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α_1-Adrenoceptor function in the rat hippocampus as assessed by noradrenaline-stimulated inositol phospholipid breakdown after destruction of noradrenergic neurons by neonatal 6-hydroxydopamine treatment

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Neonatal 6-hydroxydopamine treatment of male Sprague-Dawley rats resulted in a large depletion of noradrenaline 84–87 days later, as demonstrated by an 82% reduction in the intra-synaptosomal deamination of [^{14}C]noradrenaline within noradrenergic synaptosomes with only marginal effects upon the extrasynaptosomal deamination. The hippocampal stimulation of inositol phospholipid breakdown by noradrenaline was, however, unaffected by the neonatal 6-hydroxydopamine treatment.

Systemic administration of the monoamine neurotoxin 6-hydroxydopamine to newborn rats results in a number of changes in the pattern of postnatal development of noradrenergic innervation of the central nervous system, with a depletion of noradrenaline in the hippocampus, cerebral cortex and spinal cord, but an increased innervation in the pons-medulla area being found (Clark et al 1972; Sachs & Jonsson 1972, 1975; Jonsson et al 1979). At the same time, a number of adaptive changes occur in the affected regions. Thus in the cerebral cortex, neonatal 6-hydroxydopamine treatment results in an increased noradrenaline (NA) turnover being found in the remaining noradrenaline neurons two to three months after the lesion (Jonsson et al 1979). In addition, these authors found an increased binding of the α_1- and β-adrenergic radioligands [^{3}H]WB4101 and [^{3}H]dihydroalprenolol, respectively, in cerebral cortical membrane preparations from these rats (Jonsson et al 1979). Binding experiments, however, give little information themselves, particularly in lesion experiments, unless they are combined with tests for receptor function (for discussion, see Fowler 1984). Thus, Jonsson et al (1979) demonstrated that the increase in [^{3}H]dihydroalprenolol binding was accompanied by an increased NA-stimulated cAMP formation, but no technique was available with which to assess the α_1-adrenergic function. More recently, it has been demonstrated that in both the cerebral cortex and hippocampus, stimulation of α_1-adrenoceptors by NA leads to an increased breakdown of inositol phospholipids (PI) into inositol-1,4,5-triphosphate and diacylglycerol (see e.g. Minneman & Johnson 1984; Fowler et al 1986). In the present study, the effect of neonatal 6-hydroxydopamine treatment upon NA-stimulated inositol phospholipid breakdown (PI breakdown) in the rat hippocampus has been studied.

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Materials and methods

The rats used were part of a study into the effect of noradrenergic denervation upon animal behaviour. Male neonatal Sprague-Dawley rat pups (Anticimex, Sollentuna, Sweden) were injected with 6-hydroxydopamine hydrobromide (100 mg kg^{-1} s.c., dissolved in 0·9% saline with 0·2 mg ml^{-1} ascorbic acid as antioxidant) on days 1, 3, 5 and 7 after birth. Control rats received an equal volume of saline–ascorbic acid on the same days. This procedure has been shown to give a complete and long lasting depletion of noradrenaline in cortical areas (Clark et al 1972; Archer et al 1986). Possible systematic errors were minimized by the use of a cross-fostering method. After weaning at 3 weeks, the rats were housed 3–4 animals per cage. On day 78 after birth, half of the control and 6-hydroxydopamine-treated rats were injected with a single dose of the 5-HT agonist 5-methoxy-*N*,*N*-dimethyltryptamine (1 mg kg^{-1} s.c., dissolved in saline) and were tested behaviourally. The animals were decapitated on days 84–87 after birth, and the hippocampal and frontal cortical regions dissected, and placed in Krebs-Henseleit bicarbonate (KHB) buffer and 0·32 M sucrose, respectively. The single 5-methoxy-*N*,*N*-dimethyltryptamine treatment did not appear to affect the observed noradrenaline stimulated PI breakdown, and so the data presented here are for animals with and without this treatment taken together.

