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THE RELEASE OF DOPAMINE FROM SYNAPTOSOMES FROM RAT STRIATUM BY THE IONOPHORES X 537A AND A 23187

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SUMMARY

The antibiotics X 537A and A 23187 are negatively charged divalent cation ionophores. X 537A may, in addition, be an ionophore for amines including catecholamines. The effects of these ionophores were examined on the uptake and release of dopamine by synaptosomes prepared from rat corpus striatum. Both X 537A and A 23187, at concentrations less than 0.5 μM, release both endogenous and [^{3}H]-dopamine from synaptosomes. They had virtually no effect on the uptake of exogenous dopamine. These compounds act by different mechanisms. X 537A causes divalent ion-independent release in which a large fraction of the effluent consists of deaminated products. X 537A, in addition, releases [^{3}H]dopamine from rat adrenal medullary chromaffin granules. The results suggest that X 537A causes release of dopamine from intrasynaptosomal storage vesicles and perhaps is acting as a catecholamine carrier across the vesicular membrane. A 23187, on the other hand, causes a Ca^{2+}-dependent release in which only a small fraction of the catechol in the effluent is deaminated. A 23187 has little effect on the release of [^{3}H]dopamine from chromaffin granules. These results suggest that A 23187 carries Ca^{2+} into the synaptosomes and thereby initiates exocytotic release.

INTRODUCTION

Ionophoric antibiotics are able to make cations soluble in a lipid phase and transport cations across lipid membranes [1, 2]. Recently, two carboxylic ionophores X 537A (lasalocid) and A 23187 have been extensively investigated [3–9]. At physiologic pH, both are negatively charged lipophilic agents of molecular weight 591 and 524, respectively, (see refs 3 and 9 for chemical structures). X 537A has a high affinity for divalent ions and for amines including norepinephrine and epinephrine. It has a low affinity for alkali metal cations. A 23187 also has a high affinity for divalent ions. No information is available concerning its interactions with amines although there is virtually no interaction with alkali metal ions. X 537A and A 23187 cause release of

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*-tetraacetic acid.

histamine from mast cells. A 23187 causes a Ca^{2+}-dependent release; there have been conflicting reports concerning the Ca^{2+} dependence of X 537A induced release [7, 8].

Because 10–15 % of the nerve terminals in the corpus striatum are dopaminergic [10], this tissue provided an enriched source for catecholaminergic synaptosomes (pinched off nerve terminals). Synaptosomes from rat striatum have a saturable, high affinity uptake system for dopamine which concentrates the amine in the synaptosome [11]. Catecholamines in sympathetic nerves are stored in intraterminal vesicles and can be released by a number of different mechanisms including Ca^{2+}-dependent exocytosis and interference with storage in vescicles. The present studies investigate the effects of antibiotics X 537A and A 23187 on the efflux of dopamine from synaptosomes prepared from rat corpus striatum. The experiments demonstrate that the ionophores increase dopamine release and examine the possibility that the ionophoric characteristics of these antibiotics are responsible for their effects.

MATERIAL AND METHODS

Animals. 175–250 g Sprague–Dawley male rats were used in all experiments. Unless otherwise indicated, 1 h before killing rats were injected subcutaneously with pheniprazine (Lakeside Laboratories Inc., Milkaukee, Wisc.) an inhibitor of monoamine oxidase (monoamine: O_2 oxidoreductase: EC 1.4.3.4) to prevent metabolism of dopamine.

Preparation of P_2 fraction from the corpus striatum. Rats were killed by decapitation and the anterior striatum removed [12]. The tissue was homogenized in 20 vol. of 0.3 M sucrose with a teflon pestle smooth glass homogenizer (Kontes 22; Vineland, N.J.) (6 strokes, 500 rev./min) and the homogenate centrifuged at $1000 \times g_{av}$ for 10 min. The supernatant was then centrifuged at $20\,000 \times g_{av}$ for 20 min, and the pellet (Fraction P_2) suspended in 0.3 M sucrose 1 ml/100 mg original tissue.

Purification of radioactive catecholamines. [^{3}H]Dopamine (New England Nuclear Corp., Boston, Mass.; 5 Ci/mmole), was purified by alumina chromatography before using [13].

Incubation medium. Unless otherwise indicated, the incubation medium contained 154 mM NaCl, 3.85 mM KCl, 1.5 mM $CaCl_2$, 0.65 mM $MgSO_4$, 10 mM dextrose and 0.1 mg/ml ascorbic acid. The pH was 7.4 at 37 °C. In many experiments divalent ions were omitted or altered. Where indicated, 2 mM ethylene-glycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA, sodium salt) or EDTA (sodium salt) was added to chelate trace Ca^{2+} or polyvalent ions.

[^{3}*H*]*Dopamine uptake.* To measure uptake, 25–50 μl synaptosomes (the resuspended P_2 fraction) containing 50–75 μg protein, was preincubated for 2 min in 2 ml incubation medium at 37 °C in the presence or absence of drug. Uptake studies were initiated by the addition of 0.1 μM [^{3}H]dopamine. Uptake was rapidly terminated by millipore filtration of the incubation medium [11] and the filters were washed with dopamine-free buffer containing 1 % bovine serum albumin. The filtering and washing procedure took less than 10 s. The filters were dissolved in Bray's phosphor and the radioactivity counted in a Beckman scintillation counter. Blanks, which consisted of 0.33 mM nonradioactive dopamine added to the incubation medium, were 5–10 % of the control values and were routinely subtracted from the experimental values [11].

