Biochemical Pharmacology, Vol. 18, pp. 339–346. Pergamon Press. 1969. Printed in Great Britain

INHIBITION OF PHOSPHOLIPID-FACILITATED CALCIUM TRANSPORT BY CENTRAL NERVOUS SYSTEM-ACTING DRUGS*

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(*Received* 5 *April* 1968; *accepted* 5 *July* 1968)

Abstract—Narcotic analgesics effectively inhibit the binding of Ca^{2+} to phospholipids *in vitro*. Morphine and nalorphine also inhibit the Ca^{2+} transported by phospholipids obtained from guinea pig brain subcellular fractions. The inhibition of Ca^{2+} transported was related to ionization of the drug and was compared with the analgesic potency of the narcotic drugs. About 69 per cent of morphine-^{14}C was bound to phosphatidic acid and this binding was inhibited by divalent ions (3–29 per cent). Various CNS-acting drugs were also effective in inhibiting the transport of Ca^{2+} ions by phosphatidic acid. It is postulated that an alteration in the binding of ions to phospholipids within the neuronal membrane may be involved in the pharmacological action of CNS-acting drugs.

RECENTLY this laboratory[1] reported that morphine stimulated the incorporation of $^{32}P_i$ into various phospholipids of the cerebral cortex and inhibited the incorporation of $^{32}P_i$ into phosphatidylcholine. After the development of tolerance to morphine, an adaptation to the phospholipid effect occurred.[2] The significance of these observations certainly depends upon the physiological function of the phospholipids in neural tissue. An important hypothesis[3,4] in this regard concerns the fact that phospholipids may act as carriers for the transport of ions across cell membranes. It is also important to note the data on phospholipids-ionic complexes[5–8] and the need for phospholipid integrity of axonal cell surfaces to maintain normal membrane resistance, excitability and potentials.[9–11]

Kakunaga *et al.*[12] have demonstrated that Ca^{2+} markedly antagonizes the analgesic effect of morphine and that chelating agents (EDTA-2 Na; CDTA-2 Na) enhance the analgesia provided by morphine. The calcium salts of chelating agents as well as other cations had no effect on the analgesic response to morphine. It, therefore, seemed quite possible that the narcotic analgesics as well as other CNS-acting organic bases might compete with cations for anionic sites on the phospholipid molecules in cellular membranes and thus alter ion conductance within the neuron.

That lipids extracted from nerves and muscle could promote the transport of Ca^{2+} from an aqueous Ringers phase into a lipid solvent phase was first demonstrated by Wooley.[13–14] Therefore, the main purpose of this study was to determine whether the narcotic analgesics as well as certain CNS-acting drugs would alter phospholipid-facilitated Ca^{2+} transport.

* A preliminary report of these experiments appears in the minutes of *The Annual Meeting of the Committee on Problems of Drug Dependence*, February 19–22, 1968, Indianapolis, Ind.

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MATERIALS AND METHODS

Ca^{2+} transport system. The methods used were similar to those described by Feinstein,[16] where 2-ml aliquots of $CHCl_3$:methanol (2:1) containing 0·8–2·6 μmole/ml of phospholipid were shaken at 300 oscillations/min for 10 min in 5-ml glass-stoppered tubes with 1·0 ml of Ringers solution containing 116 mM NaCl, 2·5 mM KCl, 1·0 mM $CaCl_2$ and 1·0–1·2 μc/ml of ^{45}Ca (or 0·2 μc/μmole/ml of morphine-^{14}C) in 2 mM Tris-HCl (pH 7·4). Narcotic analgesics were made up in Ringers solution to provide a final concentration of 0·6 mM. Centrifugation (2500 rpm × 10 min) provided an upper aqueous phase (1·67 ml) and a lower $CHCl_3$ phase (1·33 ml). Duplicate aliquots from each phase were evaporated on planchets and the radioactivity was determined in a Nuclear-Chicago (model 4338) gas flow counter.

Assays and materials. Phospholipid-phosphorus was determined by the method of Bartlett[17] on each phospholipid solution. The phosphorus values were multiplied by 25 to provide the total quantity of phospholipid present. The micromoles of phospholipid were calculated, assuming an average molecular weight of 750.