PI breakdown was measured by the methods of Berridge et al (1982) and Watson & Downes (1983) with slight modifications as described elsewhere (Fowler et al 1986). Briefly, the hippocampi from three rats were pooled and miniprisms (0·35 × 0·35 mm) sliced by use of a McIlwain tissue chopper. The miniprisms were washed three times with KHB buffer and incubated for 60 min in a volume of 3 ml with 30 μCi [^{3}H]myo-inositol at 37 °C under an atmosphere of 95% O_2 : 5% CO_2. The slices were then washed with warm KHB buffer, reincubated for 10 min at 37 °C, washed again and then reincubated in the presence of 50 μM pargyline in order to inhibit irreversibly the monoamine oxidase activity.

The miniprisms were then washed extensively and 30 µl aliquots incubated with KHB-buffer containing noradrenaline (0–100 µM), Li^+ (8·8 mM, with the Na^+ concentration reduced by a concomitant amount to preserve isotonicity), and ascorbic acid (0·1 mg ml^{-1}) as antioxidant for 30 min at 37 °C. The ambient potassium concentration during the incubation was 5·88 mM. The reactions were stopped by the addition of 0·94 ml chloroform–methanol (1 : 2 v/v) and the radiolabelled inositol phospholipid (Lipid) and inositol phosphate (InsP) fractions isolated as described elsewhere (Berridge et al 1982; see Fowler et al 1986).

The activity of monoamine oxidase (MAO) extra- and intra-NA-synaptosomally was assayed essentially as described elsewhere (Ask et al 1983; Fagervall & Ross 1986). Briefly, the frontal cortices were homogenized (1 : 20 w/v) in 0·32 M sucrose and centrifuged at 800*g* for 10 min. The synaptosome-rich supernatants were assayed for MAO activity with 0·25 µM [^{14}C]NA in the presence of 0·12 µM citalopram (in order to prevent uptake and deamination of NA within 5-HT synaptosomes), and in the absence or presence of the selective NA-uptake inhibitor maprotiline (3 µM). The deamination in the presence of maprotiline was defined as the extra-NA-synaptosomal deamination, and the deamination in the absence of maprotiline minus the deamination in the presence of this compound defined as the intra-NA-synaptosomal deamination (see Ask et al 1983; Fagervall & Ross 1986).

Myo-[2-^{3}H]inositol (specific activity 16 Ci $mmol^{-1}$), in a sterile water solution, was obtained from New England Nuclear GmbH, Dreieich, West Germany. (−)-Noradrenaline[8-^{14}C] hydrogen tartrate (57 mCi $mmol^{-1}$) was obtained from Amersham International plc, Amersham, UK. Citalopram hydrochloride and maprotiline hydrochloride were gifts from Lundbeck and Co. A/S, Copenhagen, Denmark, and Ciba-Geigy AG, Basel, Switzerland, respectively. 6-Hydroxydopamine hydrobromide, 5-methoxy-*N*,*N*-dimethyltryptamine and (−)-noradrenaline bitartrate were obtained from the Sigma Chemical Co., St Louis, MO, USA. All other reagents were standard laboratory reagents of analytical grade whenever possible.

Results and discussion

The degree of the noradrenergic depletion 84–87 days after neonatal 6-hydroxydopamine treatment was assessed by measuring the intra- and extra-NA-synaptosomal deamination of 0·25 µM [^{14}C]NA in synaptosome-rich homogenates of the frontal cortex. There was a small reduction in the extra-NA-synaptosomal deamination, but a large (82%) reduction in the intra-NA-synaptosomal deamination (Fig. 1), consistent with a severe depletion of NA, and consistent with the literature for the effects of repeated 6-OHDA treatment postnatally (Clark et al 1972; Jonsson et al 1979; Archer et al 1986). Since the [^{14}C]NA has to be accumulated within the NA-synaptosome before it can

