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Effect of ethanol on dibutyryl cyclic adenosine monophosphate- and theophylline-induced stimulation of dopamine biosynthesis by rat striatal slices

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Potassium depolarization of striatal slices results in an acceleration of dopamine (DA) synthesis [1, 2] which seems to be mediated in part by a kinetic activation of the rate-limiting enzyme tyrosine hydroxylase [3]. Thus, an increased enzyme activity was found when tyrosine hydroxylase prepared from K^+-depolarized striatal slices was assayed *in vitro* in the presence of sub-saturating concentrations of tyrosine and pterin cofactor [3]. This activation appears to be mediated by an increase in affinity of the enzyme for the pterin cofactor and a decreased affinity for the end-product inhibitor DA. An increase in impulse flow in the nigro-neostriatal pathway of the rat results also in an increase in DA synthesis and causes a kinetic activation of striatal tyrosine hydroxylase [4]. We have recently shown [2] that ethanol (0.2 to 0.8%, w/v) specifically blocks the increase in DA synthesis observed in striatal slices after K^+-depolarization, while having no effect on DA synthesis measured in non-depolarized striatal slices. Moreover, ethanol (0.2 to 0.8%, w/v) was able to block the kinetic activation of tyrosine hydroxylase produced by K^+-depolarization, while having no effect on tyrosine hydroxylase prepared from non-depolarized slices [3]. It was interesting to find out the mechanism(s) underlying these interesting effects of ethanol.

Recent experimental evidence is consistent with the possibility that alterations in endogenous levels of cyclic AMP which occur during depolarization [5] might in part be responsible for the increase in DA synthesis [1, 2] and for the kinetic activation of tyrosine hydroxylase produced after incubating striatal slices in a K^+-enriched medium [3]. Thus, DA synthesis in striatal slices is increased by the addition of dibutyryl cyclic adenosine monophosphate (dB-cAMP) to the incubation media [6]. Moreover, tyrosine hydroxylase in high speed supernatant prepared from rat striatum is activated by cyclic AMP [7]. The kinetic activation of the enzyme produced by cyclic AMP is similar to that which we have reported to occur after K^+-depolarization of the striatal slices [3]. It is possible then that ethanol is inhibiting the K^+-induced DA synthesis and K^+-induced activation of tyrosine hydroxylase by altering cyclic AMP production and/or its action. In search of support for this proposal, we have studied in this paper the effect of ethanol on the increase in DA biosynthesis induced by dB-cAMP. Since theophylline is an agent known to inhibit phosphodiesterase [8] and to alter tissue levels of cyclic AMP [9], we have studied also the synthesis of DA by striatal slices in the presence of theophylline and in the presence and absence of ethanol.

Striatal tissue slices (0.2 mm in thickness) were prepared with a Sorvall tissue chopper from the striatum of adult, Sprague–Dawley rats of both sexes. Tissue slices weighing about 30–40 mg were incubated at 37° in beakers containing 5 ml of Krebs–Ringer phosphate (KRP), pH 7.4, saturated with 95% O_2 + 5% CO_2. After a 10-min preincubation period, labeled L-tyrosine-[^{14}C] (U) with a sp. act. of 5 mCi/m-mole was added to the media, producing a final tyrosine concentration of 2.5×10^{-5} M. Thereafter, the slices were incubated an additional 30 min, the beakers were chilled on ice, and the slices were separated from the media by centrifugation at 12,100 *g* for 10 min in a Sorvall refrigerated centrifuge. The slices were then homogenized in 15% trichloroacetic acid (TCA); each incubation media 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, dB-cAMP and theophylline were added 5 min after 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 (2×10^{-4} M), an inhibitor of tyrosine hydroxylase. Similar blank values were obtained by incubating striatal slices which had been initially precipitated with 15% TCA. A typical sample to blank ratio usually had a value between ten and twelve.

