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REGULATION OF TRANSMITTER SYNTHESIS AND RELEASE IN MESOLIMBIC DOPAMINERGIC NERVE TERMINALS

EFFECT OF ETHANOL*

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Abstract—Slices from rat olfactory tubercle were incubated in freshly oxygenated Krebs–Ringer phosphate (KRP) and in the presence of L-tyrosine[^{14}C-U] as dopamine (DA) precursor. Thereafter, the newly synthesized [^{14}C]DA and the [^{14}C]DA released into the incubation media were isolated by Alumina column, and ion-exchange, chromatography. The presence of K^+ depolarizing concentrations (25–70 mM) in the incubation media markedly increased the formation of [^{14}C]DA from [^{14}C]tyrosine, following a rather complex and biphasic pattern. Dibutyryl cyclic AMP (dB-cAMP) and theophylline also increased the formation of newly synthesized [^{14}C]DA. Ethanol (0.2 to 0.4%, w/v) significantly blocked the stimulation of [^{14}C]DA biosynthesis that was induced by low K^+ depolarizing concentrations (25 mM) and by dB-cAMP (5×10^{-4} M) or theophylline (1×10^{-3} M). In contrast, only higher ethanol concentrations (0.8 to 1.1%, w/v) blocked the increase in DA formation induced by high K^+ depolarizing concentrations (40 and 55 mM). Potassium depolarization (40 mM) markedly evoked the release of newly synthesized [^{3}H]DA or [^{3}H]DA previously taken up by the slices. The release was shown to be dependent upon the presence of Ca^{2+} and inhibited by an excess of Mg^{2+} (12 mM). Ethanol (0.8 to 1.1%, w/v) produced no effect on K^+-induced release of [^{3}H]DA. The model described in this paper can be used as a simple experimental tool to study neurotransmitter synthesis and release from nerve terminals belonging to the mesolimbic dopaminergic system. The results reported suggest the existence of at least two mechanisms by which neuronal depolarization increases transmitter formation in mesolimbic dopaminergic terminals. Ethanol, at relatively low concentrations, seems to produce a specific inhibitory effect upon the mechanism that predominates under low depolarizing conditions. The possibility is raised that the effects described for ethanol may play a role in the neuropharmacological responses induced by this agent *in vivo*.

It has been proposed recently that some of the effects elicited by ethanol in the CNS might be mediated through a disturbance of the regulatory mechanisms normally operative in central catecholaminergic neurons to adjust transmitter synthesis to meet demands incurred by altered impulse flow [1]. This proposal is supported by the fact that the addition of ethanol (0.2 to 0.8%, w/v) to striatal slices inhibits both *in vitro* activation of tyrosine hydroxylase induced by K^+ depolarization and the *in vitro* increase in dopamine (DA) synthesis induced by K^+ depolarization or by addition of dibutyryl-cAMP to the incubation medium [2–4]. In addition, it has been possible to demonstrate a regional specificity for the ethanol effects on monoamine synthesis regulation within the brain: ethanol inhibits the potassium-dependent increase in catecholamine synthesis in dopamine-rich areas such as striatum or olfactory tubercle but not in noradrenaline-rich areas such as the hippocampus [5]. In more recent studies, chronic ethanol administration to rats has been found to modify the modulation of brain DA biosynthesis by autoreceptors located in dopaminergic nerve terminals [6].

Most of the studies described above were conducted in brain areas rich in nerve terminals belonging to the nigro-striatal dopaminergic system. The studies by Ungerstedt [7] and others [8, 9], however, have shown the existence of at least three major ascending DA neuronal pathways in rat brain. One of these pathways, the mesolimbic DA system, has received considerable attention lately with regard to speculations concerning the pathophysiology of schizophrenia and the sites of action of antipsychotic drugs [10, 11]. Recent studies have also implicated the mesolimbic system in the locomotor behaviors produced by various drugs [12, 13]. Thus, it seemed of high interest to develop an *in vitro* system to study whether ethanol, at relevant pharmacological concentrations, is able to modify those factors that control the DA life cycle in mesolimbic terminals in the brain. This paper describes a study in which we have used tissue slices from olfactory tubercles to study the neurotransmitter life cycle in nerve terminals belonging to the dopaminergic mesolimbic system

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as well as the way in which DA synthesis and release in these terminals are modified by the addition of ethanol.

