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EFFECT OF METABOLIC INHIBITORS AND OUABAIN ON AMPHETAMINE- AND POTASSIUM-INDUCED RELEASE OF BIOGENIC AMINES FROM ISOLATED BRAIN TISSUE*

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Abstract—In experiments on isolated rat cerebral cortex, it was observed that *d*-amphetamine released norepinephrine from cerebral cortex into the incubation medium. Amphetamine-induced release of norepinephrine was markedly potentiated in the presence of the metabolic inhibitors sodium cyanide, 2,4-dinitrophenol and iodoacetate. Release of norepinephrine by the metabolic inhibitors in the absence of amphetamine was minimal. Amphetamine-induced release of dopamine from corpus striatum was also potentiated by sodium cyanide. The EC_{50} for amphetamine-induced release of dopamine was 20 μM with amphetamine alone and 0.58 μM in the presence of sodium cyanide. Release of norepinephrine and dopamine by elevated concentrations of potassium chloride was again potentiated by the metabolic inhibitors. Similarly, ouabain (10–25 μM) produced minimal release of norepinephrine but potentiated amphetamine- and potassium-induced release of catecholamines. The metabolic inhibitors markedly reduced the ATP content of the chopped tissue during the incubation while ouabain had no effect. The results suggest that the potentiation of amphetamine- and potassium-induced release of biogenic amines from isolated brain tissue is not simply due to depletion of tissue ATP levels but may be related to sodium or potassium transport. The Na^+, K^+-ATPase sodium pump is inhibited either: (1) when ATP levels are reduced by metabolic inhibitors, or (2) when Na^+, K^+-ATPase is inhibited by ouabain. Inhibition of the sodium pump would lead to an increase in the intraneuronal sodium concentration. A model is described in which norepinephrine and sodium are co-transported across the membrane during amphetamine-induced release of the biogenic amine. Elevation of the intraneuronal sodium concentration would provide increased levels of the co-substrate for transport and could account for the potentiation of release produced by metabolic inhibitors and ouabain.

The role of ATP in the release of biogenic amines is complex and not well understood. Reduction in ATP content by metabolic inhibitors has been used to determine ATP requirements for release of catecholamines. ATP is apparently required for release of catecholamines from the adrenal gland by acetylcholine and potassium chloride since metabolic inhibitors markedly reduce release of catecholamines in this system [1, 2]. On the other hand, metabolic inhibitors enhance nerve-stimulated release of norepinephrine from sympathetic nerves [3, 4]. Since ATP is required for the extrusion of sodium from the nerve ending, it has been suggested that the augmented release is due to enhanced intraneuronal sodium, which in turn results in increased intraneuronal calcium [3, 4]. Since calcium is required for stimulus secretion coupling, the increased intraneuronal calcium could potentiate release by nerve stimulation.

Although release of norepinephrine and dopamine by amphetamine in central nervous system (CNS) nerve endings is well known [5–8], the effect of metabolic inhibitors on amphetamine-induced release has not been studied. The present study was designed to determine whether release of catecholamines by amphetamine was altered either when ATP production was inhibited by metabolic inhibitors or when ATP utilization by Na^+, K^+-ATPase was inhibited by ouabain.

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METHODS

Release of biogenic amines by d*-amphetamine and potassium.* The procedure for studying release of biogenic amines has been previously described [7, 8]. The method involved incubating chopped brain tissue with 1.0 μM solutions of either [^{3}H]-*dl*-norepinephrine (8 μCi) or [^{3}H]dopamine (1.0 μCi). The [^{3}H]amine was accumulated within the nerve endings, the unbound and nonspecifically bound [^{3}H]amine was washed from the tissue, and the [^{3}H]amine released into the incubation medium after a 30-min incubation with *d*-amphetamine or potassium chloride was measured. It has been previously demonstrated that release of [^{3}H]norepinephrine by amphetamine is linear for 30 min [9]. [^{3}H]catecholamines in the tissue and medium fractions were separated from ^{3}H-deaminated metabolites by cation exchange chromatography on Dowex 50, Na^+ [10].

