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REGIONAL INHIBITORY EFFECT OF ETHANOL ON MONOAMINE SYNTHESIS REGULATION WITHIN THE BRAIN

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Abstract—Catecholamine biosynthesis from labeled tyrosine was examined in hippocampal and olfactory tubercle slices. The presence of depolarizing concentrations of potassium (25–55 mM) resulted in significant increases in catecholamine synthesis in both tissue preparations. Following depolarization of slices in 25 mM potassium, the major portion of newly synthesized catecholamine was retained in the tissue whereas incubation in 55 mM potassium resulted in a significant portion of the newly synthetized catechols being released into the media. Addition of ethanol in concentrations of 0.2–0.8% (w/v) to tissue slices prepared from brain regions rich in dopaminergic nerve terminals, such as olfactory tubercles, caused a dramatic reduction in dopamine synthesis from labeled tyrosine induced by potassium depolarization. The ability of ethanol to inhibit the potassium-induced acceleration of catecholamine biosynthesis appeared to be related to the dopaminergic innervation of the tissue. Catecholamine synthesis in slices of depolarized hippocampi, tissue which is relatively free from substantial dopaminergic innervation, was unaffected by ethanol in concentrations as high as 1.2% (w/v). These results are discussed in terms of the selectivity and mechanisms of the ethanol-induced effects.

In recent studies we have reported that ethanol (0.2–0.8%, w/v) added to striatal slices inhibits both the *in vitro* activation of tyrosine hydroxylase induced by K^+-depolarization, and the *in vitro* increase in dopamine synthesis induced by K^+-depolarization or by addition of dibutyryl-cAMP to the incubation medium [1–3]. In view of these observations, it seemed possible that some of the CNS effects elicited by administration of ethanol might be mediated through a disturbance of the regulatory mechanisms that normally operate in catecholaminergic neurons to adjust transmitter synthesis to meet demands incurred by altered impulse flow.

In order to determine the selectivity of this action of ethanol, we have analyzed whether the above *in vitro* effects of ethanol on catecholamine biosynthesis displayed any regional specificity within the brain or whether they were restricted to dopaminergic terminals. Specifically, we have determined whether similar effects can be observed in noradrenergically innervated brain regions. In previous experiments we had demonstrated that dopamine synthesis in two dopamine rich brain areas, the striatum and the olfactory tubercle, was inhibited by addition of ethanol to the incubation media. Dopamine synthesis in mesolimbic dopaminergic terminals was relatively less sensitive to the inhibitory effects of ethanol compared to nigro-striatal dopaminergic nerve terminals [4], but the effects of ethanol were not investigated in non-dopaminergic regions of brain. Hippocampal slices were chosen for the current study because this tissue possesses very few, if any, dopaminergic nerve terminals and is densely innervated with noradrenergic nerve terminals of the dorsal noradrenergic pathway. This paper describes a study in which we have used the *in vitro* tissue slice system to compare the effect of ethanol on the regulation of catecholamine biosynthesis in two distinct brain structures: the olfactory tubercle and the hippocampus.

MATERIALS AND METHODS

Studies on catecholamine formation. Male Sprague-Dawley rats, weighing about 200–250 g were decapitated and the olfactory tubercles and the hippocampi were rapidly dissected. Slices from olfactory tubercles and hippocampus (0.23 mm in thickness) were prepared with a Sorvall tissue chopper. Tissue slices, weighing about 60–80 mg, were incubated at 37° in beakers containing 2 ml of either Krebs–Ringer-MOPS‡ (KRM), pH 7.4, or KRM-high K^+ (25 or 55 mM K^+), pH 7.4, saturated with 95% O_2 + 5% CO_2. After a 10-min preincubation period, labeled L-tyrosine [ring-2,6-^{3}H] (New England Nuclear, Boston, MA) was added to the beakers containing the incubation media. When olfactory tubercles were used, L-tyrosine[^{3}H] with a sp. act. of 14.2 mCi/mmole was added to the media, producing a final tyrosine concentration of 6×10^{-5} M. When hippocampal slices were used, the specific activity of the labeled L-tyrosine[^{3}H] added and the final tyrosine concentration attained were 616 mCi/mmole and 4.3×10^{-5} M respectively. After

