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MODIFICATION OF ENDORPHIN/ENKEPHALIN ANALGESIA AND STRESS-INDUCED ANALGESIA BY DIVALENT CATIONS, A CATION CHELATOR AND AN IONOPHORE

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1 The possibility that divalent cations may antagonize opiate peptide analgesia and stress-induced analgesia was examined.

2 Intracerebroventricular injection of low doses of Ca^{2+}, Mn^{2+} and Mg^{2+} antagonized β-endorphin and methionine-enkephalin analgesia. Ba^{2+} and Cd^{2+} were without effect.

3 The ionophore, A23187, significantly antagonized β-endorphin analgesia and the effect was increased when a low dose of Ca^{2+} was injected at the same time as the ionophore.

4 Ethylene glycol tetraacetic acid (but not ethylenediamine tetraacetic acid) significantly potentiated endorphin analgesia.

5 Stress-induced analgesia, as determined by increased tail-flick latencies following intraperitoneal injection of acetic acid, was effectively antagonized by naloxone, Ca^{2+} and Mn^{2+}. The frequency of writhing following acetic acid injection was increased by both naloxone and divalent metal ions, again suggesting antagonism of endogenous opiates.

6 These results confirm previous findings indicating that divalent metal ions (and especially Ca^{2+}) may be involved in the actions of opiates.

Introduction

A considerable number of reports have indicated that endogenously occurring divalent metal ions may be intimately involved in the actions of narcotic drugs (for review see Chapman & Way, 1980). Thus morphine was found to inhibit respiration in brain slices only in Ca^{2+}-free medium (Elliott, Kokka & Way, 1963). Ca^{2+} antagonized narcotic analgesia, while Ca^{2+} chelating agents potentiated analgesia (Hano, Kakunaga & Kaneto, 1964; Kakunaga, Kaneto & Hano, 1966). Furthermore, tolerance to narcotic analgesia was reduced by Ca^{2+} administration (Weger & Amsler, 1936; Kakunaga & Kaneto, 1965). Later it was shown that Ca^{2+} levels in synaptosomes are reduced by acute morphine treatment (Ross, Lynn & Cardenas, 1976). Subsequent results indicated that decreases in Ca^{2+} were located in synaptic vesicle and possibly synaptic plasma membrane fractions (Harris, Yamamoto, Loh & Way, 1977; Yamamoto, Harris, Loh & Way, 1978). A number of workers have demonstrated that narcotics inhibit Ca^{2+} influx into isolated brain tissues (e.g., Kakunaga, 1966; Guerrero-Munoz, Cerreta, Guerrero & Way, 1979). Divalent metal ions are known to alter opiate receptor binding *in vitro* (Pasternak, Snowman & Snyder, 1975) and there is also evidence that opiate actions on certain enzyme systems, e.g., guanylate cyclase (Minneman & Iversen, 1976) may involve Ca^{2+} metabolism.

In a detailed study of divalent cation antagonism of morphine analgesia, Harris, Loh & Way (1975) showed that this property was shared by Ca^{2+}, Mn^{2+} and Mg^{2+}. A Ca^{2+} chelator was found to potentiate morphine analgesia whilst the ionophore, X537A, significantly potentiated the antagonistic effect of a low dose of Ca^{2+}. Similar results were later reported for acetylcholine analgesia (Widman, Rosin & Dewey, 1978). In view of these results it seemed of interest to study the effects of such treatment on methionine-enkephalin and β-endorphin analgesia, since these peptides also cause antinociception via opiate receptors (Belluzzi, Grant, Garsky, Sarantakis, Wise & Stein, 1976; Loh, Tseng, Wei & Li, 1976). It has been shown that certain types of stress may produce analgesia in experimental animals via release of endogenous opiates (e.g., Akil, Madden, Patrick & Barchas, 1976). The effects of divalent cations on stress-induced analgesia were thus also examined.