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 12,100 *g* for 20 min. Separation and analysis of the tissue and the media for labeled DA were carried out as described previously [10] by absorption chromatography through Alumina columns and ion-exchange chromatography through Amberlite CG-120 columns. Eluates from the columns containing labeled DA were analyzed for ^{14}C in a Nuclear Chicago scintillation counter. Results were calculated as described previously [1] and are expressed in terms of nmoles [^{14}C]DA/g wet weight/hr 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. [^{14}C]dopamine present in the media was always a small fraction of the [^{14}C]DA content in slices (10 ± 0.6 per cent). This fraction remained the same with the different incubation media used and it was shown not to vary under the presence of dB-cAMP or theophylline. Under the experimental conditions used, 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.

The [^{14}C]tyrosine taken up by the tissue during the incubation period was also determined [11]. Tyrosine was separated from catechols and other labeled tyrosine metabolites by passage through Alumina columns and columns of Dowex 50W-X8 (H^+), 100–200 mesh. Eluates from the Dowex-50 columns were evaporated to dryness, re-dissolved in distilled water and the radioactivity was determined by liquid scintillation counting.

Incubation of striatal slices in the presence of dB-cAMP resulted in a marked increase in the total conversion of [^{14}C]tyrosine to [^{14}C]DA (Table 1). The increased formation of newly synthesized [^{14}C]DA was found to be dose-dependent. Concentrations of dB-cAMP ranging from 1×10^{-5} to 5×10^{-4} M, respectively, increased the formation of newly synthesized [^{14}C]DA from 19 to 84 per cent (Table 1). Cyclic AMP (5×10^{-4} M) which is a good substrate for phosphodiesterase and which penetrates cell membranes less readily than dB-cAMP [12, 13], produced no stimulating effect on the rate of [^{14}C]DA synthesis and rather a small inhibition was found. Similar results to those described above have been reported by other authors

Table 1. Effect of dB-cAMP, theophylline and ethanol on the synthesis of [^{14}C]dopamine by striatal slices*

Incubation media	N	Total [^{14}C]dopamine synthesis (nmoles/g wet wt/hr)	Per cent change from KRP controls
KRP (controls)	34	29.3 ± 0.8	
KRP ± dB-cAMP (1×10^{-5} M)	4	34.8 ± 1.4†	+19
KRP + dB-cAMP (5×10^{-5} M)	4	40.3 ± 1.7‡	+38
KRP + dB-cAMP (5×10^{-4} M)	15	54.0 ± 1.4‡	+84
KRP + theophylline (10^{-5} M)	4	36.0 ± 3.3†	+23
KRP + theophylline (10^{-4} M)	4	39.5 ± 4.8§	+35
KRP + theophylline (10^{-3} M)	29	47.4 ± 1.3‡	+62
KRP + ethanol (0.8%, w/v)	8	27.70 ± 0.6	−5
KRP + dB-cAMP (5×10^{-4} M) + ethanol (0.8%, w/v)	8	33.40 ± 1.9‖	+14
KRP + theophylline (10^{-3} M) + ethanol (0.8%, w/v)	9	29.50 ± 1.9‖	+1

* Striatal slices were prepared by means of a Sorvall tissue chopper and incubated in a media containing saturating concentrations of [^{14}C]tyrosine (2.5×10^{-5} M, sp. act. 5.0 mCi/m-mole and 0.625 μCi/flask) for 30 min at 37°. [^{14}C]dopamine was separated by column chromatography, and its radioactivity determined in a Nuclear Chicago scintillation counter. Results represent the mean ± S.E.M.; N represents the number of different experiments.

† $P < 0.05$ when compared to normal KRP control.

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

§ $P < 0.005$ when compared to normal KRP control.

