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EVIDENCE THAT AMPHETAMINE AND Na^+ GRADIENT REVERSAL INCREASE STRIATAL SYNAPTOSOMAL DOPAMINE SYNTHESIS THROUGH CARRIER-MEDIATED EFFLUX OF DOPAMINE*

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Abstract—Amphetamine (AMPH) releases dopamine (DA) from striatal synaptosomes and concomitantly increases DA synthesis. Since AMPH may release DA through carrier-mediated diffusion via reversal of the DA uptake system, the increase in DA synthesis might depend on a functioning uptake carrier. Consistent with such a mechanism, the uptake inhibitors nomifensine (NMF) and benztropine (BZT) completely prevented the AMPH-induced increase in DA synthesis at concentrations known to inhibit DA uptake. Changes in the Na^+ gradient across the synaptosomal membrane also promote DA release, since DA and Na^+ are cotransported by the neuronal uptake carrier. Incubation of synaptosomes in medium containing decreasing Na^+ increased DA synthesis inversely proportional to Na^+ over the range 128 to 20 mM. Similarly, incubations in the presence of 10^{-4} M ouabain to inhibit Na^+, K^+-ATPase and allow intracellular accumulation of Na^+ also increased DA synthesis. These changes in DA synthesis could also be prevented by BZT and were non-additive with the AMPH-induced increase in DA synthesis. However, a concentration of ouabain (10^{-6} M) which by itself did not increase DA synthesis, and does not promote DA release, potentiated the AMPH-induced increase in DA synthesis. Further, the increased DA synthesis promoted by all three manipulations was only marginally dependent on the presence of Ca^{2+} in the incubation medium. However, at 5 and 10 mM Na^+, a second component of increased DA synthesis was observed which was insensitive to BZT, but was prevented by Ca^{2+} removal. These results suggest that the increase in DA synthesis, and presumably DA release promoted by AMPH, lowered Na^+, and ouabain, depend on the availability of the DA carrier at the internal face of the neuronal membrane and the intracellular content of Na^+. The second component of increased DA synthesis which is evident at 5 and 10 mM Na^+ is discussed in terms of a possible Ca^{2+}-mediated change in DA synthesis which is independent of the DA carrier.

Amphetamine (AMPH) at concentrations up to 10 μM increases the synthesis of dopamine (DA) from labeled tyrosine in striatal slices and synaptosomes [1–3]. The mechanism of this effect remains unclear, but it has been suggested that AMPH may activate synthesis through its DA releasing effect [1, 4]. AMPH-induced DA release does not appear to involve exocytosis from synaptic vesicles, since it is totally or at least partially independent of Ca^{2+} [3, 5, 6], since it is relatively insensitive to agents thought to affect presynaptic DA autoreceptors [6], and since it is not inhibited by reserpine treatment to deplete vesicular DA stores [7–10]. It is therefore likely that AMPH release of DA is intimately related to cytoplasmic DA. Thus, AMPH may increase DA synthesis by decreasing the size of the cytoplasmic pool which is in inhibitory contact with the rate-limiting enzyme tyrosine hydroxylase [1]. Consistent with this suggestion, AMPH-induced synthesis activation is sensitive to pharmacological manipulations which alter the size of the cytoplasmic pool [1, 4].

It has been suggested that the mechanism by which AMPH releases DA from neuronal tissue may involve exchange diffusion [11] via reversal of the DA uptake carrier [12, 13]. According to this model, AMPH would serve as a substrate for the DA uptake carrier and would be transported into the nerve ending or synaptosome. Binding of AMPH to the carrier at the outside face of the membrane would both diminish uptake of DA and accelerate the inward movement of the carrier, thus increasing the availability of the carrier at the inside face of the membrane for the outward transport of cytoplasmic DA. This hypothesis is supported by the findings that AMPH can serve as a substrate for catecholamine uptake systems [13–15], that AMPH-induced DA release can be prevented by agents which block the DA uptake system [16, 17], and that AMPH-induced release is similar to DA uptake in its sensitivity to physical and chemical manipulations [17].

A similar mechanism for release of DA is apparently operative accompanying reversal of the inward-directed Na^+ gradient across the synaptosomal membrane. Replacement of Na^+ in the incubation

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medium by choline or sucrose, or inhibition of Na^+, K^+-ATPase by ouabain to allow intracellular Na^+ accumulation, both promote the release of DA [9, 16]. This presumably occurs because the function of the DA carrier molecule is linked to an inward-directed Na^+ gradient as DA and Na^+ are thought to be cotransported by the uptake carrier [18]. Release of DA by decreasing the Na^+ gradient can be blocked by the DA uptake inhibitors nomifensine (NMF) and benztropine (BZT), consistent with a carrier-facilitated diffusion mechanism [9, 16].