N-^{14}C-methyl-labeled morphine was prepared as described by Andersen and Woods.[18] The specific activity of the *N*-^{14}C-methyl-labeled morphine was 1·14 mc/m-mole. The labeled drug was diluted with unlabeled morphine to provide a specific activity of 0·23 mc/m-mole. All experiments were performed with the diluted drug.

The phospholipids were purchased from the Pierce Chemical Company, Rockford, Ill., except cardiolipin (General Biochemicals, Chagrin Falls, Ohio), and were subjected to silicic acid-impreganted paper chromatography. The chromatograms were developed with either diisobutyl ketone–acetic acid–water (40:25:5, v/v) or phenol–conc. NH_3 (99:1). The phospholipids were detached with Rhodamine-6G (12 μg/ml). Only phosphatidylcholine and sphingomyelin were considered pure. Phosphatidic acid contained a small quantity (about 1%) of lysophosphatidic acid. The other phosphatides consisted primarily of the specified phospholipid, but contained various quantities of related phospholipids.

$^{45}CaCl_2$ was purchased from the New England Nuclear Corp., Boston, Mass. Ten mc $^{45}CaCl_2$ in 1 N HCl with a specific activity of 22·1 mc/mg of Ca^{2+} was evaporated to dryness *in vacuo* and the residue was dissolved in 10 ml Ringers solution, pH 7·4 (1 mc/ml). Suitable aliquots were taken at the time of each experiment. All drugs were used as their commercially available salts.

Subcellular fractionation. The preparation of the guinea pig brain subcellular fractions and subsequent extraction of the phospholipids from these fractions were performed as described by Mulé *et al.*[19]

RESULTS

Effect with various phospholipids. Table 1 summarizes the data obtained on Ca^{2+} transport with various phospholipids. In the absence of the phosphatides, calcium was not transported into $CHCl_3$. Morphine inhibited the binding of Ca^{2+} to phospholipids from 0·2 to 42 per cent. The most effective inhibition was observed with phosphatidylcholine, followed by phosphatidylethanolamine and phosphatidic acid. The inhibition obtained with the other phosphatides was about 7 per cent or less and apparently was not significant. The inhibitory effect on phosphatidic acid-facilitated Ca^{2+} transport was 3 per cent when the final concentration of morphine was 0·06 mM.

Morphine not only inhibited the phospholipid-facilitated Ca^{2+} transport into $CHCl_3$, but also induced the return to the aqueous phase of Ca^{2+} previously transported. The release of Ca^{2+} from phosphatidic acid in the $CHCl_3$ phase was accomplished by adding either 1·0 mM morphine or 1 mM nalorphine to the methanol:H_2O phase and shaking for another 10 min. Morphine induced 31·5 per cent and nalorphine induced 50·7 per cent of the Ca^{2+} in $CHCl_3$ to return to the aqueous phase. Morphine (1 mM) plus nalorphine (0·5 mM) induced 50·3 per cent of the Ca^{2+} in $CHCl_3$ to return to the aqueous phase.

TABLE 1. MORPHINE INHIBITION OF PHOSPHOLIPID-FACILITATED Ca^{2+} TRANSPORT USING VARIOUS PHOSPHOLIPIDS*

Phospholipid	Bound Ca^{2+} in $CHCl_3$ (mμmoles/mg phospholipid)		
	Control	0·6 mM Morphine	Inhibition (%)
None	0		
Phosphatidylcholine	5·1 ± 0·5	3·0 ± 0·7	42·0
Phosphatidic acid	606 ± 10	520 ± 14	14·2
Sphingomyelin	1·7 ± 0·2	1·6 ± 0·4	5·9
Phosphatidylethanolamine (Folch fr. V)	138 ± 18	113 ± 12	18·1
Phosphatidylserine (Folch fr. III)	818 ± 8	763 ± 11	6·7
Cardiolipin	913 ± 17	892 ± 14	2·3
Monophosphoinositide	496 ± 11	487 ± 12	1·8
Diphosphoinositide	605 ± 10	604 ± 6	0·2
Triphosphoinositide	782 ± 9	745 ± 5	4·7

* Each value represents the mean ± S.E. of 6–12 determinations.