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

working with slices and synaptosomal preparations obtained from rat striatum [6, 7]. These authors have also ruled out the possibility that dB-cAMP-induced stimulation of [^{14}C]DA synthesis is due to the butyrate moiety, since butyric acid (5×10^{-4} M) had no significant effect on the rate of [^{14}C]DA formation in striatal slices [6]. A significant increase in the formation of newly synthesized [^{14}C]DA was also found after incubating striatal slices in the presence of theophylline. Concentrations of theophylline ranging from 1×10^{-5} to 1×10^{-3} M were found to increase the rate of [^{14}C]DA formation by striatal slices from 23 to 62 per cent (Table 1). The extent of [^{14}C]DA synthesis increase induced by theophylline is similar to that produced by dB-cAMP (Table 1).

As previously reported [2], the addition of ethanol (0.8%, w/v) directly to normal KRP produced no significant effect on the total conversion of [^{14}C]tyrosine to [^{14}C]DA by striatal slices (Table 1). However, ethanol (0.8%, w/v) almost completely blocked the increase in [^{14}C]DA formation produced by dB-cAMP (5×10^{-4} M) or by theophylline (1×10^{-3} M). In fact, the amount of [^{14}C]DA synthesized after adding dB-cAMP plus ethanol or theophylline plus ethanol to the KRP media was very similar to [^{14}C]DA synthesized in normal KRP media

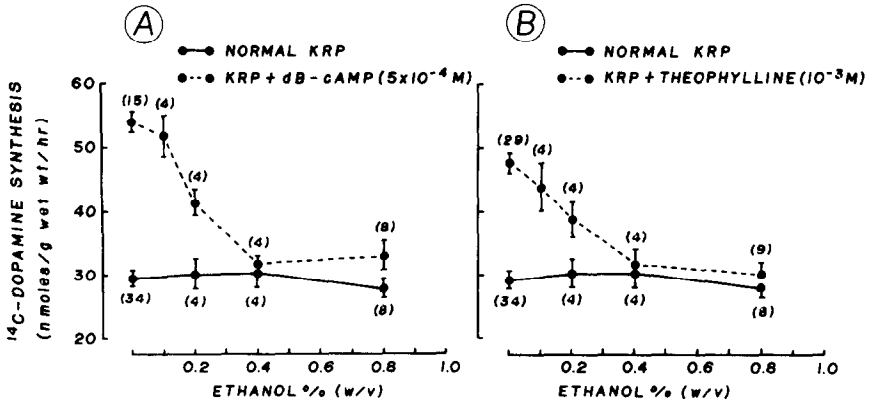

Fig. 1. Effect of different doses of ethanol on dibutyryl cyclic AMP- (curve A) and theophylline- (curve B) induced formation of newly synthesized [^{14}C]DA by striatal slices. The striatum was dissected out and slices were prepared by means of a Sorvall tissue chopper. Incubation conditions and separation of [^{14}C]dopamine were as described under Table 1. The numbers in parentheses denote the number of individual experiments and the brackets indicate the standard error of the mean.

(Table 1). The [^{14}C]tyrosine taken up by the tissues at the end of the incubation period was not significantly altered by the presence of ethanol (0.8%, w/v) in the media. The values obtained (mean ± S. E. M.; n = 6) at the end of the incubation period were, respectively, 417.2 ± 24.3, 471.7 ± 15.3, 392.4 ± 15.2 and 408.2 ± 19.2 nmoles/g wet wt/hr of [^{14}C]tyrosine taken up by slices incubated in the presence of dB-cAMP alone, dB-cAMP plus ethanol, theophylline alone and theophylline plus ethanol.