DA release by decreasing the Na^+ gradient, like AMPH-induced DA release, should therefore be dependent on a cytoplasmic pool of DA and should also increase synaptosomal DA synthesis. Further, the increased DA synthesis should be prevented by NMF and BZT and should not exhibit a strict dependence on calcium ions. The data presented below, utilizing a crude synaptosomal preparation, characterize the effects of AMPH, Na^+ replacement, and ouabain on synaptosomal DA synthesis and compare the extent to which DA uptake blockers and Ca^{2+} can interact with these effects.

MATERIALS AND METHODS

Male Sprague–Dawley rats (200–250 g) were obtained from Harlan Industries, Indianapolis, IN, and housed five per cage with *ad lib.* access to food and water. L-[1-^{14}C]Tyrosine was obtained from New England Nuclear, Boston, MA. *S*(+)-Amphetamine sulfate and ouabain octahydrate were obtained from the Sigma Chemical Co., St. Louis, MO. Nomifensine was a gift of Hoechst-Roussel Pharmaceuticals, Inc., Sommerville, NJ. Benztropine was a gift of Merck Sharp & Dohme Research Lab., Rahway, NJ.

Rats were killed by decapitation, and corpora striata were removed and homogenized in 20 vol. of 0.32 M sucrose using a Kontes glass–Teflon homogenizer (clearance 0.025 cm). The homogenate was centrifuged at 1000 *g* for 10 min, and the resulting supernatant fraction was removed and centrifuged at 39,000 *g* for 20 min. The resulting crude synaptosomal pellet, containing synaptosomes, mitochondria, and membrane fragments, was gently resuspended in 0.32 M sucrose.

Synaptosomal conversion of L-[1-^{14}C]tyrosine to DA was determined by measuring the evolution of $^{14}CO_2$. All assays were performed in triplicate. Incubation took place in Krebs–Ringer phosphate (KRP) medium, pH 7.4, which contained 128 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 15.8 mM $NaHPO_3$, and 11.1 mM glucose. In some experiments these concentrations were modified as described. In addition, the incubation medium contained 8×10^{-6}M L-[1-^{14}C]tyrosine, plus any other required agents. The incubations were carried out in polypropylene tubes containing 0.45 ml of the incubation medium. Incubations were initiated by adding 0.05 ml of the synaptosomal suspension, containing the equivalent of 5–10 mg of tissue (wet weight), to each tube. Tubes were sealed with tight fitting rubber caps from which were suspended center wells containing 0.1 ml NCS (Amersham Corp. Arlington Heights, IL). Samples were incubated for 20 min at 37°. The time between the addition of synaptosomes and the start of 37° incubation was strictly controlled at 0.5 min, since preincubation at 0° in Krebs–Ringer phosphate caused a decline in synthesis rates. Preincubations at 0° in sucrose did not affect synthesis rates. Incubations were stopped by placing the samples in ice and injecting 0.5 ml of 10% trichloroacetic acid. Blanks consisted of cerebellar synaptosomes incubated as above in the presence of 10^{-3} M 3-iodotyrosine, an inhibitor of tyrosine hydroxylase.

For concentration–response curves, data were analyzed by analyses of variance. Following significant main effects, posthoc comparisons were made using the Newman–Keuls test, and the nature of significant interactions was further defined using tests of simple main effects [19]. All other comparisons were made using paired *t*-tests.

RESULTS

Effects of AMPH on DA synthesis. In agreement with previous findings, the addition of AMPH at concentrations up to 10 μM significantly increased striatal synaptosomal DA synthesis. In the data shown in Fig. 1, a maximal increase of DA synthesis of 53% occurred at 5 μM AMPH with an EC_{50} near 0.2 μM. AMPH at 100 μM significantly inhibited synthesis. In subsequent experiments, 5 μM AMPH was utilized.

Effects of DA uptake inhibitors on the increase in DA synthesis induced by AMPH. Two agents which block DA uptake, nomifensine (NMF) [20, 21] and benztropine (BZT) [22], were utilized to evaluate the participation of a functional DA uptake system in AMPH-induced increases in striatal synaptosomal DA synthesis. NMF, at concentrations above 10 μM, completely inhibited the increase in DA synthesis by AMPH (Fig. 2). The EC_{50} for this effect was 2 μM. At concentrations that inhibited the effect of AMPH,

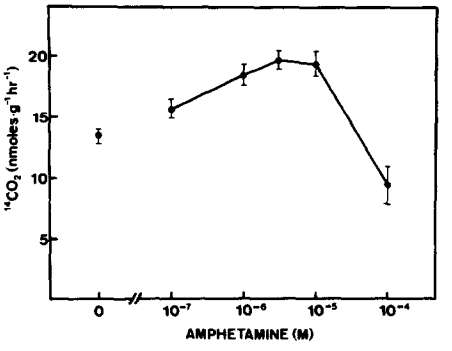

Fig. 1. Effects of increasing AMPH concentration on striatal synaptosomal DA synthesis. Values presented are the means ± S.E.M. of four experiments. ANOVA indicated that AMPH significantly altered DA synthesis over the concentration range employed ($P < 0.001$), and the Neuman–Keuls test indicated that all concentrations of AMPH significantly altered DA synthesis. An AMPH concentration of 5×10^{-6} M maximally increased DA synthesis from 13.1 ± 0.5 to 20.0 ± 0.8 nmoles/g/hr, with an EC_{50} near 0.2 μM.