[^3H]Dopamine efflux from synaptosomes. Synaptosomes were loaded with [^{3}H]dopamine by incubating 4 ml of the P_2 suspension in 26 ml buffer without divalent ions in the presence of 0.13 μM [^{3}H]dopamine for 5 min at 37 °C. Because the concentration of [^{3}H]dopamine was at the apparent K_m for the high affinity uptake system of the dopaminergic synaptosomes [11], dopamine was probably selectively taken up by the synaptosomes. The mixture was cooled to 0–5 °C and centrifuged at 20 000 $\times g_{av}$ for 15 min. The pellet was rinsed in 5 ml 0.3 M sucrose without resuspending and then resuspended in 30 ml 0.3 M sucrose. The synaptosomes were recentrifuged at 20 000 $\times$ g_{av} for 20 min and the pellet resuspended in 4 ml 0.3 m sucrose. The washings and centrifugations were all done at 0–5 °C.

In a typical efflux experiment, 10–50 μl of the [^{3}H]dopamine-containing synaptosomes (20–100 μg protein) was added per ml to incubation medium at 37 °C shaken in a tissue incubator. At various times 1-ml aliquots were removed, filtered, washed, and the radioactivity counted as described for the uptake experiments. Zero time aliquots were removed immediately after adding the synaptosomes to the incubation medium. Immediately after loading the synaptosomes with [^{3}H]dopamine, 60–70 % of the [^{3}H]dopamine (5–7 pmoles/100 μg protein) was in the synaptosomes at zero time. Although the synaptosomes were kept at 0–5 °C in sucrose, they slowly lost [^{3}H]dopamine. In the course of a day, the zero values decreased with a half-time of approximately 4 h. The data in a given efflux experiment is expressed relative to the zero time value in the experiment (zero time equals 1.0). By using this procedure, in spite of the gradual decrease in the absolute level of the zero time point, the efflux curves were reproducible. Generally the amount of free [^{3}H]dopamine in the medium was less than $5 \cdot 10^{-9}$ M.

Analysis of deaminated metabolites in effluent. [^{3}H]Dopamine-containing synaptosomes were prepared from rats not treated with pheniprazine. The filtrate from an efflux experiment was collected in test tubes at 0–5 °C. 0.25 ml of the filtrate was diluted with 2.25 ml H_2O (0–5 °C). The radioactivity of 1 ml of this dilute effluent was counted directly in 10 ml Aquasol (New England Nuclear Corp.) in a scintillation counter. The amount of deaminated products in another 1 ml was determined according to the method of Moss et al. [14].

Efflux of endogenous dopamine. 100 μl of the P_2 suspension prepared from the rat corpus striatum was added to 1.2 ml incubation medium at 37 °C in a shaking tissue incubator in the presence or absence of drug. After a given time the solution was transferred to centrifuge tubes, cooled to 0–5 °C and centrifuged at 20 000 $\times g_{av}$ for 15 min. The pellet was resuspended in 400 μl 0.1 M $HClO_4$ and recentrifuged at 20 000 $\times g_{av}$ for 10 min. The catecholamine content in 100 μl of the supernatant was determined using the radiometric catecholamine assay of Coyle and Henry [15]; because dopamine is virtually the only catecholamine present [15], the periodate cleavage was omitted.

Efflux from adrenal chromaffin granules. Adrenal glands from 20 rats not treated with pheniprazine were cleaned of fat and loose connective tissue, minced with small scissors and homogenized with a Kontes 22, teflon pestle smooth glass homogenizer (6 strokes, 500 rev./min) in 10 ml sucrose, 0.3 M (0.5 °C). Iproniazide phosphate ($1 \cdot 10^{-5}$ M) was present to inhibit monoamine oxidase. A P_2 fraction was prepared as described for synaptosomes and the pellet resuspended in 0.2 ml 0.3 M sucrose per adrenal gland. Chromaffin granules within the fraction were loaded with [^{3}H]dopa-

mine by incubating 5 ml of the resuspended P_2 fraction in 25 ml incubation medium consisting of 0.3 M sucrose, 25 mM Tris–HCl, 5 mM ATP (disodium salt), 5 mM $MgCl_2$, and $1 \cdot 10^{-5}$ M [^{3}H]dopamine (43 Ci/mole, prepared by adding nonradioactive dopamine to alumina purified [^{3}H]dopamine, 5 Ci/mmole). The pH of the medium was 7.1 at 37 °C. The mixture was incubated for 15 min at 37 °C, cooled and centrifuged at $26\,000 \times g_{av}$ for 10 min. The pellet was washed by resuspending in 10 ml 0.3 M sucrose and recentrifuging at $26\,000 \times g_{av}$ for 10 min. The pellet was resuspended in 0.2 ml 0.3 M sucrose per adrenal gland. Efflux of [^{3}H]dopamine was examined by diluting the suspension 15-fold into medium consisting of 0.3 M sucrose and 25 mM Tris–HCl (pH 7.1) at 37 °C. The incubations were performed in a shaking tissue incubator. At various times 1-ml aliquots were removed and added to centrifuge tubes containing 1 ml 0.3 M sucrose at 0.5 °C. The radioactivity remaining in the chromaffin granules was determined by centrifuging the sample at $26\,000 \times g_{av}$ for 10 min, resuspending the pellet in 1.0 ml 0.1 M $HClO_4$ and measuring the radioactivity in the solution in 10 ml Aquasol in a liquid scintillation counter.

Bulk phase partition experiments. Organic phase–aqueous phase partition experiments were performed using the method of Pressman [3] to investigate the effects of X 537A and A 23187 on the partitioning of [^{2}H]dopamine and $^{45}Ca^{2+}$. $^{45}CaCl_2$ was obtained from the New England Nuclear Corp., 16.9 Ci/g and the specific activity lowered with nonradioactive $CaCl_2$.

Proteins. Protein content of samples was determined using the method of Lowry et al. [16] with bovine serum albumin as standard.

Drugs and chemicals. X 537A and A 23187 were dissolved in ethanol. Reserpine (Sigma, St. Louis, Mo.) was dissolved in dimethylsulfoxide. Concentrated solutions of drug were diluted 100–200-fold into the incubation media. Controls consisted of an equivalent dilution of solent.