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Methods

Assessment of analgesia

Male ICR mice (Simonsen Laboratories, Gilroy, CA) weighing between 20 and 26 g and maintained on standard laboratory food and tap water were used for all the experiments. Analgesia was assessed by the tail-flick method of D'Amour & Smith (1941). An initial reaction time (1.5 to 2.1 s) to a painful thermal stimulus was first established. β-Endorphin or Met-enkephalin were then injected intracerebroventricularly (i.c.v.) in a volume of 5 μl as described by Harris *et al.* (1975), except that ether anaesthesia was not used. Analgesia was determined 5 min after Met-enkephalin injection and 15 min after β-endorphin injection. A 3.5 s increase in reaction time was taken as a positive analgesic response. Six animals were injected with each of three to five doses of drug and each experiment was performed two or more times, so that a minimum of 12 animals per dose was used. A log dose-response curve was then plotted on probability paper and the median antinociceptive dose (AD_{50}) and the 95% confidence limits (C.L.) were estimated by the method of Litchfield & Wilcoxon (1949). This method was also used to determine the significance of differences from control values after various treatments.

In order to correct for variations in analgesic sensitivity in different batches of animals, control curves for saline-treated animals were produced each day.

Effect of divalent cations, chelators and an ionophore on enkephalin/endorphin analgesia

The chloride salts of the different cations listed in Table 1 were dissolved in 0.9% w/v NaCl solution (saline). Disodium edetate (EDTA) was dissolved in saline and the pH adjusted to 7.4. EGTA was dissolved in 5.0 M NaOH, diluted with saline and the pH adjusted to 7.4. A23187 was dissolved in a small volume of dimethyl sulphoxide (DMSO). This stock solution was then diluted 1 in 400 with saline or saline plus Ca^{2+} with rapid mixing. Solutions were injected i.c.v. as described above for enkephalin/endorphin. The doses used (corresponding to those used by Harris *et al.*, 1975) caused no obvious signs of toxicity such as catalepsy, although motility was often somewhat increased or decreased.

The control latency was determined for each animal shortly before injection of the appropriate agent, and was then redetermined 40 min after injection (test latency). The mean control and test latencies for groups of 6 animals were compared by an unpaired Student's *t* test (two tailed). This procedure was repeated before each AD_{50} determination. When it was established that no significant change in latency was being produced, the effect of the cation, chelator or ionophore on endorphin or enkephalin analgesia was examined in a separate experiment. The agents were injected 40 min before testing and the opiate peptide was then administered at the same injection site 15 min (for β-endorphin) or 5 min (for Met-enkephalin) before testing. Initially it had been determined that double i.c.v. injection of saline had no effect on tail-flick latency and produced no overt behavioural changes.

Time course of Ca^{2+} antagonism of β-endorphin analgesia

$CaCl_2$ (44.1 μg) was injected i.c.v. in a group of 72 mice and appropriate doses of β-endorphin injected i.c.v. 0.5, 2, 6 and 24 h later. The AD_{50} was then determined 15 min later for each of the four groups of 18 animals. The control group received 5 μl saline instead of Ca^{2+}.

In order to estimate brain Ca^{2+} levels after i.c.v. injection, mice were injected with 44.1 μg $CaCl_2$ containing 0.4 μCi ^{45}Ca. The animals were then decapitated at intervals of 0.5, 2, 6 and 24 h after injection. The brains were removed and homogenized in 5.0 ml water and duplicate 1.0 ml aliquots added to 10 ml Scintiverse (Fisher, Santa Clara, CA) for scintillation counting.

Effect of divalent cations and naloxone on tail-flick latency

In order to determine possible hyperalgesic effects of divalent cations more precisely, the intensity of the heat source was decreased so as to give control tail-flick latencies between 4.0–5.0 s. Saline or divalent cations were then injected i.c.v. as described above and the test latency determined 10 and 40 min later. The mean control and test latencies for groups of 6 mice were compared by means of an unpaired Student's *t* test (two tailed). The effects of intraperitoneal (i.p.) injections of naloxone were similarly examined.