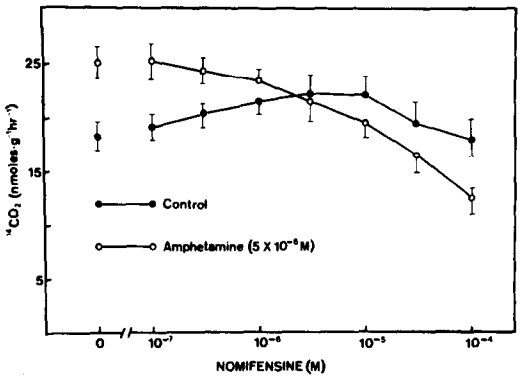

Fig. 2. Effect of nomifensine on striatal synaptosomal DA synthesis in the presence and absence of 5×10^{-6} M AMPH. Values are the means ± S.E.M. of four experiments. AMPH alone significantly ($P < 0.001$) increased DA synthesis from 17.7 ± 0.9 to 25.1 ± 1.5 nmoles/g/hr. Nomifensine alone significantly increased DA biosynthesis by 22 ± 3% (mean ± S.E.M.; N = 5) with an EC_{50} near 6×10^{-7} M, and at 1×10^{-5} M completely blocked the increase in DA synthesis promoted by AMPH with an EC_{50} near 2×10^{-6} M.

NMF alone increased synthesis, with a maximal activation of 22% at 10 μM. BZT at a concentration of 10 μM also completely inhibited the effect of AMPH (Fig. 3). The EC_{50} for this effect was 2.5 μM. BZT by itself did not increase DA synthesis, in contrast to NMF. For this reason, BZT was used as a DA uptake blocker in subsequent experiments.

Effects of Na^+ *gradient manipulations on DA synthesis.* Since reversal of the Na^+ gradient across the synaptosomal membrane provides an alternate method of releasing cytoplasmic DA via the DA uptake system, the effect of manipulations of the Na^+ gradient on DA synthesis was determined. To decrease the Na^+ gradient, Na^+ was replaced in the incubation medium with choline or with sucrose, or ouabain was added to inhibit Na^+, K^+-ATPase. In experiments in which Na^+ replacement was desired, 31.6 μM Tris–HCl replaced the Na_2HPO_4 as the buffering medium. At fixed Na^+ concentrations, DA synthesis was identical whether phosphate or Tris was utilized (data not shown). Total replacement of Na^+ in the incubation medium with choline increased synthesis by 63 ± 7% (mean ± S.E.M. for four determinations) relative to controls containing 145 mM Na^+. Similar results were obtained using sucrose to replace Na^+ (data not shown). Increasing Na^+ from 0 to 145 mM resulted in a multiphasic effect on DA synthesis (Fig. 4). DA synthesis increased as the Na^+ concentration was increased from 0 to 10 mM and then declined at Na^+ concentrations greater than 10 mM. Ouabain, at a concentration of 0.1 mM, also increased DA synthesis by near 60% (Table 1). The effect of ouabain appeared to be independent of Na^+ concentration and non-additive with the effect of decreasing Na^+ on DA synthesis.

Effects of BZT on Na^+ *gradient-induced activation.* BZT was used to determine whether the DA uptake system is involved in the increase in DA synthesis caused by Na^+ gradient reversal. BZT was used at a concentration of 10^{-5} M, the highest concentration which did not inhibit control synthesis levels. The increase in DA synthesis following total Na^+ replacement was not significantly inhibited by BZT (Fig. 4). On the other hand, the increased DA synthesis obtained by partial Na^+ replacement was inhibited significantly at all Na^+ concentrations tested, with inhibition becoming complete as the Na^+ concentration approached 40 mM. The increased DA