X 537A was a gift from Hoffmann-La Roche Inc. and A 23187 was a gift from Eli Lilly Co. Other chemicals were of reagent grade and readily obtained commercially.

RESULTS

Release of dopamine. In the absence of drug there were at least two phases in the efflux of dopamine from synaptosomes, an initial rapid one and a later slower one. Both X 537A (Fig. 1) and A 23187 (Fig. 2) at less than 0.1 μM concentrations increased the release of [^{3}H]dopamine from synaptosomes. Extrapolating the slow phase efflux back to zero time, one finds that X 537A decreased or eliminated the fraction in the slow phase. X 537A also increased the rate of the slow phase efflux. In the presence of A 23187 it was usually not possible to define distinct fast and slow phases in the efflux. X 537A and A 23187 also caused a net decrease in endogenous catecholamine (Table I); thus, these compounds were not simply causing an exchange diffusion between intra- and extrasynaptosomal dopamine.

Importance of divalent ions for release. Physiologic release of neurotransmitter from nerve terminals and the adrenal medulla is a Ca^{2+}-dependent process that appears to involve release of transmitter from intraterminal storage vesicles directly into the extracellular space (exocytosis) [17–19]. Because of their ability to transport divalent ions across biological membranes, both X 537A and A 23187 may be causing

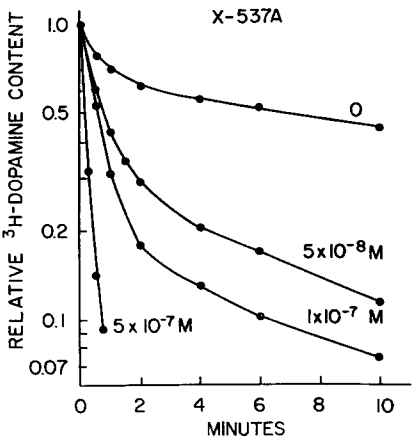

Fig. 1. Release of [^{3}H]dopamine by X 537A. Synaptosomes containing [^{3}H]dopamine were added to incubation medium at 37 °C without divalent ions, with 2 mM EDTA, in the presence or absence of drug. 1-ml aliquots containing 90 μg synaptosomal protein were removed at various times and the [^{3}H]dopamine in the synaptosomes determined as described in Material and Methods. Each point represents duplicate determinations.

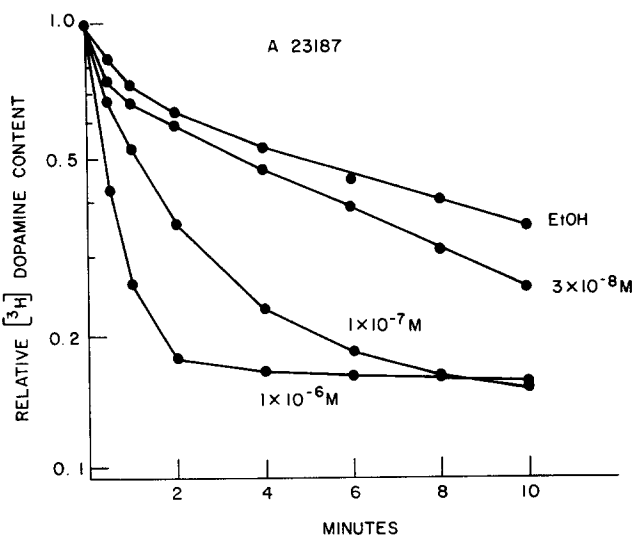

Fig. 2. Release of [^{3}H]dopamine by A 23187. Synaptosomes containing [^{3}H]dopamine were added to incubation medium with 1.5 mM Ca^{2+} and 0.65 mM Mg^{2+} at 37 °C in the presence or absence of drug. 1-ml aliquots containing 26 μg protein were removed at various times and the [^{3}H]dopamine in the synaptosomes determined as described in Material and Methods. Each point represents duplicate determinations.

TABLE I

RELEASE OF ENDOGENOUS DOPAMINE

Synaptosomes (65 μg protein) were added to incubation medium (1.2 ml) at 37 °C in the presence or absence of drug. The mixture was immediately (zero time) or at 3 or 4 min cooled to 0–5 °C, centrifuged and the pellet assayed for catecholamine as described in Material and Methods. The incubation medium in the X 537A experiment contained EDTA (2 mM) and no divalent ions. The incubation medium in the A 23187 experiment contained $CaCl_2$ (1.5 mM) and $MgSO_4$ (0.65 mM). The results are expressed as the amount of endogenous dopamine remaining in the synaptosomes after the indicated incubation.

X 537A		Endogenous dopamine after 4 min incubation (pmoles/65 μg protein)
Control (1 % ethanol, v/v)		24.7±0.8
0.1 μM		7.6±1.6*
0.5 μM		3.7±0.4*
A 23187	**Endogenous dopamine (pmoles/65 μg protein)**	
	0 min	3 min
Control (1 % ethanol, v/v)	25.2±1.5	24.7±1.8
0.1 μM		24.5±1.7
0.5 μM		13.2±0.6**

* $P < 0.001$ compared to controls at 4 min.
** $P < 0.001$ compared to controls at 3 min.