Effect of naloxone and divalent cations on stress-induced analgesia

Tail-flick latencies (between 2.4–3.0 s) were determined for groups of 6 mice. The animals were then injected i.p. with 1% acetic acid (10 ml/kg) and the tail-flick latency again determined at a given interval after injection for each group. The mean test latencies at the different times after injection were then compared with the corresponding mean control latencies by means of an unpaired Student's *t* test (two tailed).

Naloxone was injected i.p. in a volume of 10 ml/kg,

while divalent cations were injected i.c.v. in a volume of 5 μl, both 10 min before acetic acid injection. Animals injected i.p. received a second dose of naloxone subcutaneously (s.c.) 15 min after acetic acid. Acetic acid-treated groups not receiving naloxone or cations, were injected i.p. and s.c. or i.c.v. with saline.

Effect of naloxone and divalent cations on acetic acid writhing

Individual mice were placed in a large glass observation tank immediately following i.p. injection of 1% acetic acid (10 ml/kg). The number of completed writhes (Fennessy & Lee, 1975) was noted for the 10 min period between 5 and 15 min after injection. The mean number of writhes/10 min period for groups of 6–8 mice injected either i.p. with naloxone (5 min before acetic acid) or i.c.v. with divalent metal ions (15 min before acetic acid) was compared with control groups similarly injected with saline.

Drugs

The chemicals used and their suppliers were as follows: naloxone hydrochloride, a gift from Endo Laboratories Inc. (Garden City, N.Y.); β-endorphin, a gift from Professor C.H. Li (University of California, San Francisco, CA); methionine-enkephalin, ethylene glycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA) and ethylene-diamine tetraacetic acid (EDTA), Sigma Chemical Co. (St Louis, Mo); A23187, a gift from Lilly Research Laboratories (Indianapolis, IND); chloride salts of the different ions ($CaCl_2.2H_2O$, $MgCl_2.6H_2O$, $MnCl_2.4H_2O$, $BaCl_2.2H_2O$, $CdCl_2.2.5H_2O$), Baker and Adamson (Morristown, N.J.), Fisher Scientific (Fair Lawn, N.J.) and Mallinckrodt, Inc. (St Louis, Mo.).

Results

Effects of divalent cations on tail-flick response and endorphin/enkephalin analgesia in mice

The effects of various divalent cations on tail-flick latency and endorphin/enkephalin analgesia are shown in Table 1. At the doses used, none of the ions produced any significant changes in tail-flick latency. The analgesic effects of β-endorphin were significantly antagonized by 44.1 μg (0.3 μmol) of Ca^{2+}, 60.9 and 81.3 μg (0.3 and 0.4 μmol) of Mg^{2+} and 19.7 and 59.3 μg (0.1 and 0.3 μmol) of Mn^{2+}, while Met-enkephalin analgesia was also antagonized by Ca^{2+} (44.1 μg) and Mn^{2+} (19.7 μg). Mn^{2+} was the most potent antagonist, producing in excess of a 10 fold increase in the β-endorphin AD_{50} value. Ba^{2+} and Cd^{2+} did not cause any change in β-endorphin antinociception at the doses used.

Table 1 Effect of divalent cations on the tail-flick response to thermal stimulus and the analgesic potency of β-endorphin and Met-enkephalin in mice

