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MORPHINE INHIBITION OF CALCIUM FLUXES, NEUROTRANSMITTER RELEASE AND PROTEIN AND LIPID PHOSPHORYLATION IN BRAIN SLICES AND SYNAPTOSOMES

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Abstract—Morphine (1–100 μM) was found to inhibit several concomitant events in brain slices and synaptosomes which are augmented by depolarizing agents. Thus, $^{45}Ca^{2+}$ uptake, amino acid neurotransmitter release, increases in 3′,5′ cyclic AMP levels and $^{32}P_i$ incorporation to proteins and lipids induced by veratrine (25 μM) and by potassium (56 mM), were each inhibited in a dose related manner. These inhibitory actions of morphine were all prevented by naloxone (1 μM).

Evidence is presented that morphine binding to a receptor on the synaptic membrane affects intracellular mechanisms involved in neurotransmitter release possibly via a second messenger system. An enhancing action of GTP on the inhibitory influences of morphine suggests that its actions are mediated at least in part, via a coupling of the receptor to adenyl cyclase in the outer membrane. This is supported by its inhibitory action on the capacity of depolarizing agents to increase cyclic AMP levels.

There have been many reports of the action of morphine in reducing the release and turnover of acetylcholine and biogenic amines both *in vivo* and *in vitro* [1–5]. Similarly, enkephalins have been shown to inhibit the release of these neurotransmitters and all of the inhibitory actions of morphine are prevented by naloxone indicating that they are specific and receptor mediated [6–10]. In addition, naloxone-sensitive suppression by morphine of amino acid neurotransmitter release evoked *in vivo* by depolarizing agents and by sensory stimulation has been reported [11, 12]. Parallel effects of morphine on Ca^{2+} uptake and amino acid neurotransmitter release from synaptosomes incubated *in vitro* have been demonstrated [13]. The present paper describes further features of this inhibition of Ca^{2+} fluxes and neurotransmitter release by morphine, and examines parallel effects of the drug on related systems including adenylate cyclase and protein and lipid phosphorylation, with a view to further defining the mode of action of morphine.

MATERIALS AND METHODS

Preparation of synaptosomes. Synaptosomes were prepared from cerebral cortex of female Sprague–Dawley rats, by the method of Gray and Whittaker [14] as modified by Bradford *et al.* [15].

$^{45}Ca^{2+}$ uptake by synaptosomes. The synaptosomal pellet was suspended in 1 ml of Tris buffered Krebs–Ringer medium of the following composition (mM); NaCl, 120.0; KCl, 5.0; $MgSO_4$, 1.0; glucose, 10.0; Tris–HCl, 20.0; pH 7.4. Incubation was performed in 5 ml conical flask at 37° for 30 min in a shaking water bath. A gas phase of oxygen was provided. Morphine sulphate (final concentration 1–100 μM) was present for the last 15 min of incubation. The depolarizing agent and $^{45}CaCl_2$ were present for the last 20 min. $^{45}Ca^{2+}$ was added to give a final concentration of 1.2 mM, with specific activity 0.8 mCi/mmole.

Ice-cold EGTA solution of composition (mM); NaCl, 120.0; KCl, 5.0; EGTA, 30.0; $MgCl_2$, 1.2; Tris–HCl, 20.0; NaH_2PO_4, 1.2; pH 7.4, was added to terminate the reaction, and the synaptosomal suspensions were sedimented in a bench ultracentrifuge [16]. The supernatants were retained for amino acid analysis, and the pellets were washed three times in 1 ml of K^+-free Na^+ solution, composition (mM); NaCl, 137.0; $MgCl_2$, 1.2; Na_2HPO_4, 1.2; Tris, 20.0; glucose, 10.0; pH 7.4. The resulting pellets were digested at 50° in an appropriate volume of 1 M NaOH for 20 min. Aliquots were taken for radioactivity and protein determination.

$^{32}P_i$ incorporation into synaptosomes. Synaptosomal pellets were suspended in Krebs–Ringer medium and 0.5 ml aliquots (4 mg/ml) were incubated at 37° for 5 min in the presence of morphine before the addition of drugs and $^{32}P_i$. The $^{32}P_i$ (Amersham International) was added to give a specific activity of 200 μCi/ml Ci. The synaptosomal suspension was precipitated by the addition of 1.5 ml of 15% TCA. The mixture was left on ice for about 15 min with frequent "vortex mixing" and the suspension was sedimented in a bench-top ultracentrifuge. The resulting protein was then taken for radioactivity counting.