The protein content per sample was determined by the Biuret method [11] and both medium and tissue

radioactivities were based on the amount of tissue (mg protein) present in the sample. The results were generally expressed as a percentage of [^{3}H]amine in the incubation medium calculated as:

$$\frac{[^3\text{H}]\text{amine in medium} \times 100}{([^3\text{H}]\text{amine in medium}) + ([^3\text{H}]\text{amine in tissue})}$$

The EC_{50} (median effective concentration) for release was estimated as the concentration of drug which gave half the maximal response (adjusted for "spontaneous release"). Statistical comparisons were made by Student's *t*-test.

Measurement of tissue ATP content. Samples of chopped cerebral cortex were incubated as in the release experiments described above. At the end of the final incubation the tissue was centrifuged and 1 ml of 0.4 M $HClO_4$ (4°) was added to the pellet in each tube. The tissue was homogenized and the homogenate centrifuged at 10,000 *g* for 10 min. ATP was measured immediately in the supernatant by the luciferin–luciferase fluorescence assay using a Beckman-LS-100 liquid scintillation spectrometer to measure the resulting fluorescence [12].

Substances. d-Amphetamine sulfate was obtained from Smith, Kline & French Laboratories, Philadelphia, PA. [^{3}H]*dl*-norepinephrine[7-^{3}H]hydrochloride (7.7 to 13 Ci/m-mole) and [^{3}H]dopamine hydrochloride (0.5 Ci/m-mole) were obtained from Amersham/Searle Corp., Arlington Heights, IL. The luciferase enzyme was isolated from desiccated firefly lanterns obtained from Sigma Chemical Co., St. Louis, MO. Adenosine 5'-triphosphate disodium salt (ATP), ouabain octahydrate (strophanthin-G), 2,4-dinitrophenol and iodoacetic acid were also obtained from Sigma Chemical Co. Sodium cyanide was obtained from Mallinckrodt Chemical Works, St. Louis, MO.

RESULTS

Effect of metabolic inhibitors on release of [^{3}H]catecholamines from chopped cerebral cortex by amphetamine and potassium chloride. The three metabolic inhibitors, sodium cyanide, dinitrophenol and iodoacetate, had no effect on the release of [^{3}H]norepinephrine during a 30-min incubation (Fig. 1). The addition of either 1 μM amphetamine or potassium chloride leads to a significant increase in the amount of [^{3}H]norepinephrine released from nerve endings in cerebral cortex. This release was markedly potentiated by all three metabolic inhibitors. When concentration effect curves were determined for amphetamine with each of the metabolic inhibitors, it was observed that both the maximal effect as well as the potency of amphetamine was increased by each of the inhibitors (Figs. 2–4). Similar results were observed when the experiments were conducted in a physiological salt solution with calcium omitted from the media (Fig. 5). When the release of [^{3}H]dopamine from corpus striatum was studied, it was observed that sodium cyanide markedly potentiated the release of [^{3}H]DA by amphetamine but the maximal effect was unchanged (Fig. 6). The release of [^{3}H]norepinephrine by amphetamine and potassium chloride was directly proportional to release of total tritium in all experiments.

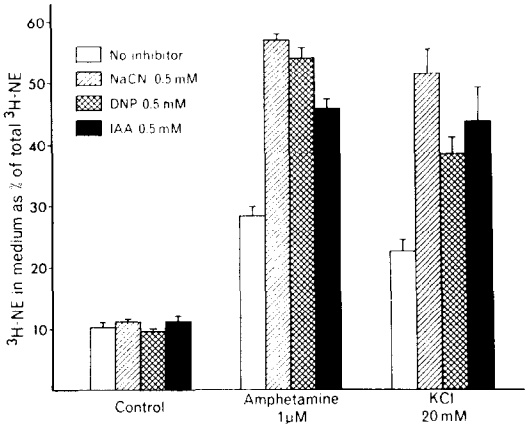

Fig. 1. Potentiation of amphetamine- and potassium-induced release of [^{3}H]norepinephrine by metabolic inhibitors. Chopped cerebral cortex tissue which had accumulated [^{3}H]norepinephrine ([^{3}H]NE) was incubated for 10 min with either 1 μM amphetamine or 20 mM KCl. In some experiments, either sodium cyanide (NaCN), dinitrophenol (DNP) or iodoacetic acid (IAA) was also present during the 30-min incubation. Total tritium in the tissue and medium was 99.5 ± 3.6 × 10^3 dis./min/mg of protein. Each value represents the mean ± S.E.M. of four to eight experiments.