MATERIALS AND METHODS

Studies on dopamine synthesis. Sprague–Dawley rats of both sexes, weighing about 200–250 g, were decapitated, and the left and right olfactory tubercles were rapidly dissected, as described by Murrin and Roth [14]. Olfactory tubercle slices (0.2 mm in thickness) were prepared with a Sorvall tissue chopper. Tissue slices weighing about 20 mg were incubated at 37° in beakers containing 2 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] sp. act. 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, the beakers were chilled on ice, and the slices were separated from the media by centrifugation at 30,000 *g* for 10 min in a Sorvall refrigerated centrifuge. The slices were then homogenized in 15% trichloroacetic acid (TCA); each incubation medium was acidified with 0.5 ml of 50% TCA. When ethanol was employed, it was added to the medium at the beginning of the preincubation period. When used, dibutyryl cyclic adenosine monophosphate (dB-cAMP) and theophylline were added 5 min after the beginning of the preincubation period. Tissue blanks were run by incubating olfactory tubercle slices as described above but in the presence of α-methyl-*p*-tyrosine (2×10^{-4} M), an inhibitor of tyrosine hydroxylase. Similar blank values were obtained by incubating olfactory tubercle tissue that had been initially precipitated with 15% TCA. A typical sample to blank ratio usually had a value between 3 and 5. Unlabeled DA, norepinephrine and tyrosine (50 μg each) were added to both tissue homogenates and media, and the precipitated protein was then removed by centrifugation at 12,000 *g* for 20 min. The tissue and media extracts were immediately frozen and kept for separation and analysis of labeled DA and its metabolites as described below.

In the experiments utilizing radioactive L-dihydroxyphenylalanine (L-DOPA) as precursor of [^{14}C]DA, following a 10-min preincubation period, labeled L-DOPA-2-[^{14}C], sp. act. 4.5 mCi/mmole, was added to the media containing the slices, producing a final L-DOPA concentration of 2.2×10^{-5} M. After an additional incubation period of 30 min, the reaction was stopped with ice-cold KRP and the slices were separated from the media by centrifugation at 12,000 *g* for 10 min. The slices and each incubation medium were processed then with TCA using the same procedure as described above. To each tissue homogenate and medium fraction were added 50 μg each of nonradioactive DA, noradrenaline and L-DOPA. The fractions were frozen and kept for separation of cetecholamines from L-DOPA as described below. Tissue blanks were run by incubating labeled L-DOPA with olfactory tubercle slices that had been previously precipitated with 15% TCA.

Results were calculated as described previously [15] and are expressed in terms of nmoles [^{14}C]DA·g wet wt^{-1}·hr^{-1}, unless stated differently. Total [^{14}C]DA synthesized represents the sum of the [^{14}C]DA content in slices plus the [^{14}C]DA content in the medium.

Studies on dopamine release. The release of exogenously taken up [^{3}H]DA or newly synthesized [^{3}H]DA from olfactory tubercle slices was followed essentially as described before for striatal slices [16]. Briefly, 5–7 mg of the olfactory tubercle slices was incubated for 30 min at 37° in 2 ml KRP, pH 7.4, saturated with oxygen and containing either [^{3}H]DA (3×10^{-7} M) or [^{3}H]tyrosine (2×10^{-7} M). Thereafter the slices were 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. Stimulation of release was carried out for 1 or 2 min by switching the superfusion to iso-osmotic KRP containing either 55 mM K^+ or 40 mM K^+.

When the release from olfactory tubercle slices of exogenously loaded [^{3}H]DA was to be studied, an initial superfusion period of 15 min was allowed before release was stimulated with K^+. Potassium-induced release of newly formed [^{3}H]DA from the slices was started after 10 min of superfusion with normal KRP. These procedures allowed for a steady and constant basal release prior to stimulation. Samples containing the released material were collected every minute into tubes containing 1 ml of 50% TCA and carrier DA (50 μg). The slices were recovered at the end of the superfusion period, and catechols were extracted from the tissues with 15% TCA. Samples were immediately frozen and kept for chromatographic analysis as described below. Release of radioactive DA is expressed as the percentage of total [^{3}H]DA found in the tissue and in different collecting tubes at the end of the superfusion period.

Chromatographic procedures and radioactivity determinations. Separation and analysis of the tissue and the media for labeled DA and its metabolites were carried out by adsorption chromatography and ion-exchange chromatography as described previously [15, 16]. Alumina columns were used to concentrate the catecholamines and deaminated metabolites and to separate them from tyrosine and *O*-methylated metabolites. Long (12×0.4 cm) columns of Dowex 50W-X4 (Na^+), 100–200 mesh, were used to separate DA from deaminated metabolites and from L-DOPA. Analysis through Dowex columns showed that labeled DA accounted for 80–85 percent of the labeled catechols synthesized after normal KRP incubation or after K^+ stimulation. Eluates from chromatography columns containing labeled catechols were analyzed for ^{14}C and ^{3}H in a Nuclear Chicago Scintillation counter to a constant deviation of 1.5 percent. Reported values have not been corrected for recovery.

The [^{14}C]tyrosine taken up by the tissue during the

incubation period was separated from catechols and other labeled tyrosine metabolites by passage through Alumina columns and columns of Dowex 50W-X8 (H^+), 100–200 mesh, as described previously [17]. Eluates from the columns containing labeled tyrosine were analyzed for ^{14}C as described above.

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 and 16 mM Na_2HPO_4, (pH 7.4). Krebs–Ringer phosphate-high K^+ was made by replacing proportions of NaCl with equimolar amounts of KCl. Other modifications of the KRP are described in the text.

L-Tyrosine-[^{14}C-U], L-tyrosine-[3,5-^{3}H] and [^{3}H]dopamine were obtained from the New England Nuclear Corp., Boston, MA U.S.A.