Effect with subcellular phospholipids. The inhibitory effect of morphine and nalorphine on calcium transported by phospholipids extracted from guinea pig brain subcellular fractions appears in Table 2. Morphine had the greatest inhibitory effect

TABLE 2. MORPHINE AND NALORPHINE INHIBITION OF PHOSPHOLIPID-FACILITATED Ca^{2+} TRANSPORT USING PHOSPHOLIPIDS EXTRACTED FROM GUINEA PIG BRAIN SUBCELLULAR FRACTIONS*

Subcellular fraction phospholipids	Bound Ca^{2+} in $CHCl_3$ (mμmoles/mg phospholipid)					
	Control	0·6 mM Morphine	Inhibition (%)	Control	0·6 mM Nalorphine	Change (%)
Homogenate	1·9 ± 0·3	1·6 ± 0·2	− 15·7	2·6 ± 0·2	1·8 ± 0·1	− 30·7
Crude nuclear	1·6 ± 0·1	1·4 ± 0·2	− 12·5	1·6 ± 0·1	1·3 ± 0·2	− 18·7
Crude mitochondrial	2·2 ± 0·1	2·0 ± 0·1	− 9·0	1·8 ± 0·1	1·8 ± 0·2	0
Microsomal	3·6 ± 0·2	2·6 ± 0·2	− 27·7	1·1 ± 0·2	1·0 ± 0·1	− 9·0
Soluble supernatant	15·3 ± 0·3	12·9 ± 0·4	− 15·7	3·7 ± 0·3	3·7 ± 0·2	0

* Each value is the mean ± S.E. of 3–8 determinations.

with the microsomal phospholipids (27·7) and the smallest percentage inhibition, which lacks significance (9·0), was obtained with the phospholipids from the crude mitochondrial fraction. The morphine effects observed with the phospholipids from

the remaining fractions were quite similar (12·5–15·7 per cent inhibition). Nalorphine had no effect on the transport of Ca^{2+} by phospholipids from the crude mitochondrial or soluble supernatant fractions. However, nalorphine was quite effective in inhibiting Ca^{2+} transport by the phospholipids extracted from the brain homogenate. There did not appear to be any significance associated with the effect on the crude nuclear and microsomal fractions. The percentage of Ca^{2+} bound to the phosphatides or transported into the $CHCl_3$ phase was less than 2 per cent for the phospholipids extracted from the subcellular fractions. This may reflect the high levels of zwitterionic phospholipids (phosphatidylcholine, phosphatidylethanolamine, ethanolamine plasmalogen, sphingomyelin) present in the cell fractions.[20] In effect, this reduces the total net negative charge on the phospholipid molecules and thus prevents an appreciable binding of calcium to the phospholipids for transport.

The relationship between the transport of calcium by phosphatidic acid and pH is shown in Fig. 1(a). As the pH increases from 6·4 to 9·0, the percentage of calcium transported by phosphatidic acid (pK_a 4·1, phosphate group) increases from 57 to 94

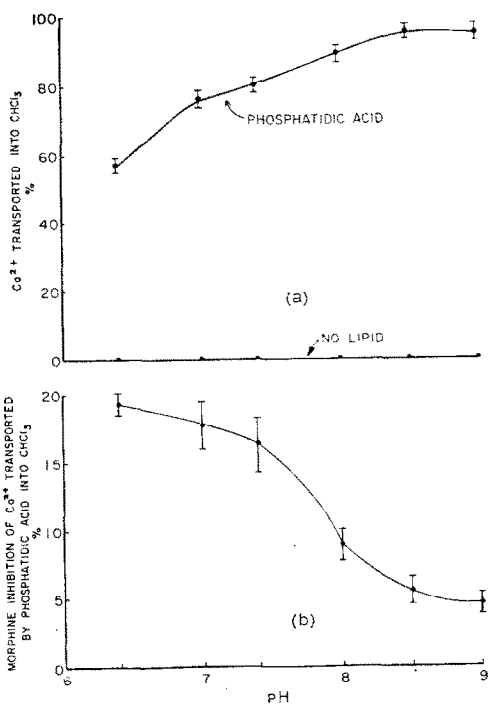

FIG. 1(a) The effect of pH on Ca^{2+} transported by phosphatidic acid (1·1–1·4 μmole/ml) into the $CHCl_3$ phase and the absence of transport without lipid. Each value is the mean ± S.D. of 6–12 determinations. (b) The effect of pH on morphine (0·6 mM) inhibition of Ca^{2+} transported by phosphatidic acid (1·1–1·4 μmole/ml) into $CHCl_3$. Each value is the mean ± S.D. of 6–12 determinations.

