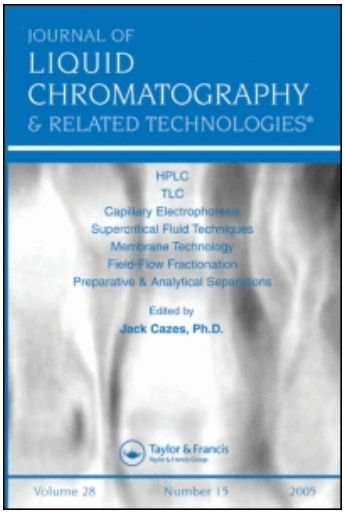

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Measurement of Extracellular Calcium Ions within the Hypothalamus of the Freely-Moving Cat: A Novel Approach

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MEASUREMENT OF EXTRACELLULAR CALCIUM IONS WITHIN THE HYPOTHALAMUS OF THE FREELY-MOVING CAT: A NOVEL APPROACH

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ABSTRACT

In order to investigate the kinetics of calcium ion (Ca^{++}) activity within the hypothalamus by high performance liquid chromatography, standard push-pull guide cannulae were implanted stereotaxically above the hypothalamus of the cat. Following post-operative recovery, an isotonic artificial cerebrospinal fluid (ACSF) was perfused in the site at a rate of 25 ul/min over successive intervals of 5.0 min. In the mid-point of a sequence of repeated push-pull perfusions, verapamil (4.0 ug/ul) was added to the perfusate. Samples collected from the hypothalamus of the freely-moving cat were analyzed for calcium concentration by an HPLC conductivity detector. The results showed that verapamil perfused within the hypothalamus of the cat caused an efflux of calcium ion into the perfusate which was detected by HPLC. This study proposes a new approach to precisely determine the calcium concentration in extracellular fluid in awake freely-moving animals.

INTRODUCTION

Calcium ions play a pivotal role in a large number of neurophysiological processes. The critical role of this cation in a variety of membrane-linked processes such as neurotransmitter release has been very well documented (1,2). Now there is also growing evidence that Ca^{++} is involved in some of the physiological and behavioral manifestations provoked by drugs such as ethanol (3,4,5,), and morphine (6,7). Thus, an exact knowledge of the Ca^{++} ion concentration in certain regions of the brain can contribute significantly to the better understanding of the neurophysiological mechanisms of drug actions and eventually central ionic mechanisms underlying drug-induced behaviors. Various techniques have been used to measure the concentration of Ca^{++} ions and other ions in the brain. In most of the previous studies the experimental animal was sacrificed in order to measure the central nervous system (CNS) tissue content of ions (7,8) or anesthetized when collecting samples. Since both, anesthesia and the trauma induced from sacrificing the animal, can affect the level of ions in the brain, these techniques have imposed some limitations.

To avoid these limitations we utilized the brain push-pull perfusion technique in freely-moving animals to collect extracellular fluid for the measurement of Ca^{++} ion concentration. This technique has been used as a reliable investigational tool for examining the dynamic activity of neurosubstances in the CNS and the kinetics of neurotransmitters (9,10,11,12). The advantage of this technique is that the change in the animal's behavior after drug administration can be correlated with a change in ion or neurotransmitter activity in a specific region of the brain (13,14,15).

The purpose of this study is to propose a novel combined technique (brain perfusion and ion chromatography) to study the dynamic activity of Ca^{++} ions in the brain. The present experiments were undertaken to investigate the characteristic kinetics of calcium ions within the hypothalamus of the cat using verapamil as a pharmacological tool for blocking calcium channels.

METHOD

Animals and Surgery

Adult female cats (Felis domesticus) were housed individually in a colony room at an ambient temperature of 22-24°C on a 12 hour light cycle. Animals were anesthetized with 30-35 mg/kg I.V. sodium pentobarbital, and an array of four 20-gauge thin-walled stainless-steel tubes (Small Parts) was implanted aseptically 4-5 mm above the rostral and caudal hypothalamus (16) following standard procedures (17,18). The guide cannulae were secured to the calvarium with a layer of cranioplast cement and protected by a pedestal which was then affixed to the skull according to procedures described previously (16). Post-operatively, penicillin (Parke-Davis) was administered intramuscularly for seven days with at least 15 days elapsing prior to the first experiment.

Drug Preparation

Perfusion solutions were prepared immediately before each experiment using pyrogen-free artificial cerebral spinal fluid (ACSF) containing the chloride salts of (in mM): Na^+ 127, K^+ 2.6, Ca^{++} 1.3, and Mg^{++} 0.9 (18,19) and passed through a 0.22 um Swinnex millipore filter. Verapamil-HCl (Sigma) was prepared in a concentration of 4.0 ug/ul (17).