Effect of metabolic inhibitors on ATP content of chopped cerebral cortex. Cerebral cortex was incubated with physiological salt solution as described in the release experiments. The ATP content of the tissue was determined immediately after the end of the 30-min incubation and it was found that all three metabolic inhibitors reduced the content of ATP (Fig. 7). Of the three metabolic inhibitors, iodoacetate was the most effective in reducing the ATP content. KCl (20 mM) reduced ATP content in the absence and presence of each of the metabolic inhibitors.

Effect of ouabain on release of [^{3}H]catecholamines and on ATP content in chopped cerebral cortex. Ouabain (10 and 25 μM) caused a slight release of [^{3}H]norepinephrine from chopped cerebral cortex tissue. However, both concentrations markedly potentiated the release of [^{3}H]norepinephrine by 1.0 μM amphetamine and by 20 mM KCl (Fig. 8).

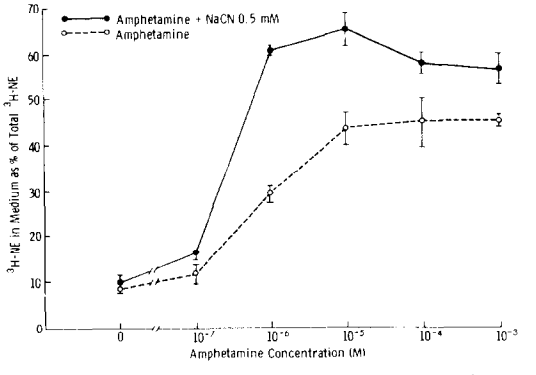

Fig. 2. Potentiation of amphetamine-induced release of [^{3}H]norepinephrine from cerebral cortex by 0.5 mM sodium cyanide (NaCN). Total tritium in the tissue and medium was 88.4 ± 1.2 × 10^3 dis./min/mg of protein. Each value represents the mean ± S. E. M. of three experiments.

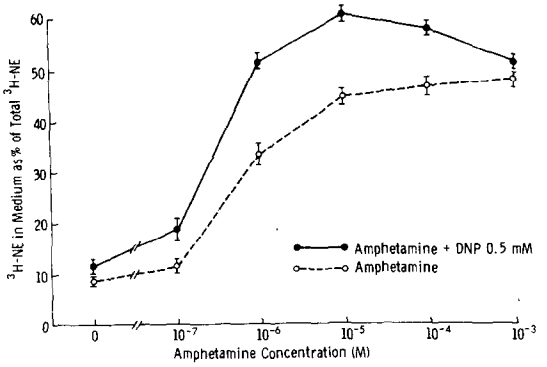

Fig. 3. Potentiation of amphetamine-induced release of [^{3}H]norepinephrine from cerebral cortex by 0.5 mM dinitrophenol (DNP). Total tritium in the tissue and medium was 88.3 ± 1.2 × 10^3 dis./min/mg of protein. Each value represents the mean ± S. E. M. of three experiments.

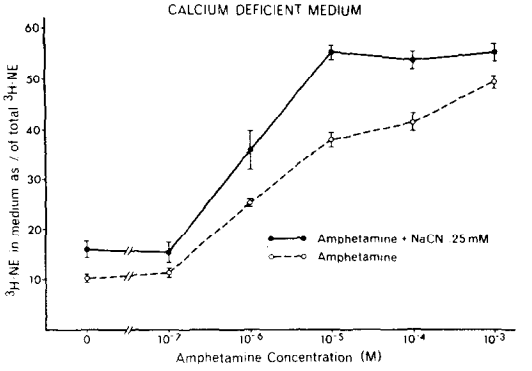

Fig. 5. Potentiation of amphetamine-induced release of [^{3}H]norepinephrine from cerebral cortex by 0.25 mM sodium cyanide in a calcium-free medium. Calcium was omitted from the incubation medium throughout the entire experiment. Total tritium in the tissue and medium was 65.4 ± 1.7 × 10^3 dis./min/mg of protein. Each value represents the mean ± S. E. M. of four to eight experiments.

