Factors Influencing ^{45}Ca Metabolism in Brain and Other Organs *in Vivo*[1] (36017)

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Although calcium ions participate in a number of vital cellular processes, there is a particularly intimate association between calcium ions and neural function, both at the axon and the synapse (1). We have been concerned with processes involved in calcium transport into isolated subcellular particles (2–4) and slices (5) from brain, and it thus seemed of interest to extend earlier studies (6–8) on the distribution of calcium in the whole animal, to consider the subcellular localization in brain *in vivo*, and to study the effects on the subcellular localization of ^{45}Ca of certain pharmacological agents that cause gross changes in neural activity.

Methods and Materials. Male Sprague-Dawley rats (Holtzman Farms), 150–160 g, received, via the tail vein, 25 μCi ^{45}Ca ($^{45}CaCl_2$, 0.7–1.7 Ci/mmole; New England Nuclear Corp.) and were then decapitated at various intervals. The organs and pieces of tissue were rapidly removed, blotted, and weighed. Bone (tibia) was cleaned, ashed at 550°, and then dissolved in 0.1 *N* HNO_3. Other tissues were homogenized in water, with the protein precipitated and ^{45}Ca extracted by the addition of trichloroacetic acid (TCA) to a final concentration of 5%. Blood was collected in heparinized vessels and the TCA extraction was performed on measured volumes. Tissue extraction was continued for at least 30 min at room temperature prior to centrifugation, and the radioactivity in portions of the supernatant material was measured by liquid scintillation counting using Bray's (9) solution. Data are expressed (dpm), with correction for quenching made by the external standard method. Resuspension of the extracted precipitate in 5% TCA, or digestion with formic acid, revealed that less than 0.5% of the total activity escaped the initial extraction. The ^{45}Ca content was expressed on the basis of the initial wet weight of the tissue, with no correction for remaining blood.

To measure the subcellular distribution of ^{45}Ca in brain, ^{45}Ca was injected as indicated above and 2 hr later the brains were removed and fractionated by discontinuous density gradient centrifugation according to the method of Grey and Whittaker (10) as modified by Lust and Robinson (3). Fraction P contains the nuclei and cell debris, Fraction S is the postmitochondrial supernatant material and Fractions A, B, C, and E are the area above the myelin layer, the myelin layer, the synaptosomal layer, and the mitochondrial pellet, respectively. Fraction D is that portion of the gradient lying under the synaptosomal layer and above the mitochondrial pellet. After collecting the fractions with Pasteur pipets and measuring the volumes, the fractions were homogenized and samples were taken for measurement of protein, and, after extraction with TCA, for measurement of ^{45}Ca content. In some experiments, liver mitochondria were separated in 0.25 *M* sucrose as described by Schneider and Hogeboom (11). Protein content was determined by the method of Lowry *et al.* (12) using bovine serum albumin as the standard.

Data are presented as averages of individual observations together with the standard error of the mean. Statistical significance was calculated using Student's *t* test for paired

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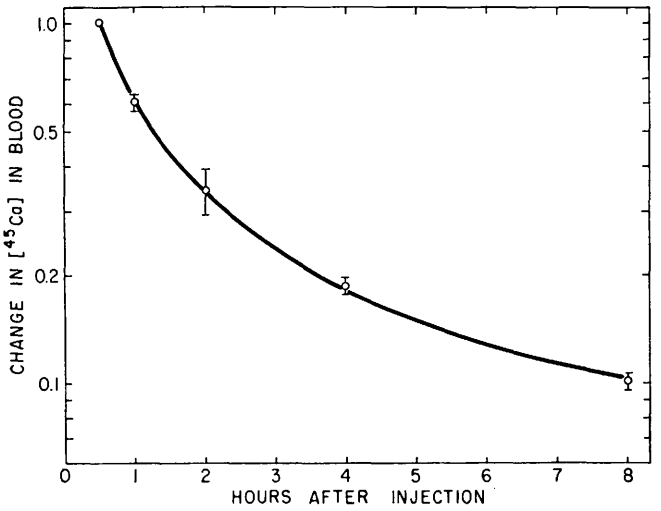

FIG. 1. Time course of the loss of ^{45}Ca from blood of rats: Results are presented as the ratio between the concentration of ^{54}Ca in the blood at each time interval and the concentration of ^{45}Ca in the blood at 30 min after the injection of $^{45}CaCl_2$. Symbols = mean ± SEM ($n = 5$).

observations; the criterion for significance was $p<0.05$.