Ion†	*Dose* (μg)	*Control latency*‡ (s)	*Test latency* (s)	AD_{50} *(95% C.L.)* (μg)
				β-Endorphin§
Saline	—	1.9 ± 0.09	2.0 ± 0.1	0.29 (0.18–0.46)
Ca^{2+}	14.7	1.8 ± 0.05	1.8 ± 0.08	0.39 (0.27–0.55)
	44.1	1.7 ± 0.07	1.7 ± 0.09	0.91 (0.51–1.63)*
Mg^{2+}	60.9	1.6 ± 0.1	1.6 ± 0.06	0.67 (0.36–1.23)*
	81.3	1.8 ± 0.08	1.9 ± 0.08	0.88 (0.47–1.64)*
Mn^{2+}	19.7	1.9 ± 0.08	2.0 ± 0.07	1.04 (0.55–1.98)*
	59.3	1.7 ± 0.06	1.6 ± 0.12	3.3 (2.13–5.11)**
Ba^{2+}	1.4	1.7 ± 0.08	1.6 ± 0.12	0.22 (0.15–0.32)
Cd^{2+}	2.2	1.8 ± 0.11	1.9 ± 0.13	0.27 (0.14–0.50)
				Met-enkephalin§
Saline	—	1.7 ± 0.08	1.8 ± 0.13	106 (81.5–137.8)
Ca^{2+}	44.1	1.7 ± 0.09	1.5 ± 0.06	269 (216.9–333.6)*
Mn^{2+}	19.7	1.6 ± 0.04	1.5 ± 0.08	280 (207.4–378)*

Values are given ± s.e.mean.
†Injected i.c.v. 40 min before testing.
‡Determined before injection of ions.
§Injected i.c.v. 15 min (β-endorphin) or 5 min (Met-enkephalin) before testing.
Significantly different from control: *$P < 0.05$; **$P < 0.01$.

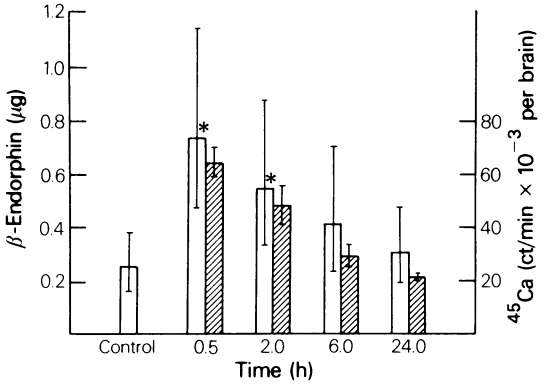

Figure 1 Time course of Ca^{2+} antagonism of β-endorphin analgesia and removal of ^{45}Ca from brain. Mice were injected i.c.v. with 44.1 μg Ca^{2+} and AD_{50} for β-endorphin determined at intervals up to 24 h later. Control injected with saline. To determine rate of removal of ^{45}Ca, mice were injected with 44.1 μg Ca^{2+} containing 0.4 μCi ^{45}Ca ($n = 6-8$ mice per group). Open columns represent AD_{50} for β-endorphin. Vertical lines show 95% C.L. Hatched columns show ^{45}Ca levels. Vertical lines represent s.e.mean.
Significantly different from saline control: $^*P < 0.05$.

Time course of Ca^{2+} antagonism of β-endorphin antinociception and decay of ^{45}Ca levels in brain

Pretreatment of animals with 44.1 μg Ca^{2+} caused a significant antagonism of β-endorphin analgesia (compared with saline injected controls) for up to 2 h after injection (Figure 1). At 6 and 24 h after Ca^{2+} administration no significant increases in β-endorphin AD_{50} were observed. The decrease in the degree of antagonism of analgesia with time was matched by the rate of removal of injected ^{45}Ca from the brain. Injection of saline at different intervals before determination caused no significant change, so only one control is shown.

Effects of chelators on β-endorphin and Met-enkephalin analgesia

At doses of 16.3 and 32.7 μg (44 and 88 nmol) EDTA had no effect on either tail-flick latency or β-endorphin AD_{50} (Table 2). In contrast, EGTA at a dose of 16.7 μg (44 nmol), whilst not affecting tail-flick latency, did significantly potentiate the analgesic effects of both β-endorphin and Met-enkephalin.