release of dopamine by transporting Ca^{2+} from the medium into the synaptosomes, thereby initiating exocytotic release. To investigate the importance of divalent ions for the action of these drugs, the release of [^{3}H]dopamine by X 537A and A 23187 was examined in media in the presence or absence of various divalent ions (Fig. 3). Although the control efflux at 2 min was more rapid in the presence than in the absence of divalent ions, X 537A caused virtually identical increments of efflux in the two media at 2 min. At later times the amount released by X 537A if anything is less in divalent ion containing medium. X 537A also caused equivalent release in Mg^{2+}-containing medium (0.65 mM) in the absence of Ca^{2+} with EGTA (2 mM) present to chelate trace Ca^{2+} (data not shown). Even when synaptosomes were homogenized and prepared in the presence of 2 mM EDTA to deplete divalent cations, there was no evidence for Ca^{2+} dependence of the action of X 537A. A 23187, on the other hand, did show Ca^{2+} dependence for its action (Fig. 3). Although a high concentration (1 μM) did cause release of [^{3}H]dopamine in the absence of calcium (with 2 mM EGTA present) the amount released in the presence of 1.5 mM Ca^{2+} was significantly greater. The fractional increment released in 2 min in the presence of divalent ions was 0.456±0.014 as opposed to 0.298±0.031 in the absence of Ca^{2+} with 2 mM EGTA ($n = 4$ per group, $P < 0.001$). The divalent ion specificity of the release caused by A 23187 was examined in more detail (Table II). A lower concentration of iono-

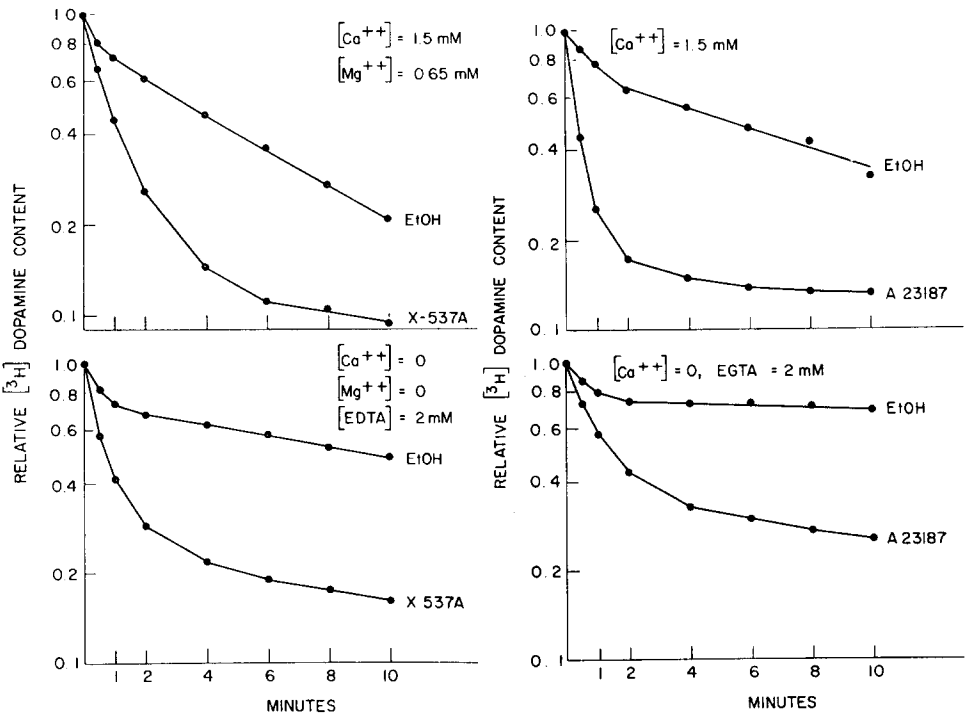

Fig. 3. Divalent ion dependencies of the actions of X 537A and A 23187. Left, X 537A. Synaptosomes containing [^{3}H]dopamine were added to incubation medium in the presence or absence of divalent ions. EDTA was present to chelate trace polyvalent cations. 1-ml aliquots (50 μg protein) were removed at various times and the [^{3}H]dopamine in the synaptosomes was determined as described in Material and Methods. Each point represents duplicate determinations. [X 537A] = 0.1 μM. Right, A 23187. Synaptosomes containing [^{3}H]dopamine were added to incubation medium in the presence or absence of Ca^{2+}. EGTA was present to chelate trace Ca^{2+}. 1-ml aliquots (22 μg protein) were removed at various times and the [^{3}H]dopamine in the synaptosomes was determined as described in Material and Methods. Each point represents duplicate determinations. [A 23187] = 1 μM.

phore (0.1 μM) in the absence of divalent ions caused no additional release over controls by 2 min. Only in the presence of Ca^{2+} did A 23187 cause additional release of [^{3}H]dopamine. Mg^{2+}, Sr^{2+}, Ba^{2+}, and Co^{2+} were not able to substitute for Ca^{2+}.

Mg^{2+} and Co^{2+} antagonize the Ca^{2+}-dependent release of neurotransmitter from the nerve terminal [20, 21]. The ability of various divalent ions to antagonize the release of [^{3}H]dopamine from synaptosomes was investigated in the presence of 5 mM Ca^{2+} (Table III). 10 mM Co^{2+} almost completely prevented the incremental release caused by A 23187.

Analysis of deaminated products in the effluent. Monoamine oxidase, a mitochondrial enzyme, is the major metabolizing enzyme for catecholamines within sympathetic nerve terminals [22]. It acts on cytoplasmic catecholamines but not on catecholamine in storage vesicles. Moss et al. [14] have shown in whole brain synaptosomes that reserpine, which interferes with storage in vesicles, causes release of catecholamine products in which a substantial fraction is deaminated; veratridine, which causes Ca^{2+}-dependent exocytosis of catecholamines [23, 29], causes release

TABLE II

THE EFFECT OF VARIOUS DIVALENT CATIONS ON THE [^{3}H]DOPAMINE EFFLUX INDUCED BY A 23187

Synaptosomes (95 μg protein) were added to 3 ml incubation medium at 37 °C in the presence or absence of drug. The incubation medium contained only the divalent ions indicated. In the first group, additional NaCl was added to keep the osmolality constant. 1-ml aliquots were removed at zero time and at 2 min, filtered and the radioactivity counted as described in Material and Methods. The standard errors in the last column are the square root of the sum of the squares of the standard errors of the control and drug groups. There were four determinations per group except for the groups with additional NaCl or $CaCl_2$ which had seven and eight determinations, respectively.

