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DETERMINATION OF NOREPINEPHRINE IN BRAIN PERFUSATES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A procedure for determining catecholamine levels in brain perfusates is described. A single-step extraction on alumina followed by reversed-phase ion-pair chromatography and electrochemical detection was employed. Levels of norepinephrine are reported in push-pull perfusates of the rat hypothalamus under baseline and various ion substitution conditions. The resulting estimated norepinephrine release values are correlated with the behavior of the animal. The data are discussed in terms of the validity of the measures and the noradrenergic mediation of behavior.

INTRODUCTION

The catecholamines, norepinephrine, epinephrine and dopamine, are of considerable importance in clinical as well as basic neurobiological research [1]. The development of sensitive assays for these compounds and their metabolites [2] has led to renewed interest in their role in neurological diseases [3], sympathetic neuronal function, and development [4, 5], and in the transmission and modulation of central neural information [6]. We were interested in adapting a single assay technique for the catecholamines in a variety of tissues and physiological fluids. Of the available methods, high-performance liquid chromatography (HPLC) with electrochemical detection (ElCD) is the method of choice because of its resolution, selectivity, sensitivity and simplicity [7].

Our interest in the function of catecholamines in the central nervous system has led us to develop a highly specific and sensitive assay for norepinephrine, epinephrine and dopamine in brain perfusates. Other HPLC—ElCD methods have been successfully applied to the measurement of catecholamines in brain

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perfusates without a pre-purification step [8]. The procedure presented here utilizes a well-documented single-step extraction procedure [9] with an improved chromatographic step resulting in a relatively high capacity factor for norepinephrine as well as the resolution required for determination of the catecholamines in a variety of samples. We have been able to successfully apply this method to brain perfusates, spinal fluid and plasma. In this report, we present the data obtained from the measurement of norepinephrine in push-pull perfusates from the medial preoptic/anterior hypothalamus (POAH) of the rat. Data on spinal fluid and plasma catecholamines will be presented elsewhere.

EXPERIMENTAL

Reagents

Norepinephrine (NE), epinephrine (E) and dopamine (DA) (Sigma, St. Louis, MO, U.S.A.), 3,4-dihydroxybenzylamine (DHBA) (Aldrich, St. Regis, WI, U.S.A.), and sodium octyl sulfate (Eastman, Rochester, NY, U.S.A.) were used. Acid-washed activated alumina was purchased from Bioanalytical Systems (West Lafayette, IN, U.S.A.) but was customarily re-activated according to the method of Anton and Sayre [9]. All other chemicals were of reagent grade and all solutions were prepared from HPLC-grade water obtained from Fisher (Fairlawn, NJ, U.S.A.). All solutions were filtered through a 0.22-μm filter (Millipore, Bedford, MA, U.S.A.) before use.

Stock solutions of catecholamines and DHBA, 1 mg/ml in 0.1 *M* perchloric acid containing 1.3 m*M* EDTA and 7.9 m*M* sodium metabisulfite, were prepared bimonthly and stored at 4°C. Working standards were prepared daily by making appropriate dilutions of the stock solutions in perchloric acid. Solutions of 1.5 *M* Tris, (pH 8.6) and 0.1 *M* phosphate buffer, pH 7.4, both containing 52 m*M* EDTA, were prepared monthly.

In vivo brain perfusates

Fifteen rats were implanted with push-pull cannulae constructed according to the method of Sparber [10] in the right medial POAH. After 1 week post-operative recovery and habituation to the perfusion chamber, experiments were begun. Rats were perfused with filtered Krebs—bicarbonate solution, pH 7.4, aerated with oxygen—carbon dioxide (95:5) at a constant flow-rate of 20.6 μl/min by means of a Harvard syringe pump for a 30-min washout period followed by a 1-h baseline period. Solutions with altered ion concentrations (0.75 m*M* cobalt replacing calcium in five rats or 30 m*M* potassium replacing equimolar amounts of sodium in three rats) were then switched into the push flow remotely through 3-way valves in eight rats, while in seven others, a second hour of baseline measures were taken. In all rats, an attempt was made to correlate behavior with NE release. Their behavior was classified on a minute-by-minute basis by observation as waking, waking-with-movement (includes locomotion and grooming) and sleeping. Samples were collected in 20-min intervals by means of 3-way valves into tubes on ice containing 600 μl 0.1 *M* perchloric acid. The perchloric acid was partially replaced by 412 μl of perfusate in each 20-min interval. The perfusate took exactly 9.5 min to reach

the collection tube from the brain. Thus, no pharmacological measures were employed to limit NE oxidation. While some auto- and/or enzymatic oxidation of NE probably took place, we were more interested in maintaining a preparation free of artificial pharmacological intervention. After collection, the sample was stored at −80°C until analysis. Cannula placements were verified histologically; only those placements in the medial preoptic/anterior hypothalamus are included in the data presented here.

