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CALCIUM DEPLETION OF SYNAPTOSOMES AFTER MORPHINE TREATMENT

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1 It was confirmed that morphine administration *in vivo* produced a selective decrease in the calcium content of synaptosomal fractions prepared from rat cerebral cortices. This effect was prevented by naloxone.

2 After morphine or naloxone, there were no changes in the sodium, potassium and magnesium contents of any of the seven subcellular fractions tested.

3 Since the selective calcium loss was found to occur from fractions similar to those reported to exhibit opiate receptor binding, it is concluded that calcium may play a role in the action of narcotic agonist drugs.

Introduction

Much evidence points to the involvement of several contributory factors in the phenomena of tolerance to and dependence on opiate drugs. Previous studies on the role of calcium in the action of opiates (Elliott, Kokka & Way, 1963; Kakunaga, Kaneto & Hano, 1966) have been confirmed and extended in recent work from this laboratory. Calcium levels in brain decrease after opiate agonist treatment; this effect is stereospecific and can be blocked by naloxone which in contrast, has no effect on reserpine-induced depletion of brain calcium (Ross, Medina & Cardenas, 1974; Cardenas & Ross, 1975; Ross, 1975a). Tolerance develops to calcium depletion by morphine and can be prevented by cycloheximide (Ross & Lynn, 1975; Ross, 1975b). It seemed important, therefore, to define the subcellular locus of the opiate-induced calcium depletion and at the same time to study the effect of morphine on other cations in subcellular fractions from brain.

Methods

Administration of drugs

All drugs were administered by the intraperitoneal route with control animals receiving equivalent volumes of 0.9% w/v NaCl solution (saline). Morphine was administered as the sulphate salt (25 mg/kg) and animals killed 30 min later. Naloxone hydrochloride (1 mg/kg) was administered either alone (animals killed after 45 min) or 15 min before administration of morphine. No significant differences were observed for any of the cations after naloxone treatment for 15 or 45 min, compared to saline controls.

Preparation of subcellular fractions

Fractions were prepared from brains of male Sprague-Dawley rats (175–200 g) according to the procedure outlined by Gurd, Jones, Mahler & Moore (1974), with the following modifications. Saline control or drug-treated animals were killed by decapitation at the times given in the tables. The whole brains were immediately removed, cortices dissected free and immersed in ice cold saline to remove traces of blood. A 10% homogenate was prepared in ice cold 0.32 M sucrose with a Teflon-glass homogenizer, then pelleted at 1000 *g* for 10 minutes. The resulting pellet was washed once with sucrose and pelleted a second time. The resulting pellet constituted P_1 and was saved for analysis of protein and ions. The supernatants from both washes were combined and centrifuged at 17,500 *g* for 20 min to produce the 'crude' mitochondrial pellet containing synaptosomes (P_2). The crude P_2 pellet was washed twice, resuspending each time in 0.32 M sucrose and the combined supernatants centrifuged at 100,000 *g* for 60 min to produce the microsomal pellet (P_3) and supernatant (S).

The washed mitochondrial pellet (P_2) was resuspended in 0.32 M sucrose and floated over a two gradient suspension consisting of 7.5% Ficoll in 0.32 M sucrose upper phase and 13% Ficoll in 0.32 M sucrose lower phase. This suspension was then centrifuged at 63,500 *g* for 90 minutes. The material

sedimenting at each of the three interphases was collected as myelin (Myl, 0.32 M sucrose – 7.5% Ficoll), synaptosome (Syn, 7.5%–13% Ficoll) and mitochondria (Mit, 13% Ficoll). Each of the three fractions was carefully removed with a Pasteur pipette, diluted with equal volumes of ice cold 0.32 M sucrose and pelleted at 100,000 *g* for 30 minutes. The resulting myelin, synaptosomal and mitochondrial pellets were resuspended in glass distilled deionized water and thoroughly mixed by sucking up and down in a syringe equipped with a No. 14 gauge needle. After standing at 0–4°C for 15 min, the samples were pelleted at 100,000 *g* for 30 minutes. This process was repeated a second time after which aliquots of the resulting particulates were removed for ion and protein analysis. The second wash was routinely included after a washing curve determined that no further decrease in pellet content occurred for all four ions after the second wash.

Preparation of tissue samples for cation analysis

The sodium, potassium, magnesium and calcium contents were estimated for nuclear (P_1), crude microsomal (P_3), soluble (S_3), myelin (Myl), synaptosomal particulate (Syn) and mitochondrial (Mit) and mitochondrial lysate (Sm). Suitable portions of primary fractions were assayed for ion content by methods previously outlined by Hanig, Tachiki & Aprison (1972) and Cardenas & Ross (1975). Samples were removed and placed in pre-weighed disposable test tubes (Corning disPo, 16 × 100 mm culture tubes) together with 200 µl of concentrated HNO_3 (analytical grade) and the entire contents evaporated to a dry white ash over a hot plate. Incomplete ashing, evidenced by dark brown flecks of tissue, was treated with a second 200 µl volume of acid and the ashing procedure repeated. The residue was allowed to cool to room temperature then resuspended in a solution of 0.5 ml of 0.1 N HCl + 4.5 ml of 1% lanthanum oxide (in 0.1 N HCl). The solutions were then read against standard curves for the appropriate ion.