As shown in Fig. 1, the inhibitory effect exerted by ethanol on dB-cAMP- and theophylline-induced formation of [^{14}C]DA is dose-dependent. Concentrations of ethanol as low as 0.2% (w/v) produce a 50 per cent inhibition on the stimulatory effect produced by dB-cAMP and theophylline ($P < 0.005$ and $P < 0.05$ when compared to respective dB-cAMP or theophylline controls without ethanol). High concentrations of ethanol (0.4 and 0.8%, w/v) completely blocked the stimulatory effect induced by dB-cAMP or theophylline and restored the rate of [^{14}C]DA formation to that obtained after incubating striatal slices in normal KRP. Very low concentrations of ethanol (0.1%, w/v) produced a small although not significant inhibitory effect on dB-cAMP- or theophylline-induced formation of [^{14}C]DA. In contrast to these results, ethanol (0.2 to 0.8%, w/v) was found to be without effect on the rate of [^{14}C]DA formation by striatal slices incubated in normal KRP (Fig. 1).

Figure 2 shows that the inhibitory effect of ethanol on dB-cAMP- and theophylline-induced formation of [^{14}C]DA is dependent on the time of incubation used. The synthesis of [^{14}C]DA by striatal slices in normal KRP or KRP plus ethanol increases in a way proportional to the entire period studied. The amount of [^{14}C]DA synthesized in the presence of dB-cAMP or theophylline was also found to increase linearly over the time period studied. However, in the presence of ethanol, the dB-cAMP- or theophylline-induced increase in [^{14}C]DA synthesis levels off after 20 min of incubation (Fig. 2). Therefore, the inhibitory effect of ethanol only becomes readily observable after 30 min of incubation.

Dibutyryl cyclic AMP and theophylline were found to stimulate the formation of newly synthesized [^{14}C]DA and this stimulatory effect was blocked by the presence of ethanol (0.2 to 0.8%, w/v) in the incubation media. It seems unlikely that these effects are mediated through an alteration of [^{14}C]tyrosine transport into the striatal slices, since neither dB-cAMP nor theophylline nor ethanol was shown to modify the [^{14}C]tyrosine taken up by the slices at the end of the incubation period. It could be that the stimulation of DA biosynthesis by dB-cAMP or theophylline results as a consequence of these drugs triggering the release of a small strategic pool of dopamine, which in turn relieves the rate-limiting enzyme tyrosine hydroxylase from end-product inhibition. However, as mentioned above, dB-cAMP and theophylline were found not to alter the amount of [^{14}C]DA released to the incubation media. Moreover, preliminary experiments from our laboratory indicate that neither dB-cAMP (5×10^{-4} M) nor theophylline (1×10^{-3} M) increases spontaneous release of newly synthesized [^{3}H]DA from striatal slices which are being continuously superfused with KRP. Most likely dB-cAMP is stimulating [^{14}C]DA synthesis in striatal slices by mimicking the recently reported cyclic AMP-mediated kinetic activation of striatal tyrosine hydroxylase [7]. It seems also likely to explain the stimulatory effect of theophylline in terms of cyclic AMP-mediated actions at the dopaminergic terminal. Theophylline is known to inhibit phosphodiesterase [14], and it has been shown, through this inhibition, to increase the accumulation of cyclic AMP in cortical and cerebellar slices [15, 16] and in neuroblastoma cells [17]. Furthermore, the characteristics of the inhibitory effect exerted by ethanol upon drug-stimulated DA synthesis also suggest that theophylline is acting through cyclic AMP in order to increase DA biosynthesis. Ethanol inhibited dB-cAMP- and theophylline-stimulated [^{14}C]DA synthesis after a lag period of about 20 minutes in either case (Fig. 2), and the concentration of ethanol needed to produce 50 per cent inhibition in both cases was 0.2% (w/v) (Fig. 1). All these results point to the fact that theophylline, under our experimental con-