Results. The relative concentration of ^{45}Ca in the blood [(dpm/μl of blood at time t/(dpm/μl of blood at 30 min)] decreased from the first measurement, made 30 min after intravenous administration. The loss of ^{45}Ca was not a simple first order process, but followed a pattern definable as a polycomponent exponential function (Fig. 1), as previously described (13–15). For other tissues, the data may be considered on the basis either of: (i) the concentration of ^{45}Ca in the tissue with respect to that in the blood at each time interval [(dpm/mg of tissue)/(dpm/μl of blood)]; or (ii) the concentration of ^{45}Ca in each tissue with respect to its concentration at 30 min [(dpm/mg of tissue at time t)/(dpm/mg of tissue at 30 min)].

The relative concentration of ^{45}Ca in red muscle (soleus) and cardiac muscle remained fairly constant with respect to the concentration of ^{45}Ca in the blood (Fig. 2); whereas the relative concentration of ^{45}Ca in white muscle (gastrocnemius) increased markedly. In most of the other tissues examined (kidney, liver, adrenal, and spleen), the changes closely paralleled those seen in cardiac and red muscle (Fig. 3). In testicle (Fig. 3), the relative concentration of ^{45}Ca was initially low, but increased after a lag of about 1 hr to reach a value similar to that of spleen and liver.

As previously reported (16), bone (Fig. 4) differed markedly from the other tissues studied: (i) the relative content of ^{45}Ca with respect to blood increased rapidly, attaining a value of 210 at 8 hr; and (ii) the total content of ^{45}Ca in the tissue also increased with time (Fig. 4, right panel).

In brain tissue, although the relative content of ^{45}Ca increased with respect to that in the blood (Fig. 5), there was no apparent change in the content of ^{45}Ca with time (Fig. 5, right panel).

After subcellular fractionation of the brain (Table I) the fractions containing the largest percentage of ^{45}Ca were mitochondria (E); postmitochondrial supernatant material (S); cell debris, nuclei (P); and synaptosomes (C). The relative concentration of ^{45}Ca [(dpm of ^{45}Ca/mg of protein)/(dpm of ^{45}Ca/μl of blood)] in Fraction E was 3 times higher than that of ^{45}Ca in the liver mitochondria (Table II).

Injecting phenobarbital (35 mg/kg, ip) 30 min prior to the intravenous administration of $^{45}CaCl_2$ decreased significantly the relative concentration of ^{45}Ca in both synaptosomes

TABLE I. Relative Distribution of ^{45}Ca in Subcellular Components of Brain 2 hr Following Intravenous Administration of $^{45}CaCl_2$.[a]

Fraction[b]	% of Total recovered
Homogenate	(100)
P	20.4 ± 0.7
A	1.3 ± 0.1
B	5.4 ± 0.6
C	14.2 ± 2.3
D	5.8 ± 0.6
E	25.6 ± 1.0
S	25.2 ± 3.2

[a] Results expressed as the mean ± SEM of 8 determinations.

[b] P, nuclei and cell debris; A, myelin; B, between myelin and synaptosomes; C, synaptosomes; D, between synaptosomes and mitochondrial pellet; E, mitochondria; S, supernatant material.

and brain mitochondria with respect to control values, but the ratio between these two fractions remained unchanged (Table II). This is in agreement with the observation that phenobarbital decreased the concentration of ^{45}Ca in the whole homogenate. Phenobarbital had no effect on the concentration of ^{45}Ca in the liver mitochondria.

Reserpine (5 mg/kg, ip) was administered either 2 or 22 hr before the administration of ^{45}Ca: 4 hr after reserpine the animals displayed ptosis, flaccidity, and diarrhea, but after 24 hr the animals were not grossly distinguishable from controls. Four hours after administering reserpine (2 hr after administering $^{45}CaCl_2$) the concentration of ^{45}Ca in the brain mitochondria was depressed; this difference disappeared by 24 hr (Table II).

