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ON THE MECHANISM OF PRESYNAPTIC AUTORECEPTOR-MEDIATED INHIBITION OF TRANSMITTER SYNTHESIS IN DOPAMINERGIC NERVE TERMINALS*

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Abstract—The effect of apomorphine (APO) upon dopamine (DA) synthesis and release from rat striatal slices was studied. The synthesis of DA was measured by incubating the slices in Krebs–Ringer phosphate (KRP) medium of variable ionic composition containing L-tyrosine[^{14}C-U] as DA precursor. A superfusion system was used to study both spontaneous and K^+-induced release of labeled DA from striatal slices. The addition of APO directly to the normal KRP medium markedly blocked the formation of [^{14}C]DA from [^{14}C]tyrosine with an IC_{50} of 1.8×10^{-7} M. Haloperidol (4×10^{-7} M), a known DA antagonist, produced a shift to the right of the concentration–response curve for APO inhibition on DA synthesis, whereas the DA antagonist (+)butaclamol (4×10^{-7} M) completely reversed the inhibition caused by APO (2×10^{-7} M). DA uptake blockers, such as benztropine (2×10^{-6} M) or cocaine (1×10^{-5} M), did not affect the ability of APO to inhibit DA synthesis. Furthermore, the α_2-adrenergic agonist clonidine produced only a mild inhibition and the β-adrenergic agonist isoproterenol produced no inhibition of [^{14}C]DA formation. APO was able to inhibit DA formation both in the absence of calcium in the incubation medium or in the presence of high external calcium concentrations (4, 8 and 24 mM) which depress the rate of DA synthesis. Incubation conditions that cause an increase of free intraneuronal calcium concentrations, such as Na^+-free medium, the presence of ouabain (1×10^{-4} M), or K^+ depolarization, dramatically abolished or impaired the ability of APO to inhibit DA synthesis in striatal slices. It was not possible to demonstrate any change in spontaneous and K^+ (27 mM)-induced release of DA in the presence of APO concentrations that produced a marked inhibition of DA synthesis. The results reported in this work indicate that tissue slices can be used as a valuable experimental tool to study the inhibitory effect of APO on DA synthesis, and that this effect occurs through an interaction of APO with presynaptic DA autoreceptors located in striatal dopaminergic nerve terminals. The results obtained are not in keeping with the hypothesis that this autoreceptor-mediated inhibition of DA synthesis occurs through regulation of calcium influx into the DA nerve terminals. However, the possibility is raised that a sensitivity to high intraneuronal calcium concentration exists during the events that mediate APO–DA autoreceptor interaction and DA synthesis inhibition. It is further suggested that DA-synthesis-modulating autoreceptors do not participate in the modulation of DA release.

Recent findings have suggested that dopaminergic nerve terminals in the rat brain are endowed with presynaptic receptor sites which respond to external dopamine (DA) in such a way that a marked decrease in the rate of DA biosynthesis results when these receptors are activated [1, 2]. It has been possible to study these autoreceptors *in vivo* by measuring the accumulation of DOPA following inhibition of dopa decarboxylase under experimental conditions in which impulse flow in central DA neurons is inhibited. Transection of the nigro-striatal dopaminergic pathway, either by hemisection or electrothermic lesion, or administration of drugs such as γ-butyrolactone (GBL) which cause rapid and reversible abolition of dopaminergic impuse flow, all result in a rapid accumulation of DA in striatal nerve terminals and an accelerated rate of DA formation [3–5]. The increases in DA levels and DOPA accumulation are effectively blocked by administration of DA agonists, while, in turn, the blocking effects of the agonists are prevented by administration of DA-receptor-blocking agents [1, 6]. Since in these experiments the impulse flow in nigro-striatal DA neurons is blocked, it has been proposed that the reported drug-induced changes of DA synthesis must result as a consequence of their interactions with receptors located on dopaminergic nerve terminals.

Experiments *in vitro* also support the existence of these DA-synthesis-modulating autoreceptors. In synaptosomal preparations, apomorphine (APO), a DA agonist, depresses very markedly the conversion of tyrosine to DA [7–9]. Dopamine receptor blockers such as haloperidol, pimozide and spiroperidol partially reverse the inhibitory effect of APO on DA synthesis and this appears to be due to a competitive interaction between these drugs and APO at presynaptic receptor sites. Similar experi-

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ments conducted with striatal slice preparation have shown that fluphenazine, a DA antagonist, partially prevents the decrease in DA synthesis induced by exogenous DA [10]. These studies, as well as those cited above, suggest the involvement of DA autoreceptors in the modulation of DA synthesis.

The mechanism involved in the autoreceptor-mediated inhibition of tyrosine hydroxylation is still unknown. However, during cessation of impulse flow in central dopaminergic neurons, when calcium influx and transmitter release are blocked, there is an alteration in the kinetics of tyrosine hydroxylase, mainly a marked decrease in the affinity of the enzyme for the natural end-product inhibitor, DA. This time-dependent activation of the enzyme can be readily reversed by *in vivo* administration of DA agonists [2, 11]. Further studies have indicated that conditions that lower tissue calcium, such as exposure of striatal slices to calcium-free media or to media containing a calcium-chelating agent, result in increases in DA synthesis and tyrosine hydroxylase activity [12–14]. All these experiments have given rise to the hypothesis that DA autoreceptors may modulate DA synthesis and tyrosine hydroxylation by gating calcium entry into the DA nerve terminal [11, 15]. Thus, it seemed of high interest to develop an experimental system to test the validity of this hypothesis. This paper describes a study in which we have used rat striatal tissue slices to study autoreceptor-mediated modulation of neurotransmitter synthesis in nerve terminals belonging to the dopaminergic nigro-striatal system. Apomorphine was chosen for these studies as a DA receptor agonist. We have determined whether variations in external calcium concentrations and manipulations known to increase the concentration of ionized calcium within the neuron are able to modify the effect of nerve terminal autoreceptors on DA synthesis. In addition, since it is a matter of discussion whether DA autoreceptors influence DA release in a manner similar to their influence on synthesis [16], we have studied the effect of APO on spontaneous and depolarization-evoked release of DA from striatal slices.