When concentration–effect curves were determined for release of norepinephrine by amphetamine in the presence and absence of 25 μM ouabain, it was observed that both the maximal effect and the potency of amphetamine were increased by ouabain (Fig. 9). In the case of release of [^{3}H]dopamine from corpus striatum, the concentration of ouabain was reduced to 1 μM to minimize release of [^{3}H]dopamine by ouabain. At this concentration, ouabain increased the potency but had no effect on maximal release of [^{3}H]dopamine by amphetamine (Fig. 10). It should be noted that at a high concentration of amphetamine (1 mM), most of the [^{3}H]dopamine was released from the tissue into the incubation medium. Unlike the metabolic inhibitors, ouabain had no effect on the content of ATP in the isolated tissue (Fig. 11).

DISCUSSION

Although the precise mechanism by which metabolic inhibitors and ouabain potentiate release of biogenic amines is not known, it is likely that it is related to the intraneuronal concentration of sodium. It is well known that norepinephrine is transported into nerve endings by a process which has an absolute requirement for sodium [13–15]. It has been suggested that norepinephrine and sodium are co-transported by a membrane carrier [13, 15] in a manner analogous to the transport of glucose and sodium in the intestine [16, 17]. There is recent evidence that amphetamine also enters the nerve ending by a sodium-dependent carrier mechanism [18, 19]. Upon entering the nerve ending, sympathomimetic amines are thought to displace norepinephrine from intraneuronal binding sites and the displaced amine is then released by a reversal of the carrier-mediated transport system [20, 21]. This is represented diagrammatically in Fig. 12. Thus, the efflux of norepinephrine would also be sodium dependent and the rate of efflux would be a function of: (1) the norepinephrine gradient across the membrane, (2) the sodium

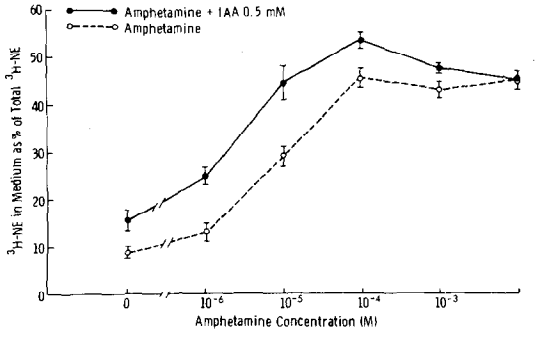

Fig. 4. Potentiation of amphetamine-induced release of [^{3}H]norepinephrine from cerebral cortex by 0.5 mM iodoacetic acid (IAA). Total tritium in the tissue and medium was 75.7 ± 2.1 × 10^3 dis./min/mg of protein. Each value represents the mean ± S. E. M. of four experiments.

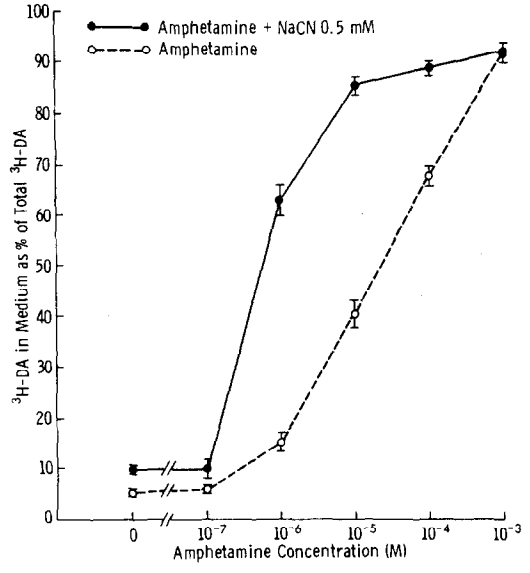

Fig. 6. Potentiation of amphetamine-induced release of [^{3}H]dopamine from corpus striatum by 0.5 mM sodium cyanide. Total tritium in the tissue and medium was 236.4 ± 6.5 × 10^3 dis./min/mg of protein. Each value represents the mean ± S. E. M. of four experiments.