Extraction of catechols

Brain perfusate (0.6 ml) and 4 ng DHBA in 20 μl 0.1 *M* perchloric acid were added to a 5-ml reaction vial containing 50 mg acid washed alumina (baked at 180°C for 3 h prior to use). A 0.5-ml aliquot of 1.5 *M* Tris, pH 8.6 was added and the vial quickly capped, vortexed briefly, and shaken for 5 min on a reciprocal shaker.

The liquid in the vials was then aspirated and the alumina then washed with 0.5—1.0 ml of water, which was aspirated again. The alumina was then transferred as a water slurry in a Pasteur pipet to a centrifugal microfilter (Bioanalytical Systems) containing a 0.2-μm filter. The alumina was dried by centrifugation at 1000 *g* for 1 min.

The catechol compounds were then eluted by adding 100 μl of 0.1 *M* perchloric acid and vortexing for 1 min. This slurry was then centrifuged at 100 *g* for 1 min yielding the final injectate.

Chromatography

A Hewlett-Packard 1084B system was used (Hewlett-Packard, King of Prussia, PA, U.S.A.); it included two reciprocating diaphragm pumps, a continuously adjustable injection system, a Supelco LC-18 column (5 μm, C_{18}-bonded silica, 250 × 4.6 mm I.D.) and precolumn (50 × 4.6 mm I.D.) (Supelco, Bellefonte, PA, U.S.A.) maintained at a constant temperature of 30°C, and a Bioanalytical Systems TL5 glassy carbon or TL3 oil-based carbon paste electrode and LC4 amperometric controller. Chromatograms were recorded on both the Hewlett-Packard graphic-integrator and a Houston Omniscribe potentiometric recorder (Houston Instruments, Austin, TX, U.S.A.).

The mobile phase was composed of 14.5 m*M* citric acid, 71.0 m*M* dibasic sodium phosphate, 1.3 m*M* EDTA and $1.7 \cdot 10^{-4}$ *M* sodium octyl sulfate (pH 6.5, using 2 *M* sodium hydroxide) and was pumped at a flow-rate of 1.0 ml/min. The mobile phase was filtered through a 0.22-μm filter and degassed in vacuo and by helium purging. The working electrode potential was + 0.9 V vs. a Ag/AgCl reference electrode. An 85-μl aliquot of the final 100-μl alumina extract was injected onto the column.

Quantitation of catecholamine concentrations

The concentration of NE in each sample, was calculated by determining its peak height ratio relative to DHBA and comparing this ratio to that obtained with synthetic standards prepared in Krebs—bicarbonate solution, pH 7.4 (the perfusion medium). These synthetic standards were prepared in three different concentrations in the range of expected sample values and carried through the same procedure as the samples. In addition, the relative recovery of catechol-

amines was determined using perfusate pools with known amounts of standards added.

RESULTS

Sample chromatograms

Fig. 1 illustrates chromatograms of a synthetic standard and a 0.6-ml brain perfusate sample taken during perfusion with 30 m*M* potassium solution. All samples were spiked with 4 ng of DHBA. Each run was done at a temperature of 30°C in order to obtain the maximum capacity factor for NE. Higher temperatures can be used for DA determinations but will decrease the retention of NE.

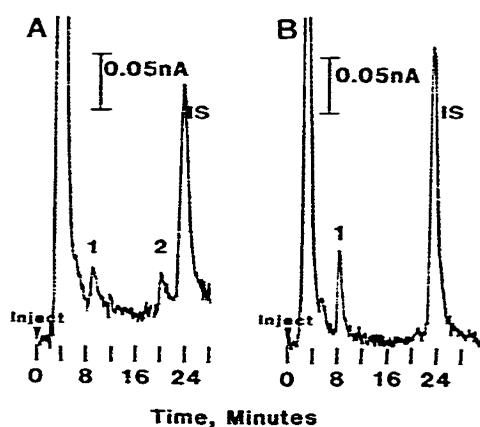

Fig. 1. Sample chromatograms obtained by the present HPLC—ElCD method. (A) Synthetic standard composed of 1 ml Krebs—bicarbonate buffer with 60 pg NE, 100 pg E and 4 ng DHBA (internal standard) added prior to alumina extraction; (B) 0.6 ml rat brain perfusate sample taken during perfusion with normal Krebs—bicarbonate solution and containing 215.8 pg/ml NE, corresponding to 129.5 pg NE released during that 20-min period. The rat was exhibiting waking-without-movement during the period corresponding to this release. A column temperature of 30°C was employed for each run. The glassy carbon electrode, at +0.9 V vs. Ag/AgCl, was used in each case. Peaks: 1 = NE; 2 = E; IS = DHBA (internal standard).

