Biochemical Pharmacology, Vol. 31, No. 16, pp. 2697–2698, 1982.
Printed in Great Britain.

0006-2952/82/162697–02 $03.00/0

Lack of morphine effect on potassium-stimulated calcium uptake by whole brain synaptosomes

(*Received* 23 *September* 1981; *accepted* 26 *February* 1982)

A number of studies have implicated calcium in the pharmacological actions of narcotic analgesics. Intracerebroventricular (i.c.v.) injection of calcium, for example, antagonizes, while ethyleneglycol-bis-[β-aminoethyl ether]-*N,N'*-tetraacetic acid (EGTA) potentiates, the analgesic actions of morphine [1]. Moreover, lanthanum, which inhibits calcium ion movements across cell membranes [2], produces an analgesia in both rats and mice which is reversed by naloxone [3]. Schmidt and Way [4] have shown that calcium enhances the hyperalgesic response following withdrawal of morphine from dependent mice while EGTA produces dose-dependent antinociceptive responses in both morphine-dependent and control mice. It was concluded [4] that morphine and EGTA may produce analgesia by depleting a small calcium pool in the nerve cell. This suggestion agrees with observations made by Cardenas and Ross [5] who showed that acute morphine administration decreases brain calcium levels. Studies examining the cellular localization of calcium depletion suggest that morphine has a rather select capacity to reduce calcium in nerve endings [6] and synaptic vesicles [7, 8].

Recent reports have also suggested that calcium flux at the nerve ending may be altered by morphine. Guerrero-Munoz *et al.* have shown that acute morphine administration, 10 mg/kg, s.c., resulted in a significant reduction in ATP-dependent $^{45}Ca^{2+}$ uptake into both intact [9] and lysed synaptosomes [10]. In addition, their work shows that ATP-dependent uptake of $^{45}Ca^{2+}$ is inhibited by *in vitro* morphine in concentrations ranging from approximately 0.1 to 1.0 μM [9, 10]. Chronic morphine administration, on the other hand, resulted in an enhanced ATP-stimulated uptake of $^{45}Ca^{2+}$. More recently, evidence has been published which indicates that morphine, 0.1 μM [11] and 9.0 μM [8], when added *in vitro* markedly inhibits $^{45}Ca^{2+}$ influx into synaptosomes after 60 and 30 mM KCl depolarization.

Previous work in our laboratories has shown that barbiturates [12, 13], chlorpromazine [14], and chlordiazepoxide [15] selectively block depolarization-induced $^{45}Ca^{2+}$ uptake into synaptosomes. However, during the course of these experiments, we also examined the effects of morphine on the uptake process and were unable to detect any significant alterations (unpublished observation). Since the results of our earlier work were not in agreement with the recent reports cited above [8, 11], we have undertaken a more complete analysis of the effects of acute and chronic morphine administration on $^{45}Ca^{2+}$ uptake by whole brain synaptosomes. Our results suggest that morphine does not significantly alter depolarization-induced $^{45}Ca^{2+}$ uptake.

Male Sprague–Dawley rats (250–350 g) were kept on a 12/12 hr light–dark cycle with food and water *ad lib.* before being randomly divided into control, acute morphine, and chronic morphine groups. Acutely treated animals received a single dose of morphine sulfate in normal saline (15 mg/kg, i.p.) and control animals were given an equal injection volume of normal saline. Animals were killed 30 min after injection. Chronically treated animals were anesthetized with diethyl ether, and one morphine pellet (75 mg of morphine base) was implanted subcutaneously [16]. One additional pellet was implanted 24 hr later. Animals were killed on the morning of the twelfth day following pellet implantation. Sham-operated control rats, which were implanted with pellets not containing morphine, were also killed at this time.