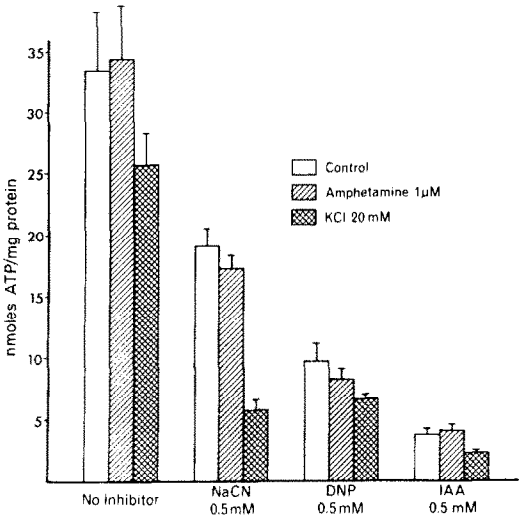

Fig. 7. Effect of amphetamine and potassium chloride in the presence and absence of the metabolic inhibitors, sodium cyanide (NaCN), dinitrophenol (DNP) and iodoacetate (IAA) on ATP content of chopped cerebral cortex. The tissue was incubated as described for release, and ATP was measured immediately after the incubation. Each value is the mean ± S. E. M. of four experiments.

gradient across the membrane and (3) the availability of the carrier on the inside of the membrane.

Sodium concentration inside the nerve membrane is normally maintained at a low level by Na^+, K^+ ATPase which is coupled to the transport of sodium out of the nerve ending and the transport of potassium into the nerve ending. ATP inside the nerve ending is required for the functioning of this transport process.

Using this model, it is possible to explain the marked potentiation of amphetamine-induced release of norepinephrine by metabolic inhibitors at concentrations of the inhibitors which did not markedly increase the efflux of amines. It is well known that sodium cyanide inhibits cytochrome oxidase [22], 2,4-dinitrophenol uncouples oxidative phosphorylation [23], and iodoacetate inhibits glycolysis by inhibition of glyceraldehyde-3-phosphate dehydrogenase [24]. All three of these substances markedly reduced the ATP content of the chopped tissue presumably by inhibition of glucose metabolism. Since all three

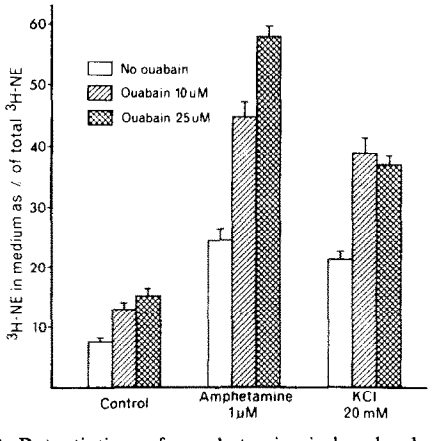

Fig. 8. Potentiation of amphetamine-induced release of [^{3}H]norepinephrine from cerebral cortex by ouabain. Total tritium in the tissue and medium was 85.0 ± 2.1 × 10^3 dis./min/mg of protein. Each value represents the mean of four experiments.

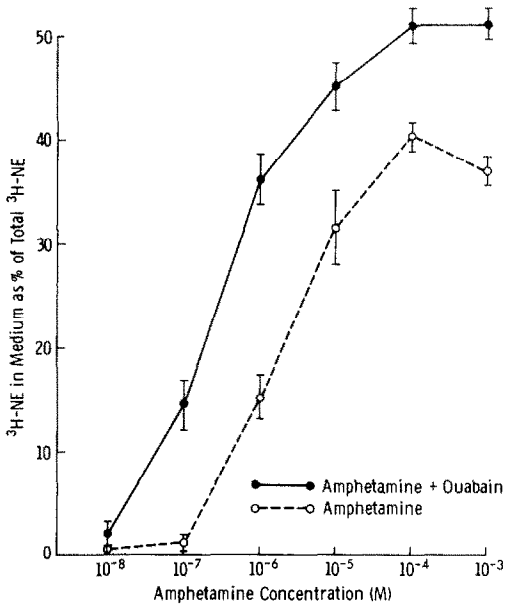

Fig. 9. Potentiation of amphetamine-induced release of [^{3}H]norepinephrine from cerebral cortex by 25 μM ouabain. Basal release was subtracted from each of the amphetamine values and was 9.0 ± 1.5 per cent for control and 14.0 ± 1.3 per cent for 25 μM ouabain. Total tritium in the tissue and medium was 149.5 ± 5.2 × 10^3 dis./min/mg of protein. Each value is the mean ± S. E. M. of four experiments.