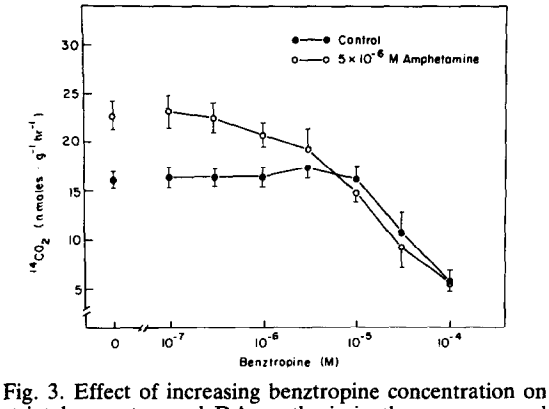

Fig. 3. Effect of increasing benztropine concentration on striatal synaptosomal DA synthesis in the presence and absence of 5×10^{-6} M AMPH. Values are the means ± S.E.M. of five experiments. AMPH alone significantly ($P < 0.001$) increased DA synthesis from 15.7 ± 0.8 to 22.8 ± 1.4 nmoles/g/hr. Benztropine completely inhibited the increase in DA synthesis promoted by AMPH with an EC_{50} near 2×10^{-6} M.

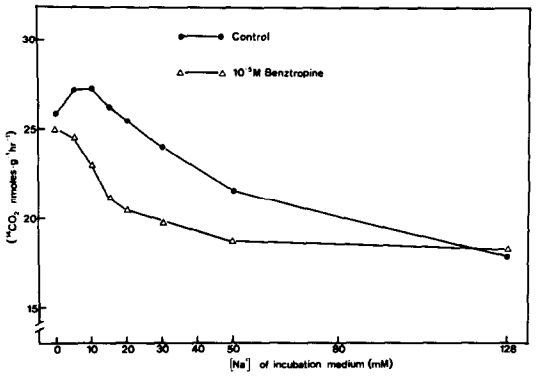

Fig. 4. Effects of increasing Na^+ concentration on striatal synaptosomal DA synthesis in the presence and absence of 10^{-5} M benztropine. The incubation was maintained isotonic with the replacement of Na^+ by choline. The data presented were obtained from a single experiment, and were essentially identical in three additional replications. In the experiment depicted, the rate of DA synthesis in the presence of 160 mM Na^+ was 16.3 nmoles/g/hr in the absence of benztropine, and 16.6 nmoles/g/hr in the presence of benztropine. Across the four experiments in the absence of benztropine, a significant increase in DA synthesis, relative to 145 mM Na^+ controls was obtained at Na^+ concentrations less than 50 mM. DA synthesis at 10 mM Na^+ (27.3 ± 1.9 nmoles/g/hr) was significantly ($P < 0.01$) greater than DA synthesis at 0 (25.8 ± 1.4 nmoles/g/hr) or 20 (25.2 ± 1.5 nmoles/g/hr) mM Na^+. Benztropine signficantly inhibited DA synthesis at all Na^+ concentrations less than 50 mM, but not at 0 mM Na^+.

Table 1. Effects of ouabain on dopamine synthesis in striatal synaptosomes

Incubation medium	$^{14}CO_2$ formation* (nmoles/g wet wt/hr)	% of Control
KRP (control; 160 mM Na^+)	16.3 ± 0.9	
+ 10^{-6} M Ouabain	16.1 ± 0.9	98.8
+ 10^{-5} M Ouabain	22.7 ± 2.3†	139
+ 10^{-4} M Ouabain	27.4 ± 2.2†	168
KRP (20 mM Na^+)‡	25.9 ± 2.9†	159
+ 10^{-4} M Ouabain	27.5 ± 2.5†	169
KRP (50 mM Na^+)‡	22.0 ± 2.1†	135
+ 10^{-4} M Ouabain	27.2 ± 2.3†§	167

* Experimental details are presented in Materials and Methods. Values represent the mean ± S.E.M. for at least three separate experiments.

† $P < 0.01$ relative to KRP (control).

‡ Isotonicity was maintained by replacing NaCl with choline chloride and by replacing Na_2HPO_4 with Tris–HCl. In the presence of 128 mM NaCl, DA synthesis was identical in Na_2HPO_4 or Tris–HCl.

§ $P < 0.01$ relative to KRP (50 mM Na^+).

synthesis promoted by 10^{-4} M ouabain was partially inhibited from 61 to 38% by 10^{-5} M BZT (Table 2).