Effects of A23187 and A23187 plus Ca^{2+} on β-endorphin analgesia

Neither vehicle (DMSO plus saline, 1:399) nor vehicle plus Ca^{2+} (7.3 μg) caused any change in tail-flick latency or β-endorphin AD_{50} (Table 3). However, when 4.1 μg A23187 (8 nmol) was injected 25 min before β-endorphin, significant antagonism of analgesia was seen, and this effect was increased when the ionophore was injected with a low dose of Ca^{2+} (7.3 μg). A23187 injected i.c.v. alone or with Ca^{2+} caused no significant change in tail-flick latency.

Assessment of possible hyperalgesic effects of divalent cations and naloxone

Ca^{2+}, Mg^{2+} and Mn^{2+} at i.c.v. doses that caused significant antagonism of opiate analgesia, failed to produce changes in tail-flick latencies after the inten-

Table 2 Effect of chelators on the tail-flick response and the analgesic potency of β-endorphin and Met-enkephalin in mice

Chelator†	*Dose* (μg)	*Control latency*‡ (s)	*Test latency* (s)	*AD_{50} (95% C.L.)* (μg)
				β-Endorphin§
Saline	—	1.8 ± 0.06	2.0 ± 0.12	0.33 (0.19–0.56)
EDTA	16.3	1.8 ± 0.08	2.0 ± 0.09	0.28 (0.19–0.41)
	32.7	1.9 ± 0.08	2.1 ± 0.09	0.27 (0.16–0.46)
EGTA	16.7	1.9 ± 0.08	2.2 ± 0.13	0.13 (0.09–0.19)*
				Met-enkephalin§
Saline	—	1.7 ± 0.08	1.8 ± 0.12	106 (81.5–137.8)
EGTA	16.7	1.7 ± 0.1	2.0 ± 0.08	57 (39.0–83.2)*

Values are given ± s.e.mean.
†Injected i.c.v. 40 min before testing.
‡Determined before injection of ions.
§Injected i.c.v. 15 min (β-endorphin) or 5 min (Met-enkephalin) before testing.
Significantly different from control: $^*P < 0.05$.

Table 3 Effect of A23187 and Ca^{2+} on the tail-flick response and the analgesic potency of β-endorphin in mice

Treatment†	*Dose* (μg)	*Control latency*‡ (s)	*Test latency* (s)	*β-endorphin AD_{50} (95% C.L.)*§ (μg)
Saline	—	1.9 ± 0.13	2.0 ± 0.08	0.27 (0.19–0.39)
Saline/ DMSO	—	1.7 ± 0.08	1.9 ± 0.11	0.33 (0.22–0.50)
Ca^{2+}	7.3	1.8 ± 0.04	1.9 ± 0.05	0.31 (0.17–0.56)
A23187	4.1	1.9 ± 0.07	2.1 ± 0.09	0.96 (0.61–1.52)*
Ca^{2+} +A23187	7.3 + 4.1	1.9 ± 0.1	2.1 ± 0.12	1.61 (1.04–2.50)*

Values are given ± s.e.mean.
†Injected i.c.v. 40 min before testing.
‡Determined before injection of ions.
§Injected i.c.v. 15 min before testing.
Significantly different from control: $*P < 0.05$.

sity of the heat source had been reduced to give control latencies in the range 4.0–5.0 s (Table 4). A higher dose of 58.8 μg Ca^{2+} did produce a small but significant decrease in latency similar to that reported by Schmidt & Way (1980). Naloxone, however, at doses of up to 5 mg/kg also failed to alter tail-flick latencies.

Effect of divalent cations and naloxone on stress-induced analgesia

Injection of 1% acetic acid (i.p.) produced significant increases (24–47%) in test latencies compared with control latencies for up to 1 h after injection (Figure 2). No increase in latency was observed for animals which were either not injected or were injected i.p. with physiological saline. This effect of acetic acid was antagonized by naloxone (1.5 mg/kg, i.p. plus 1.5 mg/kg, s.c.), Ca^{2+} (44.1 μg, i.c.v.) and Mn^{2+} (59.3 μg, i.c.v.). No significant antagonism of stress-induced analgesia was observed when animals were injected i.c.v. or i.p. and s.c. with saline, although in the latter case there was a small reduction in acetic acid effect, presumably due to dilution of acetic acid by saline injected i.p.