RESULTS

Effect of ethanol on dopamine synthesis. The rate of formation of [^{14}C]DA from [^{14}C]tyrosine, studied in olfactory tubercle slices incubated in normal KRP medium, was linear for up to 45 min when using a saturating [^{14}C]tyrosine concentration of 2.5×10^{-5} M. The synthesis of [^{14}C]DA, over a 30 min period, in the presence of increasing external concentrations of [^{14}C]tyrosine, followed Michaelis–Menten kinetics; the apparent K_m value for [^{14}C]tyrosine was 3.8×10^{-6} M. The absence of Ca^{2+} from the KRP increased significantly the net or total formation rate of newly synthesized [^{14}C]DA by olfactory tubercle slices (Table 1). The omission of Ca^{2+} from the incubation media also markedly raised the tissue/medium ratios of [^{14}C]DA, probably due to the inhibitory effect of lack of Ca^{2+} on the release of newly formed DA (see below). As shown in Table 1, the addition of ethanol (0.8% and 1.1%, w/v) directly to normal KRP did not significantly affect the total rate of conversion of [^{14}C]tyrosine to [^{14}C]DA by olfactory tubercle slices. Further, the presence of ethanol did not either block or potentiate Ca^{2+}-free induced formation of [^{14}C]DA (Table 1). These results are in essential agreement with previous reports by us in which striatal slices were used as an experimental tool to study the effects of ethanol [3].

A depolarizing concentration of K^+ (55 mM) significantly increased both the rate of formation of [^{14}C]DA by olfactory tubercle slices and the amount of newly synthesized [^{14}C]DA present in the media (Table 1). As a result of this, the net or total rate of formation of [^{14}C]DA was markedly increased (151 per cent) by K^+ depolarization. Omission of Ca^{2+} or the addition of Mg^{2+} (12 mM) to the KRP–high K^+ (55 mM) medium significantly blocked the K^+-induced formation of newly synthesized [^{14}C]DA (data not shown). Potassium ions (55 mM) also markedly lowered the tissue/medium ratio of [^{14}C]DA from 7.8 to 1.6 (Table 1, column 6). This last effect was probably due both to the stimulatory effect of high K^+ on the release of newly synthesized DA and to its inhibitory effect on the re-uptake of the monoamine [16, 18]. The synthesis of [^{14}C]DA was also studied in olfactory tubercles incubated under normal and depolarizing conditions and using

Table 1. Effect of ethanol on dopamin synthesis by slices from rat olfactory tubercle*

Incubation medium	N	Rate of [^{14}C]dopamine synthesis [nmoles·g (wet wt)$^{-1}$·hr^{-1}] Tissue	Medium	Total	Tissue/Medium
KRP (controls)	17	10.1 ± 0.3	1.3 ± 0.2	11.3 ± 0.3	7.8
KRP + ethanol (0.8%, w/v)	4	9.3 ± 0.6	2.4 ± 0.7	11.7 ± 0.2	3.9
KRP + ethanol (1.1%, w/v)	3	9.2 ± 0.7	1.9 ± 0.2	11.2 ± 0.6	4.9
KRP, Ca^{2+}-free	12	15.2 ± 0.5†	0.6 ± 0.2	15.8 ± 0.5†	25.3
KRP, Ca^{2+}-free + ethanol (0.8%, w/v)	4	14.6 ± 0.7†	1.4 ± 0.5	16.0 ± 0.8†	10.4
KRP, Ca^{2+}-free + ethanol (1.1%, w/v)	4	13.1 ± 1.1†	2.0 ± 0.4‡	15.0 ± 1.0†	6.5
KRP-high K^+ (55 mM)	6	16.2 ± 0.6†	10.1 ± 0.3†	26.2 ± 0.7†	1.6
KRP-high K^+ (55 mM) + ethanol (0.8%, w/v)	9	15.4 ± 0.9†	9.4 ± 0.7†	25.2 ± 0.9†	1.6
KRP-high K^+ (55 mM) + ethanol (1.1%, w/v)	5	9.6 ± 0.8§	8.3 ± 0.6†‖	17.9 ± 0.7†,§	1.2

* Slices of olfactory tubercle were prepared by means of a Sorvall tissue chopper and were incubated in media containing saturating concentrations of [^{14}C]tyrosine (2.5×10^{-5} M, sp. act. 5.0 mCi/mmole and 0.313 μCi/flask) for 30 min at 37°. [^{14}C]Dopamine was separated by column chromatography, and its radioactivity was determined in a Nuclear Chicago Scintillation counter. Results are means ± S.E.M.; N represents the number of different experiments.

† $P < 0.001$ when compared to normal KRP controls.

‡ $P < 0.02$ when compared to KRP, Ca^{2+}-free.

§ $P < 0.001$ when compared to KRP-high K^+ (55 mM) without ethanol.

‖ $P < 0.05$ when compared to KRP-high K^+ (55 mM) without ethanol.