Interactions between AMPH and decreasing Na^+ *gradient.* The increases in synaptosomal DA synthesis promoted by AMPH and Na^+ replacement were partially additive between 100 and 30 mM Na^+ (Fig. 5), i.e. for example at 50 mM Na^+, DA synthesis was 22.3 ± 1.6 nmoles/g/hr in the absence of AMPH, and 25.4 ± 1.2 nmoles/g/hr in the presence of 5×10^{-6} M AMPH ($P < 0.01$). The latter rate of DA synthesis was also significantly greater ($P < 0.001$) than synthesis in the presence of 145 mM Na^+ in the presence (22.9 ± 1.6 nmoles/g/hr) or absence (16.3 ± 0.8 nmoles/g/hr) of 5×10^{-6} M AMPH. Below 30 mM Na^+, AMPH did not cause a further increase in DA synthesis, but rather at 5 and 10 mM Na^+, AMPH caused a slight but significant inhibition. AMPH had no effect on synaptosomal DA synthesis at 0 mM Na^+. AMPH also did not affect the level of synthesis promoted by 10^{-4} M ouabain (Fig. 6). However, as shown in Fig. 6, a concentration of ouabain (10^{-6} M), which was by itself subthreshold for increasing synaptosomal DA synthesis, significantly (ANOVA, $P < 0.001$) potentiated the increase in DA synthesis promoted by AMPH and increased the maximum rate of DA synthesis in the presence of 5×10^{-6} M AMPH from 141 ± 8.8% of control to 159 ± 7.9% ($N = 3$; $P < 0.05$). Furthermore, AMPH significantly (ANOVA, $P < 0.01$) increased DA synthesis in the presence of a sub-optimal but activating concentration of 10^{-5} M ouabain (Fig. 6).

Ca^{2+} *dependency of synthesis activation.* The Ca^{2+} dependency of AMPH-induced synthesis activation was examined, to help determine the relationship of synthesis activation to AMPH-induced DA release, which is at least partially Ca^{2+} independent. For comparison, the Ca^{2+} dependency of synthesis activation caused by decreasing the Na^{2+} gradient was also examined.

The increase in synthesis promoted by AMPH was found to be partially dependent on Ca^{2+}. Results are summarized in Table 3, in which the increase in DA synthesis was reduced from 53 ± 6.9 to 39 ± 6.6% by the removal of Ca^{2+} from the incubation medium. Similar results were obtained when 0.5 mM EGTA was added to ensure total removal of Ca^{2+} (Table 3).

The effect of Ca^{2+} removal on the increase in DA synthesis obtained as a function of decreasing Na^+

Table 2. Interaction of ouabain with benztropine or amphetamine on synaptosomal dopamine synthesis

Incubation medium	$^{14}CO_2$ evolution* (nmoles/g wet wt/hr)	% of Control
KRP (control)	17.2 ± 1.1	
+ Ouabain	27.7 ± 2.0†	161
+ 10^{-5} M Benztropine	17.3 ± 1.0	101
+ Ouabain + benztropine	23.7 ± 1.3†‡	138
+ 5×10^{-6} M Amphetamine	24.6 ± 1.2†	143
+ Ouabain + amphetamine	28.0 ± 2.1†§	163

* Experimental details are presented in Materials and Methods. Values represent the means ± S.E.M. for at least three separate experiments.

† $P < 0.01$ relative to control.

‡ $P < 0.01$ relative to ouabain alone.

§ $P < 0.05$ relative to amphetamine alone.

Table 3. Effects of Ca^{2+} removal and EGTA on striatial synaptosomal dopamine synthesis as a function of added amphetamine or ouabain or Na^+ removal

Changes to incubation medium	% of Control* KRP (control)	KRP ($-CaCl_2$)	KRP† ($-CaCl_2 + 5 \times 10^{-4}$ M EGTA)
No change	100 ± 4.9	102 ± 6.9	106 ± 5.7
+ 10^{-4} M Ouabain	181 ± 9.2‡	162 ± 8.3§	158 ± 9.1
+ 5×10^{-6} M Amphetamine	153 ± 6.9‡	139 ± 6.6§	140 ± 6.3
+ 0 mM Na^+	161 ± 6.8‡	159 ± 6.1	157 ± 7.4
+ 10 mM Na^+	179 ± 8.8‡	160 ± 7.3§	150 ± 6.9

* Values represent the mean ± S.E.M. for four experiments. KRP (control) level of synthesis was 16.3 ± 0.8 nmoles/g/hr.

† Addition of 5×10^{-4} M EGTA failed to alter levels of synthesis observed in the absence of Ca^{2+}.

‡ Significantly different, by paired *t*-test, from "No change" ($P < 0.01$).

§ Removal of Ca^{2+} significantly decreased the level of synthesis relative to the appropriate value in the presence of Ca^{2+} ($P < 0.01$).

concentration appeared to contain two components (Fig. 7). Ca^{2+} removal had no effect on the progressive increase in DA synthesis observed as Na^+ was decreased from 145 to 20 mM, or on DA synthesis levels in the complete absence of Na^+. However, Ca^{2+} removal completely prevented the secondary component of low Na^+-induced increase in DA synthesis at Na^+ concentrations of 5 and 10 mM. The ouabain-induced increase in DA synthesis was also partially dependent on the presence of Ca^{2+} (Table 3). Addition of 0.5 mM EGTA to the incubation medium in the absence of Ca^{2+} did not alter the effects of Ca^{2+} removal alone (Table 3).