Linear range and limits of detection

The linear range of the method for perfusates was determined by adding various amounts of the catecholamines to 1-ml aliquots of a perfusate pool. The assay was linear in the range 60 pg/ml—20 ng/ml for NE, 100 pg/ml—20 ng/ml for E, and 80 pg/ml—10 ng/ml for DA. The limit of detection in all cases was determined at a minimum signal-to-noise ratio of 3:1. Since the minimum amplitude of the noise encountered was 0.01 nA, a signal of 0.03 nA corresponded to the minimum detectable amount injected which was typically 30 pg of NE, 45 pg of E and 39 pg of DA.

Precision

Repeated determinations (n = 20) of NE, E and DA in pooled 1-ml perfusate

samples gave the following coefficients of variation (C.V.): NE, C.V. = 3.2% at a concentration of 333 pg/ml; E, C.V: = 4.4% at a concentration of 167 pg/ml; DA, C.V. = 5.2% at a concentration of 80 pg/ml. The relative recovery of catecholamines from pooled perfusates relative to the synthetic Krebs—bicarbonate standard was 92.1% based on sixteen determinations.

Capacity factor for NE

The capacity factor (k'), calculated as $(t_R - t_0)/t_0$, where t_R and t_0 are the analyte and void volume retention times, respectively, varied between 2.80 and 3.50 for NE, depending on the age of the column. Thus, as can be seen in Fig. 1, at a column temperature of 30°C, NE is completely resolved from the elution front, allowing for accurate quantitation.

Interferences

The following compounds were found not to interfere with the assay: α-methyl-DA, α-methyl-NE, *l*-dopa, dihydroxyphenylacetic acid, 4-hydroxy-3-methoxy-phenylglycol, normetanephrine, ascorbic acid and uric acid. A contaminant, present on the alumina that is eluted with perchloric acid, has been seen occasionally. This unknown component has a retention time near NE in this system. Although it has not been a problem in our studies, it could possibly interfere with the determination of NE if it is present in large quantities and if NE is near the detection limit. We have found that the most satisfactory solution to this problem has been to pre-elute the alumina several times with 100 μl of perchloric acid and to run reagent blanks each day.

Experimental applications

Fig. 2 shows the effects of perfusion with solutions of altered ion con-

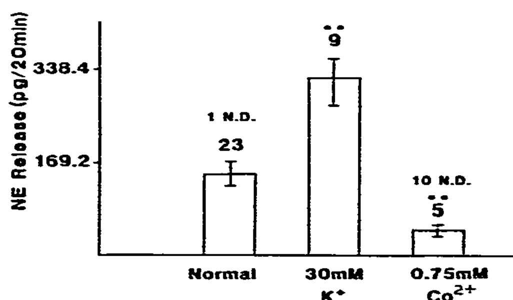

Fig. 2. Estimation of the ion dependent in vivo release of rat hypothalamic NE in pg/20 min ± S.E.M. using the present HPLC—EICD method. Rats were perfused with normal Krebs—bicarbonate solution at a rate of 20.6 μl/min for 60 min, followed by a Krebs solution containing elevated levels of potassium (30 m*M* K^+) replacing sodium, or with a Krebs solution containing cobalt (0.75 m*M* Co^{2+}) to replace calcium for an additional 60 min. The addition of depolarizing concentrations of K^+ significantly elevated NE release as would be expected if NE release was truly being measured. Furthermore, the significant inhibition of spontaneous NE release due to the elimination of calcium, indicates that this release is calcium sensitive. The numbers above the standard error bars indicate the number of determinations where detectable levels of NE were found. The numbers above these indicate the number of samples in which NE could not be detected. ** = $P < 0.01$ by two-tailed *t*-test, vs. normal.