Effect of naloxone and divalent cations on acetic acid-induced writhing

The frequency of writhing following acetic acid injection was significantly increased following i.p. injection of naloxone (2 mg/kg) and i.c.v. injection of Ca^{2+} (7.3 μg) and Mn^{2+} (3.9 μg) (Table 5). Pre-injection of saline either i.p. or i.c.v. had no significant effect on the frequency of writhing compared with control animals receiving acetic acid alone, although there was some slight reduction after i.p. injection presumably due to a dilution effect as above. Lower doses of metal ions were used than before because it was observed that the higher doses of Ca^{2+} and Mn^{2+} usually caused somewhat reduced motility in mice and this may thus have interfered

Table 4 Effect of divalent cations and naloxone on tail-flick responses in mice

Treatment†	*Route*	*Dose*	*Control latency*‡ (s)	*Test latency for 10 min* (s)	*Test latency for 40 min* (s)
Saline	i.c.v.	—	4.7 ± 0.23	4.7 ± 0.16	4.5 ± 0.27
Ca^{2+}	i.c.v.	44.1 μg	4.6 ± 0.17	4.0 ± 0.16	4.0 ± 0.18
	i.c.v.	58.8 μg	4.5 ± 0.14	3.5 ± 0.11*	3.6 ± 0.18*
Mg^{2+}	i.c.v.	81.3 μg	4.4 ± 0.29	4.2 ± 0.22	4.6 ± 0.23
Mn^{2+}	i.c.v.	59.3 μg	4.1 ± 0.10	4.2 ± 0.29	4.2 ± 0.16
Cd^{2+}	i.c.v.	2.2 μg	4.7 ± 0.28	4.7 ± 0.10	5.3 ± 0.23
Saline	i.p.	—	4.2 ± 0.24	4.3 ± 0.19	4.2 ± 0.22
Naloxone	i.p.	5 mg/kg	4.3 ± 0.12	4.0 ± 0.17	4.1 ± 0.18

Values are given ± s.e.mean.
†Injected i.c.v. or i.p. 10 or 40 min before testing.
‡Determined before injection of ions or naloxone.
Significantly different from control: $*P < 0.05$.