per cent. Over the same pH range, the percentage of phosphatidic acid ionized increases from 99·500 to 99·999. In Fig. 1(b) the relationship of morphine to pH and the inhibition of Ca^{2+} transported is given. An increase in pH from 6·4 to 9·0 causes a decrease in the inhibitory effect of morphine from 19·5 to 4·5 per cent. This

agrees quite well with the decrease in the percentage of morphine ionized (pK_a 8·05, nitrogen), which at pH 6·4 is 98 per cent, at pH 7·4 is 80 per cent and at pH 9·0 is 13 per cent.

Comparisons of analgesia with inhibition of Ca^{2+} transport. Table 3 shows the comparison between the inhibition of calcium transported by phosphatidic acid and

TABLE 3. COMPARISON OF ANALGESIC POTENCY WITH THE INHIBITION OF PHOSPHATIDIC ACID-FACILITATED Ca^{2+} TRANSPORT BY NARCOTIC DRUGS

Drugs	Relative inhibition of Ca^{2+} transported*	Relative analgesic potency in man†	Relative analgesic potency in mice‡
Meperidine	0·77	0·1–0·2	0·2
Normorphine	0·88	0·25	0·05
Morphine	1·0	1·0	1·0
Codeine	1·36	0·07–0·1	0·15
Nalorphine	1·54	1·0	0·81§
Methadone	1·66	1·0	0·80
Heroin	1·68	2–3	2·3
Naloxone	1·70	<0–5	<0·015§
Oxymorphone	1·94	10	12·3
Levorphan	2·02	5	4·2
Dextrorphan	2·37		0·012§
Cyclazocine	2·64	40	4·4

* The final concentration of each narcotic analgesic was 0·6 mM. All data were calculated relative to the inhibitory effect of 0·6 mM morphine.

† The potency data in man were obtained from W. R. Martin, in *Physiological Pharmacology* (Eds. W. S. Root and F. G. Hoffmann), vol. 1, p. 275, Academic Press, New York (1963); for nalorphine, from A. S. Keats and J. Telford, *J. Pharmac. exp. Ther.* **117**, 190 (1956); for cyclazocine, from L. Lasagna, T. J. DeKornfeld and J. W. Pearson, *J. Pharmac. exp. Ther.* **144**, 12 (1964); for oxymorphone, from B. N. Eddy and L. E. Lee, *J. Pharmac. exp. Ther.* **125**, 116 (1959); for naloxone, from L. Lasagna, *Proc. R. Soc. Med.* **58**, 978 (1965); for normorphine, from T. J. DeKornfeld and L. Lasagna, *J. Pharmac. exp. Ther.* **124**, 260 (1958).

‡ The potency values were calculated relative to morphine from the analgesic activity ED_{50} data (S.Q. in mice, mg/kg) as reported by L. B. Mellett and L. A. Woods, in *Progress in Drug Research* (Ed. E. Jucker), vol. 5, p. 157, Birkhäuser Verlag, Basel and Stuttgart (1963). The data on phenylquinone-induced writhing were obtained from J. Pearl and L. S. Harris, *J. Pharmac. exp. Ther.* **154**, 319 (1966).

§ Data obtained by inhibition of phenylquinone-induced writhing and calculated relative to morphine by the same analgesic test. The data on dextrorphan represent the unpublished results kindly provided by Dr. H. Blumberg of Endo Laboratories, Inc., N.Y.

the analgesic potency of the drugs relative to morphine in man and mice. A good correlation existed for some of the narcotic drugs, i.e. meperidine and normorphine were less effective than morphine in inhibiting Ca^{2+} transport and are weaker analgesics than morphine. Nalorphine, methadone, heroin, levorphan, oxymorphone and cyclazocine are equal to or more potent analgesics than morphine and were greater inhibitors of Ca^{2+} transport relative to morphine. However, codeine, a weaker narcotic analgesic, was a more potent inhibitor of Ca^{2+} transport than morphine. This might be explained by the higher percentage ionization of codeine (pK_a 8·2) in comparison to morphine at pH 7·4, Naloxone, which at best is a very weak analgesic and is currently considered primarily as a potent narcotic antagonist, and dextrorphan, the dextro isomer of levorphan, which apparently does not possess analgesic properties, [21]were