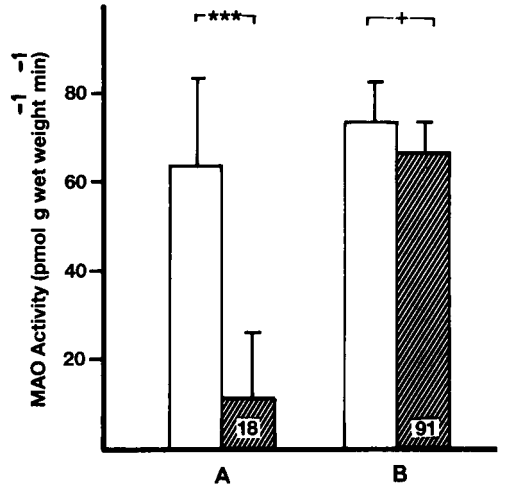

FIG. 1. Deamination of 0·25 µM [^{14}C]noradrenaline A, within and B, outside noradrenergic synaptosomes from rat frontal cortex. Data are means ± s.d. (n = 12) for control (unhatched bars) and neonatal 6-hydroxydopamine-treated (hatched bars) rats. The values within the bars are the mean values for the 6-hydroxydopamine-treated rats as a % of the mean values for the control rats. +, $P = 0{\cdot}057$; ***, $P < 0{\cdot}001$, two-tailed *t*-test.

be deaminated, it seems likely that the effect of neonatal 6-hydroxydopamine upon the intra-NA-synaptosomal deamination of [^{14}C]NA is due primarily to the large reduction in NA uptake found after this treatment (see Sachs & Jonsson 1975). Nevertheless, the measurement of intra- and extra-NA-synaptosomal deamination of [^{14}C]NA represents a simple and effective method for the estimation of NA-depletion after 6-hydroxydopamine treatment. An analagous method for the measurement of striatal dopamine deamination has similarly demonstrated that hemitransection of rats results in a decreased intrasynaptosomal deamination of dopamine without effect on the extrasynaptosomal deamination (Oreland et al 1984).

Rat hippocampal PI breakdown has been assessed by measuring the accumulation of [^{3}H]inositol phosphates (InsP) formed from prelabelled inositol phospholipids (Lipid) after inhibition of inositol-1-phosphatase by Li^+ (Berridge et al 1982). NA is able to stimulate PI breakdown measured in this way via activation of α_1-adrenoceptors both in the cerebral cortex (see e.g. Minneman & Johnson 1984) and in the hippocampus (Janowsky et al 1984; Fowler et al 1986). In the latter tissue, the EC50 for NA is in the range 2–3 µM, and the stimulation is antagonized by the α_1-antagonist prazosin with a pA_2 of 9·2 (Fowler et al 1986).

Stimulation of hippocampal PI breakdown by NA has been used in the present study to determine whether or not α_1-adrenergic receptor function has been changed after neonatal 6-hydroxydopamine treatment. The treatment did not appear to change the incorporation of the [^{3}H]myo-inositol into the tissue (as seen by un-