Analysis of cations

The concentrations of sodium, potassium, magnesium and calcium were determined with the aid of a Perkin-Elmer Atomic Absorption Unit Model 403. The following wave lengths and slit settings were recorded for sodium (295 nm) (4), potassium (383 nm) (4), magnesium (284 nm) (4) and calcium (211 nm) (4). The remainder of the instrument was preset in accordance with the procedure outlined in the Perkin-Elmer Analytical Method Manual, 1973. In all determinations of calcium, proper amounts of lanthanum were included to reduce phosphate interference (Hanig & Aprison, 1967). Appropriate blanks for reagents, sucrose and Ficoll-sucrose solutions were carried throughout the procedure and all samples were multiple blank corrected before calculating final concentrations.

Analysis of protein

Protein content was determined for each particulate and soluble fraction by an automated version of the method of Lowry, Rosebrough, Farr & Randall (1951).

Reagents

Drugs and chemicals used in this study and their sources are as follows: morphine sulphate (Eli Lilly Laboratories, Indianapolis, Indiana), naloxone hydrochloride (Endo Laboratories, Garden City, New York), sucrose (Grade 1) and Ficoll (Sigma Chemical Company, St. Louis, Mo.). Lanthanum and calcium standards were obtained from Research Chemical Corporation (Sun Valley, Calif.) and Fischer Scientific Company (Fairlawn, New Jersey). Sodium and potassium were obtained as their chloride salts from Mallinkrodt Chemical Works (St. Louis, Mo.). Unless otherwise specified, all reagents used were analytical grade.

Statistics

Group and paired comparisons were made with Student's *t*-test as outlined by Snedecor & Cochran (1969).

Results

Effects of morphine and naloxone on subcellular distribution of sodium and potassium

The effects of morphine sulphate (25 mg/kg) and naloxone hydrochloride (1 mg/kg) on sodium and potassium contents in subcellular fractions of brain tissue are outlined in Tables 1 and 2. Sodium and potassium were found predominantly in the soluble fractions (S + Sm) with 73.8% of the total content as sodium and 79.3% of the total as potassium. In the particulate fractions (P_1, P_3, Myl, Syn, Mit) sodium represented 45.9% of the total monovalent ion content with potassium representing 54.1% of the total.

Morphine treatment alone or in combination produced no significant changes in sodium or potassium content in any subcellular fraction. Treatment with naloxone alone also failed to produce any significant alteration of either ion.

Effects of opiate treatment on subcellular distribution of magnesium and calcium

In contrast to subcellular localization of monovalent ions, calcium and magnesium were found predominantly in the particulate fractions which contain 76.4% of the magnesium and 73.8% of the calcium. Magnesium was the predominant cation representing 73% of the total particulate fraction.

Morphine, alone or in combination with naloxone, had no significant effect on subcellular distribution of magnesium (Table 3). In contrast, acute morphine treatment revealed a highly significant calcium depletion (73%) in the synaptosomal particulate and to a lesser extent in the myelin fraction (18%) (Table 4). No significant effects were observed in any of the other subcellular fractions. Naloxone alone did not alter the soluble particulate ratios of calcium but when administered before morphine effectively prevented the depletion of calcium from synaptosomal particulates.

Table 1 Effect of opiate ligands on subcellular distribution of sodium from rat cortex

	Sodium (μmol/g) (mean ± s.e. mean)						
	P_1	P_3	*S*	*Myl*	*Syn*	*Mit*	*Sm*
Control	1.60 ±0.11	8.27 ±0.28	37.6 ± 0.79	0.163 ±0.011	1.70 ±0.01	2.65 ±0.13	2.82 ±0.24
Morphine 25 mg/kg (30 min)	1.34 ±0.10	7.51 ±0.31	39.99 ± 0.69	0.136 ±0.008	1.52 ±0.18	2.68 ±0.07	3.06 ±0.07
Naloxone 1 mg/kg (15, 45 min)	1.40 ±0.05	7.66 ±0.32	40.48 ± 0.69	0.178 ±0.015	1.36 ±0.07	2.98 ±0.09	2.78 ±0.20
Naloxone + morphine	1.41 ±0.01	7.82 ±0.80	40.02 ± 0.69	0.155 ±0.009	1.44 ±0.50	3.02 ±0.09	2.84 ±0.34

Ion values for pellets and soluble fractions are expressed as μmol per g wet wt. of pellet or equivalent wet wt. of tissue for 500 μl of soluble fractions. Values represent the means ± s.e. mean for $n = 8–10$ analyses. Each analysis was performed on a sample of brain tissue represented by two cortices.