To assess the effect of the analeptic agent pentylenetetrazol, 75 mg/kg was injected in the scruff of the neck 30 min before terminating the experiment (90 min after administering ^{45}Ca). Within 10–15 min, the animals developed sporadic clonic convulsions and remained in a state of hyperexcitability until they were decapitated. Under these conditions the relative concentration of ^{45}Ca in the mitochondrial fraction (E) increased significantly without a change in that of ^{45}Ca in the synaptosomal fraction (C) (Table II).

Morphine (10 mg/kg, ip) had little effect on the distribution of ^{45}Ca in brain (Table II) when administered 30 min before the ^{45}Ca.

Discussion. After the intravenous administration of $^{45}CaCl_2$ three patterns of distribution of ^{45}Ca were seen, represented by: (i) brain (Fig. 5), (ii) bone (Fig. 4); and (iii) other tissues (Figs. 1–3). Assuming that the amount of calcium injected (calc to be $<0.001\%$ of the plasma Ca^{2+} content) did not affect the homeostatic mechanisms of the animals, the localization of the ^{45}Ca at the indicated time intervals then reflects the exchange between certain calcium pools of the tissues and blood.

With bone, the content of ^{45}Ca increased

TABLE II. Effects of Various Agents on the Relative Concentration of ^{45}Ca in Subcellular Fractions of Rat Brain.[a]

Agent[b]	Homogenate[c]	Fraction C[d] synaptosomes	Fraction E[d] mitochondria	Ratio E/C	n	Liver mitochondria[d]	n
Control	0.476 ± 0.025	3.64 ± 0.20	11.29 ± 0.82	3.17 ± 0.27	12	5.85 ± 0.31	4
Phenobarbital	0.366 ± 0.043[e]	2.59 ± 0.39[e]	9.26 ± 0.54[e]	3.9 ± 0.60	5	6.49 ± 0.32	4
Reserpine							
(4 hr prior)	0.442 ± 0.025	2.82 ± 0.56	7.12 ± 0.62[e]	3.38 ± 0.15	5	5.87	2
(24 hr prior)	0.458 ± 0.025	3.28 ± 0.35	10.21 ± 1.10	3.31 ± 0.65	5	6.72	2
Pentylenetetrazol	0.484 ± 0.085	3.45 ± 0.20	13.97 ± 0.20[e]	4.09 ± 0.25[e]	4		
Morphine	0.495 ± 0.019	4.06 ± 0.53	12.89 ± 1.42[e]	2.93 ± 0.24	4		

[a] Results expressed as the mean ± SEM of n determinations.

[b] Administered as indicated in text.

[c] (dpm of ^{45}Ca/g of brain)/(dpm of ^{45}Ca/ml of blood).

[d] (dpm of ^{45}Ca/mg of protein)/(dpm of ^{45}Ca/μl of blood).

[e] $p < 0.05$ with respect to control.

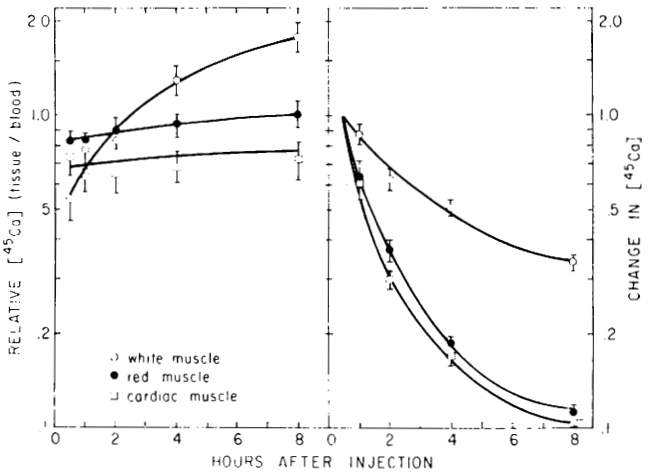

FIG. 2. Changes in the concentration of ^{45}Ca in muscle of rats after administration of $^{45}CaCl_2$: red muscle = soleus; white muscle = gastrocnemius; symbols = mean ± SEM (n = 5).

rapidly with respect to blood, while the total content of ^{45}Ca increased slowly (Fig. 5), in accord with previous investigations (16, 17). By contrast, in most other tissues studied (*e.g.*, liver, adrenal, heart, red muscle), there appeared to be a rapid exchange between blood and tissue consistent with a continuous equilibrium with at least a fraction of the tissue pool(s) of calcium.