Whole brain synaptosomes were prepared as previosuly described [12]. The accumulation of $^{45}Ca^{2+}$ by synaptosomes was studied as described by Leslie *et al.* [14]. A 0.5-ml aliquot of the synaptosomal preparation from each treatment group, containing approximately 0.3 to 0.5 mg protein, was added to 0.5 ml of incubation medium (NaCl, 136 mM; KCl, 5 mM; $CaCl_2 \cdot 2H_2O$, 1.2 mM; $MgCl_2$, 1.3 mM; glucose, 10 mM; Tris base, 20 mM; pH adjusted to 7.65 with 1 M maleic acid) and allowed to equilibrate for 12 min at 30° in a Dubnoff metabolic shaker. Morphine sulfate, 0.1 or 0.5 μM (final concentration), was added to some of the incubating samples (for 12 min) to examine the effects of *in vitro* morphine addition. For depolarized synaptosomes, $^{45}Ca^{2+}$ loading was initiated by adding 0.5 ml of depolarizing medium (KCl, 213 mM; $CaCl_2 \cdot 2H_2O$, 1.2 mM; $MgCl_2$, 1.3 mM; glucose, 10 mM; Tris base, 20 mM; pH adjusted to 7.65 with 1 M maleic acid) containing $^{45}Ca^{2+}$ (sp. act. = 2 μCi $^{45}Ca^{2+}$/μmole $^{40}Ca^{2+}$). This resulted in a 1.5-ml incubation volume containing 74 mM KCl. After a 2-min depolarizing period, $^{45}Ca^{2+}$ uptake was terminated by adding 5.0 ml of ice-cold EGTA stopping solution (NaCl, 136 mM; KCl, 5 mM; $MgCl_2$, 1.3 mM; glucose, 10 mM; Tris base, 20 mM; EGTA, 3mM; pH adjusted to 7.65 with 1 M maleic acid). Each sample was immediately filtered on presoaked (250 mM KCl) Millipore cellulose acetate filters, 0.45 μm, using a Millipore microfiltration manifold. Each filter was washed with 8.0 ml of 0.32 M sucrose and placed in a scintillation vial to which 15 ml of Beckman Ready-Solv™ was added. Samples were counted in a Beckman LS 9000 liquid scintillation counter. Nondepolarized samples were handled in the same manner except that after the 12-min incubation period 0.5 ml of incubation medium (5 mM KCl) containing $^{45}Ca^{2+}$ (2 μCi $^{45}Ca^{2+}$/μmole $^{40}Ca^{2+}$) was added. Net $^{45}Ca^{2+}$ influx (Δ_k) into synaptosomes was calculated by subtracting nondepolarized (5 mM KCl) values from depolarized (74 mM) values. Synaptosomal protein concentrations were assayed by the method of Oyama and Eagle [17].

Table 1 shows that neither acute nor chronic morphine administration altered synaptosomal $^{45}Ca^{2+}$ uptake. Likewise, *in vitro* additon of morphine, 0.1 to 0.5 μM, had no effect on $^{45}Ca^{2+}$ accumulation by nondepolarized or depolarized synaptosomes. Similar *in vitro* experiments were also performed using synaptosomes isolated from whole brains of untreated ICR mice (20–25 g). Morphine, added to synaptosomes in concentrations of 0.86, 4.20 and 8.70 μM, had no significant effect on $^{45}Ca^{2+}$ uptake (data not shown). As stated above, these findings do not agree with recent reports which indicate that morphine added *in vitro* in a concentration of 0.1 μM [11] or 9.0 μM [8] significantly inhibits $^{45}Ca^{2+}$ uptake into synaptosomes induced by KCl depolarization. Our results do agree, however, with those obtained by Harris [18] who reported that morphine, 10 μM, *in vitro* does not alter synaptosomal $^{45}Ca^{2+}$ uptake in response to KCl stimulation. The reason for these discrepancies is not clear. Our experimental conditions were similar although not entirely comparable to those employed in the work cited above [8, 11]. End *et al.* [11] employed a centrifugation technique to isolate synaptosomes after $^{45}Ca^{2+}$ uptake. This method is considered to be inefficient for quenching $^{45}Ca^{2+}$ [19, 20] but still should provide similar qualitative results as compared to those obtained using filtration. Since calcium uptake by synaptosomes appears to be a multiphasic process [20, 21], it is important to utilize

Table 1. Effects of acute and chronic morphine administration, with *in vitro* morphine addition, on KCl-stimulated $^{45}Ca^{2+}$ uptake by whole brain synaptosomes*

		$^{45}Ca^{2+}$ (μmoles/g protein)		
	N	Depolarized	Nondepolarized	Δ_k
Control	5	5.40 ± 0.88	2.89 ± 0.57	2.60 ± 0.33
Control + morphine (0.1 μM)	5	6.05 ± 1.11	2.67 ± 0.56	3.39 ± 0.61
Control + morphine (0.5 μM)	5	5.80 ± 0.59	2.98 ± 0.43	2.82 ± 0.31
Acute (morphine, 15 mg/kg)	4	5.06 ± 0.76	2.39 ± 0.44	2.67 ± 0.42
Acute + morphine (0.1 μM)	3	5.77 ± 1.18	3.29 ± 0.67	2.48 ± 1.10
Acute + morphine (0.5 μM)	4	5.42 ± 0.71	2.74 ± 0.39	2.68 ± 0.48
Chronic	4	5.20 ± 1.31	2.80 ± 0.93	2.40 ± 0.64
Chronic + morphine (0.5 μM)	4	5.51 ± 1.40	2.49 ± 0.98	3.02 ± 0.86
Sham control	5	4.54 ± 0.71	2.23 ± 0.52	2.31 ± 0.49
Sham + morphine (0.5 μM)	5	5.30 ± 0.86	1.95 ± 0.46	3.36 ± 0.61

* Values are means ±S.E.M. of experiments in which each experiment was performed using duplicate samples. Depolarized data represent synaptosomal $^{45}Ca^{2+}$ accumulation in the presence of 74 mM KCl. Nondepolarized data represent $^{45}Ca^{2+}$ accumulation by resting synaptosomes (5 mM KCl). The Δ_k values were calculated as the difference between appropriate KCl-depolarized and nondepolarized data and represent the net synaptosomal $^{45}Ca^{2+}$ uptake in response to depolarization. Morphine sulfate, 0.1 or 0.5 μM, was added *in vitro* to samples from each of the three treatment groups (control, acute and chronic) to determine the membrane effects of an *in vitro* morphine challenge.