Added salt	Percentage of [^{3}H]dopamine remaining after 2 min		
	Control	A 23187 ($1 \cdot 10^{-7}$ M)	Drug group minus control group
NaCl (15 mM)	63.5±1.2	61.3±0.7	−2.2±1.4
$MgCl_2$ (10 mM)	60.7±2.7	58.7±1.2	−2.0±3.0
$CaCl_2$ (10 mM)	60.2±2.2	34.1±2.3	−26.1±3.2*
$SrCl_2$ (10 ,mM)	57.0±2.7	53.7±2.1	−3.3±3.4
$BaCl_2$ (10 mM)	58.3±1.7	58.8±1.3	+0.5±2.1
$CoCl_2$ (10 mM)	66.4±1.0	68.1±0.7	+1.7±1.2

* $P < 0.001$ compared to the absence of divalent ions.

TABLE III

EFFECT OF VARIOUS DIVALENT CATIONS ON THE EFFLUX OF [^{3}H]DOPAMINE FROM SYNAPTOSOMES IN THE PRESENCE OF Ca^{2+} AND A 23187

Synaptosomes (40 μg protein) were added to 2.5 ml incubation medium at 37 °C in the presence or absence of drug. The incubation medium contained $CaCl_2$ (5 mM) and other divalent ions as indicated. In the first group, additional NaCl was added to keep the osmolality constant. 1-ml aliquots were removed at zero time and at 2 min, filtered and the radioactivity counted as described in Material and Methods. The results are expressed as the percentage of [^{3}H]dopamine remaining in synaptosomes after 2 min. The standard errors in the last column are the square root of the sum of the squares of the standard errors of the control and drug groups. There were four determinations per group.

Added salt	Control	Antibiotic A 23187 ($1 \cdot 10^{-7}$ M)	Drug group minus control group
NaCl (15 mM)	58.4±1.7	39.9±3.9	−18.5±4.3
$MgCl_2$ (10 mM)	62.1±2.9	51.5±1.7	−10.6±3.4
$SrCl_2$ (10 mM)	52.6±2.0	41.6±1.5	−11.0±2.5
$BaCl_2$ (10 mM)	59.5±3.0	46.6±3.0	−12.9±4.2
$CoCl_2$ (10 mM)	66.2±3.1	64.4±2.1	− 1.8±3.7*

* $P < 0.02$ compared to the group with additional NaCl.

in which a smaller fraction consists of deaminated products. By measuring the fraction of deaminated products of dopamine released by X 537A and A 23187 one can obtain an indication of whether the drugs are causing release from vesicles into the cytoplasm

TABLE IV

EFFECT OF DRUGS ON RELEASE OF [^{3}H]DOPAMINE AND DEAMINATED PRODUCTS

Synaptosomes were prepared from rats not treated with a monoamine oxidase inhibitor. Efflux of ^{3}H from synaptosomes (35 μg protein) preincubated with [^{3}H]dopamine was measured after a 5 min incubation in buffer at 37 °C and analyzed as described in Meterial and Methods. Zero times values of total ^{3}H and ^{3}H-labeled deaminated products were subtracted. Percentages in last column represent fraction of deaminated products in the increment released over controls. There were five determinations per group.

	Total ^{3}H released pmoles/35 μg protein	^{3}H-Labeled deaminated products released pmoles/35 μg protein	^{3}H-Labeled deaminated products % of increment released
Control (1 % ethanol, v/v)	1.64±0.05	0.144±0.016	
X 537A (0.1 μM)	2.09±0.09*	0.234±0.019*	20.1
A 23187 (0.5 μM)	2.39±0.11*	0.241±0.019*	12.9
Veratridine (0.1 mM)	2.25±0.11*	0.225±0.017*	13.3
Reserpine (0.2 μM)	1.99±0.04*	0.247±0.006*	29.4

* $P < 0.01$ compared to control.

and then into the medium or directly from vesicles into the medium. In the present experiments, synaptosomes from rats which were not treated with a monoamine oxidase inhibitor were used. The filtrate from an efflux experiment was analyzed for deaminated products (Table IV). In a parallel experiment (data not shown) [^{3}H]-dopamine added to the incubation medium was not metabolized which indicated that dopamine once released from the synaptosomes was not further deaminated by free mitochondria in the medium. This result allows one to substract the zero time values of total and deaminated catechol from the 5-min values to obtain the amount of the total and deaminated catechol released in 5 min. All the drugs examined caused a significant increase over controls in both the total and deaminated products released. With reserpine, 30 % of the incremental release over controls was deaminated (dimethylsulfoxide, the solvent for reserpine, did not affect efflux), whereas, with veratridine, only 13 % was deaminated. X 538A gave a fractional release of deaminated products that was intermediate between reserpine and veratridine which suggests that X 537A causes, at least in part, a net efflux of dopamine from vesicles into the cytoplasm before the dopamine leaves the synaptosome. In contrast, the fraction of deaminated products in the effluent released by A 23187 was identical to the fraction of deaminated products released by veratridine, a compound that causes exocytotic release. Hence, the data are consistent with the possibility that A 23187 causes exocytotic release as is suggested by the Ca^{2+} dependence.