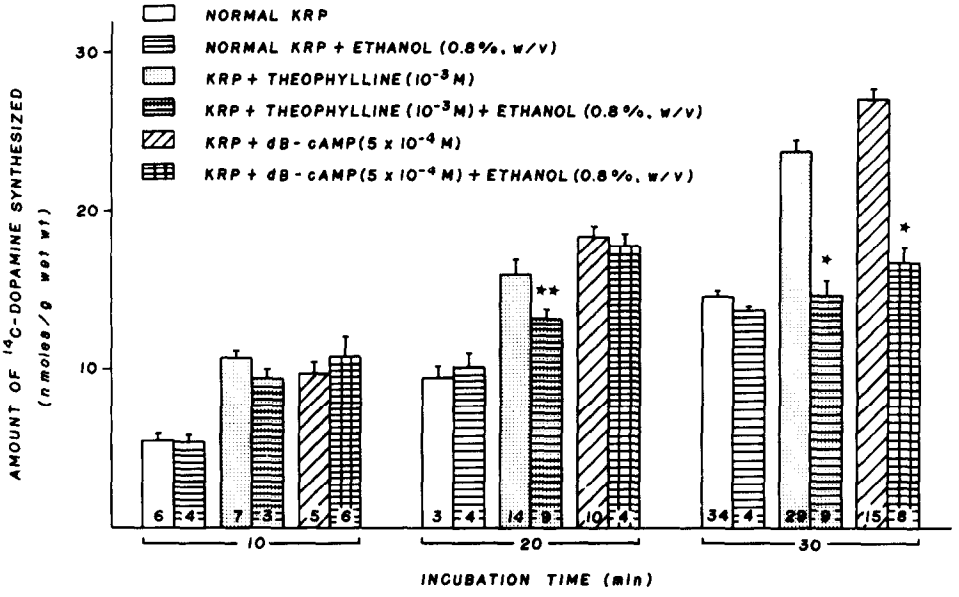

Fig. 2. Time course of ethanol effect on dB-cAMP- and theophylline-induced formation of [^{14}C]DA by striatal slices. Incubation conditions were as described under Table 1. At the times indicated, the incubation was terminated and [^{14}C]dopamine was separated by column chromatography. The number of individual experiments is indicated at the bottom of each column. The brackets indicate the standard error of the mean. A single asterisk (*) indicates $P < 0.001$, and a double asterisk (**) $P < 0.05$ when compared to respective controls without ethanol.

ditions, most likely is increasing the accumulation of cyclic AMP which, in turn, stimulates DA biosynthesis. The marked stimulatory effect on [^{14}C]DA synthesis produced by theophylline also suggests that endogenously synthesized cyclic AMP normally plays an important role in the short-term regulation of DA synthesis at the dopaminergic terminal. The results also indicate that ethanol might be inhibiting dB-cAMP- and theophylline-induced DA synthesis by the same mechanism, namely through an alteration of exogenous and endogenous cyclic AMP actions at the dopaminergic nerve terminal.

One interesting observation is the fact that the inhibitory effect of ethanol on drug-stimulated DA synthesis is only demonstrable after 20 min, whereas ethanol is known to be quite permeable to membranes. It could be that ethanol modifies the stability of the enzymes and/or accelerates the breakdown of some of the intermediates believed to form part of the chain of reactions leading from cAMP to kinetic activation of striatal tyrosine hydroxylase enzyme [18]. However, so far there is no sound experimental evidence to support this.

Ethanol inhibited dB-cAMP- and theophylline-induced [^{14}C]DA synthesis even at concentrations as low as 0.2% (w/v) or 43.4 mM (Fig. 1). These ethanol concentrations are compatible with a mild intoxication *in vivo* [19, 20]. The possibility should be considered then that ethanol effects on cyclic AMP-mediated changes in DA biosynthesis are somehow related to the intoxicating action of ethanol and to the acquisition of tolerance and physical dependence to this drug.

In conclusion, our experiments indicate that ethanol interferes with cyclic AMP actions that lead to an increase in DA biosynthesis at the dopaminergic nerve terminal. This probably explains why ethanol blocks K^+-induced activation of tyrosine hydroxylase [3] and K^+-induced DA synthesis in rat striatal slices [2]. Further work is needed in order to clarify the mechanism responsible for this ethanol inhibition of cyclic AMP-mediated action at the dopaminergic terminals.

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