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‡ MOPS, morpholino-propane sulfonic acid.

addition of labeled tyrosine, the slices were incubated for an additional 20 min, the beakers were chilled on ice, and the slices were separated from the media by passage under vacuum through membrane filters which contained a nylon mesh top (pore size, 35 μm). The slices collected on the nylon mesh were then homogenized in 10% trichloroacetic acid (TCA), and each incubation medium was acidified with 5 ml of 10% TCA. When ethanol was employed, it was added to the media at the beginning of the preincubation period. Preliminary experiments demonstrated that the concentration of ethanol in the incubation medium remained constant throughout the time course of the incubation. Tissue blanks were run by incubating tissue 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 tissue slices which had been initially precipitated with 10% TCA.

Chromatographic procedures and radioactivity determinations. Unlabeled dopamine, norepinephrine and L-dopa (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. Separation and analysis of the tissue and the media for labeled dopamine and noradrenaline were carried out as described previously by adsorption chromatography through Alumina columns [1, 5]. Eluates from the columns containing labeled catechols were analyzed for ^{3}H in a Packard Tri-carb liquid scintillation spectrometer. The counting efficiency under the conditions of these experiments was 29.5 per cent. Analysis through Amberlite and Dowex columns demonstrated that labeled dopamine and noradrenaline account for 80–85 per cent of the labeled catechols synthesized or released after K^+ stimulation [1, 4, 5]. Reported values have not been corrected for recovery. Results are expressed, unless stated otherwise, as cpm of [^{3}H] catechols synthesized per mg of wet tissue during the 20-min incubation period. The [^{3}H]tyrosine taken up by the tissue during the incubation period was also separated from the catechols and determined as described previously [1, 3].

Solutions and chemicals. The Krebs–Ringer-MOPS (KRM) used in these experiments had the following composition: 1.28 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 15.8 mM MOPS at pH 7.4, and 11.1 dextrose. Potassium-enriched KRM

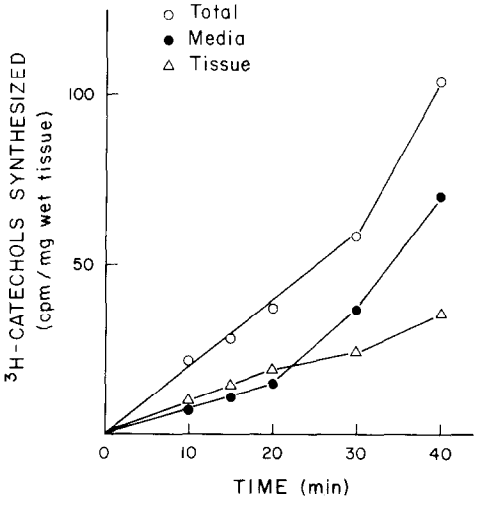

Fig. 1. Time–course of formation of [^{3}H]catechols in hippocampal slices. Hippocampal slices were prepared by means of a Sorvall tissue chopper and incubated in a medium containing saturating concentrations of [^{3}H]tyrosine (4.3×10^{-5} M, sp. act. 616 mCi/mmole and 53 μCi/flask) for different time periods at 37°. [^{3}H]Catechols were separated by column chromatography, and radioactivity was determined in a Packard Tri-carb scintillation spectrometer. Results represent the average of two different experiments.