Effects on the uptake of [^{3}H]dopamine and the release of recently taken up dopamine. The uptake of [^{3}H]dopamine into synaptosomes from rat striatum involves a saturable high affinity uptake system [11, 24]. To investigate whether either X 537A

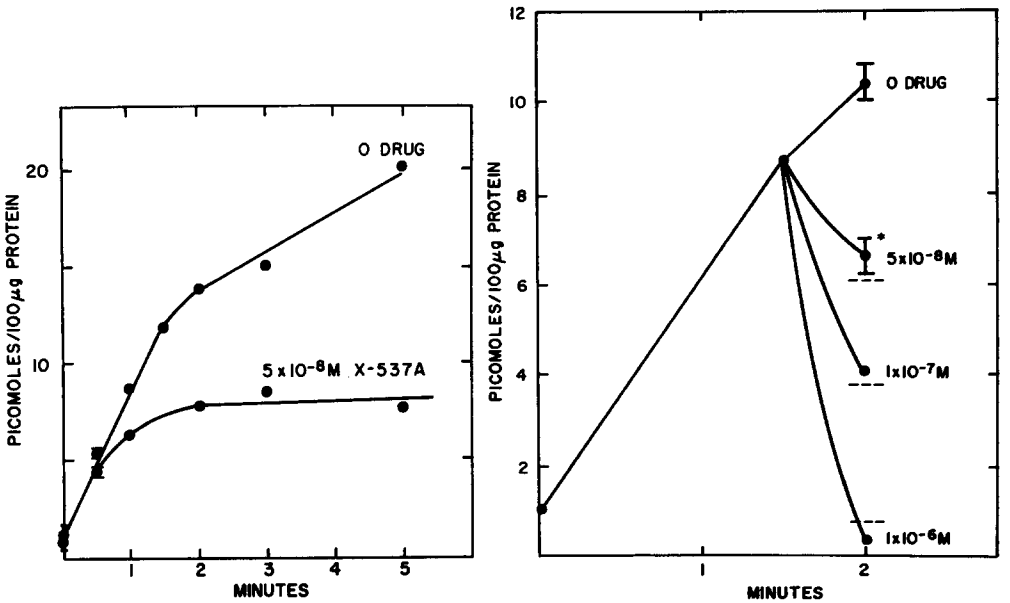

Fig. 4. Left, The effects of X 537A on the uptake of [^{3}H]dopamine. Synaptosomes were preincubated for 2 min in the presence or absence of drug before the addition of [^{3}H]dopamine (0.1 μM). Right, Release of recently taken up [^{3}H]dopamine by X 537A. X 537A was added at 1.5 min during a 2 min incubation with [^{3}H]dopamine (0.1 μM). The dashed lines at 2 min represent the amount of [^{3}H]-dopamine taken up when synaptosomes were preincubated in the presence of drug before a 2 min incubation with [^{3}H]dopamine. The incubation medium contained EDTA (2 mM) and no divalent ions. Each point represents 2–4 determinations with 75 μg synaptosomal protein per determination. $\star P < 0.001$ compared to 0 drug at 2 min.

or A 23187 affect the uptake of [^{3}H]dopamine, synaptosomes were incubated in 0.1 μM [^{3}H]dopamine in the presence or absence of ionophore. In the absence of ionophore, uptake was linear for 1.5 min and continued throughout the 5 min incubation. X 537A ($5 \cdot 10^{-8}$ M) had little effect on the initial uptake at 30 s but caused uptake to level off by 1–2 min (Fig. 4, left). The effect of X 537A was also examined when the ionophore was added at 1.5 min during a 2 min incubation with [^{3}H]dopamine (Fig. 4, right). X 537A caused a net efflux even in the presence of [^{3}H]dopamine (0.1 μM) in the medium. The [^{3}H]dopamine content finally attained at 2 min in the presence of drug is approximately the same whether the drug is added at the beginning (dashed lines in Fig. 4, right) or at 1.5 min in a 2 min incubation. The steady-state level depends upon the balance between the normal uptake mechanism and efflux induced by X 537A. A 23187 (0.1 μM) also had little effect on the initial rate of uptake and was able to cause release of recently taken up [^{3}H]dopamine (Fig. 5). The release of [^{3}H]dopamine in the presence of [^{3}H]dopamine (0.1 μM) in the medium by both X 537A and A 23187 is further evidence that these compounds cause a net efflux rather than a simple exchange diffusion since the specific activity of [^{3}H]dopamine must be higher in the medium than in the synaptosomes.

The effects of X 537A are reversible. When synaptosomes are incubated in $1.5 \cdot 10^{-7}$ M X 537A and then diluted to $0.75 \cdot 10^{-7}$ M with ionophore-free buffer, the

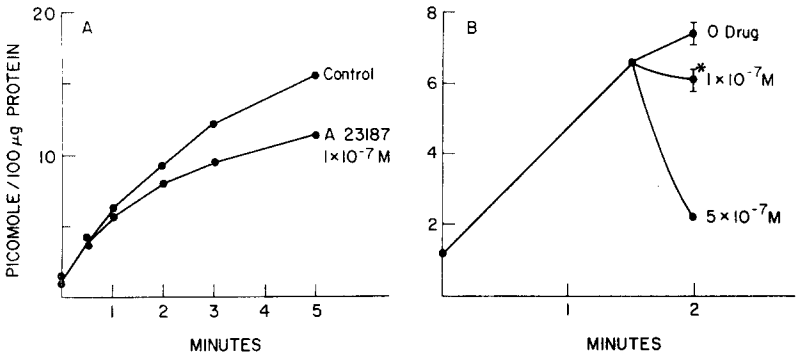

Fig. 5. (A) The effect of A 23187 on the uptake of [^{3}H]dopamine. Synaptosomes were preincubated for 2 min in normal medium in the presence or absence of drug before the addition of [^{3}H]dopamine (0.1 μM). (B) Release of recently taken up [^{3}H]dopamine by A 23187. A 23187 was added at 1.5 min during a 2 min incubation with [^{3}H]dopamine (0.1 μM) in normal incubation medium. Each point represents 2–4 determinations with 69 μg protein per determination. *$P < 0.05$ compared to 0 drug at two min.

steady-state level attained upon addition of [^{3}H]dopamine was identical to that attained with continuous incubation in $0.75 \cdot 10^{-7}$ M X 537A (data not shown).