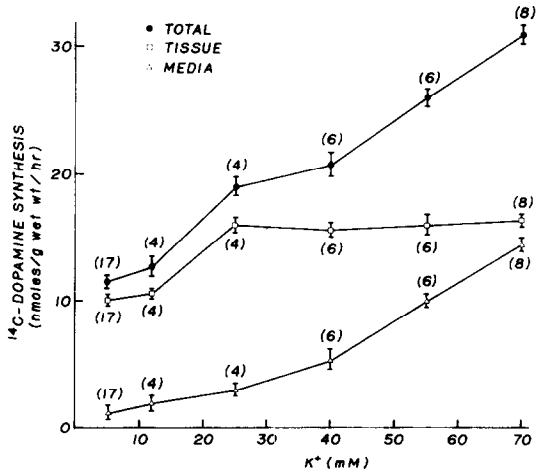

Fig. 1.[^{14}C]Dopamine biosynthesis in olfactory tubercle slices as a function of external K^+ concentrations. The olfactory tubercle was dissected and slices were prepared by means of a Sorvall tissue chopper. Thereafter, the slices were incubated for 30 min at 37° in medium containing saturating concentrations of [^{14}C]tyrosine (2.5×10^{-5} M, sp. act. 5.0 mCi/mmole and 0.313 μCi/flask) in the presence of increasing external K^+ concentrations (from 4.8 to 70 mM). At the end of the incubation period, the [^{14}C]DA was separated by column chromatography and radioactivity was determined as described in Table 1. The numbers in parentheses denote the numbers of individual experiments and the brackets indicate the S.E.M.

L-DOPA [2-^{14}C] as a precursor of [^{14}C]DA. The rate of DA formation from L-DOPA-[2-^{14}C] did not change when the slices were subjected to K^+ (55 mM) depolarization (data not shown). These results indicate that the stimulatory effect of K^+ on the rate of [^{14}C]DA formation, as shown in Table 1, was probably exerted at the level of the rate-limiting enzyme in the synthesis of DA, tyrosine hydroxylase.

Ethanol (0.8%, w/v) added directly to the KRP–high K^+ (55 mM) medium did not modify the K^+-induced rate of formation of [^{14}C]DA (Table 1). These results differ substantially from those reported previously with striatal slices where ethanol (0.2 to 0.8%, w/v) was found to specifically inhibit the increase in DA synthesis induced by K^+ (55 mM) depolarization [3]. It was decided then to study the increased rate of DA formation by means of detailed concentration–response curves in the presence of both ethanol and depolarizing conditions. Figure 1 illustrates the conversion of [^{14}C]tyrosine to [^{14}C]DA in olfactory tubercle slices in the presence of increasing external concentrations of K^+. Total [^{14}C]DA synthesized per hr by the slices, as shown in Fig. 1, represents the sum of the [^{14}C]DA synthesized per hr in the tissue plus that in the medium. The rate of [^{14}C]DA formation varied as a function of the external K^+ concentration, following a rather complex pattern (Fig. 1). External K^+, at concentrations up to 12 mM, did not modify significantly the rate of total [^{14}C]DA synthesis. Raising the external K^+ concentration from 12 mM to concentrations that undoubtedly produce neuronal depolarization [19] resulted in a marked and biphasic increase in the rate of total [^{14}C]DA synthesis (Fig. 1). From 12 to 40 mM K^+ there was a substantial rise in [^{14}C]DA synthesis which seemed to reach a plateau around 25 mM. The initial increase in the rate of [^{14}C]DA formation seems to correlate well with the amount of [^{14}C]DA that was present in the tissue. In fact, most of the [^{14}C]DA newly synthesized under these last experimental conditions was localized in the tissue (Fig. 1). Thereafter, and from 40 mM up to 70 mM K^+, there was a new substantial increase in the rate of [^{14}C]DA formation that correlated very well with the amounts of [^{14}C]DA being released to the media (Fig. 1). The effects of various concentrations of ethanol (0.2 to 1.1%, w/v) on the increase in the rate of DA formation induced by different external K^+ concentrations were then studied (Fig. 2). Only a relatively high concentration of ethanol (1.1%, w/v) blocked K^+ (55 mM)-induced synthesis of [^{14}C]DA (Table 1 and Fig. 2). However, stimulation of DA synthesis by K^+ was more sensitive to

Table 2. Effects of various concentrations of ethanol on dibutyryl cyclic AMP- and theophylline-induced stimulation of [^{14}C]dopamine synthesis by slices from rat olfactory tubercle*