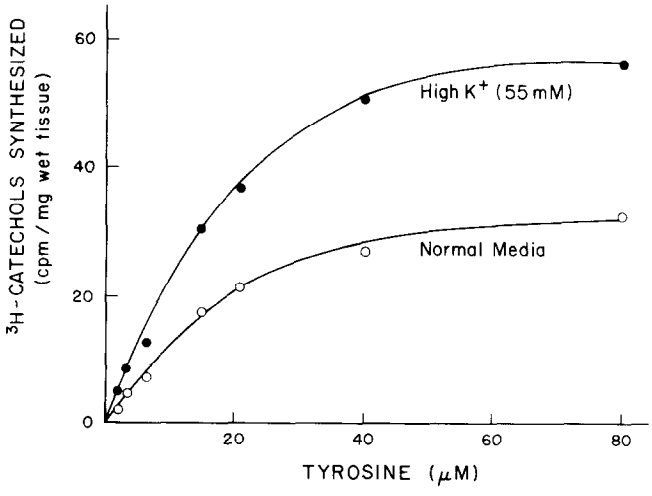

Fig. 2. [^{3}H]Catechol formation in hippocampal slices as a function of external [^{3}H]tyrosine concentration. The striatum was removed by dissection and slices were prepared by means of a Sorvall tissue chopper. Incubation of the slices was carried out for 20 min at 37° both in normal and high K^+ (55 mM) media and in the presence of increasing external [^{3}H]tyrosine concentrations. Separation of [^{3}H]catechols was as described under Fig. 1. Results represent the average of two different experiments.

containing 55 mM or 25 mM KCl was prepared by replacing a portion of the NaCl with an equimolar amount of KCl.

RESULTS

[^{3}H] *Catechol synthesis in hippocampal slices.* Figure 1 shows the time–course for the formation of [^{3}H]catechols in hippocampal slices. In this experiment, hippocampal slices were incubated at different time periods in a KRM medium in the presence of [^{3}H]tyrosine. A linear relationship was found for up to 20 min, whether the [^{3}H]catechols synthesized were measured in the tissue or in the medium. Thereafter, and up to 40 min, [^{3}H]catechol formation seemed to proceed at a faster rate.

A Michaelis–Menten relationship was found when the synthesis of [^{3}H]catechols in the hippocampus was followed for 20 min in the presence of increasing external concentrations of [^{3}H]tyrosine (Fig. 2). Under these experimental conditions, saturation and maximal velocities were attained at a concentration of 4×10^{-5} M tyrosine. [^{3}H]Catechol formation was increased significantly when the slices were incubated in the presence of depolarizing conditions with K^+ (55 mM). However, even under this last condition, maximal velocities of [^{3}H]catechol formation were obtained in the presence of 4×10^{-5} M tyrosine.

As shown in Fig. 3, potassium depolarization markedly increased the rates of [^{3}H]catechol formation and release in hippocampal slices. The rate of release was found to be highly dependent on the presence of Ca^{2+} in the incubation medium. Omission of Ca^{2+} from the KRM-high K^+ (55 mM) medium completely abolished both [^{3}H]catechol formation and release induced by K^+-depolarization. By contrast, [^{3}H]catechol formation of slices in normal KRM medium was unaffected by the omission of Ca^{2+} from the medium (Fig. 3).

Effect of ethanol on [^{3}H]*catechol synthesis in olfactory tubercle and hippocampal slices.* The formation of [^{3}H]catechols from [^{3}H]tyrosine was followed both in hippocampal and olfactory tubercle slices, in the presence of depolarizing conditions and in the absence and presence of ethanol (0.8 and 1.6%, w/v). As shown in Fig. 4, incubation of hippocampal slices in a KRM-high K^+ (55 mM) medium resulted in a substantial increase in the rate of total [^{3}H]catechol formation and release. Since the total [^{3}H]catechols synthesized represent the sum of the [^{3}H]catechol content in slices plus the [^{3}H]catechol content in the medium, it can be safely concluded that the net formation of [^{3}H]catechols was increased by K^+-depolarization. Ethanol, added to the incubation medium at two different concentrations (0.8 and 1.6%, w/v), was not able to modify this increased formation and the release of newly synthesized catechols that occurred in hippocampal slices subjected to depolarizing conditions of incubation. In addition, ethanol did not alter the rate of [^{3}H]catechol formation measured in hippocampal slices incubated in normal KRM medium.