MATERIALS AND METHODS

Studies on dopamine synthesis. The conversion of [^{14}C]DA to [^{14}C]tyrosine in striatal slices was determined essentially as described previously [13, 17]. Briefly, striatal tissue slices (0.20 mm in thickness) were prepared with a Sorvall tissue chopper from the striata of adult, male, Sprague–Dawley rats. Tissue slices weighing about 30–40 mg were incubated at 37° in beakers containing 5 ml of either Krebs–Ringer phosphate (KRP), pH 7.4, or modified KRP, pH 7.4, previously saturated with 100% oxygen. After a 10-min preincubation period, labeled L-tyrosine[^{14}C-U], with a specific activity of 5 mCi/mmole, was added to the media, producing a final tyrosine concentration of 2.5×10^{-5} M. Thereafter, the slices were incubated for an additional 30 min, and the incubation was stopped by the addition of 2.0 ml of 50% trichloroacetic acid (TCA) to the beakers. The slices plus media were then subjected to homogenization, and the precipitated protein was then removed by centrifugation at 12,400 *g* for 15 min. The supernatant fraction was then immediately frozen and kept for separation and analysis of labeled catechols as described below. When used, the different drugs were added to the medium at the beginning of the preincubation period. Tissue blanks were run by incubating striatal slices as described above, but in the presence of α-methyl-*p*-tyrosine (2.3×10^{-4} M), an inhibitor of tyrosine hydroxylase. A typical sample-to-blank ratio usually had a value around 10.

Separation and analysis of the tissue and the media for labeled catechols were carried out by adsorption chromatography as described previously [13, 18]. Alumina columns were used to concentrate the catecholamines and deaminated metabolites. Analysis through long Amberlite CG-120 columns has shown that labeled DA accounts for 85% of the labeled catechols synthesized [13]. Eluates from chromatography columns containing labeled catechols were analyzed for ^{14}C in a Nuclear Chicago scintillation counter. The reported values have not been corrected for recovery. The rate of [^{14}C]DA formation was calculated as described previously [13, 19] and is expressed in terms of [^{14}C]DA · (g wet wt)$^{-1}$ · hr^{-1}. The [^{14}C]DA synthesized represents the sum of the [^{14}C]DA content in slices plus the [^{14}C]DA content in the medium. In some experiments, the rat of [^{14}C]DA synthesis is expressed as percent. However, the nmoles of [^{14}C]DA · (g wet wt)$^{-1}$ · hr^{-1} which correspond to 100% are adequately indicated in the table and figure legends. In some experiments, the [^{14}C]tyrosine taken up by the tissue during the incubation period was analyzed. For this purpose, the slices were separated from the media by centrifugation at 30,000 *g* for 10 min in a Sorvall refrigerated centrifuge. The [^{14}C]tyrosine taken up by the tissue was separated from catechols and other labeled metabolites by chromatography columns as described previously [20]. Eluates from the columns containing labeled tyrosine were analyzed for ^{14}C as described above.

Studies on dopamine release. The release from striatal slices of [^{3}H]DA that had been exogenously taken up was followed essentially as described previously [18]. Briefly, 8–10 mg of the striatal slices was incubated for 30 min at 37° in 2 ml KRP, pH 7.4, saturated with oxygen and containing [^{3}H]DA (1×10^{-7} M). The slices were then transferred to lucite superfusion chambers (2-ml capacity), washed with 10 ml KRP, and then superfused with KRP solution that was being continuously oxygenated and prewarmed to 37°. A constant flow of 4 ml/min was maintained by means of a peristaltic pump (DESAGA, Heidelberg, Germany), and a two-way system was set up to switch to different superfusing solutions without disrupting the flow. An initial superfusion period of 12 min was allowed before release stimulation. These procedures allowed for a constant and steady basal release prior to stimulation. Stimulation of release was carried out for 1 min by switching the superfusion to iso-osmotic KRP containing either 27 mM K^+ or 55 mM K^+. Two successive K^+ stimulations (S_1 and S_2) were used with an interval of 19 min between them. When used, APO and (+)butaclamol, were added to the super-

fusion system 8 min prior to the second K^+ stimulation (S_2). Samples containing the released material were collected every minute and analyzed for radioactivity. At the end of the superfusion period the slices were homogenized in 15% TCA and centrifuged at 12,000 *g* for 15 min, and the clear supernatant fractions were analyzed for radioactivity. Stimulus-induced release of radioactive DA was expressed as percentage of the total radioactivity found in the tissue and in the different collecting tubes at the end of the superfusion period. To evaluate the effects of APO and (+)butaclamol on stimulated DA release, the ratio S_2/S_1 was determined and compared with the same ratio obtained in the absence of a drug.

Solutions and chemicals. The Krebs–Ringer phosphate used in these experiments had the following composition: 128 mM NaCl, 4.8 mM KCl, 0.75 mM $CaCl_2$, 1.20 mM $MgSO_4$, 16 mM glucose, 16 mM Na_2HPO_4 (pH 7.4), and sodium ascorbate, 20 mg/liter. Krebs–Ringer phosphate-high K^+ was made by replacing proportions of NaCl with equimolar amounts of KCl. Krebs–Ringer MOPS (KR MOPS), pH 7.4, was made by replacing Na_2HPO_4 with equimolar amounts of morpholinopropane sulfonic acid. In the Na^+-free media, NaCl was replaced isoosmotically either by choline chloride or by sucrose. Other modifications of the KRP are described in the text.