Incubation medium	N	Rate of [^{14}C]dopamine synthesis [nmoles·(g wet wt)$^{-1}$·hr^{-1}] Tissue	Medium	Total
KRP (controls)	17	10.1 ± 0.3	1.3 ± 0.2	11.3 ± 0.3
KRP + dB-cAMP	8	23.2 ± 0.5†	1.6 ± 0.3	25.0 ± 0.6†
KRP + dB-cAMP + ethanol (0.2%, w/v)	5	19.8 ± 0.4‡	2.3 ± 0.1	22.0 ± 0.5‡
KRP + dB-cAMP + ethanol (0.4%, w/v)	8	18.4 ± 1.0‡	2.3 ± 0.4	20.7 ± 0.7§
KRP + dB-cAMP + ethanol (0.8%, w/v)	4	17.4 ± 0.4§	0.9 ± 0.2	18.2 ± 0.7§
KRP + theophylline	9	16.0 ± 0.3†	1.9 ± 0.3	17.9 ± 0.2†
KRP + theophylline + ethanol (0.2%, w/v)	8	15.3 ± 0.6	0.8 ± 0.2‖	16.1 ± 0.5‡
KRP + theophylline + ethanol (0.4%, w/v)	11	13.1 ± 0.7‡	2.0 ± 0.3	15.1 ± 0.3§
KRP + theophylline + ethanol (0.8%, w/v)	7	10.2 ± 0.3§	2.9 ± 0.4	13.1 ± 0.3§

* Olfactory tubercles were dissected and slices were prepared by means of a Sorvall tissue chopper. Incubation conditions and separation of [^{14}C]dopamine are described under Table 1. Dibutyryl cyclic AMP and theophylline were used, respectively, at concentrations of 5×10^{-4} M and 1×10^{-3} M. Results are means ± S.E.M.; N represents the number of different experiments.

† $P < 0.001$ when compared to normal KRP control.

‡ $P < 0.005$ when compared to respective dB-cAMP or theophylline controls without ethanol.

§ $P < 0.001$ when compared to respective dB-cAMP or theophylline controls without ethanol.

‖ $P < 0.025$ when compared to respective theophylline control without ethanol.

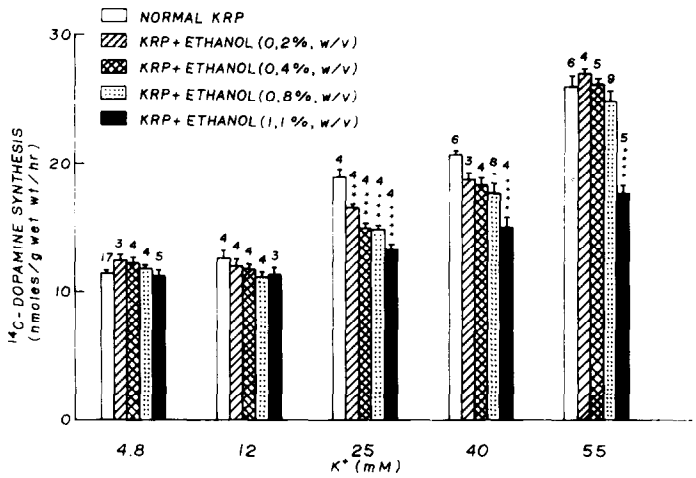

Fig. 2. Effect of various concentrations of ethanol on potassium-induced synthesis of [^{14}C]DA by olfactory tubercle slices. The olfactory tubercle was dissected and slices were prepared by means of a Sorvall tissue chopper. Incubation conditions and separation of [^{14}C]DA are described in the legend of Fig. 1. External K^+ concentrations ranging from 4.8 to 55 mM were used. The number of individual experiments is indicated at the top of each column. The brackets indicate the S.E.M. One asterisk (*) indicates $P < 0.025$; two asterisks (**) indicate $P < 0.01$; three asterisks (***) indicate $P < 0.005$; and four asterisks (****) indicate $P < 0.001$ when compared to respective controls without ethanol.

the inhibitory effect of ethanol when the K^+ external concentrations used to depolarize the slices were reduced. Thus, [^{14}C]DA formation induced by K^+ (40 mM) was significantly blocked by both 0.8 and 1.1% (w/v) ethanol (Fig. 2). Furthermore, concentrations of ethanol as low as 0.2% (w/v) significantly inhibited the effect of 25 mM K^+ on the rate of DA formation (Fig. 2). In contrast to these results, ethanol (0.2 to 1.1%, w/v) was without effect on the rate of DA formation in olfactory tubercle slices incubated in normal KRP or KRP containing 12 mM K^+. Hillman and McIlwain [19] have reported that 12 mM K^+ does not produce neuronal depolarization in brain slices.

Incubation of olfactory tubercle slices in the presence of dB-cAMP or theophylline also resulted in a marked increase in the rate of total conversion of [^{14}C]tyrosine to [^{14}C]DA (Table 2). The increased formation of newly synthesized [^{14}C]DA was concentration-dependent. Concentrations of dB-cAMP ranging from 1×10^{-5} to 5×10^{-4} M, respectively, increased the rate of formation of total DA from 25 to 125 percent. Theophylline, in a concentration range of 1×10^{-5} to 1×10^{-3} M, accelerated the rate of DA synthesis from 25 to 61 percent. [^{14}C]Dopamine in the medium was always a small fraction of the [^{14}C]DA content in the tissue, and it did not vary in the presence of dB-cAMP or theophylline. As shown in Table 2, ethanol exerted a concentration-dependent inhibitory effect on dB-cAMP- and theophylline-induced formation of [^{14}C]DA. Concentrations of ethanol as low as 0.2% (w/v) produced, respectively, a 27 and 21 percent inhibition of the stimulatory effect produced by dB-cAMP (5×10^{-4} M) and theophylline (1×10^{-3} M) ($P < 0.005$ when compared to respective dB-cAMP or theophylline controls without ethanol). Higher concentrations of ethanol (0.8%, w/v) were able to block respectively 49 and 73 percent of the stimulatory effects induced by dB-cAMP and theophylline (Table 2).