Effects of X 537A and A 23187 on release of catecholamine from rat chromaffin granules. Because X 537A and A 23187 may be acting on nerve terminal storage vesicles, the effect of these drugs was also examined on chromaffin granules from rat adrenal. Chromaffin granules in chromaffin cells in the adrenal medulla are similar although not identical to storage vesicles in sympathetic nerves [25]. The uptake of [^{3}H]dopamine into the resuspended adrenal P_2 fraction occurred only in the presence of ATP (5 mM) and $MgCl_2$ (5 mM) and was blocked completely by reserpine (1 μM) (data not shown). Since catecholamine uptake by chromaffin granules requires ATP and is blocked by reserpine [26], these results suggest that dopamine entered the chromaffin granules. Dopamine may have been metabolized by dopamine-β-hydroxylase (3,4-dihydroxyphenylethylamine; ascorbate: oxygen oxidoreductase, EC 1.14.17.1) to norepinephrine within the granule. However, analysis of the radioactivity by cation exchange resin chromatography [27] revealed that over 95 % of the radioactivity taken up remained as dopamine. X 537A, 0.1 μM, caused a rapid efflux of radioactivity whereas A 23187 caused an equivalent efflux only at a 100-fold greater concentration (Table V). Reserpine at a concentration that completely inhibited uptake into the granules had no effect on efflux. The concentrations of X 537A and A 23187 indicated in Table V represent the amounts added two the medium. Because both compounds are lipophilic, the compounds may have partitioned into the lipid present; thus, the concentrations indicated may not represent true solution concentrations. However, there is a glaring discrepancy between the relative effectiveness of the drugs in granules as compared to synaptosomes. In synaptosomes the drugs were equipotent in Ca^{2+}-containing medium. The results suggest that in the synaptosome experiments at low ionophore concentrations, the primary effect of X 537A was to cause efflux from intrasynaptosomal vesicles whereas the primary effect of A 23187 was not directly on the vesicles.

Bulk phase partition experiments. X 537A causes norepinephrine and epinephrine as well as divalent ions to partition from an aqueous phase into toluene–

TABLE V

IONOPHORE-INDUCED RELEASE OF [^{3}H] DOPAMINE FROM CHROMAFFIN GRANULES

A suspension of chromaffin granules that had been incubated in [^{3}H]dopamine was added to an incubation medium consisting of 0.3 M sucrose, 25 mM Tris–HCl, pH 7.1 at 37 °C in the presence or absence of drug. At zero time and at 10 min 1-ml aliquots containing granules from 0.3 adrenal glands were removed, cooled centrifuged and the radioactivity in the pellets counted as described in Material and Methods. The results are expressed as the percentage of the zero time controls, which contained 116 pmoles [^{3}H]dopamine.

	Percentage remaining in chromaffin granules after 10 min
Control	54.3±1.7
X 537A (0.1 μM)	12.9±0.9*
X 537A (1.0 μM)	6.9±0.9*
A 23187 (0.1 μM)	58.6±0.9
A 23187 (1.0 μM)	32.8±0.9*
A 23187 (10 μM)	13.8±0.9*
Reserpine (1 μM)	57.8±1.7

* $P < 0.001$ compared to controls at 10 min.

TABLE VI

EFFECT OF IONOPHORES ON THE PARTITIONING OF [^{3}H]DOPAMINE INTO AN ORGANIC PHASE

Partition experiments were performed as described in Material and Methods. The aqueous phase (1 ml) contained [^{3}H]dopamine (10 μM), triethanolamine (10 mM), pH 7.4 and additions as indicated. The organic phase (1 ml) was toluene–butanol (70:30, v/v) with or without ionophore. There were four samples per group.

Additions to aqueous phase	Organic phase	Percent of total dopamine in organic phase
– (Control)	–	0.70±0.07
KCNS (20 mM)	–	0.78±0.02
–	X 537A ($5 \cdot 10^{-5}$ M)	26.2±0.02**
–	A 23187 ($5 \cdot 10^{-5}$ M)	0.73±0.03
–	A 23187 ($5 \cdot 10^{-4}$ M)	1.59±0.16*

* $P < 0.01$ compound to control.
** $P < 0.001$ compound to control.

butanol (70:30, v/v) [3]; A 23187 causes divalent ions to partition from an aqueous phase into toluene–butanol (70:30, v/v) [5]. These partition experiments suggest ionophoric capabilities for these compounds. To determine the ionophoric possibilities with dopamine, bulk phase partition experiments were performed with [^{3}H]dopamine (10 μM) in the aqueous phase (Table VI). In the absence of ionophore virtually no dopamine partitioned into the organic phase. X 537A (50 μM) but not A 23187 (50 μM) drew [^{3}H]dopamine into the organic phase. Even when the concentration of A 23187 was increased 10-fold, the amount of dopamine that partitioned into the organic phase was just two times that of the control. Thus, it is possible for X 537A but unlikely for A 23187 to act as a dopamine ionophore. X 537A may act as a lipid-soluble anion that forms an ion pair with the positively charged dopamine in the organic phase. Because another lipid-soluble anion, thiocyanate, did not cause dopamine to partition into the organic phase, the interaction of X 537A and dopamine in the organic phase appears to be more specific than ion pair formation.

Co^{2+} antagonized the effect of Ca^{2+} in inducing the A 23187 dependent [^{3}H]-dopamine release. One possible explanation is that Co^{2+} competes with Ca^{2+} for free A 23187 in the membrane. To investigate the possibility of such a competition, bulk phase partition experiments with $^{45}CaCl_2$ (50 μM) in the aqueous phase were performed. Without ionophore only 0.11 ± 0.04 % ($n = 4$) appeared in the organic phase. With A 23187 (50 μM) in the organic phase, 5.11 ± 0.14 % ($n = 4$) of the Ca^{2+} appeared in the organic phase. Co Cl_2 (50 μM) reduced the partitioning in the presence of A 23187 to 0.38 ± 0.03 % ($n = 4$, $P < 0.001$ compared to the absence of $CoCl_2$). $MgCl_2$ and $BaCl_2$ (50 μM) did not interfere with the A 23187 induced partitioning of $^{45}Ca^{2+}$ into the organic phase. Hence the effect of Co^{2+} to inhibit the Ca^{2+}-dependent, A 23187 induced efflux of [^{3}H]dopamine from synaptosomes could be explained by competition between Co^{2+} and Ca^{2+} for the ionophore.