L-Tyrosine[^{14}C-U] and [^{3}H]dopamine were obtained from the New England Nuclear Corp., Boston, MA, U.S.A. The following drugs were used: (−)apomorphine HCl, Laboratorio Chile, Santiago, Chile; α-methyl-*p*-tyrosine, Regis Chemical Co., Morton Grove, IL, U.S.A.; (+) and (−) enantiomers of butaclamol, Ayerst Laboratories Inc. New York, NY, U.S.A.; cocaine HCl, Merck Co. Rahway, NJ, U.S.A.; haloperidol HCl, Beta Co.; isoproterenol HCl, Sigma Chemical Co., St. Louis, MO, U.S.A.; ouabain, Calbiochem Corp., San Diego, CA, U.S.A.; and benztropine and clonidine HCl, donated by Dr. R. H. Roth, Yale University, New Haven, CT, U.S.A.

RESULTS

Effect of apomorphine on dopamine synthesis. Previous experiments from our laboratory have shown that the conversion of labeled tyrosine to DA can be readily determined in striatal slices subjected to different incubation conditions [13, 17]. Under the experimental conditions reported here, the apparent K_m for [^{14}C]tyrosine was 2.4×10^{-6} M and the synthesis of [^{14}C]DA was linear for up to 45 min when using a [^{14}C]tyrosine concentration of 2.5×10^{-5} M. Apomorphine, added directly to striatal slices incubated in normal KRP, markedly inhibited the conversion of [^{14}C]tyrosine to [^{14}C]DA (Fig. 1). The inhibitory effect of APO was shown to be concentration dependent with an IC_{50} (concentration causing 50% inhibition) of 1.8×10^{-7} M. The presence in the incubation media of DA uptake blockers, such as benztropine (2×10^{-6} M) or cocaine (1×10^{-5} M), did not affect the ability of APO to inhibit [^{14}C]DA synthesis (data not shown). In fact, the extent of DA synthesis inhibition produced by APO (4×10^{-7} M) was the same in the absence or presence of DA uptake blockers. Neither benztropine nor cocaine modified *per se* the rate of [^{14}C]DA formation measured in normal KRP.

The abilities of DA-receptor-blocking drugs to reverse the inhibition of [^{14}C]DA synthesis induced by APO was then studied. Other investigators, conducting similar experiments in striatal slices, have been unable to show any reversal by DA antagonists added *in vitro* of the APO-mediated inhibition [21]. However, under our experimental conditions, the addition of a fixed concentration of haloperidol (4×10^{-7} M) to the incubation medium produced a shift to the right of the concentration–response curve for APO inhibition of DA synthesis (Fig. 1). The IC_{50} for APO was found to increase almost 11-fold in the presence of haloperidol, that is, to a value of 19×10^{-7} M (Fig. 1). Haloperidol, at the concentration used (4×10^{-7} M), produced no significant effect *per se* on the rate of [^{14}C]DA formation by striatal slices. In fact, a wide range of haloperidol concentrations tested (5×10^{-8} M to 1×10^{-6} M) seemed to produce no appreciable effects upon DA synthesis (data not shown). Butaclamol was also studied in its ability to reverse the inhibitory effect of APO on DA synthesis (Fig. 2). Butaclamol possesses isomeric forms which differ in their pharmacological properties. Only the (+) enantiomer can act as a "neuroleptic" agent and is able to displace DA agonists

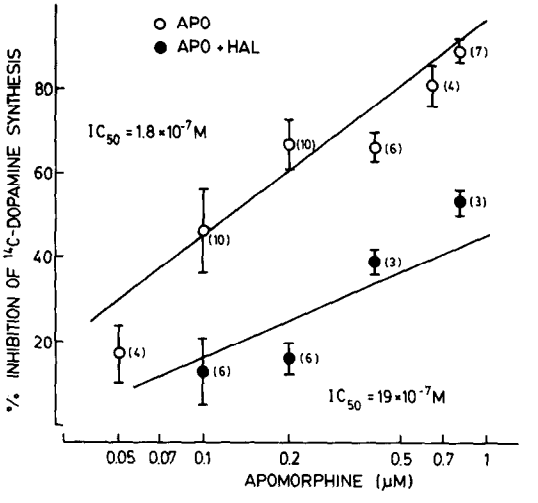

Fig. 1. [^{14}C]Dopamine biosynthesis in rat striatal slices as a function of external APO concentrations. Slices from corpus striatum were incubated in KRP medium containing saturating concentrations of [^{14}C]tyrosine in the presence of increasing external APO concentrations and in the absence (○) or presence (●) of a fixed haloperidol concentration (4×10^{-7} M). Incubation conditions and separation of [^{14}C]DA are described in the text. Striatal slices incubated in KRP alone had synthesized 38.5 ± 4.0 nmoles of [^{14}C]DA · (g wet wt)$^{-1}$ · hr^{-1} (N = 6; mean ± S.E.M.), whereas slices incubated in KRP + haloperidol had synthesized 42.2 ± 0.3 nmoles of [^{14}C]DA · (g wet wt)$^{-1}$ · hr^{-1} (N = 15; mean ± S.E.M.). APO values (○) refer to slices incubated in KRP alone, and APO + HAL values (●) refer to slices incubated in KRP + haloperidol. The numbers in parentheses denote the number of individual experiments and the brackets indicate the S.E.M.