both effective inhibitors of Ca^{2+} transport. The results obtained with naloxone in this system may be due to a higher pK_a value for naloxone in comparison to morphine; thus a greater percentage of the drug could be in the ionized form at pH 7·4, The observation with dextrorphan was expected, since the chemical and physical properties of dextrorphan are identical to those of the narcotic analgesic, levorphan. The inhibition of Ca^{2+} transport, however, did not strictly adhere to percentage ionization of the narcotic drugs, since heroin and nalorphine had pK_a values of 7·8 in comparison to morphine (8·05), but were better inhibitors of Ca^{2+} transport than morphine. It must also be noted that non-analgesics, such as those listed in Table 5, were effective inhibitors of phosphatidic acid-facilitated Ca^{2+} transport.

Binding of morphine-^{14}C to phosphatidic acid. A study of the binding of morphine-^{14}C to phosphatidic acid and the effect of various divalent ions on this binding appear in Table 4. In the absence of ions, about 69 per cent of the morphine was bound to

TABLE 4. EFFECT OF 1MM DIVALENT IONS ON THE BINDING OF 1 MM MORPHINE-^{14}C (INITIAL CONCENTRATION) TO PHOSPHATIDIC ACID (0·8–1·3 μmole/ml)*

Ion	Morphine-^{14}C (μmoles)		
	Ringers phase	$CHCl_3$ phase	Inhibition (%)
None	0·302 ± 0·03	0·667 ± 0·03	
Zn^{2+}	0·642 ± 0·05	0·471 ± 0·05	29·4
Mg^{2+}	0·379 ± 0·02	0·502 ± 0·04	24·7
Fe^{2+}	0·447 ± 0·04	0·516 ± 0·03	22·6
Ca^{2+}	0·413 ± 0·05	0·582 ± 0·02	20·5
Ba^{2+}	0·330 ± 0·03	0·531 ± 0·04	20·4
Sr^{2+}	0·343 ± 0·07	0·578 ± 0·05	13·3
Ringers, pH 7·4 minus Ca^{2+}	0·356 ± 0·04	0·648 + 0·06	2·8

* Each divalent ion was substituted for $CaCl_2$ in the standard Ringers solution, pH 7·4. The morphine values in $CHCl_3$ were corrected for the passage of the drug into the organic phase in the absence of phospholipid. Each value is the mean ± S.E. of 4–8 determinations.

phosphatidic acid in the $CHCl_3$ phase. The binding or transport of morphine into $CHCl_3$ by phosphatidic acid was inhibited by divalent ions in the following order: $Zn^{2+} > Mg^{2+} > Fe^{2+} > Ca^{2+} > Ba^{2+} > Sr^{2+} >$ Ringers minus Ca^{2+}. However the percentage difference between the inhibitory effect of Zn^{2+} and Ca^{2+} was only 9 per cent. Strontium was much less effective (13·3 per cent) and the Na^+ and K^+ ions of the Ringers solution had practically no effect on the binding of morphine to phosphatidic acid.

Effect of CNS drugs on Ca^{2+} transport. Table 5 provides the data on various amine-containing CNS drugs which affect the transport of Ca^{2+} by phosphatidic acid. Chlorpromazine, imipramine, meprobamate, chlordiazepoxide and amphetamine inhibited the transport of Ca^{2+} by phosphatidic acid from 2·4 to 42·2 per cent. The barbiturates, barbital and pentobarbital, increased the transport of Ca^{2+}. This was as expected, since barbiturate acids ionize as anions and may thus provide additional sites for transport.

Norepinephrine and epinephrine also inhibited the transport of Ca^{2+} by phosphatidic acid (about 13 per cent).

TABLE 5. EFFECT OF VARIOUS CNS DRUGS ON PHOSPHATIDIC ACID-FACILITATED Ca^{2+} TRANSPORT*

Drug	Bound Ca^{2+} in $CHCl_3$ (mμmoles/mg/phospholipid)		
	Control	0·6 mM Drug	Change (%)
Chlorpromazine (Thorazine)	390 ± 14	313 ± 12	− 19·7
Imipramine (Tofranil)	390 ± 14	286 ± 17	− 26·7
Meprobamate (Equanil, Miltown)	531 ± 12	518 ± 16	− 2·4
Chlordiazepoxide (Librium)	531 ± 12	440 ± 20	− 17·1
Amphetamine (Benzedrine)	574 ± 20	332 ± 28	− 42·2
Barbital (Veronal)	560 ± 10	590 ± 15	+ 5·3
Pentobarbital (Nembutal)	560 ± 10	603 ± 18	+ 7·7

* Each value is the mean ± S.E. of 3–9 determinations.

