points were smaller than the point symbols and were omitted. In A and B, in the presence of MgATP all concentrations of *P*-286 gave differences compared to the absence of *P*-286 that were significant ($P < 0.001$). In C, * $P < 0.001$ vs 0 carbachol, 0 *P*-286; ** $P < 0.001$ vs carbachol alone.

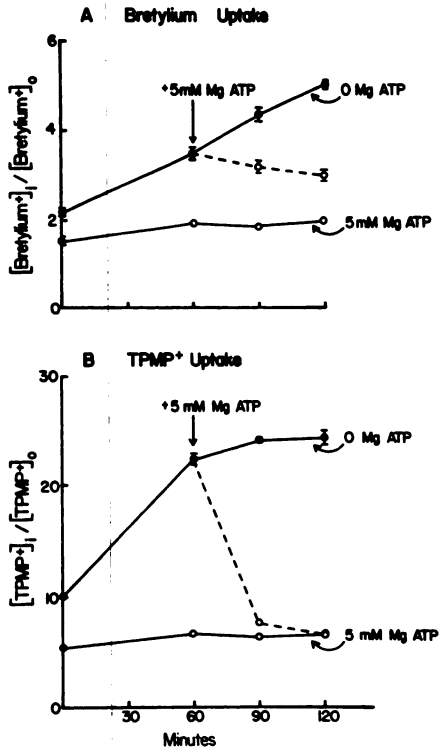

FIG. 3. *Time course of bretylium (A) and TPMP⁺ (B) uptakes into chromaffin granules*

Chromaffin granules were incubated in the presence or absence of ATP and Mg^{2+} in a solution containing both [^{14}C]bretylium and [^{3}H]TPMP⁺. Some granules were incubated for 60 min in the absence of MgATP, after which time 5 mM MgATP was added; the uptake of bretylium and TPMP⁺ was determined at later times (dashed lines). The standard error of the mean bars of some points were smaller than the point symbols and were omitted. At each time point the uptake of bretylium and TPMP⁺ in the absence of MgATP was significantly different from their uptake in the presence of MgATP ($P < 0.001$–0.02).

take to within 80% of the uptake in the absence of ATP and Mg^{2+} (Fig. 4A). As previously reported (5), the inhibition of TPMP⁺ uptake by ATP and Mg^{2+} is completely reversed by FCCP (Fig. 4B).

DISCUSSION

N,N-Diisopropyl-*N'*-isoamyl-*N'*-diethylaminoethylurea (*P*-286), bretylium, and guanethidine all interfere with adrenal medullary or sympathetic nerve function. The present study examined possible relationships between the effects of these drugs on sympathetic nerve or cell function and the interaction with chemiosmotic systems in chromaffin granules.

The Mg^{2+}-dependent ATPase associated with the chromaffin granule membrane is an electrogenic H^+ pump which, in the presence of anions which are permeant across the granule membrane (e.g., Cl^-), decreases the intragranular pH (4). In the absence of permeant anions, the pump does not alter the intragranular pH, but creates an inside positive potential which is in the direction of increasing the H^+ electrochemical gradient (5, 6). Because granules are normally unstable in the presence of ATP, Mg^{2+}, and a permeant anion, experiments with ATP and Mg^{2+} are usually performed in the absence of permeant anions as in the present study. *P*-286 inhibited ATP, Mg^{2+}-stimulated uptake of catecholamine into isolated chromaffin granules in the absence of permeant anions, a finding that was first made by Ferris *et al.* (12). In the present study, inhibition of catecholamine uptake was closely correlated with a reduction in the ATP, Mg^{2+}-induced potential across the granule membrane. Over the concentration range where *P*-286 reduced the potential in the presence of ATP and Mg^{2+} by 25 mV, catecholamine uptake was inhibited by approximately 65%. In a previous study the H^+ translocator FCCP also reduced granule membrane potential without altering the H^+ concentration gradient and also inhibited catecholamine influx (3). The inhibition of ATP, Mg^{2+}-stimulated catecholamine uptake by *P*-286 (and FCCP) is probably caused by the reduction in the granule membrane potential and the equivalent reduction in the H^+ electrochemical gradient. The mechanism by which *P*-286 reduced the membrane potential is unclear. *P*-286 does not inhibit the granule membrane ATPase (12). It is a tertiary amine and it may function as a H^+ translocator which can cause a H^+ conductance in membranes.