Finally, the effects of Ca^{2+} removal and the interaction of BZT with decreasing Na^+ on synaptosomal DA synthesis were assessed (Fig. 7). BZT at a concentration of 10^{-5} M in the presence of Ca^{2+} significantly (ANOVA, $P < 0.001$) inhibited the increase in DA synthesis promoted by decreasing the Na^+ concentration in the incubation medium (Fig. 7) essentially identical to the data presented in Fig. 4. The effect of BZT was significant (Neuman–Keuls, $P < 0.01$) compared to no BZT control at all Na^+ concentrations of 50 mM and below, but not in the complete absence of Na^+ (Fig. 7). In the absence of added Ca^{2+}, BZT further inhibited the increase in DA synthesis promoted by decreasing Na^+ (ANOVA; $P < 0.01$), with the inhibition achieving significance (Neuman–Keuls, $P < 0.05$) at 5 and 10 mM Na^+ (Fig. 7). No significant further inhibition, however, was obtained in the complete absence of Na^+.

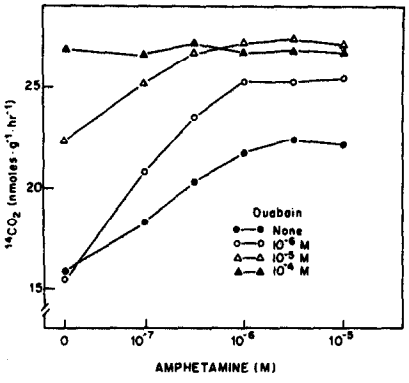

Fig. 6. Effects of ouabain on the amphetamine-induced increase in striatal synaptosomal DA synthesis. The data presented above are from a single experiment and were essentially identical in two additional replications. See Materials and Methods and Results for additional details.

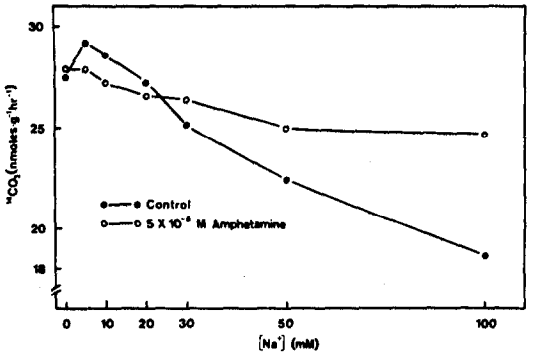

Fig. 5. Effects of increasing Na^+ concentration on striatal synaptosomal DA synthesis in the presence and absence of 5×10^{-6} M AMPH. The incubation medium was maintained isotonic with the replacement of Na^+ by choline. The data presented were obtained from a single experiment and were essentially identical in two additional experiments. In the experiment depicted, DA synthesis at 145 mM Na^+ was 16.9 nmoles/g/hr in the absence of AMPH and 23.7 nmoles/g/hr in the presence of AMPH. Across the three experiments, AMPH singificantly ($P < 0.05$) increased DA synthesis at Na^+ concentration greater than 30 mM, and significantly ($P < 0.01$) inhibited DA synthesis at 5 and 10 mM Na^+. AMPH had no effect on synthesis at 0 mM Na^+.

DISCUSSION

The results presented above support the hypothesis that AMPH increases DA synthesis in synaptosomes by releasing cytoplasmic DA through facilitated diffusion via reversal of the DA carrier

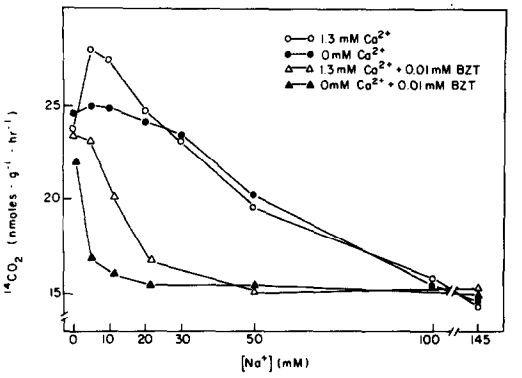

Fig. 7. Effects of increasing Na^+ concentrations on striatal synaptosomal dopamine synthesis in the presence and absence of exogenous 1.3 mM $CaCl_2$ and 10^{-5} M BZT. The incubation medium consisted of a modified KRP in which 31.6 mM Tris–HCl replaced Na_2HPO_4. Isotonicity was maintained by adding choline chloride in place of NaCl. The data presented are from a single experiment and were identical in two additional replications. In three replications, the absence of $CaCl_2$ in the incubation medium in the presence or absence of BZT significantly ($P < 0.01$) altered the response to decreasing Na^+ only at 5 and 10 mM Na^+.

system. (1) The effects of AMPH on synthesis depended on the function of the DA uptake system, (2) an alternate method of releasing DA via the uptake system, decreasing the Na^+ gradient across the synaptosomal membrane, also increased DA synthesis, and (3) the increased synthesis in both cases was only partially Ca^{2+} dependent.