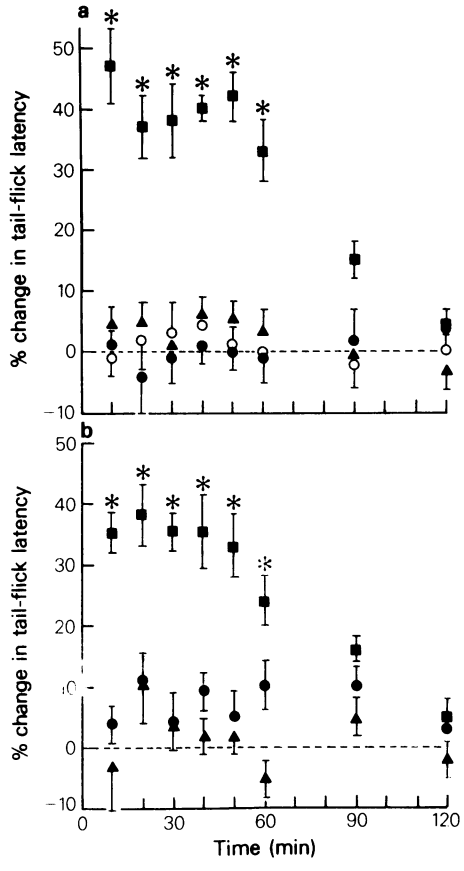

Figure 2 Time course of stress-induced analgesia following i.p. injection of 1% acetic acid in mice. Mean control tail-flick latencies were determined for groups of 6 mice. The animals were then injected i.p. with 1% acetic acid and the mean test latency again determined for each group after a given time interval. Vertical lines represent s.e.mean. (a) Control (not injected) (●); saline injected i.c.v. 10 min before acetic acid (■); 44.1 μg Ca^{2+} (▲) or 59.3 μg Mn^{2+} (○) injected i.c.v. 10 min before acetic acid. (b) Control (not injected) (●); saline injected i.p. 10 min before and s.c. 15 min after acetic acid (■); naloxone (1.5 mg/kg) injected i.p. 10 min before and s.c. 15 min after acetic acid (▲). Test latency significantly increased compared with control latency: $^*P<0.05$.

with the writhing tendency. Doses were thus chosen which had no apparent effect on motility but did increase writhing.

Discussion

The results presented in this paper suggest that the effects of certain divalent cations, chelating agents and a cation ionophore on endorphin/enkephalin analgesia may closely resemble their effects on morphine and acetylcholine analgesia.

Table 5 Effect of naloxone and divalent metal ions on the frequency of writhing following i.p. injection of acetic acid in mice

Treatment	*Route*	*Dose*	*No. writhes/10 min*§
Saline†	i.p.	—	34.8 ± 3.1
Naloxone†	i.p.	2 mg/kg	45.3 ± 2.5*
Saline‡	i.c.v.	—	41.6 ± 2.3
Ca^{2+}‡	i.c.v.	7.3 μg	51.3 ± 2.0*
Mn^{2+}‡	i.c.v.	3.9 μg	57.7 ± 3.6*

Values are given ± s.e.mean.
†Injected 5 min before acetic acid.
‡Injected 15 min before acetic acid.
§Determined from 5–15 min after acetic acid injection.
Significantly different from control: $^*P<0.05$.

None of the divalent cations had any effect on tail-flick latency at the doses used. This was also the finding of Widman *et al.* (1978) although Harris *et al.* (1975) observed that a similar dose of Mn^{2+} slightly reduced tail-flick latency. Ca^{2+}, Mg^{2+} and Mn^{2+} were all found to antagonize β-endorphin analgesia, and Ca^{2+} and Mn^{2+} also inhibited the antinociceptive effect of Met-enkephalin (Mg^{2+} was not tested). Morphine (Harris *et al.*, 1975) and acetylcholine (Widman *et al.*, 1978) analgesia was also antagonized by these ions. In all cases parallel shifts of agonist dose-response curves were observed. In the present study Mn^{2+} was a more potent antagonist of opiate peptide analgesia than Ca^{2+} or Mg^{2+}, producing in excess of a tenfold increase in AD_{50} values for β-endorphin. Similar increases were reported for morphine AD_{50} values by Harris *et al.* (1975), while Widman *et al.* (1978) found that Mn^{2+}, Ca^{2+} and Mg^{2+} were essentially equipotent in antagonizing acetylcholine antinociception. Sr^{2+} and Ba^{2+} failed to antagonize opiate peptide analgesia, just as they failed to antagonize morphine or acetylcholine analgesia. It has been suggested (Harris *et al.*, 1975) that since Sr^{2+} and Ba^{2+}, like Ca^{2+}, stimulate the release of neurotransmitters (Elmqvist & Feldman, 1965) while Mg^{2+} and Mn^{2+} inhibit release (Douglas & Rubin, 1964; Meiri & Rahamimoff, 1972), it is unlikely that the antagonism of analgesia seen in these studies is due to increased release of neurotransmitter caused by the divalent ions. Thus it was argued that a postsynaptic action of Ca^{2+} and other ions in antagonizing analgesia was more likely than a presynaptic action. Another way in which Ca^{2+}, Mg^{2+} and Mn^{2+} have similar effects on opiates is that they are all reported to enhance agonist binding (Pasternak *et al.*, 1975) with Mn^{2+} again being the most potent of the cations.