P-286 inhibits acetylcholine-induced secretion from

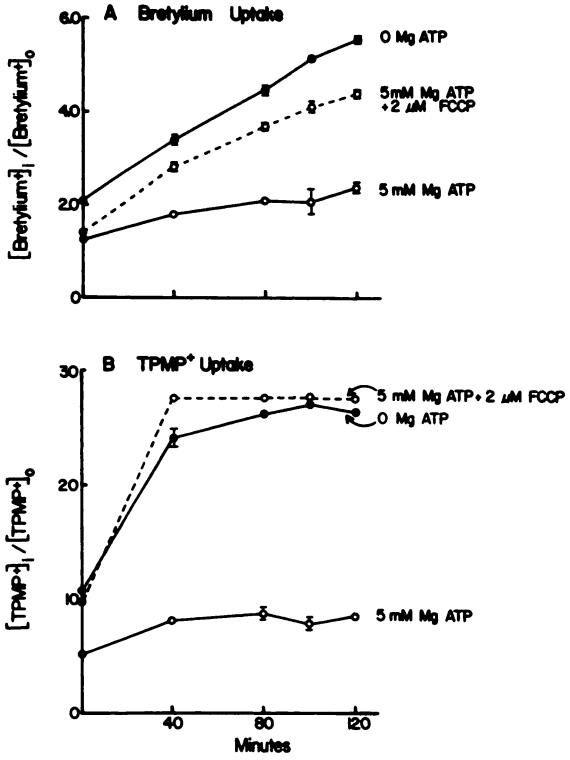

FIG. 4. *Effect of FCCP on the uptake of bretylium and TPMP⁺ into chromaffin granules in the presence of ATP and Mg^{2+}*

Chromaffin granules were incubated in the presence or absence of ATP and Mg^{2+} in a solution containing both [^{14}C]bretylium and [^{3}H]TPMP⁺. FCCP (2 μM) was present where indicated at the beginning of the incubation. The standard error of the mean bars of some points were smaller than the point symbols and were omitted. At each time point the uptake of both bretylium and TPMP⁺ in the absence of ATP and Mg^{2+} was significantly different ($P < 0.001$) from their uptake in the presence of ATP and Mg^{2+} (without FCCP). Except for bretylium uptake at zero time, the uptake of bretylium and TPMP⁺ at each time point in the presence of ATP and Mg^{2+} with FCCP was significantly different ($P < 0.01$–0.001) from their uptake in the presence of ATP and Mg^{2+} without FCCP.

the adrenal medulla (12, 13). In the present study the effect of *P*-286 on catecholamine secretion was reexamined in monolayers of bovine chromaffin cells maintained in tissue culture. *P*-286 inhibited carbachol-induced secretion of catecholamine over a similar concentration range over which it reduced the granule membrane potential and the H^+ electrochemical gradient. *P*-286 might inhibit energy metabolism in the chromaffin cells or may alter the properties of the plasma membrane of chromaffin cells. Nevertheless, the correlation of the inhibition of secretion with changes in the granule membrane potential raises the possibility that the granule membrane potential or the H^+ electrochemical gradient across the granule membrane plays a role in secretion.

Bretylium at high concentrations causes release of norepinephrine from sympathetic nerves and at lower concentrations inhibits physiological release of norepinephrine (14). Guanethidine causes release of norepinephrine from sympathetic nerves which can result in profound depletion of norepinephrine stores (15). Both drugs probably are taken up into storage vesicles within sympathetic nerves. In the present study neither drug altered catecholamine influx or efflux in isolated chromaffin granules, and neither drug altered the H^+ electrochemical potential across the granule membrane. The virtual absence of effects of bretylium and guanethidine on catecholamine influx into chromaffin granules confirms previous observations by Carlsson *et al.* (22) and is consistent with the resistance of the adrenal medulla to catecholamine-depleting effects of these agents (23, 24).

To date there is no direct evidence that chemiosmotic processes occur in sympathetic nerve vesicles. However, norepinephrine uptake into sympathetic storage vesicles from splenic nerve (25), from heart (26), and from brain (27) is stimulated by ATP and Mg^{2+}. Furthermore, the ATP, Mg^{2+}-stimulated norepinephrine uptake in splenic nerve vesicles is inhibited by a H^+ ion translocator (25). Thus, it is likely that chemiosmotic processes similar to those in chromaffin granules occur in sympathetic nerve storage vesicles. If the functioning of these processes closely resembles that of chromaffin granules, then based upon the present study, it is unlikely that bretylium and guanethidine alter sympathetic nerve function by altering the presumed chemiosmotic processes in sympathetic nerve storage vesicles.

[^{14}C]Bretylium, a quaternary ammonium compound, accumulated in chromaffin granules. The rate of accumulation of bretylium responded to changes in granule membrane potential in a manner consistent with a positively charged compound crossing the membrane. The effects of changes in membrane potential on bretylium accumulation qualitatively paralleled the effects on accumulation of the more lipid-soluble cation, $TPMP^+$. Bretylium was taken up by granules more slowly than $TPMP^+$. The data suggest that bretylium crosses the membrane by a solubility-diffusion mechanism without a specific transport system, and demonstrate that chemiosmotic processes in storage vesicles can affect the disposition of drugs.

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