changed Lipid and basal InsP counts) (Fig. 2). PI breakdown was stimulated by NA (as seen for either InsP d min^{-1} (Fig. 2A) or for the data calculated as InsP/(Lipid + InsP) (Fig. 2C), the latter being a more useful representation of PI breakdown as it is independent of aliquot volume and the prelabelling time with the [^{3}H]myo-inositol used (J. A. Court, C. J. Fowler, J. M. Candy, P. R. Hoban & C. J. Smith, unpublished results)) for both the control and the 6-hydroxydopamine-treated rats. There was, however, no difference between the degrees of stimulation found for the control and 6-hydroxydopamine-treated rats (Fig. 2). Thus, neonatal 6-hydroxydopamine treatment does not result in any changed hippocampal α_1-adrenergic responsiveness, as assessed by NA-stimulated PI breakdown 84–87 days after lesion. This finding is at variance with the finding of Jonsson et al (1979) that there was a slight increase in the specific binding of the α_1-adrenergic antagonist [^{3}H]WB4101 to cerebral cortical membranes 2–3 months after neonatal 6-hydroxydopamine treatment. This discrepancy may simply be a reflection of the difficulty of comparing antagonist binding with an agonist-mediated response (for discussion, see e.g. Menkes et al 1983), although the increase in [^{3}H]dihydroalprenolol binding found in the 6-hydroxydopamine-treated animals was paralleled by an increased responsiveness of cAMP formation to NA-stimulation (Jonsson et al 1979). While it is possible that the small degree of supersensitivity suggested by the [^{3}H]WB4101 experiments might be lost during the long incorporation and incubation times used for the assessment of PI breakdown, the most likely explanation for the discrepancy between the binding data and the functional data presented here is that the increased [^{3}H]WB4101 binding reported in cortical membranes by Jonsson et al (1979) is either not found in the hippocampus, or alternatively merely reflects an increased binding to recognition sites not coupled to the PI breakdown response. A similar discrepancy between [^{3}H]WB4101 binding and NA-stimulated PI breakdown has been found in a regional distribution study (Fowler et al 1986) and may be a reflection of the ability of [^{3}H]WB4101 to bind to sites other than the α_1-adrenoceptor site (Norman et al 1985). Thus, the present results underline the conclusion reached earlier (Fowler 1984) that in lesion experiments, it is essential to compliment radioligand binding data with measures of receptor function.

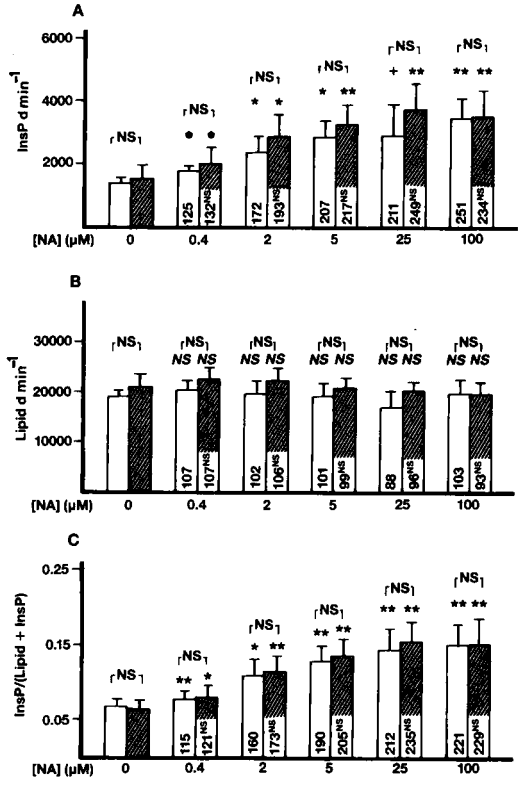

FIG. 2. Noradrenaline-stimulated PI breakdown in hippocampal miniprisms from control (unhatched bars) and neonatal 6-hydroxydopamine-treated (hatched bars) rats. Data are means ± s.d. (n = 4, each group comprising hippocampi from 3 rats) for: Panel A, InsP d min^{-1}; Panel B, Lipid d min^{-1}; Panel C, InsP/(Lipid + InsP). +, $P = 0{\cdot}061$; *, $P < 0{\cdot}05$; **, $P < 0{\cdot}01$ (NS, not significant) with respect to the corresponding value at [noradrenaline] = 0 (two-tailed paired t-test). Comparisons between the control and lesioned values for each noradrenaline concentration were determined by a two-tailed t-test (NS: $P > 0{\cdot}10$). Values within the bars are the mean values as a % of the values for the same treatment group at [noradrenaline] = 0 (NS, not significantly different from the % value for the control group, $P > 0{\cdot}20$, two-tailed Mann-Whitney U-test).