It is possible that the above inhibition by ethanol

Table 3. Effect of ethanol on K^+-induced release of [^{3}H]dopamine newly synthesized by rat olfactory tubercle slices*

Superfusion medium	N	Release of [^{3}H]dopamin (% of newly synthesized [^{3}H]dopamine) Release 1 min before K^+ stimulation	Release during K^+ stimulation	Release 1 min after K^+ stimulation
KRP normal	6	0.28 ± 0.05	5.25 ± 0.25	1.32 ± 0.34
KRP + ethanol (1.1%, w/v)	3	0.29 ± 0.01†	5.13 ± 0.05†	0.62 ± 0.17†

* Slices from olfactory tubercles were incubated in normal KRP for 30 min at 37° in the presence of [^{3}H]tyrosine and then transferred to the superfusion system. Spontaneous and K^+-induced release from the tissues was then followed in the superfusion system in either the absence or presence of ethanol. Potassium stimulation was carried out for 1.0 min. The tissues exposed to KRP, or KRP + ethanol, had previously synthesized, respectively, 135,961 ± 7560 and 139,867 ± 21,212 cpm of [^{3}H]dopamine (mean ± S.E.M.). Values in the table are means ± S.E.M.; N represents the number of different experiments.

† Not significantly different when compared to respective control without ethanol.

of the stimulated formation of [^{14}C]DA could have been produced through an inhibitory effect of ethanol on [^{14}C]tyrosine uptake by olfactory tubercle slices. However, data not shown in the paper indicate that the presence of ethanol (0.8 and 1.1%, w/v) in the media did not modify significantly the amounts of [^{14}C]tyrosine taken up by olfactory tubercle slices incubated in the different media.

Effect of ethanol on dopamine release. It was particularly interesting to study whether ethanol was able to modify depolarization-induced release of DA from olfactory tubercle slices using an experimental system specially designed for this purpose [16]. In these experiments, the slices were incubated in the presence of [^{3}H]tyrosine or [^{3}H]DA, and the K^+-induced release of newly formed [^{3}H]DA or exogenously taken up [^{3}H]DA was followed both in the presence and absence of ethanol, using the superfusion system described in Materials and Methods. Under these experimental conditions, K^+ (40 mM) stimulation produced a marked increase in the release of [^{3}H]DA (Table 3 and Fig. 3) which was shown to be highly dependent on the presence of Ca^{2+} and totally abolished by the addition of Mg^{2+} (12 mM) to the superfusion media (data not shown). The presence of ethanol (1.1%, w/v) in the superfusion media did not modify the K^+-evoked release of newly synthesized [^{3}H]DA (Table 3) or the K^+-induced release of labeled DA from olfactory tubercle slices previously loaded with [^{3}H]DA (Fig. 3).

DISCUSSION

Our work [3, 17] and that of others [20, 21] indicate that it is possible to study DA synthesis and release in brain areas containing nigro-striatal nerve terminals by resorting to striatal slices as an experimental tool. We have now used slices from rat olfactory tubercles to study transmitter life cycle regulation in nerve terminals belonging to the mesolimbic system. The results presented in this paper suggest that similar factors are involved in the regulation of DA synthesis and release in dopaminergic terminals located in brain areas such as striatum and olfactory tubercle. It is possible that the acceleration in DA synthesis rate after neuronal depolarization arises primarily as a result of the removal of tyrosine hydroxylase from end-product inhibition subsequent to the release of transmitter [15]. The acceleration of transmitter synthesis could also be mediated, in part, by kinetic activation of the rate-limiting enzyme, tyrosine hydroxylase, i.e. increased enzyme activity was found when tyrosine hydroxylase, prepared from K^+-depolarized striatal slices, was assayed *in vitro* in the presence of subsaturating concentrations of tyrosine and pterin cofactor [2]. This activation appeared to be mediated by an increase in affinity of the enzyme for the pterin cofactor and a decreased affinity for the natural end-product inhibitor DA. The results reported in this paper suggest that ethanol is able to modify DA synthesis regulation in mesolimbic dopaminergic nerve terminals, possibly by disturbing the above regulatory mechanisms.