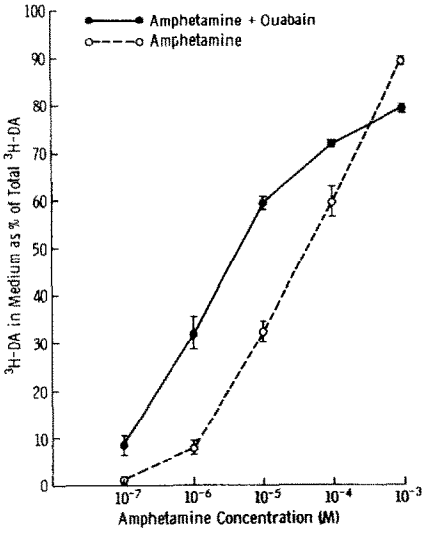

Fig. 10. Potentiation of amphetamine-induced release of [^{3}H]dopamine from corpus striatum by 1 μM ouabain. Basal release was subtracted from each of the amphetamine values and was 4.0 ± 2.2 per cent for control and 13.4 ± 2.2 per cent for 1 μM ouabain. Total tritium in the tissue and medium was 615 ± 12 × 10^3 dis./min/mg of protein. Each value is the mean ± S. E. M. of four experiments.

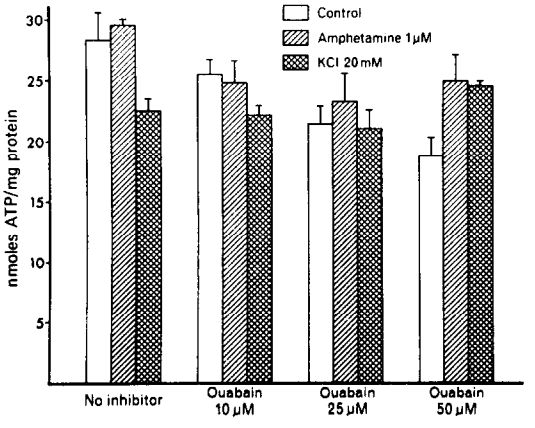

Fig. 11. Effect of amphetamine and potassium chloride in the presence and absence of ouabain on ATP content of chopped cerebral cortex. The tissue was incubated as described for the release experiments, and ATP was measured immediately after the incubation. Each value is the mean of four experiments.

inhibitors were used at maximally effective concentrations [22–24], the greater inhibition of glycolysis by iodoacetate compared to inhibition of oxidative metabolism by cyanide and 2,4-dinitrophenol suggests that anaerobic metabolism is a more important source of ATP under the conditions of this study.

Reduction in the content of ATP at the site of Na^+, K^+-ATPase would result in inhibition of the enzyme with a consequent inhibition of sodium transport out of the nerve ending and potassium transport into the nerve ending [25]. This would lead to an increase in intraneuronal Na^+ and a decrease in intraneuronal

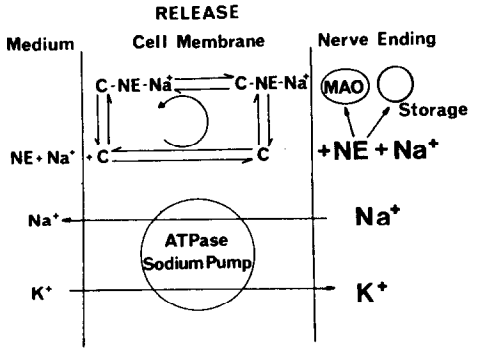

Fig. 12. Diagram of a postulated mechanism by which norepinephrine is released from nerve endings. Neuronal uptake occurs when the external sodium and norepinephrine concentration are high relative to the respective free intraneuronal concentrations. Neuronal uptake also occurs when the carrier is facing the outside of the nerve ending. When the following occurs, there is an increased probability that the carrier would mediate the efflux rather than the influx of biogenic amines: (1) amphetamine is transported into the nerve ending and as a result the carrier is on the inside of the membrane, (2) amphetamine displaces norepinephrine from binding sites and the norepinephrine is now available to bind to the carrier, and (3) intraneuronal sodium is increased by inhibition of the sodium pump coupled to Na^+-K^+-ATPase. This could occur either by inhibiting the formation of ATP by metabolic inhibitors or by inhibition of Na^+, K^+ ATPase by ouabain.