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Activities of novel polyhydroxylated cardiotonic steroids purified from nuchal glands of the snake, *Rhabdophis tigrinus*

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Seven novel polyhydroxylated steroids were isolated from the nucho-dorsal glands of the snake, *Rhabdophis tigrinus*. Biological activities of these steroids in inhibiting $(Na^+ + K^+)$ATPase and in producing positive inotropic action were examined in comparison with those of ouabain and gamabufotalin. Gamabufotalin was approximately 10 times more potent than ouabain in inhibiting $(Na^+ + K^+)$ATPase. Two compounds, compounds III and XIII, of the seven, produced nearly equipotent enzyme inhibitory activity to ouabain. The activity of the remaining five was relatively low among the compounds tested. All compounds exhibited more or less positive inotropic action in the papillary muscle preparations. The ranking order of the potency was: gamabufotalin > ouabain and compound IV > compound III and XIII > compound I, II, XII and XIV.

We have reported (Akizawa et al 1985a, b) that nuchodorsal glands of the snake, *Rhabdophis tigrinus*, contain novel steroids. These are analogues of the known bufodienolides, but differ from them in the polyhydroxylation and configuration of the hydroxy group. It is generally known that bufodienolide toxins obtained from the family of *Bufonidae* possess a potent inhibitory activity on $(Na^+ + K^+)$ATPase and produce a positive inotropic action (Flier et al 1980; Ku et al 1974). However, it is not known whether the newly identified steroids produce an inhibitory effect on $(Na^+ + K^+)$ATPase and a positive inotropic action. Therefore the present experiments were undertaken.

Materials and methods

Preparation of steroids from nuchal glands. The preparation of steroids from nuchal glands of *Rhabdophis tigrinus*, was as described by Akizawa et al (1985a, b). In brief, the faint yellowish, sludgy secretion was collected by pressing the glands with forceps and was then lyophilized. A sample of the lyophilizate was extracted with 5% acetonitrile and the extract chromatographed (HPLC: JASCO, Tri Rotar-II) on an ODS column (Hibar Lichrosorb RP-8, 4 × 250 mm, elution; 5 to 50% CH_3CN/60 min at linear gradient, flow rate; 1 ml min^{-1}, monitored at 300 nm). The purity of each fraction was determined by HPLC, mass spectrometry (JOEL, JMS-D-300) and ^1H NMR (JOEL, FX-270). Chemical structures were assigned with the aid of ^{13}C NMR (JOEL, FX-270) by comparison with those of structurally known bufodienolides such as gamabufotalin, bufotalin, arenobufagin, cinobufagin and marinobufagin. Seven novel steroid structures (compounds I, II, III, IV, XII, XIII and XIV) were identified. Tentative chemical structures of these steroids are shown in Fig. 1.

$(Na^+ + K^+)$ATPase (EC 3.6.1.3) assay

Canine kidney $(Na^+ + K^+)$ATPase was obtained from Sigma (specific activity: 1·6 μmol min^{-1} (mg protein)$^{-1}$). This preparation also contained a ouabain-insensitive (Mg^{2+})ATPase which was less than 3% of the total steady-state ATPase activity. $(Na^+ + K^+)$ATPase was prepared for assay by dissolution in the buffer containing 25 mM mannitol, 20 mM Tris-HCl and 1 mM Na_2EDTA (pH 7·4).

Five to seven concentrations of the test solution or vehicle (0·1 ml each) were incubated for 5 min in a shaking water bath at 37 °C in a 0·7 ml reaction media containing (final mM concentration): NaCl 100; KCl 10; $MgCl_2$ 3; Na_2EDTA 0·2; Tris-HCl 3·2 (pH 7·4 at 22 °C). Then, $(Na^+ + K^+)$ATPase solution (0·03 u/0·1 ml) was added. Prolonged incubation caused decreased activity in the absence of ATP. Therefore, the incubation time was exactly 8 min. Reactions were initiated by the addition of γ-labelled [^{32}P]ATP (0·15 μCi ml^{-1}) to give a final concentration of 1 mM. Total volume of the reaction mixture was 1 ml. The enzyme reaction was stopped with 1 ml of ice-cold 10% TCA solution after 30 min reaction time. The samples were kept on ice for 10 min before 3 ml of TCA solution containing charcoal

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