Most of the [^{14}C]DA newly synthesized following low K^+ depolarizing concentrations (25 to 40 mM) remained localized in the tissue and, in relative terms, only a small fraction of it was released to the medium (Fig. 1). In addition, the increase in DA synthesis induced by 25 mM K^+ was inhibited significantly by relatively low ethanol concentrations (0.2 and 0.4%, w/v) (Fig. 2). Since previous experiments have shown that ethanol (0.2 to 0.8%, w/v)

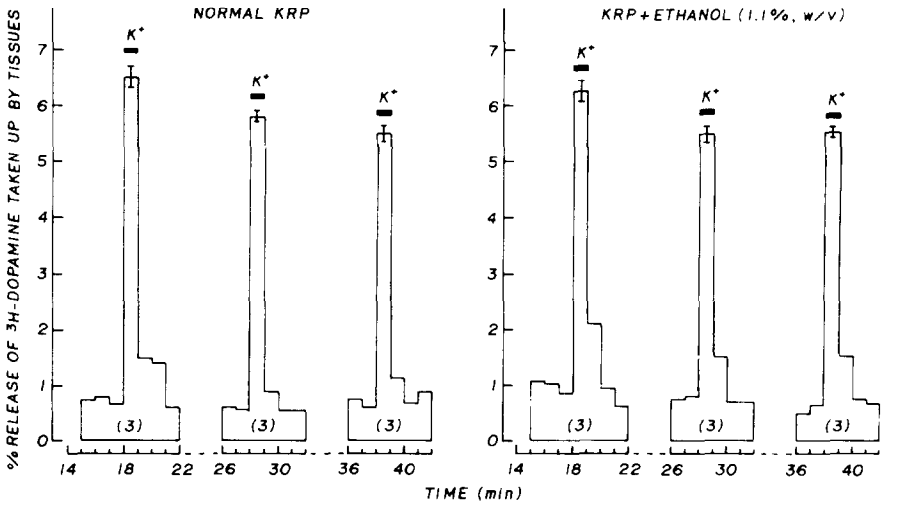

Fig. 3. Effect of ethanol on K^+-induced release of exogenous [^{3}H]DA from olfactory tubercle slices. Slices were incubated in normal KRP for 30 min at 37° in the presence of [^{3}H]DA and then transferred to a superfusion system. Release of [^{3}H]DA was then measured in either normal KRP (left side of figure) or KRP + ethanol (right side of figure). Potassium stimulation (40 mM) was carried for 1.0 min and is shown by the black bars at the top of the figure. Three succesive K^+ stimulations were used. The tissues had taken up an average of 510,025 ± 21,802 and 639,462 ± 46,615 cpm of [^{3}H]DA (means ± S.E.M.). The figure presents the means ± S.E.M. from three different experiments. The brackets indicate the S.E.M.

inhibits K^+ depolarization induced kinetic activation of tyrosine hydroxylase in striatal slices [2], it is tempting to suggest that the increase observed in DA synthesis by slices in low K^+ depolarizing concentrations (25–40 mM) can be accounted for by a mechanism which involves mainly a kinetic activation of tyrosine hydroxylase, of the type discussed above and which is not dependent upon transmitter release. In fact, in recent experiments with striatal slices, K^+ depolarizing concentrations of 20 mM resulted in a significant increase (280 per cent) in tyrosine hydroxylase activity and increasing the external K^+ concentrations (up to 100 mM) produced no further augmentation in the activity of the enzyme [22]. In contrast, following high K^+ depolarizing concentrations (40–70 mM K^+) most of the increased newly formed [^{14}C]DA was released to the medium (Fig. 1). Furthermore, under these last experimental conditions, low ethanol concentrations (0.2 to 0.4%, w/v) did not inhibit the increase in DA synthesis induced by high K^+ (40–55 mM) (Fig. 2). Ethanol (up to 1.1%, w/v) also was found not to modify K^+-evoked release of newly synthesized or exogenously taken up labeled DA (Fig. 3 and Table 3). It might be, then, that removal of tyrosine hydroxylase from end-product inhibition subsequent to the release of transmitter is the primary mechanism that increases DA synthesis in the presence of high external K^+ (40–70 mM). In any event, the findings reported in this paper suggest the existence, at least, of two mechanisms responsible for the increase in DA synthesis in olfactory tubercles following K^+ depolarization. One mechanism seems to be dependent upon transmitter release and is insensitive to the presence of ethanol. In contrast, the other mechanism seems to be inhibited by relatively low concentrations of ethanol (0.2 and 0.4%, w/v) and does not depend upon transmitter release. This last mechanism would predominate during low K^+ depolarizing concentrations (25–40 mM K^+).

The findings reported in this paper (Table 2) support the view that alterations in endogenous levels of cyclic AMP could mediate, in part, the events that lead to an increase in transmitter biosynthesis following depolarization of central dopaminergic neurons [23–26]. Such a view, derived mainly from studies performed with nigro-striatal dopaminergic neurons, can now be extended to the mesolimbic dopaminergic system. Furthermore, our experiments indicate that ethanol might interfere with cyclic AMP actions that lead to an increase in DA biosynthesis at the mesolimbic dopaminergic nerve terminal. Concentrations of ethanol as low as 0.2% (w/v) significantly inhibited the stimulatory effect produced by dB-cAMP and theophylline (Table 2). This probably explains why ethanol specifically blocked K^+-induced DA synthesis in rat olfactory tubercle slices (Fig. 2).