HPLC Instruments

The HPLC system consisted of a dual-piston pump (LKB, 2150), a conductivity detector (Waters, model 430) with IC-Pak cation column (Waters) and an integrator (Shimadzu, C-R3A).

Mobile Phase

The mobile phase was made by adding 35 ul/L of ethylenediamine (Sigma) to deionized water and adjusting the pH to 6.20 with gold label nitric acid (Sigma).

Sample Preparation and Analysis

To analyze the sample, a volume of 50 ul of perfusate collected from the hypothalamus with $BaCl_2$ (internal standard) was filtered through a sample prep cartridge (Sep-Pak C-18) to remove any insoluble contaminants; they were rinsed through to a final volume of 1.0 ml with distilled water. 200 ul of prepared sample was injected onto an IC-Pak cation column and detected with a conductivity detector. The detector range and the gain were set to 10 and to 0.005 uS, respectively.

Experimental Protocol

After post-operative recovery and prior to perfusions of a test site with ACSF or verapamil, the functional reactivity of the test site within the hypothalamus was verified by micro-injection of 5.0 ug of norepinephrine hydrochloride in a volume of 1.0 ul which produces a well-defined hypothermia (18).

To perfuse an isolated region of the hypothalamus, a standard concentric push-pull cannula assembly was lowered through the guide tube to a pre-determined depth. The outer or pull cannula and the inner or push cannula consisted of 23- and 29- gauge thin-walled stainless-steel needle tubing, respectively. Each cannula was connected by polyethylene tubing to a calibrated gas-tight 1.0 ml Hamilton syringe, mounted on a multi-channel infusion-withdrawal pump (Harvard Apparatus, model 9352).

The test site was perfused at a rate of 25 ul/min with ACSF. Each perfusion in a series of 5 perfusions lasted for 5 min with a 5 min interval between each (20). After establishing a baseline with two 5.0 min perfusions, verapamil was added to the third perfusion in a concentration of 4.0 ug/ul. Each sample of perfusate was transferred to a plastic vial and analyzed for calcium concentration. Successive experiments, carried out on the same animal, were separated by an interval of 48 hours or longer.

RESULTS AND DISCUSSION

To confirm the validity of our experimental values, a series of calcium standards was run prior to injecting the actual samples collected from the brain. Based on the initial concentration of 1.3 mM Ca^{++} in the perfusate, standards of 80%, 100%, and 120% Ca^{++} were analyzed. All standards contained the same concentrations of Mg^{++} (0.9 mM) and Ba^{++} (2.6 mM). Table 1 displays the values from the injection and repeat injection of standard solutions, as calculated by a calibrated integrator. For each standard (80%, 100%, and 120%), ten samples were injected twice to verify the validity of the calibration to within 90%. Blank standards (without Ca^{++}) revealed no artifacts in the calcium peak window, and no measurable calcium carry-over occured with proper rinsing of HPLC syringes. As Table 1 illustrates, the validity of calibrations was confirmed to within 90%.

A representative set of liquid chromatograms which shows the quantitative estimates of Ca^{++} ion concentration in the brain perfusate is shown in Figure 1. The elevation of Ca^{++} ion concentration in the perfusate when the site was perfused with verapamil (Figure 1B) is noticeable. Figure 2 illustrates the mean $\pm$ SEM of Ca^{++} ion concentration in the perfusate collected from the anterior hypothalamus of cats perfused with artificial cerebral spinal fluid (Sample 1, 2, 4 and 5) and 4.0 ug/ul of verapamil (Sample 3). Perfusion of verapamil, a Ca^{++} channel antagonist (21), within the anterior hypothalamus/preoptic area of the freely-moving cat blocked the Ca^{++} influx into the neuronal membrane which lead to an efflux of Ca^{++} ions into the perfusate collected from the hypothalamus (Figure 2).

This study showed that, in conjunction with the brain perfusion technique, ion chromatography can be easily used for quantification of Ca^{++} ion concentration in

TABLE 1. CALCIUM STANDARDS CALCULATED BY TWO POINT CALIBRATION

Std Conc:	80% (1.04 mM)		100% (1.30 mM)		120% (1.56 mM)	
Injection:	1st	2nd	1st	2nd	1st	2nd
# 1	1.0198	1.0361	1.2795	1.3091	1.6528	1.6211
# 2	0.9736	0.9455	1.2899	1.3248	1.5446	1.5380
# 3	1.0177	1.0503	1.2743	1.2524	1.7043	1.6764
# 4	1.0036	1.0286	1.3489	1.3157	1.6020	1.6011
# 5	1.0751	1.0604	1.2480	1.2330	1.5164	1.5071
# 6	1.0538	1.0801	1.2615	1.2679	1.5595	1.5402
# 7	1.0068	1.0164	1.2779	1.2928	1.6206	1.6606
# 8	0.9841	0.9541	1.2605	1.2726	1.5889	1.5603
# 9	1.0244	0.9981	1.3110	1.2920	1.5771	1.6011
# 10	1.0084	1.0139	1.3262	1.3480	1.6500	1.6334
AVERAGE:	1.0175		1.2893		1.5978	
STD DEV:	0.0354		0.0318		0.0536	