Potassium depolarization also markedly increased the extent of [^{3}H]catechol formation and release when olfactory tubercle slices were used for such a study (Fig. 4). Under the experimental conditions reported, the synthesis of [^{3}H]catechols was linear for up to 45 min when using a saturating [^{3}H]tyrosine concentration of 4×10^{-5} M [4]. More than 50 per cent of the total, newly synthesized [^{3}H]catechols formed during K^+-depolarization was accounted for by the [^{3}H]catechols present in the medium (Fig. 4). In fact, the ratio of tissue/medium [^{3}H]catechols after 55 mM K^+-depolarization was less than 1.0 as compared to a ratio of close to 4.0 when incubations of the tissue slices were carried out in a normal KRM medium. This is due both to the stimulatory effect of high K^+ (55 mM) on the release of newly syn-

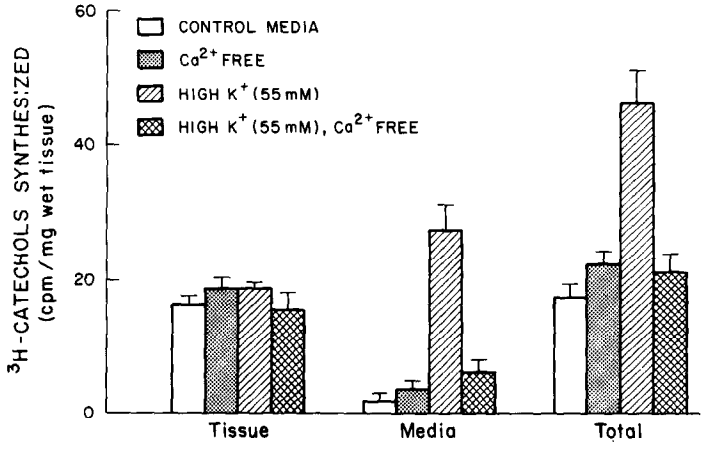

Fig. 3. Effect of potassium depolarization on [^{3}H]catechol formation and release in hippocampal slices. The hippocampus was removed by dissection and slices were prepared by means of a Sorvall tissue chopper. The slices were incubated in the different media containing saturating concentrations of [^{3}H]tyrosine (4.3×10^{-5} M, sp. act. 616 mCi/mmole and 53 μCi/flask) for 20 min at 37°. The separation and determination of [^{3}H]catechols are as described under Fig. 1. Each result is the mean ±S.E.M. of three different experiments.