Preparation and incubation of brain slices. Female Sprague–Dawley rats (200 g) were stunned and deca-

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‡ Abbreviations used: P_i, inorganic phosphate; TTX, tetrodotoxin.

pitated. Brains were rapidly removed and placed on a warmed glass plate [17] and cortex slices (40–80 mg) were rapidly cut (2 min in total) from the cerebral hemispheres using a razor blade and recessed guide [18]. Only one slice was taken from each. This was halved and then incubated in Krebs–Tris medium which had the composition (mM), NaCl, 138.0; KCl, 5.0; $MgSO_4$, 1.0; Tris–HCl, 20.0; glucose, 10.0; $CaCl_2$, 1.2; pH 7.4.

$^{45}Ca^{2+}$ uptake by cortex slices. Brain slices (approximately 30 mg wet wt) were incubated at 37° in 4.5 ml Ca^{2+} –Krebs–Tris medium (1.2 mM Ca^{2+}) in 5 ml beakers in a shaking water bath for 30 min. Pure oxygen was bubbled continuously into the medium throughout the incubation. Weight measurements showed a <5% loss of volume due to evaporation.

Test compounds, dissolved in the Krebs–Tris buffer, were added 5 min prior to addition of $^{45}Ca^{2+}$. The $^{45}Ca^{2+}$ was added after 30 min of incubation (Amersham International), to give a final specific activity of 0.14 Ci/mmol. One minute later the depolarizing agent was added and after a further 10 min incubation with $^{45}Ca^{2+}$ the slices were transferred to 25 ml beakers for 15 sec, each containing 2 ml of EGTA stop-solution (described above). The slices were then washed in three separate 2 ml aliquots of K^+-free Na^+ medium, of the composition described above, at room temperature. Slices were then digested in 1 ml of 1 M NaOH at 50° for 30 min. The suspension was aliquoted and samples taken for analysis of K^+ content by atomic absorptiometry and ^{45}Ca radioactivity was measured by scintillation counting as described elsewhere [19].

Incorporation of $^{32}P_i$ into brain slices. Cerebral slices were each suspended in 4.5 ml of Ca^{2+}–Krebs–Tris medium for 30 min. Subsequently $^{32}P_i$ was added (Amersham International) to give a final specific activity of 200 μCi/per incubation.

The incubation was allowed to proceed for an hour and then the depolarizing agent was added. The incubation was terminated 1 min later. For total $^{32}P_i$ incorporation into the slice, the tissue was rapidly washed in 1 ml of ice-cold K^+ free Na^+ medium, digested in 1 ml of 1 M NaOH and samples were taken for counting of radioactivity. When the incorporation of $^{32}P_i$ into total protein and lipid fractions were estimated, the procedure described by Abdul Ghani *et al.* [20] was followed. The lipid fraction was removed by extraction with ethanol/methanol mixtures and acidified diethyl ether [20]. All supernatants were counted as was the final NaOH digest.

Amino acid analysis. Samples were analysed for amino acid content as described by Norris *et al.* [21] using a Rank Hilger Chromaspek J180 autoanalyser with fluorimeter J143.

3′,5′ *Cyclic AMP assay.* Cerebrocortical synaptosomes were suspended in 0.32 M sucrose containing 4 mM Tris–HCl pH 7.4 [22]. The preparation was stored at −20° until use.

The standard incubation mixture (150 μl) consisted of 0.3 mM ATP, 30 mM Tris–HCl, 0.5 mM 3-isobutyl-1-methylxanthine, and 10 mM $MgCl_2$. The reaction was initiated by the addition of synaptosomal fractions (25 μg protein). Incubation was continued for 10 min at 37°, the relevant drugs being present for the last 5 min. The synaptosomal suspension was then centrifuged in a bench ultracentrifuge and the supernatant was retained for analysis.