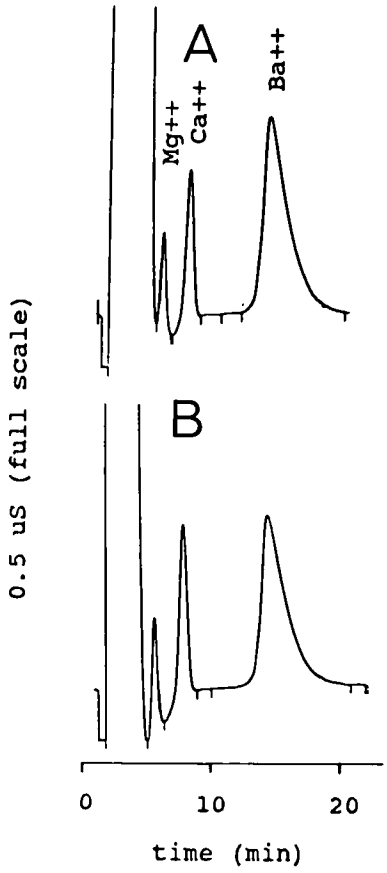

Figure 1. A representative set of liquid chromatograms which illustrate the quantitative estimates of Ca^{++} ions in the perfusate collected from the hypothalamus of the cat. The upper portion (A) shows the elution of Ca^{++} ion when the site was perfused with artificial cerebral spinal fluid. The lower portion (B) illustrates the elution of Ca^{++} ion and internal standard ($BaCl_2$) when the site was perfused with 4.0 ug/ul verapamil. Note the elevation of Ca^{++} ion concentration in the lower portion (B).

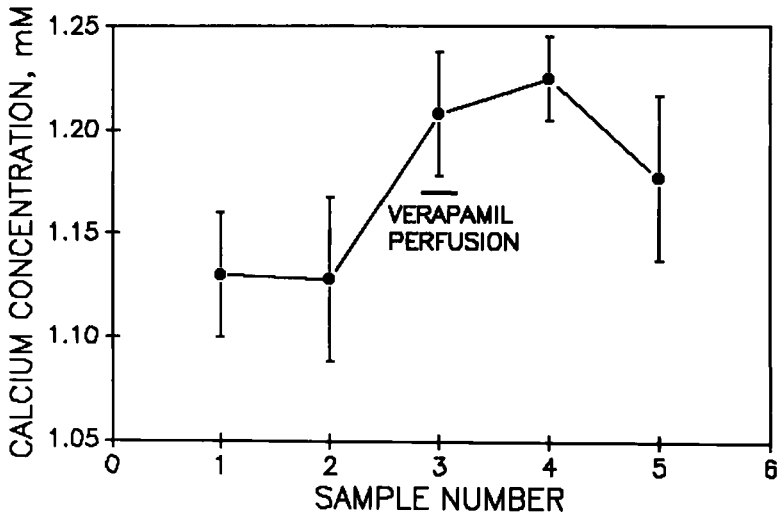

Figure 2. Mean ± S.E.M. of Ca^{++} ion concentration in perfusates collected from the anterior hypothalamus of the cat perfused with artificial cerebral spinal fluid. (Samples 1, 2, 4 and 5) and verapamil (Sample 3).

the brain of a freely-moving animal. The simple relationship that exists between conductivity and concentration of an ion makes this technique universal. This method has several advantages: a) the HPLC assay described here is rapid, relatively inexpensive, easy to perform, and quite precise, b) a small amount of sample (20 ul) of perfusate is adequate for quantification of the cation, c) no brain mass is removed, d) the temporal course of drug effects can be followed, e) the animal may be used as its own control, f) effects of several different drugs or doses may be examined in the same animal, g) the brain region of interest can be examined exclusively, h) effects of a certain drug on kinetics of ion activity within different structures of the brain can be compared in the same animal and, i) the

animal is awake and the preparation is intact with normal oxygen consumption and intact circulation, an obvious advantage over in-vitro studies.(10,12).

In conclusion, a practical HPLC method to determine Ca^{++} ion concentration in the brain of an awake freely-moving animal has been demonstrated. This method is rapid, easy to perform, relatively inexpensive, requires a small sample volume, and can be used for other ion concentration quantifications, such as Na^{+} and K^{+} in the brain.

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