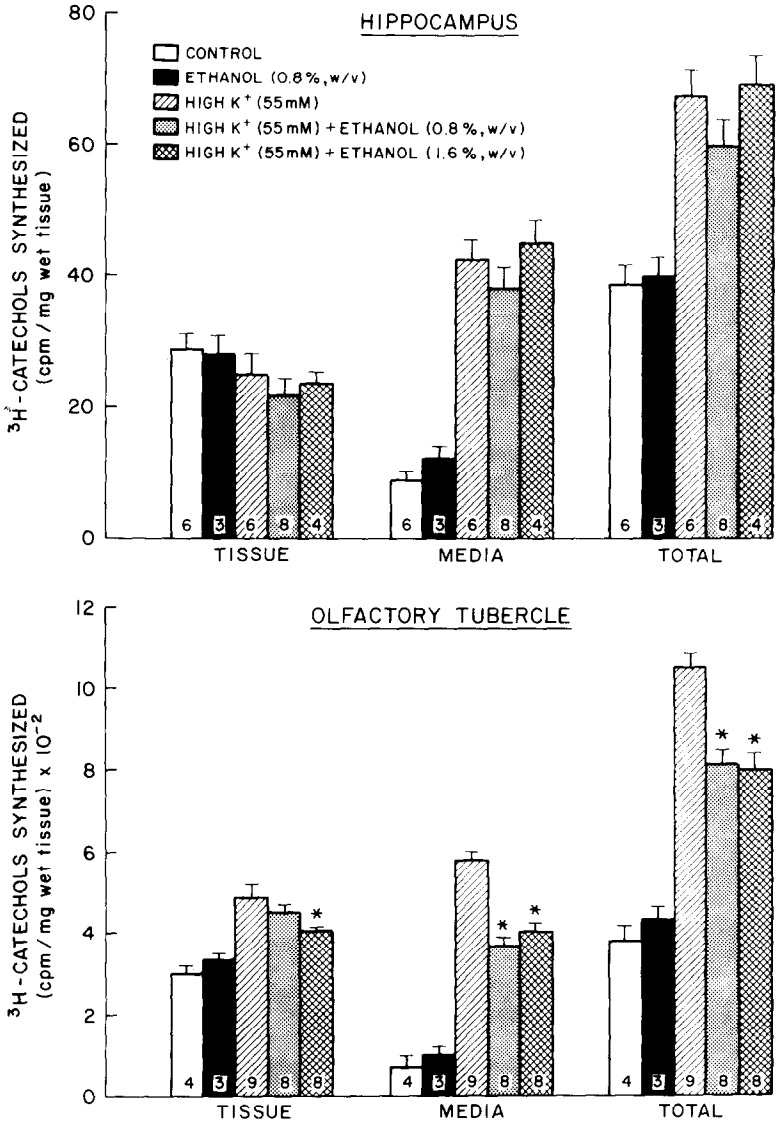

Fig. 4. Effect of ethanol on [^{3}H]catechol synthesis in olfactory tubercle and hippocampal slices. The hippocampus and olfactory tubercle were removed by dissection and slices were prepared by means of a Sorvall tissue chopper. Thereafter, the slices were incubated in the different media containing saturating concentrations of [^{3}H]tyrosine for 20 min at 37° (see legend to Fig. 3 for details). An external K^+ concentration of 55 mM was used as the depolarizing condition. At the end of the incubation period, the [^{3}H]catechols were separated by column chromatography, and radioactivity was determined as described under Fig. 1. The number of individual experiments is indicated at the bottom of each column. The brackets indicate the standard error of the mean. The single asterisk (*) indicated $P < 0.001$ when compared to respective controls without ethanol.

thesized catechol and to its inhibitory effect on the reuptake of the monoamine [6, 7] by the tissue slices. In contrast to the results obtained with hippocampal slices, ethanol (0.8 and 1.6%, w/v) was found to significantly block the K^+-induced formation of [^{3}H]catechols in olfactory tubercle slices while it had no effect on the [^{3}H]catechol formation studied in the presence of normal KRM medium. However, it is difficult to determine whether the inhibitory effect of ethanol on K^+-induced formation of total catechols was due to a direct effect of ethanol upon the synthesis or to its effect upon the release of newly formed catechols. The data presented in the lower panel of Fig. 4 suggest that the latter possibility may have been the case since ethanol markedly reduced the increase in the amount of [^{3}H]catechols normally appearing in the medium after K^+-depolarization. However, other experiments in which the effects of ethanol on the release of exogenously loaded dopamine were examined indicate that ethanol in similar concentrations does not influence dopamine release [4].