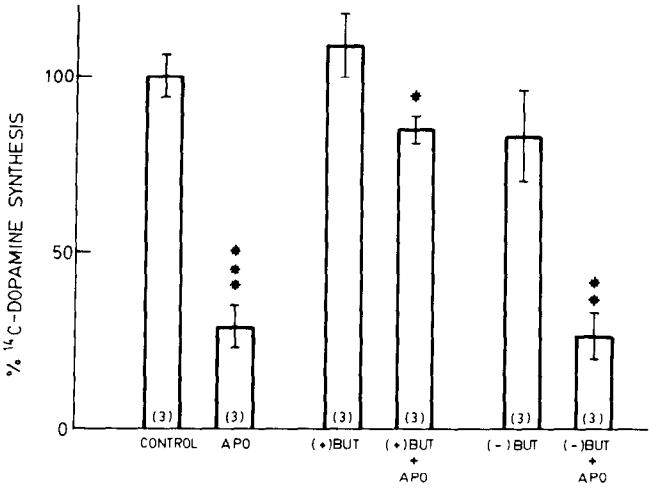

Fig. 2. Effect of apomorphine and (+)- or (−)butaclamol on [^{14}C]DA biosynthesis in striatal slices. Rat striatal slices were incubated in the different media in the presence of saturating concentrations of [^{14}C]tyrosine. Incubation conditions and separation of [^{14}C]DA are described in the text. APO was used at a concentration of 2×10^{-7} M and (+)- or (−)butaclamol at a concentration of 4×10^{-7} M. Control slices, incubated in KRP alone, had synthesized 39.5 ± 2.5 nmoles of [^{14}C]DA · (g wet wt)$^{-1}$ · hr^{-1}. Results are means ± S.E.M. of three different experiments. One asterisk (*) indicates $P < 0.05$, two asterisks (**) indicate $P < 0.025$, and three asterisks (***) indicate $P < 0.001$ when compared to respective controls without APO. One asterisk (*) also indicates $P < 0.005$ when compared to respective control without (+)butaclamol.

from receptor binding sites [9, 22, 23]. The addition of (+)- and (−)butaclamol to the incubation media did not significantly modify the rate of DA formation (Fig. 2). However, the two isomers behaved differently when their effects on DA synthesis were studied in the presence of APO (2×10^{-7} M). Whereas (+)butaclamol (4×10^{-7} M) reversed, almost completely to control values, the inhibition caused by APO on DA synthesis, (−)butaclamol (4×10^{-7} M) was not able to modify, at all, the inhibitory effect produced by APO (Fig. 2). Therefore, APO no longer inhibited DA synthesis in the presence of the (+) isomeric form of butaclamol.

Other substances were studied for their possible effects on DA synthesis in striatal slices (Table 1). The α_2-adrenergic agonist clonidine at 4×10^{-7} M produced only a mild inhibition (25%) whereas at 8×10^{-7} M it produced no effect on DA synthesis. The β-adrenergic agonist isoproterenol, studied at two different concentrations (4×10^{-7} and 8×10^{-7} M), failed to significantly inhibit [^{14}C]DA formation (Table 1).

It is possible that the above APO-mediated effects on [^{14}C]DA formation could have been produced, partially, through an inhibitory effect of APO on [^{14}C]tyrosine uptake by striatal slices. However, the presence of APO (6.4×10^{-7} M and 1.6×10^{-5} M) in the medium did not inhibit significantly the amounts of [^{14}C]tyrosine taken up by striatal slices incubated in the different media (data not shown).

Experimental modifications of the effect of apomorphine on dopamine synthesis. To learn about the mechanism involved in the APO-mediated inhibition on DA synthesis, the effects of this DA agonist were studied in the absence and in the presence of increasing external calcium concentrations. Incubation of striatal slices in calcium-free KR MOPS + EGTA*

* EGTA, ethyleneglycolbis(amino-ethylether)tetra-acetate.

Table 1. Effects of clonidine and isoproterenol on [^{14}C]dopamine synthesis by slices from rat corpus striatum*

Incubation medium	Rate of [^{14}C]dopamine synthesis [nmoles · (g wet wt)$^{-1}$ · hr^{-1}]	%
KRP (controls)	44.1 ± 2.0	100 ± 4
KRP + clonidine (4×10^{-7} M)	32.6 ± 3.6†	74 ± 9†
KRP + clonidine (8×10^{-7} M)	38.8 ± 6.3	88 ± 14
KRP + isoproterenol (4×10^{-7} M)	47.6 ± 0.9	108 ± 2
KRP + isoproterenol (8×10^{-7} M)	45.4 ± 7.2	103 ± 15

* Slices from rat corpus striatum were incubated in the different media containing saturating concentrations of [^{14}]tyrosine. Incubation conditions and separation of [^{14}C]DA are described in the text. Results are means ± S.E.M. of three different experiments. Controls represent the mean of six different experiments.

† $P < 0.05$ when compared to KRP controls.