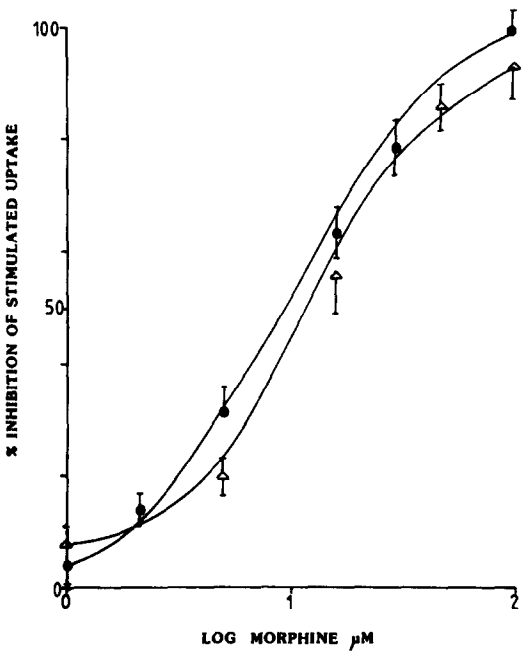

Fig. 1. Dose response curves for the inhibitory action of morphine on veratrine (25 μm) stimulated Ca^{2+} influx into both cortical brain slices and synaptosomes. Values are means ± S.E.M. and the lines were fitted by linear regression: ●, Brain slices (N = 18); △, synaptosomes (N = 12).

3′,5′ Cyclic AMP levels in the supernatant were determined using an assay kit supplied by Amersham International.

Protein determination. Both synaptosomal and brain slice protein content were determined by the method of Lowry *et al.* [23] using bovine serum albumin as the standard.

RESULTS

The effect of morphine on resting and stimulated uptake of $^{45}Ca^{2+}$

Morphine added alone in the concentration range 1–100 μM had no effect on spontaneous $^{45}Ca^{2+}$ uptake or on amino acid release from either brain slices or synaptosomes. The dose–response curve for morphine inhibition of Ca fluxes is given in Fig. 1.

Using 25 μM veratrine as the depolarizing agent, $^{45}Ca^{2+}$ uptake increased by 29% ± 3% in brain slices and 54% ± 6% in synaptosomes. These responses were significantly ($P < 0.001$) inhibited by morphine (100 μM), the levels of $^{45}Ca^{2+}$ uptake approaching those of untreated controls. The IC_{50} for morphine inhibition of 25 μM veratrine-evoked $^{45}Ca^{2+}$ uptake was 9.4 μM (5.3–16.7) in brain slices and 11.4 μM (8.7–14.9) in synaptosomes (Table 1).

High K^+ (56 mM) also stimulated Ca^{2+} uptake and this response was found to be sensitive to morphine,

Table 1. IC_{50} values for the inhibition by morphine of both stimulated Ca^{2+} uptake and endogenous glutamate release, in cerebrocortical brain slices and synaptosomes

Stimulating agent	Brain slices N = 18		Synaptosomes N = 12	
	Ca^{2+} influx	Evoked glutamate release	Ca^{2+} influx	Evoked glutamate release
Veratrine 25 μM	9.4 μM (5.3–16.7)	18.4 μM (16.3–19.8)	11.4 μM (8.7–14.9)	26.0 μM (16.1–41.9)
Potassium 56 mM	4.8 μM (2.3–10.1)	3.9 μM (2.0–7.6)	2.4 μM (0.3–19.2)	0.5 μM (0.02–11.6)

Tabulated data are for μM morphine added, and were estimated from dose response curves over a concentration range 1–100 μM morphine (see Fig. 1). In brain slices Ca^{2+} influx and evoked glutamate release were inhibited by 100% by 100 μM morphine, and by 72–79% by 50 μM morphine. In synaptosomes Ca^{2+} influx and evoked glutamate release were inhibited by 91% and 79% respectively by 50 μM morphine, and by 86% and 55% at 15 μM morphine. Evoked glutamate release was calculated as the difference between control and stimulated levels of glutamate in the incubation medium. Stimulation increased glutamate release by 9-fold in synaptosomes and by 2-fold in brain slices.

Data were collated from 6 experiments (brain slices) or 4 experiments (synaptosomes), each experiment involving triplicate samples for each condition. The 95% confidence limits (in parentheses) were calculated by the method of Litchfield and Wilcoxan [43].

with estimated IC_{50} values of 4.8 μM (2.28–10.1) in brain slices and 2.4 (0.3–19.2) in synaptosomes (Table 1). All inhibitory actions of morphine on veratrine or K^+-induced Ca^{2+} uptake were abolished by 1 μM naloxone (Table 2).