The effect of ethanol on catechol formation, in dopaminergic and noradrenergic nerve terminals, was studied following the exposure of hippocampal and olfactory tubercle slices to 25 mM external K^+

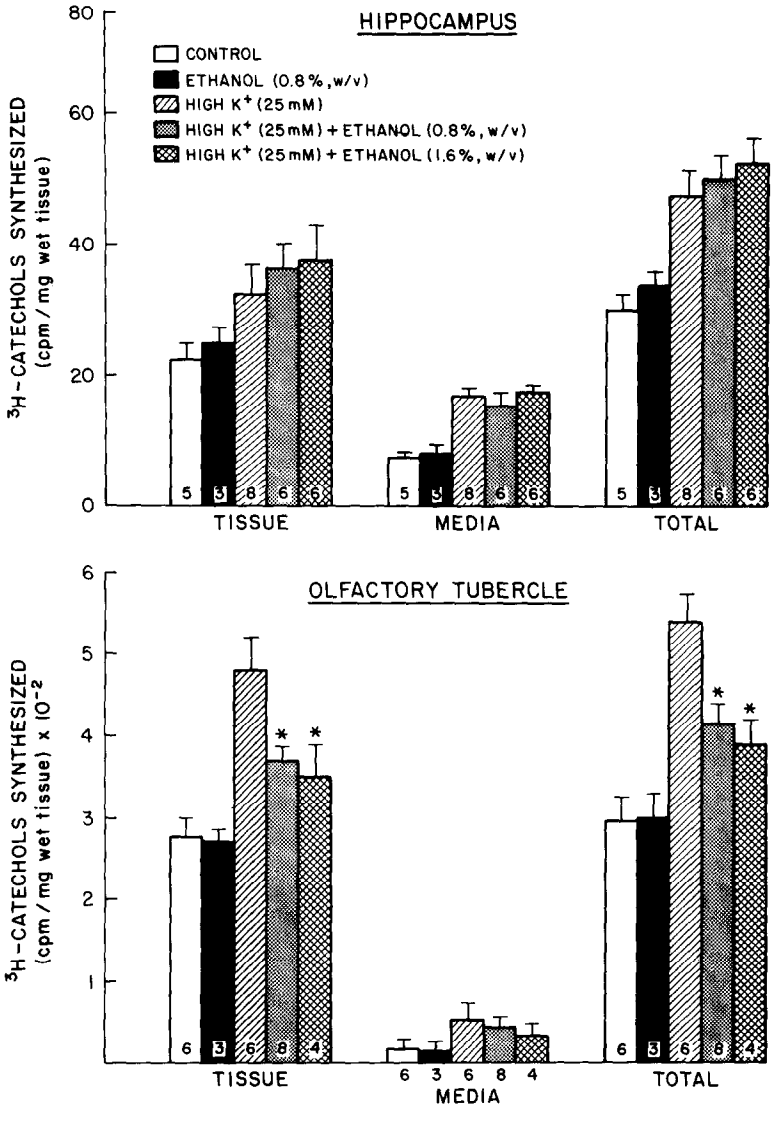

Fig. 5. Effect of ethanol on [^{3}H]catechol synthesis in olfactory tubercle and hippocampal slices. The hippocampus and olfactory tubercle were removed by dissection and slices were prepared by means of a Sorvall tissue chopper. Thereafter, the slices were incubated in the different media containing saturating concentrations of [^{3}H]tyrosine for 20 min at 37°. An external K^+ concentration of 25 mM was used as the depolarizing condition. At the end of the incubation period, the [^{3}H]catechols were separated by column chromatography, and radioactivity was determined as described under Fig. 1. 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 $P < 0.001$ when compared to respective controls without ethanol.

concentrations. Under these experimental conditions, most of the increased [^{3}H]catechol formation that resulted after K^+-depolarization could be accounted for by the [^{3}H]catechols present in the tissue (Fig. 5). In fact, the tissue/medium ratio of [^{3}H]catechols was close to 9.0 after incubating olfactory tubercle slices in a KRM-high K^+ (25 mM) medium (compared to a ratio of less than 1.0 obtained when incubations were performed in a high K^+ (55 mM) KRM medium). However, even under this latter condition, ethanol (0.8 and 1.6%, w/v) significantly antagonized the K^+-induced formation of [^{3}H]catechols in olfactory tubercle slices, whereas it produced no inhibitory effect when the formation of [^{3}H]catechols was studied in hippocampal slices. The results, obtained under depolarizing conditions of 25 mM K^+, seem to further argue against the possibility that the inhibitory effect of ethanol upon K^+-induced formation of newly synthesized catechols in mesolimbic dopaminergic terminals was due to a prior action of ethanol upon the release of the newly formed monoamine.