K^+. This action alone would not markedly increase the rate of efflux of the biogenic amine. However, if amphetamine entered the neuron by the neuronal carrier and displaced norepinephrine from intraneuronal binding sites, an increase in intraneuronal sodium would be expected to potentiate the releasing effect of amphetamine. This model assumes that, in the presence of amphetamine, the low intraneuronal sodium concentration is the rate-limiting factor for the efflux of biogenic amines.

It is possible that reduced levels of ATP result in potentiation of the release of catecholamines by an action not related to Na^+, K^+-ATPase and therefore the effects of ouabain were measured at concentrations which inhibited Na^+, K^+-ATPase [26] but did not reduce the ATP content. Ouabain potentiated amphetamine-induced release of both norepinephrine and dopamine. It has been shown in isolated synaptosomes that 10 μM ouabain increases the sodium content and decreases the potassium content [27]. This is consistent with inhibition of Na^+, K^+-ATPase by ouabain and resultant inhibition of sodium efflux and potassium influx [28]. The effects of ouabain are consistent with the model in Fig. 12 and suggest that elevation of sodium within the nerve ending by direct inhibition of Na^+, K^+ ATPase leads to the observed potentiation. Attempts were made to further test this hypothesis by reducing the external sodium concentration. Lowering the sodium concentration from 143 to 50 mM resulted in a marked increase in efflux of [^{3}H]norepinephrine. It is known that reducing Na^+ to 0 in rat heart slices markedly accelerates efflux of norepinephrine [13]. This would be consistent with the model if reduction of external sodium concentration also leads to an increased efflux of norepinephrine from storage granules.

Another possible explanation for potentiation of amphetamine-induced release is that vesicular storage of [^{3}H]norepinephrine could be inhibited by a reduction in the ATP content in the nerve endings. Although this may be a contributing factor, it is known that amphetamine is capable of releasing [^{3}H]norepinephrine from reserpine-insensitive sites under conditions similar to those in the present study [28]. In addition, inhibition of storage is probably not the only mechanism for the observed potentiation since there is little evidence that ouabain has a marked effect on storage of catecholamines.

Potentiation of K^+-induced release as observed in this study and in previous studies [3, 4] may be related to the influx of calcium which occurs in the presence of increased intraneuronal sodium. Increased intracellular sodium has been shown to stimulate calcium influx in the squid axon [29]. The potentiation of release of catecholamines by carbachol produced by ouabain in the perfused adrenal gland was a function of the calcium concentration in the perfusing medium [30]. This mechanism is probably not involved in potentiation of amphetamine-induced release since the release of norepinephrine by amphetamine occurs in calcium-free medium [7] and since the potentiation of amphetamine-induced release occurred even in the absence of calcium in the incubation medium.

This potentiated efflux of amphetamine-induced release is not restricted to release of [^{3}H]norepine-

phrine. The potentiated release of [^{3}H]dopamine is even more striking. In unreported experiments we have also observed potentiation of release of [^{3}H]5-hydroxytryptamine by sodium cyanide. Much less is known about carrier-mediated transport of these amines but it is possible that there is a similar mechanism in these neurons as well.

Another possible explanation of the effects of the metabolic inhibitors and ouabain is that they inhibit neuronal uptake of spontaneously released catecholamines. If this were the mechanism, it would be expected that spontaneous release of norepinephrine would be high and all drugs which inhibit neuronal uptake would be expected to release norepinephrine. This is not likely since the effects were observed at concentrations of the metabolic inhibitors and ouabain which did not release catecholamines in the absence of amphetamine. In experiments conducted under similar conditions, desipramine inhibited neuronal uptake of norepinephrine at concentrations which did not cause release [17]. This suggests that spontaneous release is not associated with neuronal reuptake in this system *in vitro*. It is also not likely that potentiation of amphetamine- or potassium-induced release is due to inhibition of neuronal uptake since amphetamine itself inhibits neuronal uptake, and it seems unlikely that further inhibition would be responsible for the observed potentiation. If amphetamine is incubated in the presence of a specific inhibitor of neuronal uptake such as desipramine, the releasing effect of amphetamine is inhibited rather than potentiated [17].

Thus, although the evidence is indirect, the results of this study are consistent with a model for amphetamine-induced release in which the efflux of catecholamines is limited by the intraneuronal concentration of sodium.

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