Influence of GTP on morphine

The addition of GTP (1 μM–10 μM) significantly ($P < 0.005$) increased the inhibitory effect of 15 μM morphine on the Ca^{2+} uptake induced by 25 μM veratrine (Table 2).

Morphine (15 μM) decreased stimulated Ca^{2+} uptake into brain slices by 48%, and in the presence of 10 μM GTP this inhibitory action increased by 78%. In synaptosomes there was a 16% enhancement of the inhibition induced by 15 μM morphine on the addition of 10 μM GTP.

GTP, when added alone had no influence on basal or stimulated Ca^{2+} uptake into either brain slices or synaptosomes. Other related nucleotides tested, GMP, CTP or ITP (all at 10 μM) showed no enhancement of morphine inhibition (Table 2).

Action of morphine on neurotransmitter amino acid release

Morphine at low concentrations (1–15 μM) inhibited the evoked release of endogenous neurotransmitter amino acids from both brain slices and synaptosomes when 25 μM veratrine and 56 mM K^+ were employed as stimulation agents. All of these

Table 2. Effect of GTP and naloxone on morphine-induced inhibition of stimulated Ca^{2+} uptake

Condition	Brain slices N = 9	Synaptosomes N = 9
Control	11.21 ± 0.22	3.70 ± 0.14
GTP (10 μM)	11.28 ± 5.25	3.75 ± 0.13
Veratrine	15.06 ± 4.22	8.16 ± 0.23
Veratrine plus morphine	13.21 ± 0.32 (48%)	6.33 ± 0.15 (41%)
Veratrine plus GTP (10 μM)	15.14 ± 0.24	—
Veratrine plus morphine:		
plus GTP (10 μM)	12.46 ± 0.21* (67%)	5.70 ± 0.09* (55%)
GTP (1 μM)	12.07 ± 0.24* (78%)	5.61 ± 0.11* (57%)
GMP (10 μM)	13.41 ± 0.33 (43%)	6.19 ± 0.22 (44%)
GDP (10 μM)	—	6.37 ± 0.19 (40%)
CTP (10 μM)	12.99 ± 0.63 (53%)	6.44 ± 0.23 (39%)
ITP (10 μM)	13.62 ± 0.47 (37%)	6.33 ± 0.18 (41%)
Naloxone (1 μM)	15.01 ± 2.47	8.14 ± 0.64

Veratrine was present at 25 μM final concentration, and morphine at 15 μM. Tabulated data are nmoles Ca^{2+} uptake/mg protein ± S.E.M. Values in parentheses are for % inhibition by morphine or morphine plus GTP on the veratrine stimulated Ca^{2+} uptake. Data were collated from 3 experiments for both brain slices and synaptosomes, each experiment involving triplicate samples for each condition.

* $P < 0.005$ compared to veratrine plus morphine alone employing Student's two-tailed *t*-test.

Table 3. 3′,5′ Cyclic AMP levels in synaptosomes: effects of depolarizing agents and morphine

		pmol/mg Protein	Increase in levels above control (%)	Inhibition of cyclic AMP response by morphine (%)
Control		111 ± 12	—	—
Morphine	15 μM	110 ± 15	0	—
Morphine	100 μM	119 ± 17	8	—
Naloxone	1 μM	114 ± 11	2	—
K^+		170 ± 16	53 ± 5	—
K^+ plus:				
Morphine	1 μM	156 ± 13	41	23
Morphine	100 μM	125 ± 14	13	76
Morphine and Naloxone	100 μM + 1 μM	166 ± 17	50	7
Veratrine 25 μM		183 ± 16	65 ± 6	—
Veratrine (25 μM) plus:				
Morphine	15 μM	142 ± 12	30	57
Morphine	100 μM	128 ± 15	15	76
Morphine and Naloxone	100 μM + 1 μM	178 ± 17	60	7
Veratrine 75 μM		200 ± 19	80	—
Veratrine (75 μM) plus:				
Morphine	15 μM	186 ± 14	68	15
Morphine	100 μM	180 ± 15	22	22
Morphine and Naloxone	100 μM + 1 μM	204 ± 18	85	0

Data was obtained from twelve samples from six experiments and represent the mean ± S.E.M.