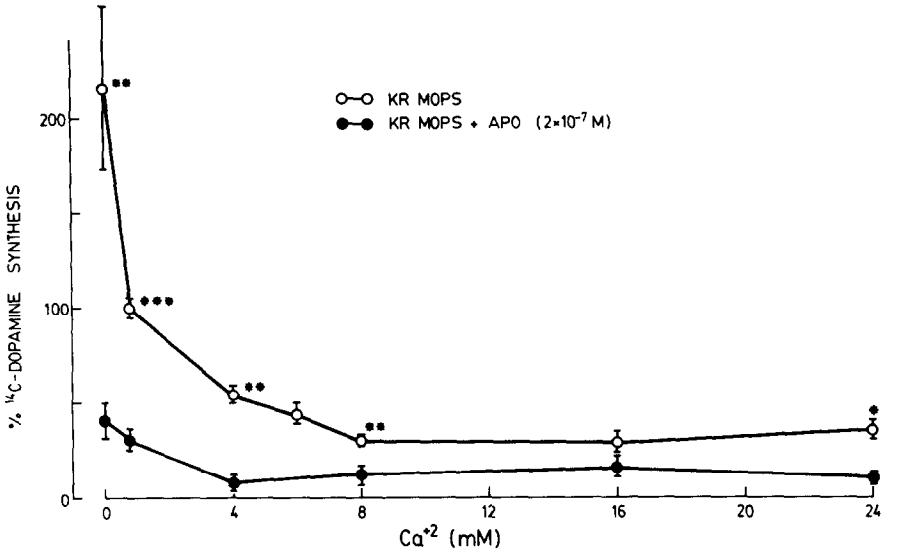

Fig. 3. [^{14}C]Dopamine biosynthesis in striatal slices as a function of external calcium concentrations and in the absence or presence of a fixed APO concentration. Incubation conditions and separation of [^{14}C]DA are described in the text. External calcium concentrations ranging from 0.75 to 24 mM were used. The calcium-free KR MOPS contained EGTA (1×10^{-4} M). Control slices, incubated in KR MOPS (Ca^{2+}, 0.75 mM), had synthesized 41.6 ± 1.8 nmoles of [^{14}C]DA · (g wet wt)$^{-1}$ · hr^{-1} (N = 18; mean ± S.E.M.) and this corresponds to a 100% value in the figure. The other points in the figure represent the mean ± S.E.M. of three different experiments. One asterisk (*) indicates $P < 0.025$, two asterisks (**) indicate $P < 0.005$, and three asterisks (***) indicate $P < 0.001$ when compared to the value obtained at the equivalent calcium concentration plus APO.

(1×10^{-4} M) for 30 min resulted in a marked increase in the rate of [^{14}C]DA formation (Fig. 3). A 116% increase in [^{14}C]DA synthesis rate was observed when compared to the rate obtained in striatal slices incubated in control KR MOPS (Fig. 3). The absence of calcium ions from the incubation medium was not able to modify the inhibitory effect of APO (2×10^{-7} M) on [^{14}C]DA synthesis. In fact, the inhibitory effectiveness of APO (2×10^{-7} M) was increased from 70 to 81% when calcium was omitted from the incubation medium (Fig. 3; compare values of APO-mediated inhibition obtained at 0 and 0.75 mM calcium).

Increasing the external calcium concentrations of the KR MOPS resulted in a decrease in the rate of DA formation (Fig. 3). MOPS buffer was used instead of phosphate buffer to avoid the formation of calcium phosphate precipitates when increasing the calcium concentration of the incubation medium. An initial rapid decline in [^{14}C]DA synthesis occurred as a function of rising external calcium concentrations from 0 and up to 8 mM. Thereafter and up to a 24 mM external calcium concentration, the rate of DA synthesis remained constantly depressed to 30% of control (Fig. 3). However, even in the presence of high external calcium concentrations (4, 8 and 24 mM), APO was able to further inhibit DA formation and therefore to potentiate the inhibition induced by calcium on DA synthesis. In other experiments (data not shown), it was found that (+)butaclamol (4×10^{-7} M) was not able to reverse or modify the inhibition on [^{14}C]DA formation produced by 6 mM calcium.

The effect of APO on DA synthesis was then studied in striatal slices subjected to experimental conditions such as absence of sodium ions or presence of ouabain in the incubation medium, which have been shown by others to increase the concentration of ionized calcium within the neuron [24–26]. Exposure of striatal slices to a sodium-free medium resulted in marked changes in the rate of [^{14}C]DA formation (Fig. 4). An increase in the rate of DA synthesis was found in slices incubated in a medium in which NaCl had been replaced iso-osmotically with choline chloride. In contrast, when sucrose was chosen as the iso-osmotic replacement for NaCl, a decrease in [^{14}C]DA formation was obtained. However, regardless of which was chosen as an iso-osmotic replacement for NaCl in the incubation medium, the presence of APO was no longer able to inhibit [^{14}C]DA synthesis in striatal slices when sodium was omitted from the incubation medium (Fig. 4). In other experiments it was found that ouabain (1×10^{-4} M), added to normal incubation medium, completely reversed the inhibitory effect of APO (2×10^{-7} M) on DA synthesis. In fact, the rate of [^{14}C]DA formation measured in slices incubated in KRP was the same as that obtained from slices incubated in KRP plus ouabain and plus APO (Table 2). Ouabain, at the concentration used, produced no effect *per se* on DA synthesis.

Since K^+ depolarization produces alterations in calcium flux within the nerve terminal [26], a study was carried out in which the inhibition of DA synthesis by APO was studied in the presence of external K^+-depolarizing concentrations. As reported previously [13, 20], incubation of striatal slices in KRP-high K^+ (25 and 40 mM) medium resulted in a marked increase in the rate of [^{14}C]DA formation (Fig. 5). Dopamine synthesis was inhibited at