EGTA was found to potentiate the analgesic effects of endorphin (and enkephalin) while EDTA was without effect. Similar results were obtained by Harris *et al.* (1975) and Widman *et al.* (1978), prompting these authors to speculate that Ca^{2+} rather than Mg^{2+} was being affected since EGTA has a much higher affinity for Ca^{2+} than Mg^{2+} (Williams, 1970).

The ionophore A23187 when injected alone significantly inhibited β-endorphin analgesia and this effect was increased when a low dose of Ca^{2+} was injected at the same time. Again these results resemble those of Harris *et al.* (1975) (who used the ionophore X537A) and Widman *et al.* (1978) (who used A23187), although in these studies significant antagonism of analgesia was only observed when the ionophore was injected with a low dose of Ca^{2+}. Ionophores may act by increasing Ca^{2+} flow across membranes (Pressman, 1973) or by releasing intracellular Ca^{2+} stores (Robinson, Russell & Thorn, 1976). Since the flow of Ca^{2+} into cells appeared to be increased by the ionophores in the previous studies, it was reasoned that analgesia may be caused by a decreased flow of Ca^{2+} across cell membranes. Alternatively, it might be argued that the ionophores are directly increasing neurotransmitter release, thus tending to overcome or mask the analgesic effect, which may thus not directly involve Ca^{2+} fluxes. Recently, however, it has been shown that Met-enkephalin fails to inhibit A23187-induced release of noradrenaline from brain slices (Göthert, Pohl & Wehking, 1979) while morphine does not affect A23187-induced release of noradrenaline or uptake of ^{45}Ca (Chapman & Way, unpublished data). In all of these cases K^+-stimulated effects are inhibited by opiates. It thus seems that normal entry of Ca^{2+} via Ca^{2+} channels during stimulation-secretion coupling may be necessary for opiate action, suggesting a direct effect on Ca^{2+} flux.

Having demonstrated divalent cation antagonism of exogenously added endorphin and enkephalin analgesia, experiments were next performed in order to examine the effects of cations on analgesic effects due to endogenously occurring opiates. No significant decreases in tail-flick latency were observed when naloxone was injected (i.p.), even when the control latency was increased in order to make detection of such decreases easier. Similarly, i.c.v. injection of divalent cations failed to alter reaction times, except when a higher dose of Ca^{2+} than was necessary to antagonize endorphin analgesia was used. It thus appeared that under our test conditions, endogenous opiates were not being mobilized to any substantial degree.

Numerous papers have shown that when various forms of stress are applied to animals, significant analgesia can be produced and in one such report Kokka & Fairhurst (1977) demonstrated that the frequency of writhing following i.p. injection of acetic acid in rats is increased after naloxone administration. We observed a similar effect of naloxone in mice, suggesting antagonism of endogenous opiates. Likewise, an increase in writhing frequency was observed following i.c.v. injection of low doses of Ca^{2+} and Mn^{2+}. The antagonism of stress-induced analgesia by such low doses of cations is presumably related to the relatively low level of analgesia produced by acetic acid injection, since doses of 14.7 μg of Ca^{2+} or lower failed to antagonize the much more pronounced analgesia due to β-endorphin (Table 1). A corresponding increase in tail-flick latency was also noted for up to about 1 h following acetic acid administration and this effect was again reversed by treatment with naloxone, Ca^{2+} or Mn^{2+}.

In summary, we have observed that divalent metal ions and an ionophore antagonize, and a cation chelator potentiates endorphin/enkephalin analgesia. Furthermore, divalent cations antagonize stress-induced analgesia (which may involve both endorphins and enkephalins). These observations, taken with other results for morphine and acetylcholine analgesia lend weight to the hypothesis that opiates may exert their actions by inhibiting ion fluxes across membranes.

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