DISCUSSION

Two ionophoric compounds X 537A and A 23187 at less than micromolar concentrations induced an efflux of [^{3}H]dopamine and endogenous dopamine from synaptosomes prepared from rat corpus striatum. The mode of action of these compounds appears to be different. X 537A acted independently of divalent ions in the medium and caused release of deaminated metabolites comparable to that observed with reserpine. These data suggest that X 537A caused a net efflux of dopamine from the intrasynaptosomal storage vesicles into the cytoplasm, where a fraction of the dopamine was acted upon by monoamine oxidase. Dopamine and its metabolites then passed through the neuronal membrane into the medium. Consistent with this proposal is the finding that X 537A (0.1 μM) also caused release of [^{3}H]dopamine from chromaffin granules. (Antibiotic X 537A also causes an efflux of [^{3}H]epinephrine and endogenous epinephrine from bovine adrenal chromaffin granules (Holz, R. W. and Brownstein, M., unpublished observations.) The effect of X 537A on the shape of the [^{3}H]dopamine efflux curves from synaptosomes also supports this conclusion. In the control curves it is tempting to attribute the fast initial phase to efflux of dopamine from the cytoplasm and the later slower phase to efflux from the vesicle, either directly into the medium or the first into cytoplasm and then rapidly across the neuronal medium. That the slow phase may be associated with vesicular

release is suggested by the observation that Ca^{2+} increased the rate of the slow phase efflux. Qualitatively, X 537A acted rapidly to reduce the fraction of [^{3}H]dopamine in the slow phase, presumably in the vesicles. If one extrapolates the slow phase to earlier times and subtracts the extrapolated values from the rapid initial efflux, one finds in the absence and at the two lower concentrations of ionophore exponential declines within the first minute with virtually equal half times ($t_{\frac{1}{2}} = 0.4$–0.5 min). X 537A had its major effect in reducing the fraction in the slow phase and in increasing the rate of efflux in the slow phase. These experiments, coupled with the finding that X 537A solubilized dopamine in an organic phase, suggest that X 537A acted as a catecholamine carrier which transported dopamine from inside the storage vesicle outward across the vesicular membrane*,**. Once in the cytoplasm, the amine rapidly crossed the neuronal membrane perhaps by utilizing the intrinsic catecholamine transport system.

A 23187 (0.1 μM) showed an absolute Ca^{2+} dependence for its releasing action during a 2 min efflux; Mg^{2+}, Sr^{2+}, Ba^{2+} and Co^{2+} could not substitute for Ca^{2+}. The fraction of deaminated products in the A 23187 induced release equaled the fraction in the veratridine induced release. Veratridine causes Ca^{2+}-dependent exocytotic release from sympathetic nerves [23, 29]. Since A 23187 can transport Ca^{2+} across biological membranes, the results in the present experiments suggest that A 23187 acted at least in part by transporting Ca^{2+} from the medium into the cytoplasm of synaptosomes. Ca^{2+} then initiated release, presumably by exocytosis. (The effects of X 537A and A 23187 on the release of norepinephrine from peripheral sympathetic nerves have recently been examined and the results are consistent with the present study [28].) Consistent with this interpretation for an indirect action of A 23187 was its striking inability, compared to antibiotic X 537A, to directly release catecholamine from chromaffin granules, although the drugs were approximately equipotent in causing release from synaptosomes. Finally, because antibiotic A 23187 was unable to solubilize dopamine into an organic phase, it is unlikely that it is itself an ionophore for dopamine.

At higher concentrations (0.5–1.0 μM) antibiotic A 23187 caused release of [^{3}H]dopamine that persisted in the absence of Ca^{2+}. Even in the presence of 2 mM EDTA at times later than 2 min, there was an increased efflux in the presence of 1 μM of A 23187 (data not shown). High concentrations of A 23187 may release Ca^{2+} from intrasynaptosomal mitochondria or other intrasynaptosomal stores of calcium. Indeed, A 23187 does release Ca^{2+} from mitochondria in vitro [5]. It should be noted that the release induced by A 23187 is the first demonstration of Ca^{2+}-dependent release from dopaminergic terminals.

These experiments give one an indication of the time course of the storage of dopamine into intrasynaptosomal storage vesicles after the dopamine is taken up by

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* It is likely that both X 537A and A 23187 transport ions as a neutral complex in an electrically silent manner. In order for a transport cycle to be completed with either ionophore, the ionophore complexed to another cation or assicoated with H^+ must diffuse back in the direction opposite to the initial transport step and release the cation. Hence, there are also possibilities for concentration changes of other cations including H^+.

** Recent work on artificial bilayers makes this suggestion very plausible. X 537A enormously increases the permeability of bilayers to [^{14}C]dopamine [Kafka, M. S. and Holz, R. W., in preparation].

the synaptosome. Although X 537A acts immediately to induce efflux, X 537A had no effect on the uptake of [^{3}H]dopamine into synaptosomes in the first 30 s of an incubation with [^{3}H]dopamine. However, by 1 min the uptake attained a steady-state value, whereas the control continued to take up [^{3}H]dopamine. The evidence suggests that the steady state is a result of a balance of an unperturbed uptake system with an increased efflux induced by X 537A. If the ionophore, indeed, acts primarily to increase efflux from the storage vesicle, then the absence of effects in the first 30 s suggests that the [^{3}H]dopamine does not enter the storage vesicle within this period.

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