Effects of DA uptake blocking agents on AMPH-induced increase in DA synthesis. The results of experiments using DA uptake blockers indicate that AMPH-induced synthesis activation is dependent on the function of the DA uptake system. Two agents known to block DA uptake, NMF and BZT, completely inhibited, at a concentration of 10 μM, AMPH-induced synthesis activation (Figs. 2 and 3). This concentration of NMF, which maximally inhibits DA uptake into synaptosomes [23], has been shown to antagonize AMPH-induced DA release [16]. Similarly, 10 μM BZT is within the range of concentrations which would be expected to block DA uptake ($K_i = 0.32$ μM [22]). The objection might be raised that the DA uptake blockers inhibited AMPH-induced synthesis activation merely by preventing entrance of AMPH into the synaptosome via the uptake system, thus precluding some intracellular action of the drug.

However, because of its high lipophilicity AMPH can accumulate inside neuronal tissue through passive diffusion, independent of active transport [24, 25]. Because NMF exerted a mild activating effect on synaptosomal DA synthesis by itself (Fig. 2), BZT was utilized in the remainder of the experiments. This increase in synthesis promoted by NMF was surprising, since it has been shown that NMF exerts little [26] or no [16] DA releasing effect on synaptosomes. The ability of NMF to increase striatal synaptosomal DA synthesis has also been reported by Cerrito and Raiteri [27]. Those authors reported that the increase in synthesis is abolished under superfusion conditions, and suggested that NMF increases synthesis by preventing reuptake of spontaneously released DA. The latter explanation seems unlikely, however, since the IC_{50} of NMF for inhibition of 0.1 μM DA uptake was near 80 nM [16] whereas in the present experiments (Fig. 2) the EC_{50} for the effect of NMF on DA synthesis was near 6 μM. Levels of DA in the synaptosomal medium following incubation with NMF never achieved 0.1 μM (data not shown).

Effects of decreasing Na^+ gradient on DA synthesis. Decreasing the Na^+ gradient across the synaptosomal membrane provides an alternate release of DA via the uptake system [9, 16]. Reversal of the Na^+ gradient can be achieved by isotonic replacement of Na^+ in the incubation medium with choline, or a decrease in the Na^+ gradient can be initiated by addition of ouabain to inhibit the Na^+, K^+-ATPase. Both manipulations promote release of DA [9, 16, 28], and the data presented above indicate that both manipulations also increase synaptosomal DA synthesis.

Total or partial replacement of Na^+ in the incubation medium increased DA synthesis (Figs. 4, 5, and 7; Table 1) inversely proportional to the Na^+ concentration over the range 20–100 mM Na^+. A similar Na^+ concentration dependence has been reported for DA release from synaptosomes [16]. Similarly, the addition of ouabain at concentrations (10 μM or greater) that have been shown to release DA from synaptosomes [9, 16, 28] also increased DA synthesis (Tables 1–3; Fig. 6), and the effects of low Na^+ and ouabain combined were non-additive (Table 1). Furthermore, the addition of BZT to the incubation medium completely prevented the low Na^+-induced increase in DA synthesis at Na^+ concentrations as low as 40 mM (Fig. 4) and partially inhibited the increase in DA synthesis promoted by ouabain (Table 2). These observations are consistent with a mechanism of release of DA under these conditions proceeding via the DA uptake system. As might be anticipated if low Na^+-, ouabain-, and AMPH-induced DA release proceed through a common mechanism, any combination of these manipulations at maximally effective concentrations to increase DA synthesis failed to produce additive effects on DA synthesis. On the other hand, both DA release by AMPH [28] and the AMPH-induced increase in DA synthesis (Fig. 6) are potentiated by a concentration of ouabain (1 μM) which by itself increases neither DA release nor DA synthesis. This latter observation would be expected if transport of DA from the nerve ending by the carrier was a function of both the intraneuronal Na^+ concentration and the intraneuronal concentration of carrier DA binding sites [28].

Although BZT completely prevented the increase in synaptosomal DA synthesis as Na^+ concentration was reduced from 100 to 40 mM, inhibition by BZT became less effective as the Na^+ concentration was further decreased, and BZT had no effect on DA synthesis at 0 mM Na^+. Similarly, the increase in DA synthesis promoted by ouabain was only partially inhibited by BZT. It would appear that more than one mechanism of DA synthesis activation are oper-

ative at the lower Na^+ concentrations as well as in the presence of optimal ouabain concentrations. This suggestion is supported by the data presented in Fig. 7, reflecting the effects of Ca^{2+} removal on DA synthesis in the presence of decreasing Na^+ concentration or in the presence of BZT. Whereas removal of Ca^{2+} from the incubation medium had no effect on DA synthesis at Na^+ concentration greater than 20 mM, Ca^{2+} removal prevented the second component of low Na^+-induced increases in DA synthesis apparent at 5–10 mM Na^+ both in the absence and presence of BZT (Fig. 7). This component of DA synthesis activation also appeared distinct by being inhibited in the presence of AMPH (Fig. 5). Since it is dependent on Ca^{2+}, and apparently not affected by BZT (Fig. 4), this component, as well as the BZT-insensitive portion of the increase in DA synthesis promoted by ouabain, most likely would not involve the DA uptake carrier directly.