It is possible that the inhibitory effect of ethanol on K^+-induced formation of newly synthesized [^{3}H]catechols was mediated through an inhibitory effect of ethanol on [^{3}H]tyrosine uptake by olfactory tubercle slices. In order to test this possibility, the [^{3}H]tyrosine taken up by the tissue slices at the end

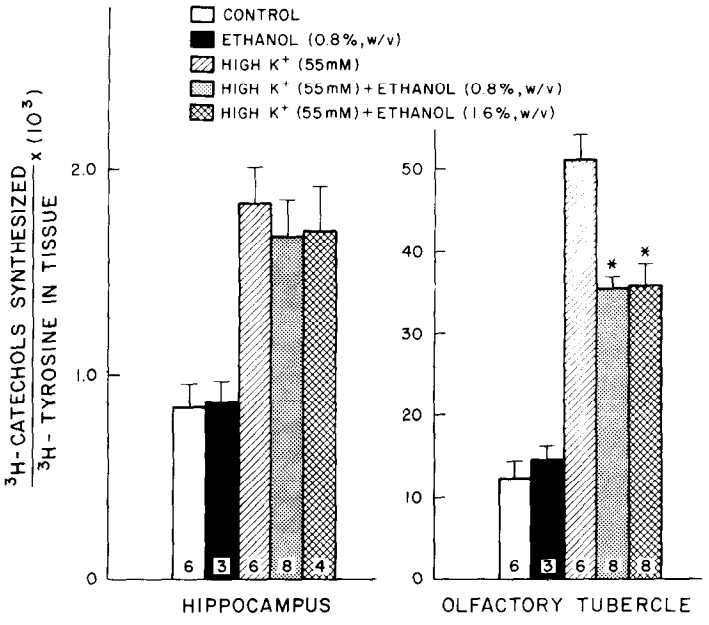

Fig. 6. Effect of ethanol on K^+-induced formation of newly synthesized [^{3}H]catechols in hippocampal and olfactory tubercle slices. Incubation conditions of the slices and separation of the [^{3}H]catechols formed are described under Figs. 3 and 4. [^{3}H]Tyrosine taken up by the tissue was separated by column chromatography; the number of individual experiments is indicated at the bottom of each column. The brackets indicated the standard error of the mean. A single asterisk (*) indicates $P < 0.001$ when compared to respective controls without ethanol.

of the incubation period was measured, and the results were expressed as conversion index [^{3}H]catechols synthesized/[^{3}H]tyrosine in tissue. The results are shown in Fig. 6 and indicate that ethanol inhibited the K^+-induced formation of newly synthesized [^{3}H]catechols in olfactory tubercle slices regardless of any effect on [^{3}H]tyrosine uptake. Again, using this conversion index, similar ethanol concentrations produced no alteration of the K^+-induced [^{3}H]catechol synthesis in hippocampal slices.

DISCUSSION

Potassium-induced depolarization of cortical and striatal slices produces an acceleration of catecholamine synthesis that seems to arise primarily as a result of the removal of tyrosine hydroxylase from end-product inhibition subsequent to the release of transmitter [1, 5]. Recent findings indicate that the acceleration of transmitter synthesis after potassium depolarization could also be mediated, in part, by a kinetic activation of the rate-limiting enzyme tyrosine hydroxylase. Thus, 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 [2]. This activation appeared to be mediated by an increase in the apparent affinity of the enzyme for pterin cofactor and a decreased affinity for the end-product inhibitor dopamine. We have recently shown that ethanol (0.2–0.8%, w/v) specifically blocks the increase in dopamine synthesis observed in striatal slices after K^+-depolarization while having no effect on dopamine synthesis measured in non-depolarized striatal slices. Although it is of interest to determine the mechanism(s) underlying these effects of ethanol [2, 3], it is also important to know whether this ability of ethanol displays any regional specificity within the brain.