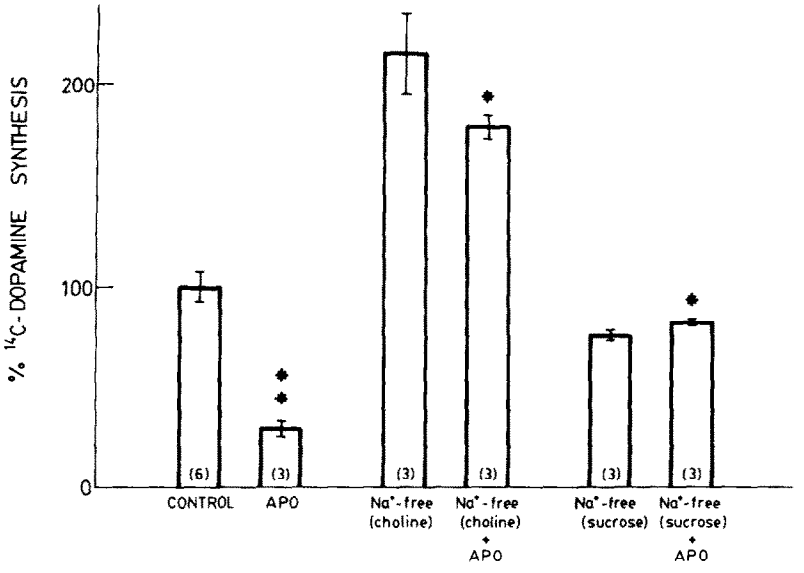

Fig. 4. Apomorphine-induced inhibition of [^{14}C]DA synthesis in striatal slices incubated in a sodium-free medium. In the Na^+-free media, NaCl was replaced iso-osmotically either by choline chloride or by sucrose. APO was used at a concentration of 2×10^{-7} M. Incubation conditions and separation of [^{14}C]DA are described in the text. The number of individual experiments is indicated at the bottom of each column. The brackets indicate the S.E.M. Control slices, incubated in KRP alone, had synthesized 36.1 ± 1.9 nmoles of [^{14}C]DA · (g wet wt)$^{-1}$ · hr^{-1}. One asterisk (*) indicates $P < 0.001$ when compared to slices incubated in normal KRP + APO and not significantly different when compared to respective control slices incubated in an Na^+-free media. Two asterisks (**) indicate $P < 0.001$ when compared to its respective control without APO.

2×10^{-7} M APO by 75% in slices incubated in normal KRP. However, the inhibitory effectiveness of APO was reduced, respectively, from 75 to 50% and from 75 to 32% in the presence of 25 mM K^+ and 40 mM K^+ in the incubation media ($P < 0.001$ in both cases) (Fig. 5).

Effect of apomorphine on dopamine release. It was interesting to study whether APO, at concentrations reported above to inhibit DA synthesis, was able to modify both spontaneous and depolarization-induced release of DA from striatal slices. In these experiments, the slices were incubated in the presence of [^{3}H]DA, and both the spontaneous and K^+-induced release of exogenously taken up [^{3}H]DA were followed in the presence and absence of APO, using the superfusion system described in Materials and Methods. Under these experimental conditions, K^+ stimulation produced a marked increase in the release of [^{3}H]DA that has been shown to be highly dependent on the presence of calcium in the superfusion medium [18]. Two successive K^+ stimulations (S_1 and S_2) were used, and APO was added to the superfusion system 8 min prior to the second stimulation. The presence of APO (2×10^{-7} and 4×10^{-7} M) in the superfusion medium did not modify the K^+ (27 mM)-induced release of labeled DA from striatal slices (Table 3). In addition, the spontaneous release of labeled DA remained unaltered in the presence of APO (data not shown). In other experiments, it was found that (+)butaclamol, at two different concentrations (5×10^{-8} and 4×10^{17} M), produced no effect upon K^+-depolarization-induced release of DA (data not shown).

Table 2. Effect of ouabain on apomorphine-induced inhibition of [^{14}C]dopamine synthesis by slices from rat corpus striatum*

Incubation medium	Rate of [^{14}C]dopamine synthesis [nmoles · (g wet wt)$^{-1}$ · hr^{-1}]	%
KRP (controls)	46.9 ± 3.4	100 ± 7
KRP + APO (2×10^{-7} M)	15.5 ± 3.4†	33 ± 7†
KRP + ouabain (1×10^{-4} M)	49.7 ± 5.2	106 ± 11
KRP + ouabain (1×10^{-4} M) + APO (2×10^{-7} M)	47.8 ± 3.3	102 ± 7

* Incubation conditions and separation of [^{14}C]DA are described in the text and under Table 1. Results are means ± S.E.M. of three different experiments. Controls represent the mean of six different experiments.
† $P < 0.001$ when compared to normal KRP control.

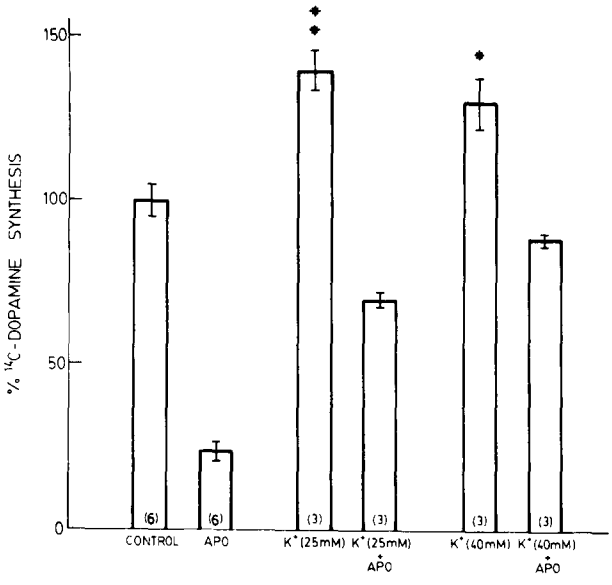

Fig. 5. Apomorphine-induced inhibition of [^{14}C]DA synthesis in striatal slices incubated under high external K^+ concentrations. Incubation conditions and separation of [^{14}C]DA are described in the text. APO was used at a concentration of 2×10^{-7} M. The number of individual experiments is indicated at the bottom of each column. The brackets indicate the S.E.M. Control slices, incubated in KRP alone, had synthesized 36.7 ± 3.6 nmoles of [^{14}C]DA · (g wet wt)$^{-1}$ · hr^{-1}. One asterisk (*) indicates $P < 0.025$ and two asterisks (**) indicate $P < 0.05$ when compared to control slices incubated in normal KRP.

DISCUSSION

Previous work from our laboratory [13, 17, 27] and that of others [12, 28] have indicated that tissue slices can be a very valuable experimental tool to study regulation of DA synthesis and release in the brain. We have now used slices from rat corpus striatum to study receptor-mediated modulation of neurotransmitter synthesis in nerve terminals belonging to the nigro-striatal dopaminergic system.