Incubation of synaptosomes in low Na^+ medium or in the presence of ouabain would be expected to alter intrasynaptosomal monovalent and divalent cation concentrations [29] which might directly influence DA biosynthesis perhaps at the level of the rate-limiting enzyme, tyrosine hydroxylase. There is some evidence that the activity of this enzyme is sensitive to specific monovalent cations [30], and shifts in the intrasynaptosomal K^+ and Na^+ distribution might contribute to the second component of DA biosynthesis activation. However, there is substantial evidence that tyrosine hydroxylase activity may be regulated, in part, by Ca^{2+}-dependent mechanisms. Tyrosine hydroxylase, isolated from striatal tissue slices previously incubated in Na^+-deficient [31] or -depolarizing [32] medium, exhibits enhanced activity, and this activation is Ca^{2+} dependent. The enzyme isolated from striatal synaptosomes previously incubated in KRP, modified to contain 10 mM Na^+, is activated via a decreased K_m for the tetrahydropterin cofactor.* And striatal tyrosine hydroxylase can be activated in cell-free extracts by a Ca^{2+}, ATP, Mg^{2+}-dependent mechanism which decreases the K_m of the enzyme for the synthetic cofactor 6-methyltetrahydropterin [33]. It therefore seems reasonable to suggest that the Ca^{2+}-dependent, second component of low Na^+-induced DA biosynthesis activation (Fig. 7) as well as the substantial Ca^{2+}-dependent component of ouabain-induced activation (Table 3) proceed through a Ca^{2+}-dependent activation of tyrosine hydroxylase.

A third component of DA synthesis activation as a function of decreasing Na^+ concentration was evident when Na^+ was completely removed from the incubation medium (Fig. 4, 5, and 7; Table 3). This component is insensitive to both Ca^{2+}, BZT and AMPH. In this regard, Bustos *et al.* [31] observed two components of tyrosine hydroxylase activation in tissue slices incubated in Na^+-free medium, one of which was not dependent on exogenous Ca^{2+}. The mechanism for this latter activation has not been identified yet but may mediate this component of DA synthesis activation.

* K. S. Strait and R. Kuczenski, unpublished observation.

It is notable that conflicting data have been reported regarding the dependence of AMPH-induced DA synthesis increases on Ca^{2+}. Whereas the AMPH-induced increase in DA synthesis in synaptosomes is at most only partially dependent on Ca^{2+} ([1, 3]; data presented above), the major portion of the increase in DA synthesis promoted by AMPH in striatal slices is Ca^{2+} dependent [34]. Uretsky *et al.* [34] have maintained that the increase in DA synthesis in tissue slices promoted by AMPH cannot depend on DA release, but reflects an effect of Ca^{2+} influx on synthesis *per se*. The effects of AMPH on DA synthesis in tissue slices are substantially greater than in synaptosomes, and other qualitative differences between DA synthesis in slices and synaptosomes can be noted. For example, ouabain increased DA synthesis in synaptosomes (data presented above) but not in slices [35], whereas removal of Ca^{2+} from the incubation medium increases DA synthesis in slices [35] but not in synaptosomes (Fig. 7). Thus, it is possible that synaptosome preparation partially disrupts some component of DA synthesis regulation which is maintained in tissue slices, and which is responsible for these discrepancies. This component may subsequently be unmasked in synaptosomes by the changes in intracellular environment promoted by ouabain or by Na^+-free medium, and may also explain the anomalies of the data presented above. Specificially, the level of DA biosynthesis in the complete absence of Na^+ was insensitive to Ca^{2+} (Fig. 7), AMPH (Fig. 5) and BZT (Figs. 4 and 7). Further, the second component of DA biosynthesis activation was apparently inhibited by AMPH (Fig. 5).

In summary, the data presented above suggest the following conclusions: (1) the AMPH-induced increase in DA synthesis in synaptosomes is dependent on the function of the DA uptake system, and (2) release of DA from synaptosomes via the uptake system increases DA synthesis. However, (3) some conditions which support DA release and promote ion fluxes may also promote additional increases in DA synthesis mediated by changes in the state of activity of tyrosine hydroxylase.

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