stituents on hypothalamic NE release in eight rats. The baseline values are composed of sixteen samples in which the rats exhibited waking-without-movement, seven samples in which there was waking-with-movement, and one sample during waking-without-movement in which the levels of NE were below the detection limit. The resulting average NE release value is thus at a level associated with waking-without-movement (Fig. 3). In three rats, a perfusate with elevated (30 m*M*) potassium concentration replacing sodium was then switched into the push flow for 1 h. An increase in locomotor activity, and in some cases, vigorous grooming, accompanied the potassium elevation in all rats and persisted for the entire hour. The level of NE release was at a value associated with waking-with-movement (Fig. 3) and was significantly different from baseline ($P < 0.01$). In the remaining five rats, an alternate perfusate containing 0.75 m*M* $CoCl_2$ replacing $CaCl_2$ was perfused for 1 h. This perfusion was accompanied by behavioral sedation in all rats, but not sleep, and persisted for the entire hour. The average NE release value was at a level below that associated with behavioral sleep and in fact, could not be detected in ten of the fifteen samples collected. The average value of the five measurable samples was significantly lower than baseline ($P < 0.01$).

Fig. 3 shows the spontaneous release of NE from the medial POAH as correlated with the behavior (arousal state) of the rat. Neither E nor DA were consistently detected in these samples. The release of hypothalamic NE correlated with movement, as the waking-with-movement values were significant-

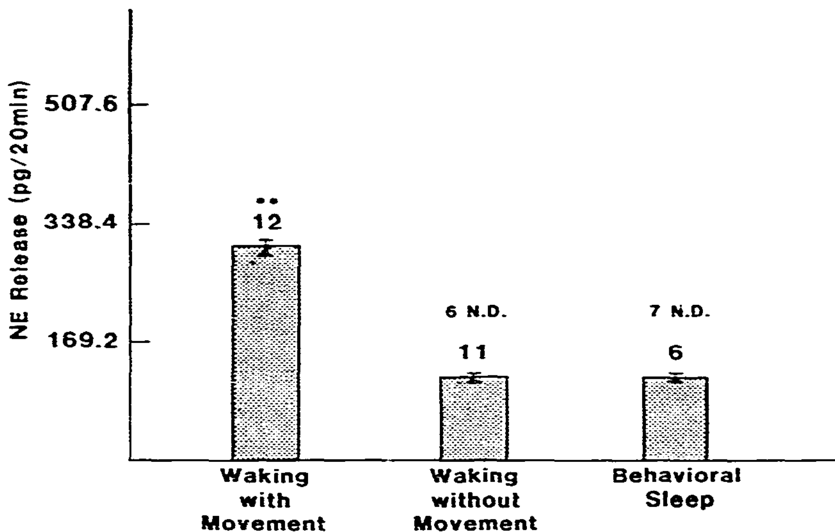

Fig. 3. Estimation of the spontaneous in vivo release of rat hypothalamic NE in pg/20 min ± S.E.M. as correlated with behavior using the present HPLC—ElCD method. Rats were perfused with normal Krebs—bicarbonate solution at a rate of 20.6 µl/min for 120 min. The rat's behavior was determined min-by-min and classified into the three categories shown (see text for details). The spontaneous release of NE was significantly greater during waking-with-movement than during waking-without-movement or behavioral sleep. The numbers above the standard error bars indicate the number of determinations where detectable levels of NE were found. The numbers above these indicate the number of samples in which NE could not be detected. ** = $P < 0.01$ by two-tailed *t*-test vs. waking-without-movement and behavioral sleep.

ly greater than either those during waking-without-movement ($P < 0.01$, two-tailed t-test) or behavioral sleep ($P < 0.01$). NE could not be detected in six waking-without-movement samples and seven behavioral-sleep samples. Since these values could not be included in the statistical measures, the aforementioned differences are biased towards less significance.

DISCUSSION

The chromatographic system employed in this study was found to be free of interferences from other acidic catechol species that may be co-extracted on alumina. Furthermore, the increased retention of NE at the high mobile phase pH employed (6.5), afforded a good separation of NE from uric acid; a constituent found in high concentrations in plasma [11] and most likely present as an extracellular constituent in brain. At this pH, protonation of acidic species is decreased, whereas protonation of basic catecholamines is unaffected. Thus, ion-pair formation of the acidic species is decreased resulting in a selective decrease in their capacity factors. An additional benefit of using a higher mobile phase pH is an improved detector response [12]. The high degree of resolution in this assay was achieved without excessive concentrations of the ion-pairing reagent, which decreases column life-time [13]. It was found that effective column life could be extended by monthly flushing with a 30% methanol in water mixture. Column viability could also be prolonged by lowering the column temperature a few degrees when retention times began decreasing.