The slow, continuous rise in the content of ^{45}Ca in brain relative to that in blood, together with the lack of change in the total ^{45}Ca content (Fig. 4), agree with previous reports on rabbit (18) and rat (7) brain. This pattern has been interpreted in terms of barrier(s) to the free exchange of calcium between blood and brain, although the site of these barrier(s) remains undefined. Graziani *et al.* (18, 19) proposed that the primary source of calcium for brain tissue is the plasma via the extracellular fluid, the cerebrospinal fluid (CSF) making only a small contribution. This proposal is supported by Woodward and Reed (8), who demonstrated that in rabbits there was a higher specific activity of ^{45}Ca in the brain than in the CSF

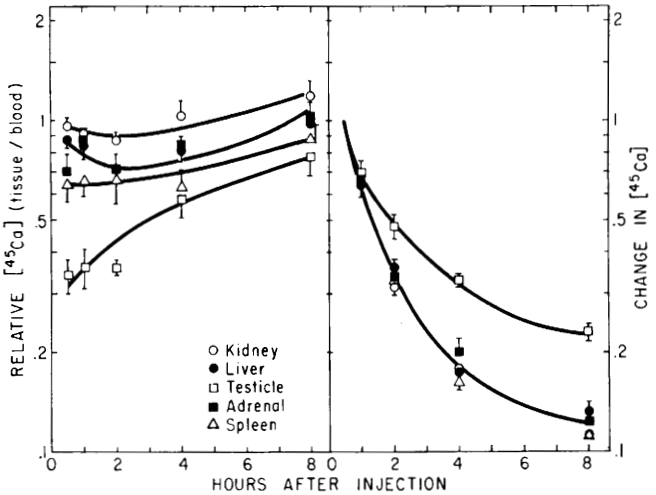

FIG. 3. Changes in the concentration of ^{45}Ca in various tissues of rat after injection of $^{45}CaCl_2$: symbols = mean ± SEM (n = 5).

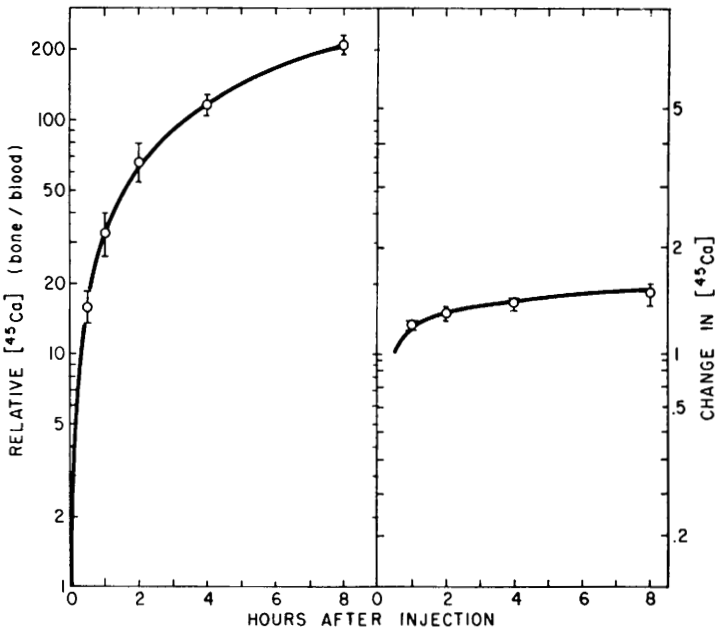

FIG. 4. Changes in the concentration of ^{45}Ca in bone of rat after injection of $^{45}CaCl_2$: symbols = mean ± SEM ($n = 5$).

after intravenous administration in accord with a measured blood–CSF concentration gradient for calcium (20).

The studies on the subcellular distribution of ^{45}Ca in brain suggest that mitochondrial fraction has a high rate of turnover of calcium (Table I), in accord with previous studies *in vitro* (3, 5). Comparable results have been reported for heart (21) and liver (22) mitochondria after administration of ^{45}Ca *in vivo*.