Table 2 Effect of opiate ligands on subcellular distribution of potassium from rat cortex

	Potassium (μmol/g) (mean ± s.e. mean)						
	P_1	P_3	*S*	*Myl*	*Syn*	*Mit*	*Sm*
Control	4.47 ±0.63	11.02 ± 0.28	59.56 ± 4.60	0.251 ±0.013	0.30 ±0.01	0.89 ±0.04	5.23 ±0.53
Morphine 25 mg/kg (30 min)	4.43 ±0.10	10.06 ± 0.92	55.20 ± 2.06	0.230 ±0.015	0.26 ±0.00	0.87 ±0.04	4.94 ±0.31
Naloxone 1 mg/kg (15, 45 min)	4.43 ±0.15	10.14 ± 0.64	57.02 ± 0.88	0.234 ±0.017	0.29 ±0.03	1.01 ±0.04	4.84 ±0.30
Naloxone + morphine	4.66 0.12	9.83 ±0.53	54.74 ± 0.88	0.248 ±0.013	0.28 ±0.02	1.01 ±0.11	4.41 ±0.06

Ion values for pellets and soluble fractions are expressed as μmol per g wet wt. of pellet or equivalent wet wt. of tissue for 500 μl of soluble fractions. Values represent the means ± s.e. mean for $n = 8–10$ analyses. Each analysis was performed on a sample of brain tissue represented by two cortices.

Cation content in pellet and supernatant after morphine treatment

To determine if a redistribution of cations had occurred during the fractionation procedure, 1.0 ml of the 10% homogenate from control and drug-treated animals was centrifuged at 100,000 *g* for 30 min (Table 5). The results for Na^+, K^+ and Mg^{2+} agreed well with the contents for each of these elements in the sum of the particulate fractions and also for the sum of the soluble fractions. For calcium, the 100,000 *g* pellet content was lower than the sum of the individual particulate fractions. This discrepancy did not appear to be compensated for by a difference between the sum of the soluble fractions and the single supernatant.

Morphine treatment did not affect the Na^+, K^+ or Mg^{2+} content of the pellet but the calcium content was significantly reduced (34.5%). This net loss of calcium (0.39 μmol/g) agrees well with the net loss from the sum of the individual particulate fractions (0.31 μmol/g).

Table 3 Effect of opiate ligands on subcellular distribution of magnesium from rat cortex

	Magnesium (μmol/g) (mean ± s.e. mean)						
	P_1	P_3	*S*	*Myl*	*Syn*	*Mit*	*Sm*
Control	0.71 ±0.02	1.78 ±0.11	0.78 ±0.05	0.52 ±0.02	0.40 ±0.02	0.566 ±0.015	0.44 ±0.02
Morphine 25 mg/kg (30 min)	0.66 ±0.02	1.86 ±0.47	0.838 ±0.01	0.63 ±0.01	0.38 ±0.01	0.633 ±0.029	0.50 ±0.06
Naloxone 1 mg/kg (15, 45 min)	0.70 ±0.02	1.76 ±0.04	0.83 ±0.01	0.44 ±0.02	0.45 ±0.02	0.667 ±0.017	0.46 ±0.02
Naloxone + morphine	0.70 ±0.02	1.83 ±0.11	0.85 ±0.01	0.48 ±0.03	0.40 ±0.03	0.634 ±0.037	0.46 ±0.04

Ion values for pellets and soluble fractions are expressed as μmol per g wet wt. of pellet or equivalent wet wt. of tissue for 500 μl of soluble fractions. Values represent the means ± s.e. mean for $n = 8–10$ analyses. Each analysis was performed on a sample of brain tissue represented by two cortices.

Table 4 Effect of opiate ligands on subcellular distribution of calcium from rat cortex

	Calcium (μmol/g) (mean ± s.e. mean)						
	P_1	P_3	*S*	*Myl*	*Syn*	*Mit*	*Sm*
Control	0.29 ±0.01	0.46 ±0.02	0.13 ±0.09	0.05 ±0.01	0.32 ±0.01	0.27 ±0.01	0.24 ±0.03
Morphine 25 mg/kg (30 min)	0.27 ±0.03	0.45 ±0.02	0.13 ±0.01	0.04 ±0.01	0.09 ±0.01	0.26 ±0.01	0.21 ±0.02
Naloxone 1 mg/kg (15, 45 min)	0.28 ±0.02	0.39 ±0.02	0.15 ±0.01	0.05 ±0.01	0.31 ±0.01	0.30 ±0.01	0.22 ±0.05
Naloxone + morphine	0.32 ±0.01	0.42 ±0.03	0.14 ±0.01	0.05 ±0.02	0.34 ±0.02	0.31 ±0.01	0.21 ±0.02

Ion values for pellets and soluble fractions are expressed as μmol per g wet wt. of pellet or equivalent wet wt. of tissue for 500 μl of soluble fractions. Values represent the means ± s.e. mean for $n = 8–10$ analyses. Each analysis was performed on a sample of brain tissue represented by two cortices.