techniques that allow for satisfactory time resolution. Rapid filtration, at the present time, represents the best technique to provide for termination of synaptosomal calcium uptake. However, rapid filtration by itself still does not represent an efficient method to measure calcium influx. A "stopping solution" should be employed to quench calcium uptake prior to filtration. EGTA or lanthanum is usually employed since they not only stop calcium uptake but also inhibit calcium binding to the synaptosomal membrane [21]. Nachshen and Blaustein [20] have recently employed the technique of adding high concentrations of unlabeled calcium to quench the uptake process. Filtration without using a "stopping solution" has been shown to quench calcium uptake but not calcium binding [21]. Thus, using a "stopping solution" gives better resolution for the measurement of calcium uptake versus calcium binding. Yamamoto *et al.* [8] used a filtration method to stop calcium uptake for their morphine work but did not quench prior to filtration with EGTA or lanthanum. This may be particularly critical in this case since they did not report on the $^{45}Ca^{2+}$ taken up by synaptosomes under resting conditions in the presence of morphine. Therefore, their results might contain a rather large binding component which could complicate the interpretation of their results. In addition, they used an extracellular concentration of 5 10^{-6} M calcium. Most studies on synaptosomal $^{45}Ca^{2+}$ uptake have used extracellular calcium concentrations of 1–2 mM.

Although our results suggest that morphine does not generally inhibit depolarization-dependent calcium uptake into synaptosomes, there does appear to be sufficient evidence to suggest that opiates may block calcium fluxes at synapses containing opiate receptors [1, 3, 4, 18, 22].

Acknowledgements—This work was supported by NIAAA Grant AA05132 and by NIAAA Research Scientist Development Award AA00044 to S. W. Leslie.

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REFERENCES

1. R. A. Harris, H. H. Loh and E. L. Way, *J. Pharmac. exp. Ther.* **195**, 488 (1975).
2. G. B. Weiss, *A. Rev. Pharmac.* **14**, 343 (1974).
3. R. A. Harris, H. H. Loh and E. L. Way, *J. Pharmac. exp. Ther.* **196**, 288 (1976).
4. W. K. Schmidt and E. L. Way *J. Pharmac. exp. Ther.* **212**, 22 (1980).
5. H. L. Cardenas and D. H. Ross, *J. Neurochem.* **24**, 487 (1975).
6. H. L. Cardenas and D. H. Ross, *Br. J. Pharmac.* **57**, 521 (1976).
7. R. A. Harris, H. Yamamoto, H. H. Loh and E. L. Way, *Life Sci.* **20**, 501 (1977).
8. H. A. Yamamoto, H. W. McCain, K. Isumi, S. Misawa and E. L. Way. *Eur. J. Pharmac.* **71**, 177 (1981).
9. F. Guerrero-Munoz, K. V. Cerreta, M. L. Guerrero and E. L. Way, *J. Pharmac. exp. Ther.* **209**, 132 (1979).
10. F. Guerrero-Munoz, M. L. Guerrero and E. L. Way, *J. Pharmac. exp. Ther.* **211**, 370 (1979).
11. D. W. End, R. A. Carchman and W. L. Dewey, *Biochem. Pharmac.* **30**, 674 (1981).
12. S. W. Leslie, M. B. Friedman, R. E. Wilcox and S. V. Elrod, *Brain Res.* **185**, 409 (1980).
13. S. V. Elrod and S. W. Leslie, *J. Pharmac. exp. Ther.* **212**, 131 (1980).
14. S. W. Leslie, S. V. Elrod, R. Coleman and J. K. Belknap, *Biochem. Pharmac.* **28**, 1437 (1979).
15. S. W. Leslie, M. B. Friedman and R. R. Coleman, *Biochem. Pharmac.* **29**, 2439 (1980).
16. S. R. Bowen, F. G. Carpenter and J. G. Sowell, *Br. J. Pharmac.* **65**, 457 (1979).
17. V. I. Oyama and H. Eagle, *Proc. Soc. exp. Biol. Med.* **91**, 305 (1956).
18. R. A. Harris, in *New Perspectives in Calcium Antagonists* (Ed. G. Weiss) p. 223. American Physiological Society (Clinical Physiology Series), Bethesda, MD (1981).
19. M. P. Blaustein, *J. Physiol. Lond.* **247**, 617 (1975).
20. D. A. Nachshen and M. P. Blaustein, *J. gen. Physiol.* **76**, 709 (1980).
21. S-E. Jansson, J. Gripenberg, M. Harkonen and P. Korpijoki, *Life Sci.* **20**, 1431 (1977).
22. S. L. Lightman, L. L. Iversen and M. L. Forslung, *J. Neurosci.* **2**, 78 (1982).