Apomorphine, a well known DA-receptor-stimulating agent, was able to markedly inhibit at relatively low concentrations (1×10^{-7} M) the conversion of tyrosine to DA when added to striatal slices (Fig. 1). These finding essentially agree with previous experiments reported by Goldstein *et al* [21]. However, these authors were unable to show any reversal of APO-mediated inhibition with DA receptor blocker agents added *in vitro* to striatal slices [7, 21] and this has made questionable the use of brain slices to study the mechanism of DA-receptor-mediated modulation of DA synthesis. In contrast, with our experimental conditions it was shown that the inhibitory effect of APO on DA synthesis was reversed quite effectively and specifically by *in vitro* addition of DA receptor blocker agents such as haloperidol and (+)butaclamol. The difference between our results and those of Goldstein *et al.* [21] might be due to the fact that these authors used a rather high haloperidol concentration (5×10^{-5} M) which probably produced *per se* an inhibitory effect on DA synthesis [9]. In other studies, we have found that

Table 3. Effect of apomorphine on K^+-induced release of exogenous [^{3}H]dopamine from rat striatal slices*

	Experimental conditions	% Release of [^{3}H]dopamine taken up by striatal slices S_1	S_2	S_2/S_1
Expt. I	Control	6.8 ± 0.7	4.3 ± 0.9	0.64 ± 0.15
	APO (2×10^{-7} M)	5.7 ± 1.2	4.4 ± 1.2	0.76 ± 0.06
Expt. II	Control	5.3 ± 1.0	4.4 ± 0.6	0.76 ± 0.08
	APO (4×10^{-7} M)	6.3 ± 0.6	4.0 ± 1.2	0.63 ± 0.16

* Striatal slices were incubated in normal KRP for 30 min at 37° in the presence of [^{3}H]DA and then transferred to superfusion chambers from which the spontaneous and K^+ (27 mM)-evoked release of the radioactive compound was followed. Two successive K^+ stimulations (S_1 and S_2) were used with an interval of 19 min in between. Where indicated, apomorphine (APO) was added to the superfusion system 8 min prior to the second K^+ (27 mM) stimulation (S_2). The tissues took up an average of 1,484,653 ± 257,809 and 1,652,294 ± 156,093 cpm of [^{3}H]DA (means ± S.E.M.) during the first experiment and an average of 758,434 ± 33,888 and 784,876 ± 22,255 cpm during the second experiment. The table presents the means ± S.E.M. from three different experiments.

DA antagonists such as spiroperidol (2×10^{-7} M) and trifluoperazine (2×10^{-6} M) partially reversed the inhibitory effect of APO in K^+-depolarized slices from olfactory tubercles.* The nature of the interaction between APO and DA antagonists has not been established by this paper. The concentration–response curve for APO determined in the presence of a fixed concentration of haloperidol showed a non-parallel shift to the right (Fig. 1), and this suggests a non-competitive interaction between haloperidol and APO. However, if in the presence of haloperidol one considers only the APO concentration interval between 2×10^{-7} and 8×10^{-7} M (Fig. 1), a parallel shift to the right of the concentration–response curve seems to occur, suggesting then a competitive interaction between haloperidol and APO. More experiments are needed in order to draw any definite conclusion regarding the nature of this interaction. The possibility exists that some of the inhibitory effects of APO reported above might have been due to a direct inhibitory effect of the drug on the enzyme tyrosine hydroxylase. However, APO caused a concentration-dependent inhibition of DA synthesis with an IC_{50} of 1.8×10^{-7} M, and higher concentrations of APO were needed to inhibit soluble tyrosine hydroxylase. Only 10^{-4} M apomorphine inhibited substantially tyrosine hydroxylase present in detergent-containing homogenates of striatal tissue,† in essential agreement with other reports [9, 21]. Therefore, in keeping with previous findings in synaptosomal preparations [7–9], it seems quite likely that the inhibitory effects of APO on DA synthesis reported in this paper were mediated through DA receptors located in the striatum.

The present results do not shed direct light on the question as to where in the striatum the DA-synthesis-modulating receptors described above are located. One possibility would be that they are located in intrinsic striatal neurons whose collaterals may impinge back on the dopaminergic nerve endings and thereby modulate DA synthesis. This possibility requires the participation of a transsynaptic phenomenon mediated via postsynaptic receptors. Alternatively, DA-synthesis-modulating receptors might exist in nerve endings of fibres that are afferent to the striatum, i.e. APO has been found to inhibit K^+-evoked release of glutamate from terminals of the cortico-striatal pathway, and this inhibition, in turn, is antagonized by haloperidol [29, 30]. However, the experimental evidence available favors the idea that the receptors involved in the modulation of DA synthesis are actually located on DA nerve terminals. First, these DA receptors have been demonstrated in striatal synaptosomal preparations whose nature excludes the preservation of the cyto-architecture of the striatum and rules out, then, the existence of intact neurons [7–9]. Second, intrastriatal administration of kainic acid to rats does not alter the APO-induced decrease in DOPA accumulation following GBL inhibition of DA cell firing, and this suggests that the APO inhibitory effect is not mediated by DA receptors located on intrinsic striatal neurons [31]. Third, as shown in this paper, the inhibitory effect of APO on DA synthesis remained unaltered in the absence of external calcium and in the presence of EGTA (Fig. 3), thereby ruling out the participation of transsynaptic phenomena via release of transmitter from intrinsic striatal neurons or from nerve terminals on fibers afferent to the striatum. Our findings support the idea that the DA-synthesis-modulating receptors described above are located on striatal dopaminergic nerve terminals. In keeping with a recent review [32], we would agree that "autoreceptor" is a more appropriate term to describe these receptors.