Table 4. Total $^{32}P_i$ incorporation into cerebral tissue

		% Incorporation Brain slices N = 9	Synaptosomes N = 15
Control		100	100
GTP	10 μM	98 ± 7	—
Morphine	100 μM	96 ± 14	109 ± 11
Veratrine	75 μM	205 ± 27	270 ± 21
Veratrine (75 μM) plus:			
Morphine	15 μM	—	241 ± 14 (17%)
Morphine	100 μM	232 ± 39 (0%)	245 ± 10 (15%)
Veratrine	25 μM	140 ± 21	217 ± 17
Veratrine (25 μM) plus:			
Morphine	15 μM	129 ± 7* (27%)	166 ± 13** (44%)
Morphine	100 μM	104 ± 12** (90%)	160 ± 9** (49%)
Morphine and Naloxone	100 μM + 1 μM	146 ± 16 (0%)	223 ± 11 (0%)
GTP	10 μM	141 ± 9 (0%)	—
Morphine and GTP	15 μM + 10 μM	118 ± 10† (55%)	—
Morphine and ITP	15 μM + 10 μM	130 ± 6 (28%)	—
K^+ 56 mM			200 ± 11
K^+ plus:			
Morphine	1 μM	—	161 ± 15 (39%)
Morphine	100 μM	—	153 ± 11 (47%)

Data represent % increases in $^{32}P_i$ incorporation, and are expressed as means ± S.E.M.

Values in parentheses are % changes from veratrine-stimulated level. Data were collated from 3 experiments for brain slices and 5 experiments for synaptosomes each experiment involving triplicate samples for each condition.

The control level of $^{32}P_i$ incorporation were:

brain slices = 29987 ± 210 dpm/mg tissue wet weight;

synaptosomes = 2681 ± 50 dpm/mg protein.

* $P < 0.005$; ** $P < 0.001$, in comparison to veratrine (25 μM) alone; † = $P < 0.001$ in comparison to veratrine (25 μM) plus morphine (15 μM) employing Student's two-tailed *t*-test.

Table 5. Incorporation of $^{32}P_i$ into lipid and protein fractions of brain slices

	% Change from control levels of incorporation		
	Lipid	Protein	Response in whole slice
Veratrine	41% (32)	71% (78)	57%
Veratrine plus Morphine	31% (42)	29%* (29)	28%*
Veratrine plus Morphine + GTP	16%* (53)	11%* (47)	13%*
Veratrine plus Morphine + Naloxone	47% (36)	69% (64)	59%

Values in parentheses represent the proportion of the total response in the whole slice recovered in each fraction. The data was from different experiments as reported in Table 6. Data were collated from 3 experiments, each experiment involving triplicate samples for each condition. Veratrine was added at 25 μM, Morphine at 15 μM, GTP at 10 μM and Naloxone at 1 μM.

* $P < 0.005$ compared to veratrine alone employing Student's two-tailed *t*-test.

inhibitory actions of morphine were reversed by 1 μM naloxone (Table 2).

Depolarizing agents and 3',5' cyclic AMP levels in synaptosomes

An increase in cyclic AMP levels was observed to occur during incubation of synaptosomes with depolarizing agents. Thus, 15 μM veratrine caused an increase of 65% ± 6 and 56 mM K^+ caused a 53% ± 5 increase (Table 3). Morphine, had no significant effect on the increase (80%) in cyclic AMP levels induced by veratrine 75 μM, although the enhancement due to 25 μM veratrine *was* reduced by morphine (100 μM). Thus, cyclic AMP levels were increased by only 15% of control values by veratrine at 15 μM when morphine was present at 100 μM (i.e. 76% inhibition of response), and by only 50% when 15 μM morphine was used (Table 3). Its potency against K^+-induced cyclic AMP increases was similar (Table 3). All of these inhibitory actions of morphine were prevented by naloxone (1 μM; Table 3).

Action of morphine on the incorporation of $^{32}P_i$-inorganic phosphate into protein and lipid fractions

Incorporation into synaptosomes. Both K^+ (56 mM) and veratrine (25 μM and 75 μM) enhanced the incorporation of $^{32}P_i$ into synaptosomal proteins two- or threefold (Table 4).