Discussion

The effects of morphine and naloxone, alone and in combination, on cation contents in subcellular fractions have been studied. The measurements of cation distribution and protein content in subcellular fractions of brain tissue closely parallel those values previously reported by Hanig, Tachiki & Aprison (1972) with one exception. The difference between Syn + Myl calcium content and the M_2 fraction reported by Hanig *et al.* (1972) may be attributed to the techniques employed to fractionate the tissue. As outlined by Whittaker (1969) and Barondes (1974) use of Ficoll-sucrose gradients preserves more of the morphology and functionality of nerve endings, protecting them from dehydration and increasing their sensitivity to osmotic shock. It may be expected then that cation contents of fractions prepared in this manner would reflect a more accurate picture of *in vivo* contents. The 100,000 *g* pellet was not prepared over a Ficoll-sucrose gradient but pelleted from a solution of 0.32 M sucrose.

Morphine treatment produced a depletion of calcium content principally in the synaptosomal fraction. The loss of calcium from individual particulate fractions, as well as the 100,000 *g* pellet, agree with our earlier reported loss of calcium (Cardenas & Ross, 1975). Naloxone effectively prevented this depletion while producing no effects on its own action.

The lack of effects of morphine or naloxone on sodium content in any of the subcellular fractions appear in disagreement with *in vitro* models of the opiate receptor recently proposed by Pert & Snyder (1974) and Simon, Hiller, Groth & Edelman (1975). Data derived from *in vitro* binding experiments suggest that sodium acts to reduce binding of opiate agonists and increase binding of opiate antagonists. If sodium ions do, in fact, regulate distinct forms of the opiate receptor, this would imply that administration of opiate agonists *in vivo* could alter the sodium content in such a manner as to shift the receptor conformation from one form to another. However, neither morphine alone nor in combination produced any significant changes in sodium levels.

Our data would suggest that the model suggested by Pert & Snyder (1974) and Simon *et al.* (1975) be viewed in a different perspective. Previous investigations (Stahl & Swanson, 1969; 1971; Swanson, Anderson & Stahl, 1974; Yoshida & Ichida, 1974) have adequately demonstrated that 100 mM Na^+ produces decreased ^{45}Ca uptake and binding in synaptosomes and a loss of ^{45}Ca bound to synaptic membranes. From this information, together with the data presented in the present paper, we would suggest that morphine action induces a Ca^{2+} dissociated membrane state (analogous to agonist binding in absence of sodium). Naloxone binding could occur to the resting state of the membrane (Ca^{2+}-associated state, uninfluenced by Na^+) (Cardenas & Ross, 1975) or in the calcium-depleted state (analogous to the presence of sodium) causing reversal (Ross, 1975a; 1976) of the calcium depletion.

The results presented in this paper confirm our earlier observation that morphine induces a loss of calcium from brain tissue, observed as a selective reduction in the calcium content of synaptosomal particulate fractions prepared from rat cerebral cortices. These findings support our working hypothesis that calcium may play a vital role in

Table 5 Cations in pellet and supernatant from homogenates of control and treated brains

	Cation content (μmol/g wet tissue)					
	Control		*Treated**		*% Change*	
Cation	*Pellet*	*Supernatant*	*Pellet*	*Supernatant*	*Pellet*	*Supernatant*
Sodium	14.6 ± 0.3	42.3 ± 0.5	13.2 ± 0.9	42.7 ± 0.4	− 1.3 9.3%†	+0.4
Potassium	27.3 ± 0.1	61.4 ± 0.1	26.6 ± 0.1	61.4 ± 0.1	− 0.67	—
Calcium	1.13 ± 0.05	0.42 ± 0.02	0.74 ± 0.01	0.42 ± 0.01	− 0.39 35.5%††	—
Magnesium	4.71 ± 0.23	1.12 ± 0.09	4.62 ± 0.22	1.10 ± 0.06	− 0.09	−0.02

* Treated animals received morphine (25 mg/kg; 30 min). Naloxone pretreatment or naloxone alone did not produce changes significantly different from controls.
† Not significantly different from controls; †† Significantly different $P < 0.01$.

mechanisms of action of morphine-like drugs. In this connection, it is interesting to note that the subcellular loss of calcium occurs with a distribution similar to opiate receptor binding, which was recently reported by Pert, Snowman & Snyder (1974).

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