Goldstein *et al.* [26] have found that the stimulation of tyrosine hydroxylase activity elicited by dB-cAMP is significantly higher in mesolimbic synaptosomes than in striatal synaptosomes. It would seem then that those factors which regulate the transmitter life cycle in dopaminergic mesolimbic terminals do not necessarily need to be the same, at least from a quantitative point of view, as those already known to regulate DA synthesis and release in dopaminergic nigro-striatal terminals. In support of this view, the stimulation of [^{14}C]DA formation induced by K^+ (55 mM), absence of Ca^{2+}, or dB-cAMP (5×10^{-4} M) is relatively higher in slices of olfactory tubercle than in striatal slices (compare Tables 1 and 2 from this paper with Table 1 from Refs. 3 and 4 respectively). Similarly, the increase

Table 4. Induced stimulation of [^{14}C]dopamine biosynthesis in rat brain slices and its inhibition by ethanol—A comparison between striatal slices and slices from olfactory tubercle*

	% Inhibition of stimulated [^{14}C]dopamine synthesis					
	KRP-high K^+		KRP + dB-cAMP		KRP + theophylline	
Ethanol (%, w/v)	Striatum†	Olfactory tubercle‡	Striatum†	Olfactory tubercle‡	Striatum†	Olfactory tubercle‡
0.1	0	0	0	0	0	0
0.2	33	0	50	20	50	27
0.4	49	0	100	31	100	42
0.8	52	5	100	49	100	73

* Striatal slices and slices from olfactory tubercle were prepared by means of a Sorvall tissue chopper and incubated in normal KRP or in a KRP medium containing either high K^+ (55 mM), dB-cAMP (5×10^{-4} M) or theophylline (1×10^{-3} M) and saturating concentrations of [^{14}C]tyrosine. [^{14}C]Dopamine was separated by column chromatography and its radioactivity was determined in a Nuclear Chicago Scintillation counter. Results are expressed as percent inhibition of either high-K^+, dB-cAMP or theophylline-induced stimulation of [^{14}C]dopamine biosynthesis. Stimulated dopamine biosynthesis studied in the absence of ethanol was given a value of 100. Striatal and olfactory tubercle slices exposed only to normal KRP had synthesized, respectively, 29 ± 0.8 (N = 34) and 11.3 ± 0.3 (N = 17) nmoles·(g wet wt)$^{-1}$·hr^{-1} of [^{14}C]dopamine (mean ± S.E.M.). Striatal slices exposed to KRP-high K^+, KRP + dB-cAMP or KRP + theophylline had synthesized, respectively, 45.0 ± 20. (N = 9), 54.0 ± 1.4 (N = 15) and 47.4 ± 1.3 (N = 29) nmoles·(g wet wt)$^{-1}$·hr^{-1} of [^{14}C]dopamine. Olfactory tubercle slices exposed to KRP-high K^+, KRP + dB-cAMP or KRP + theophylline had synthesized, respectively, 26.2 ± 0.7 (N = 6), 25.0 ± 0.6 (N = 8) and 17.9 ± 0.2 (N = 9) nmoles·(g wet wt)$^{-1}$·hr^{-1} of [^{14}C]dopamine (mean ± S.E.M.). Results represent the analysis of different experiments reported by the authors elsewhere and in this paper.

† Results were taken from the paper by Gysling *et al.* [3] and Gysling and Bustos [4].

‡ Results were taken from this paper (Tables 1 and 2).

in [^{14}C]DA synthesis induced by K^+ (55 mM) relative to the increase in transmitter formation induced by the absence of Ca^{2+} was much higher in olfactory tubercle then in striatal slices (compare Table 1 from this paper with Table 1 in Ref. 3). This has an important experimental application since it has allowed us to demonstrate that K^+ depolarization induced formation of DA in olfactory tubercle slices is highly dependent upon the presence of Ca^{2+} in the incubation medium. Previously, it was not possible to demonstrate such an effect in striatal slices since in this preparation the stimulatory effects induced by K^+ (55 mM) or by the absence of Ca^{2+} are of similar magnitude [3]. In addition, and as shown in Table 4, the sensitivity toward the inhibitory effects of ethanol upon stimulated DA biosynthesis was much different in dopaminergic mesolimbic terminals compared to nigro-striatal terminals. Ethanol, added to striatal slices at two different concentrations (0.4 and 0.8%, w/v), produced a 49–52 percent inhibition of DA biosynthesis induced by K^+ (55 mM) depolarization and totally abolished the increase in transmitter synthesis induced by dB-cAMP (5×10^{-4} M) or by theophylline (1×10^{-3} M). In contrast, the same ethanol concentration added to olfactory tubercle slices produced no inhibition of K^+ (55 mM)-induced DA formation and only inhibited by 30–70 percent the increase in DA synthesis induced by dB-cAMP or by theophylline (Table 4). We attribute the different sensitivities of mesolimbic and nigro-striatal dopaminergic terminals toward ethanol to the specific and characteristic ways by which these terminals increase DA formation upon neuronal depolarization.

Concentrations of ethanol as low as 0.2 or 0.4% (w/v) were found to specifically inhibit DA biosynthesis induced by low K^+ depolarizing concentrations (Fig. 2). These observations are consistent with the speculation that the effects described for ethanol might play a role in the intoxicating effects produced by this drug *in vivo*.

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