When added alone, morphine (100 μM) was without significant influence on the rate of $^{32}P_i$ incorporation; however, it caused a substantial inhibition of the depolarization-evoked responses in this incorporation. Thus at 15 μM morphine the enhancement due to 75 μM veratrine was reduced by 17% whilst that caused by 25 μM veratrine was inhibited to a much greater extent (44%; $P < 0.001$; Table 4). Morphine was more potent in its action against K^+-induced increases in $^{32}P_i$ incorporation (e.g. 1 μM morphine reduced this increase by 39%).

Incorporation into brain slices. As for synaptosomes, depolarization by veratrine or K^+ increased the incorporation of $^{32}P_i$ into brain slices; 75 μM veratrine causing a 110% increase in incorporation over and above that of basal levels; this increase could be blocked by TTX. Morphine at (1 μM or 100 μM) caused no change in the phosphorylation stimulated by 75 μM veratrine. However, with veratrine at 25 μM, morphine (100 μM) caused a total inhibition of stimulated phosphorylation. Naloxone, 1 μM, was able to abolish this inhibitory effect of morphine (Table 4).

The action of D-ala^2 met^5 enkephalinamide. D-Ala^2-met^5 enkephalinamide at concentrations up to 100 μM did not show any action on veratrine-stimulated Ca^{2+} uptake by synaptosomes. In brain slices the enkephalin analogue at 100 μM decreased Ca^{2+} uptake by 100% (Table 6).

DISCUSSION

Inhibition of Ca^{2+} fluxes

Morphine was found to have no effect on either basal Ca^{2+} uptake or basal amino acid release. The drug selectively inhibited only the depolarization-induced influx of Ca^{2+} and it acted in a dose-dependent manner (Fig. 1). Some investigators have found no such effects with morphine [24] whilst others have shown inhibition by morphine of K^+-evoked uptake *in vitro* when presented at concentrations of 0.1 μM [25] or 90 μM [26]. The negative results of some groups may be attributed to the high level of stimulation they employ. In this study and previous ones [13] morphine had no effect on inhibiting the Ca^{2+} influx induced by 75 μM veratrine but could block the influx due to 25 μM veratrine. This implies that the Ca^{2+} influx induced by the higher veratrine concentration was too great to be blocked by morphine even at 100 μM.

All the effects of morphine on Ca^{2+} influx could be blocked by naloxone showing that the actions of the drug are receptor mediated. Dextrophan which is the (+) isomer of levorphanol had no effect on Ca^{2+} influx (results not shown) indicating that the stereo specificity of the binding site is an important feature in the morphine interaction.

Inhibition of neuroactive amino acid release

Morphine was found to have an inhibitory effect

Table 6. Effect of D-ala^2 met^5 enkephalinamide (DAME) on stimulated Ca^{2+} influx into cerebral tissue

Condition	Brain slices N = 9	Synaptosomes N = 12
Control	16.92 ± 1.07	3.80 ± 0.13
DAME (100 μM)	17.06 ± 6.53	3.71 ± 0.13
K^+	—	6.44 ± 0.15
K^+ plus DAME (100 μM)	—	6.39 ± 0.14
Veratrine	22.17 ± 11.41	8.21 ± 0.20
Veratrine plus DAME (100 μM)	15.97* ± 1.64 (100%)	—
DAME (15 μM)	21.98 ± 11.32 (0%)	8.36 ± 0.13 (0%)

Tabulated results are in nmoles Ca/mg protein ± S.E.M. Values in parentheses represent the % inhibition of stimulation.

Veratrine was used at 25 μM and K^+ at 56 mM. Data were collated from 3 experiments for brain slices and 4 experiments for synaptosomes, each experiment involving triplicate samples for each condition.

* $P < 0.005$ in comparison to the stimulated level (either veratrine or K^+) employing Student's *t*-test.

on neuroactive amino acid release as well as on Ca^{2+} influx; actions of morphine on acetylcholine release and biogenic amines have been previously reported [1–5].

The mechanism of the inhibition by morphine of neuroactive amino acid release induced by high K^+ could involve regulation of presynaptic Ca^{2+} transport since both are inhibited in parallel. However, in the case of veratrine, neurotransmitter release is not wholly dependent on external Ca^{2+} which suggests that morphine must also have a more direct effect on the neurotransmitter release mechanism. This may occur at an internal site by preventing the action of Ca^{2+} released from internal stores [27]. There is evidence that morphine enters neural cells [42].