DISCUSSION

This report describes the transport of Ca^{2+} from an aqueous phase into a lipid solvent phase by phospholipids. Narcotic analgesics and other CNS drugs were capable of inhibiting the transport of Ca^{2+} by competing with the cation for available transport sites on the phospholipid molecule. Goldman[4] postulated that the phosphate groups of the phospholipids might act as ion-exchange "gates" for the control of ion flow through cell membranes. The fact that the polar heads of the phospholipids bind monovalent and divalent ions has been shown by several investigators.[5–8,22] The affinity of the phospholipid anionic sites for calcium, however, was some 5000- to 10,000-fold greater than for sodium or potassium.[22]

A postulated series of reactions for the transport of Ca^{2+} and its inhibition by narcotic analgesics (NA) and certain CNS drugs is presented with phosphatidic acid (PA) as the model phospholipid and NA as the drug:

1) $PA \longrightarrow HPA^- + H^+$

2) $HPA^= \longrightarrow PA^= + H^+$

3) $PA^= + Ca^{++} \longrightarrow CaPA$

in the presence of a narcotic analgesic (NA) and Ca^{2+}

4) $PA^= + Ca^{2+} + NA^+ \longrightarrow CaPA + NAPA.$

At a physiological level, Frankenhaeuser and Hodgkin[23] postulated that depolarization removed calcium (i.e. physical effect of the electrical field on the distribution of Ca^{2+} in the membrane) from sites or carriers on the nerve membrane, allowing the membrane to become selectively permeable to sodium with a resultant increase in the inward sodium current. These investigators[23] summarized the actions of calcium on axons by stating that a 5-fold reduction of Ca^{2+} on the system controlling Na and K permeability was similar to a depolarization of 10–15 mV.

Bearing in mind that the experimental system utilized in this study serves primarily as a model for gaining insight into the pharmacological action of CNS-acting drugs, the results suggest the following: 1) narcotic analgesics and certain amine-containing drugs may displace Ca^{2+} from phospholipids within neuronal cell membranes, which subsequently affects ion conductance in the neuron; 2) the binding of CNS-acting drugs to phospholipids may alter metabolism and function of the phospholipids in cell membranes, thus affecting pharmacological actions of the drug.

In addition to the data in this study, several other reports seem to implicate Ca^{2+} and phospholipids in the pharmacological action of narcotic analgesics. These are:

1) morphine-, meperidine- and ohton-induced analgesia is directly antagonized by Ca^{2+} and is enhanced by chelating agents;[12] 2) morphine and nalorphine alter phospholipid metabolism in the cerebral cortex;[1,2] 3) after the administration of morphine, an increased urinary excretion of calcium occurs;[24] 4) the inhibitory effect of morphine on potassium-stimulated O_2 uptake of cerebral cortex slices can apparently only be demonstrated in a Ca^{2+}-free media;[25] and 5) Ca^{2+} antagonizes noncompetitively the inhibition by morphine of gut contractions produced by coaxial stimulation.[26]

Certain experimental data, however, are difficult to reconcile readily with an ion alteration hypothesis for the action of narcotic drugs. These are: 1) codeine, naloxone and dextrorphan are more effective inhibitors of Ca^{2+} transport than morphine, but are much weaker analgesics; 2) the inhibitory effect of narcotic analgesics on Ca^{2+} transport may be demonstrated with nonnarcotic drugs; 3) morphine had no effect on impulse transmission in isolated peripheral nerve preparations *in situ*, but meperidine and methadone decreased the size of the action potential 20–40 per cent.[27]

In conclusion then, it may be stated that an interaction of CNS-acting drugs, phospholipids and divalent ions does occur. However, the significance of this effect is difficult to assess, since an inhibition of the transport of Ca^{2+} appears to be a common property of many centrally acting drugs.

Acknowledgement—The author expresses his appreciation to Robert Janetzko for excellent technical assistance.

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