As indicated at the beginning of this paper, it has been proposed that DA autoreceptors may modulate DA synthesis by gating calcium entry into the dopaminergic nerve terminal [2, 11]. In support of this proposal, it was found that the raising of external calcium concentrations led to a decrease in DA synthesis rate which reached its lowest value in the presence of an 8 mM external calcium concentration (Fig. 3). However, APO was able to markedly inhibit DA formation even in the absence of calcium and in the presence of EGTA in the incubation medium (Fig. 3). In addition, the APO inhibitory effect was manifest even in the presence of external calcium concentrations (4, 8 and 24 mM) which seemed to maximally depress the rate of DA synthesis (Fig. 3). These observations indicate that the APO effects reported were independent of extracellular calcium and suggest that autoreceptor-mediated modulation of DA synthesis does not occur through regulation of calcium influx into the DA nerve terminals.

Incubation of striatal slices in a calcium-free medium or in a medium containing a calcium-chelating agent resulted in an increase in DA synthesis and produced changes in the kinetic properties of the rate-limiting enzyme, tyrosine hydroxylase, i.e. the apparent K_m of the enzyme for pterin cofactor was decreased significantly with no significant changes in the apparent V_{max} [14]. These findings indicate that a time-dependent activation of tyrosine hydroxylase in dopaminergic nerve terminals can occur under conditions of diminished extracellular calcium and that the increase in DA synthesis following incubation of striatal slices in a calcium-free medium might be mediated, in part, by a time-dependent activation of this rate-limiting enzyme. The results presented in this paper suggest that the factors involved in the calcium-free induced activation of tyrosine hydroxylase might be particularly sensitive to the presence of APO in such a way that APO almost totally prevents the calcium-free induced DA formation (Fig. 3). This probably explains why the increase in tyrosine hydroxylation which occurs during cessation of impulse flow in nigro-striatal dopaminergic neurons is so effectively blocked by DA receptor agonists such as APO [1, 2, 6].

Experiments on brain slices have shown that incubation in an Na^+-free medium produces both a rapid outward movement of intracellular Na^+ and a rapid reduction in the efflux of calcium, with a concomitant increase in calcium uptake and content

* K. Umezu, R. H. Roth and G. Bustos, results to be published elsewhere.

† M. Bitran, J. Fiedler and G. Bustos, unpublished observations.

of the slices [25, 33]. Recently, Blaustein *et al.* [26] have obtained similar results in experiments in which synaptosomal preparations were used. In other experiments, ouabain was found to increase the Na^+ content of brain cells, with an associated elevation of calcium uptake [33]. All these data support the existence of a sodium-calcium exchange mechanism in cerebral slices and indicate that incubation of the slices in a Na^+-free medium or in medium enriched with ouabain can produce a rapid rise in the intraneuronal calcium concentration [24–26]. The results presented in this paper demonstrate that replacement of Na^+ in the incubation medium either by choline chloride or by sucrose, or the addition of ouabain to the medium, all dramatically abolished the ability of APO to inhibit DA synthesis in striatal slices (Fig. 4 and Table 2). In addition, the inhibitory effectiveness of APO was markedly reduced when the slices were incubated in the presence of K^+-depolarizing concentrations that are known to induce an entry of calcium within the nerve terminal (Fig. 5). All these observations indicate that rising internal calcium within the DA nerve terminals dramatically prevents the inhibitory effect of APO on DA synthesis to manifest itself. It is suggested then that a step liable to be inhibited by high intraneuronal calcium exists during the events that mediate DA autoreceptor stimulation and DA synthesis inhibition. Alternatively, exposure of the slices to the previous experimental manipulations might render tyrosine hydroxylase less sensitive to the, albeit indirect, inhibitory effect of APO since changes in the physical and kinetic properties of this enzyme occur following incubation of striatal slices in a Na^+-free medium or under K^+-depolarizing conditions [34, 35]. We are currently investigating these possibilities.

Since Na^+-free media, ouabain, and K^+ stimulation all block the inhibitory effect of APO on DA synthesis, the suggestion has been made above that this blockade of APO effect is due to increasing intracellular calcium. Accordingly, one should expect that very high extracellular calcium abolishes the APO inhibition of DA synthesis. In fact, and as shown in Fig. 3, the inhibitory effect of APO was abolished in the presence of a 16 mM external calcium concentration. Nevertheless, in the presence of other high external calcium concentrations (8 and 24 mM), the inhibitory effect of APO persisted, although it was relatively less when compared to that observed in the presence of low external calcium (0.75 mM) (Fig. 3). These results are difficult to interpret since high external calcium markedly depressed the rate of DA synthesis (Fig. 3). In addition, it is not known whether high external calcium increases internal calcium concentration to the high magnitude reported for Na^+-free, ouabain, or K^+ depolarization [24–26, 33]. It is known that nerve terminals maintain under resting conditions a relatively low concentration of calcium in the cytoplasm, and this is explained, in part, by binding of this cation to membranes and proteins and by extruding calcium against a large electrochemical gradient through a sodium–calcium exchange mechanism [24, 26]. These processes are markedly disrupted by ouabain, absence of Na^+, or K^+ depolarization.

Discrepant findings have been reported concerning DA autoreceptor modulation of DA release in rat striatal slices. Some investigators have reported that DA receptor agonists such as APO decrease, while DA receptor antagonists such as haloperidol and fluphenazine increase, electrically and K^+-evoked release of DA [10, 36, 37]. In contrast, other authors have observed that evoked release of DA does not change in the presence of APO or in the presence of haloperidol, sulpiride or chlorpromazine [38, 39]. Under our experimental conditions, it was not possible to demonstrate any change in K^+ (27 mM)-induced release of DA in the presence of APO (2×10^{-7} and 4×10^{-7} M). However, similar APO concentrations produced a marked inhibition of DA synthesis measured both in resting and depolarized striatal slices (Figs. 1 and 5). In addition, the effect of APO on DA synthesis was demonstrated under conditions in which neurotransmitter release is normally inhibited (Fig. 3). All these observations suggest that DA-synthesis-modulating autoreceptors are different from those that might modulate DA release or that quite different mechanisms mediate autoreceptor modulation of DA synthesis and release.

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