The perfusion of solutions with altered ion concentrations yielded the results one would expect if, indeed, NE release (and not leakage) was being predominantly measured. Elevation of the extracellular concentration of potassium ion should cause neuronal depolarization and an enhancement of neurotransmitter release. By raising the concentration of potassium ion to 30 m*M* in our perfusing solution, we were able to more than double the amount of NE collected in the perfusate as compared to the amount of NE collected under normal ionic conditions. This indicates that the ability of the noradrenergic terminals, at the perfusion site, to respond to depolarizing stimuli was intact. Similarly, substitution of calcium ion by cobalt ion in the perfusing medium should depress neurotransmitter release, since extracellular calcium ion is an important concomitant to the release of neurotransmitter. By substituting 0.75 m*M* cobalt ion for calcium in our perfusions, we were able to lower the amount of NE collected in five perfusates to roughly one-third of that collected under normal ionic conditions. Furthermore, the levels of NE in ten perfusate samples were below the limits of detection (36 pg/20 min), whereas only 1 of 24 samples under normal ionic conditions had non-detectable levels of NE. These data indicate that at least two-thirds of the spontaneous release of NE measured under normal ionic conditions was calcium dependent. The remaining one-third, that is, the release of NE that persisted during cobalt perfusion, could be attributed to at least three factors. A percentage of the noradrenergic terminals being perfused could have been damaged, allowing NE to leak out. Alternatively, calcium ion was not completely eliminated, and the remaining calcium then, was sufficient to allow the level

of NE release that was measured during cobalt perfusion. Finally, it is possible that neurotransmitter release is not totally calcium dependent and that some physiological release of neurotransmitter can occur by alternative mechanisms [14]. The data from the ion substitution experiments indicate that the noradrenergic terminals under study, were, for the most part, functional and that measurement of spontaneous NE release by push-pull perfusion is a valid approach to the study of neurotransmission.

The alteration of the ionic composition of the perfusion medium not only resulted in changes in the release of NE in the POAH, but also in the behavior of the animals. Potassium-evoked NE release was accompanied by increased movement (locomotion and grooming) in all animals. The increase in movement persisted as long as extracellular concentrations of potassium were elevated. In no case was there a dissociation between elevated potassium concentrations, enhanced NE release and the increase in movement. Similarly, the attenuation of NE release by cobalt was accompanied by behavioral quieting, marked by absence of locomotion or grooming, in all animals. Once again, the behavioral and neurochemical effects persisted as long as cobalt was perfused. While these data indicate that the release of NE in the medial POAH of the rat is correlated with movement, it is possible that the ion substitutions affected one or more neurotransmitter systems in this brain area, which in turn mediated the behavioral effects. The experiments which examined the correlation of spontaneous NE release with the animals' behavior were performed in order to test the hypothesis that POAH NE release is correlated with movement. The spontaneous release of NE from the POAH, measured during the waking states, is in excellent agreement with previously reported values in the hypothalamus [15]. Furthermore, the spontaneous release of NE was significantly greater during waking-with-movement (locomotion and grooming) than during waking-without-movement or sleep. This is in general agreement with the findings of Van der Gugten and Slangen [15] who found that the spontaneous release of NE from anterolateral hypothalamic areas correlated with locomotion.

These data, indicate that POAH noradrenergic transmission is, at least in part, movement related. Whether the release of NE in the POAH actually mediates the movement, or is a consequence of it, is difficult to determine. Noradrenergic transmission in the POAH may mediate other physiological functions ascribed to this brain region. For instance, the POAH is important for the central integration of body temperature [16], and NE appears to be involved in this function [17]. Since increased motor activity is generally accompanied by increases in body temperature, it is possible that the increases in spontaneous NE release seen during movement, mediate any accompanying thermal changes that might occur. We are currently investigating this possibility.

The noradrenergic projections to the POAH originate from the ventral noradrenergic system [18, 19]. The ventral system has not been studied as extensively as the dorsal noradrenergic pathway originating from nucleus locus coeruleus. Recent evidence indicates that the activity of the dorsal pathway, is correlated with level of arousal, rather than movement [20]. Our data indicate, that the activity of the ventral pathway is not identical to that of the

dorsal system, and is not related to arousal per se, but to specific behaviors that accompany arousal responses.

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