However, as indicated in previous studies (21, 23, 24) there must remain some uncertainty about possible redistribution of ^{45}Ca

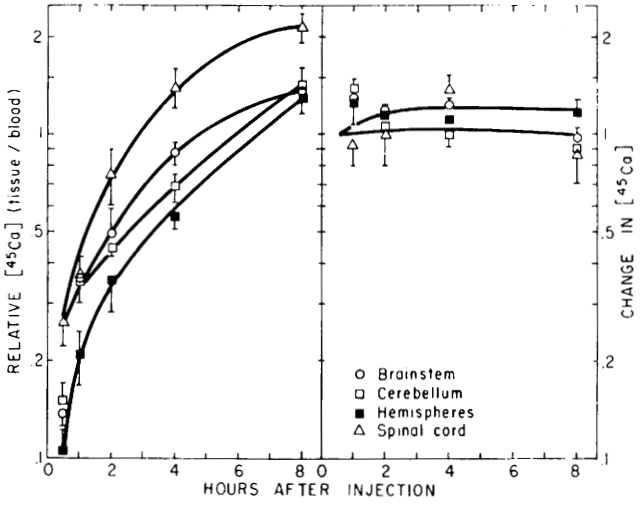

FIG. 5. Changes in the concentration of ^{45}Ca in brain tissues of rats after administration of $^{45}CaCl_2$: symbols = mean ± SEM ($n = 5$).

during the isolation procedure, an issue that appears insoluble with current techniques. Although Stahl and Swanson (24) showed that homogenization of brain slices in the presence of EDTA decreased the total ^{45}Ca bound to the particulate fractions, they found no alteration in the overall profile of the distribution. Thus, since no other methods are available to measure subcellular distribution, no reference is available to compare different approaches (*e.g.*, does isolation in the presence of EDTA more nearly reflect conditions of binding *in situ*?).

The change in specific activity of ^{45}Ca in the brain mitochondrial fraction following the administration of certain pharmacological agents is provocative. The ability of pentylenetetrazol to increase the specific activity of ^{45}Ca in the mitochondrial fraction, in the absence of a concomitant alteration in either Fraction C or the whole brain homogenate, might indicate an increased ^{45}Ca turnover in this organelle. Alternatively, these data might be explained by an increased calcium content of this fraction, accumulated at the expense of calcium associated with other portions of the subcellular fractions. However, significant changes in the relative distribution of ^{45}Ca within the whole homogenate were not detected (calculations not presented) and the content of ^{45}Ca in the homogenate did not increase. In either case, these data clearly suggest that intracellular calcium movements are altered by the administration of this agent, possibly as a result of increased neural activity.

In contrast, the effect of phenobarbital administration may be attributable to a decreased permeability of the blood–brain barrier, as has been reported to occur in response to many depressant agents (25, 26). This is supported by: (i) a decreased concentration of ^{45}Ca in both the mitochondrial and synaptosomal fractions (with the ratio E/C remaining unchanged); (ii) a decreased concentration of ^{45}Ca in the whole homogenate; and (iii) no change in the concentration of ^{45}Ca in liver mitochondria.

The marked behavioral effects attributable to reserpine administration were associated with an increased relative concentration of ^{45}Ca in brain mitochondria. It is possible that the enhanced turnover of ^{45}Ca is due to a release of catecholamines that subsequently alters membrane permeability to calcium. This would lead to an increased availability of intracellular calcium that could be accumulated by the mitochondria. Such a mechanism has been proposed to account for calcium accumulation by mitochondria in brain slices (24, 27). Recently it has been reported that reserpine did not effect the calcium content in various anatomical segments of rabbit brain, (28) supporting the proposal that reserpine induces an increased Ca^{2+} turnover, rather than accumulation.

Summary. The distribution of ^{45}Ca among various tissues of the rat was followed for 8 hr after intravenous administration of the isotope. Three major patterns of ^{45}Ca turnover were demonstrable, represented by: (i) brain; (ii) bone; and (iii) certain soft tissues: kidney, adrenal, and muscle. These differences may be explained in terms of different exchange (*e.g.*, for bone) and physiological barriers (*e.g.*, for brain). Studies on the subcellular distribution of ^{45}Ca in brain, performed 2 hr after intravenous administration of $^{45}CaCl_2$, showed that mitochondria accumulated 25% of the total ^{45}Ca; moreover, the concentration of the ^{45}Ca accumulated by the mitochondria was decreased by prior administration of phenobarbital and of reserpine, but increased by pentylenetetrazol.

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