We chose two different brain areas for this study: the hippocampus, a brain region rich in noradrenergic nerve terminals, and the olfactory tubercle, an area rich in dopaminergic nerve terminals. In recent studies we have shown that the olfactory tubercle can be a suitable tissue to study transmitter synthesis regulation in dopaminergic nerve terminals and that ethanol has an effect in inhibiting dopamine synthesis in this brain region as well as in the striatum [4]. By contrast, the hippocampus seems to be a tissue well suited to study transmitter synthesis regulation in noradrenergic terminals in the brain. In this tissue, the synthesis of monoamines showed linearity as a function of time for up to 20 min, and maximal velocities for catechol formation were reached with external tyrosine concentrations of around 4×10^{-5} M. Moreover, K^+-depolarization increased the formation of newly synthesized catecholamines in a fashion that was Ca^{2+}-dependent. In previous experiments, conducted in cortical and olfactory tubercle slices, it has been demonstrated that the stimulation of catecholamine synthesis induced by K^+-depolarization is exerted at the level of the tyrosine hydroxylase enzyme [4, 7]. The advantage of using hippocampal as opposed to cortical slices is that the hippocampus has very few, if any, dopaminergic nerve terminals.

In contrast to results previously reported for striatal slices, ethanol at two different concentrations (0.8 and 1.6%, w/v) was found to be without effect

on the K^+-stimulated catecholamine synthesis in the hippocampus. The same ethanol concentrations produced a striking inhibitory effect on the K^+-induced increase in catechol synthesis in slices of olfactory tubercle and striatum [4]. Thus, using similar experimental conditions and ethanol concentrations, it is possible to demonstrate a regional difference for the ethanol effects on catecholamine synthesis in the brain: transmitter synthesis regulation seems to be affected in dopaminergic nerve terminals whereas noradrenergic terminals seem to be unaffected. In this regard it is of interest to point out that other workers involved with investigating the effects of acute ethanol administration *in vivo* on catecholamine metabolism have come to a similar conclusion—that ethanol appears to have a selective action on dopamine neurons, leaving the noradrenaline neurons unaffected. In one study Liljequist and Carlsson [8] have shown that the administration of a small dose of ethanol (2.36 g/kg) markedly retards the formation of 3-methoxytyramine, the *O*-methylated dopamine metabolite. No effect of ethanol on the accumulation of normetanephrine, the *O*-methylated noradrenaline metabolite, was observed in this study. In another study by Bacopoulos *et al.* [9], it was found that a 2 g/kg dose of ethanol did not alter noradrenaline turnover in most of the brain regions investigated. However, the same dose of ethanol significantly reduced the turnover of dopamine in the substantia nigra and caudate nucleus. These *in vivo* results are compatible with the *in vitro* findings described in this paper in which we have shown that ethanol inhibits the potassium depolarization-dependent increase in catecholamine synthesis in dopamine-rich areas such as the olfactory tubercle but not in noradrenaline-rich areas such as the hippocampus.

Although it might appear that the ethanol concentrations used in this study are relatively high (0.8 and 1.2%, w/v), we have previously shown that concentrations of ethanol as low as 0.2% (w/v) or 40 mM are able to inhibit the potassium-dependent increase of dopamine synthesis in striatal and olfactory tubercle slices [1, 4]. However, what is interesting is that, even at these relatively high ethanol concentrations of 0.8 and 1.2% (w/v), the synthesis of catecholamines in noradrenergic-rich nerve terminals of the hippocampus is unaffected by the presence of the drug.

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