Action on adenylate cyclase

Other studies have indicated that noradrenaline may inhibit amino acid neurotransmitter release through interactions at a surface receptor which may be linked to adenylate cyclase [28] and subsequently affects intracellular phosphorylation.

Since morphine also inhibited the parallel rise in cyclic AMP levels, and GTP enhanced its actions, the drug may exert some of its effects via adenylate cyclase, including a longer term effect on neurotransmitter release. The relative affinities of narcotics for their receptors and their pharmacological potencies have been reported in accordance with their effectiveness as inhibitors of adenylate cyclase [29]. It is known that there is a selective nucleotide and ion regulation of agonist interactions with opiate receptors, and Blume *et al.* [30] reported that GTP was required for receptor-directed inhibition of adenylate cyclase in mouse neuroblastoma-glioma hybrid cells. The ability of GTP and Na^+ to affect adenylate cyclase activity is mediated by the guanine nucleotide binding protein [31].

Acute administration of morphine both *in vivo* and *in vitro* apart from affecting cAMP levels also influences the level of soluble calmodulin. Delorenzo *et al.* [34, 35] have implicated calmodulin in mediating many events at the neuronal membrane. Acute morphine increases the concentration of soluble calmodulin and evidence indicates that this is due to its removal from synaptic terminal membranes by the opiate. The membrane bound enzyme, adenylate cyclase, is found as two isoenzymes in the cerebral cortex, one of which is Ca^{2+} and calmodulin sensitive [36]. Removal of calmodulin from the membrane may well decrease cyclic AMP levels by acting on this isoenzyme of adenylate cyclase. Stimulation of cyclic AMP formation by prostaglandin E, in rat brain has been shown to be inhibited by morphine [37], and the observed inhibition correlates well with both antinociceptive potency and opiate receptor binding affinity.

Inhibition of $^{32}P_i$ incorporation

Inhibition of depolarization-induced phosphorylation was observed to occur in the presence of morphine in both brain slices and synaptosomes. Since the cyclic AMP dependent protein kinase system [38, 39] is involved in the phosphorylation of synaptic proteins, a decreased level of cyclic AMP could be expected to correlate with a reduction in phosphorylation of synaptic terminal proteins. One such protein is Synapsin I [40] a major component of the synaptic vesicle cage. The protein is phosphorylated by both cyclic AMP dependent protein kinases and calcium/calmodulin-dependent protein kinases [41]. The phosphorylation of Synapsin I by the Ca^{2+} calmodulin dependent protein kinase leads to a release of the vesicle from its cage and hence making it available for exocytosis. The effect of phosphorylating Synapsin I by cyclic AMP kinases is unknown. Thus if morphine acts to decrease the activities of adenylate cyclase and calmodulin combining with Ca^{2+} to phosphorylate protein then this may be a major mechanism by which synaptic transmission is reduced by morphine.

Relative potencies of the effects of morphine

The potency of morphine was similar whether inhibiting responses induced by veratrine (25 μM) or by K^+ (56 mM). For instance, all the responses induced by both depolarizing agents were inhibited

between 24 and 25% by morphine at 1 μM in synaptosomes and by 5 μM in brain slices. The enhancement of both Ca^{2+} uptake and glutamate release by these two depolarizing agents could be inhibited by 87–94% with 100 μM morphine. However, augmentation of cyclic AMP levels and of $^{32}P_i$ incorporation in proteins and lipids showed lesser degrees of inhibition by morphine at 100 μM. Both Ca^{2+} and phosphorylation ($^{32}P_i$ incorporation) were decreased by morphine to a similar degree and both showed increased inhibition by GTP. No other nucleotide caused this enhancement indicating a possible involvement of the G-regulatory protein of adenylate cyclase.

In summary, the data presented here show that morphine influences a range of cellular processes via naloxone-sensitive receptors including Ca^{2+} entry through voltage-sensitive channels, phosphorylation processes and neurotransmitter release. Its inhibitory action on neurotransmitter release could be due to inhibition of Ca^{2+} entry during depolarization, but may also be due to interaction with surface receptors which inhibit Ca^{2+} entry during depolarization. It may also produce its inhibitory action by influencing phosphorylation of sites concerned with neurotransmitter release, either directly at an internal site or via interaction with adenylate cyclase at surface receptors, thereby inhibiting cAMP-dependent protein phosphokinases.

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