Biochemical Pharmacology, Vol. 46, No. 7, pp. 1145–1150, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

EFFECTS OF MORPHINE AND ITS METABOLITES ON OPIATE RECEPTOR BINDING, cAMP FORMATION AND [^{3}H]NORADRENALINE RELEASE FROM SH-SY5Y CELLS

D. G. LAMBERT,* R. ATCHESON, R. A. HIRST and D. J. ROWBOTHAM
University Department of Anaesthesia, Leicester Royal Infirmary, Leicester LE1 5WW, U.K.

(*Received* 4 *May* 1993; *accepted* 29 *June* 1993)

Abstract—Opiate receptor occupation leads to a variety of intracellular events including inhibition of adenylyl cyclase and cAMP formation. We have examined the opiate binding characteristics, effects on cAMP formation and [^{3}H]noradrenaline release of morphine, morphine-6 (M6G) and -3 (M3G)-glucuronides, and fentanyl in SH-SY5Y human neuroblastoma cells. M6G and M3G are the major metabolites of morphine formed *in vivo* whose cellular action remains to be fully elucidated. In binding experiments morphine (affinity, K_{50} = 96 nM) and fentanyl (K_{50} = 99 nM) were more potent than M6G (K_{50} = 393 nM), while M3G was inactive. However, for cAMP inhibition morphine (half maximum inhibition, IC_{50} = 193 nM) and M6G (IC_{50} = 113 nM) were roughly equipotent, with fentanyl (IC_{50} = 27 nM) being more potent and producing a greater maximum inhibition (56%). M3G was inactive. These *in vitro* data are in general agreement with the *in vivo* effects of these glucuronides. Moreover, all of the opiates tested failed to inhibit K^+-evoked release of [^{3}H]noradrenaline. Whilst these data do not support a role for cAMP in neurotransmitter release, alterations in cAMP formation may still have a role to play in the mechanism of analgesia.

Morphine, a naturally occurring opiate extracted from the poppy, was one of the earliest substances used to provide clinical analgesia and is still one of the commonest opioids used in clinical practice. This opioid is glucuronidated *in vivo* at carbon positions three and six to produce morphine 3-glucuronide (M3G†) and morphine 6-glucuronide (M6G) [1]. There is good evidence to suggest that M6G may possess analgesic qualities whereas M3G may antagonize both morphine and M6G analgesia [2–6]. There are few *in vitro* studies examining the biochemical effects of morphine and its glucuronides although receptor binding profiles have been described [4, 7–9]. In these studies M6G appears less potent than morphine and M3G is inactive. It is reasonable to assume that the analgesia observed with M6G is associated with this opiate receptor occupancy and any intracellular biochemical effects consequent upon the occupancy. Typically opiate receptor occupation would result in membrane hyperpolarization [10], inhibition of L- and N-type voltage-sensitive Ca^{2+} channels [11] and an inhibition of adenylyl cyclase [12], although in a recent report NG108-15 cells showed an increase in Ca^{2+} in response to δ receptor occupation [13]. These coordinated changes in membrane potential, Ca^{2+} influx and cAMP levels could potentially depress neurotransmission [14].

In this study we have examined the opiate receptor binding profiles of morphine and its glucuronides, the subsequent inhibition of cAMP formation and any effects on potassium-evoked [^{3}H]noradrenaline release in undifferentiated SH-SY5Y human neuroblastoma cells. These cells are particularly useful in such studies as they express both μ and δ (approx. 5:1) opiate receptors [15, 16] and are capable of the uptake and release of [^{3}H]noradrenaline [17]. As a comparison we have included fentanyl, a more potent opioid, structurally related to pethidine [1].

Part of this work has appeared in abstract form [18].

MATERIALS AND METHODS

Sources of reagents and miscellaneous methods. All tissue culture media, supplements, trypsin/EDTA, sera and plasticware (Nunc) were from Life Technologies (Uxbridge, U.K.). [15,16(*n*)-^{3}H]-Diprenorphine (DPN) (31.4 Ci/mmol) and L-[7,8-^{3}H]noradrenaline (41 Ci/mmol) were from Amersham (U.K.). [2,8-^{3}H]cAMP was from NEN DuPont (Boston, MA). Morphine, M6G, M3G, fentanyl, naloxone, cAMP, pargyline and ascorbic acid were from the Sigma Chemical Co. (Poole, U.K.). All other reagents were of the highest purity available. Radioactivity was measured by liquid scintillation spectrophotometry with Optiphase-X (LKB Wallac) as a scintillant.

Cell culture. Stock cultures of SH-SY5Y cells (a kind gift from Dr J. Biedler, Sloane-Kettering Institute, NY, U.S.A.), passages 69–85, were maintained in minimum essential medium supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone and 10% foetal calf serum at 37° in 5% CO_2/humidified air. Stock cultures were passaged weekly (1:3–1:6) and refed twice weekly. For receptor binding and cAMP determinations cells were

* Corresponding author. Tel. (0533) 541414 ext. 5474; FAX (0533) 854487.

† Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide; DPN, diprenorphine.

harvested by a brief exposure to HEPES (10 mM)-buffered saline containing EDTA (0.5 mM). For [^{3}H]noradrenaline release studies, cells harvested with trypsin/EDTA were plated into six well multitrays at 2.5×10^6 cells/well and allowed to grow to confluence (5–7 days).

Measurement of [^{3}H]*DPN binding.* The binding of [^{3}H]DPN to whole cell suspensions (approx. 200 μg) was performed at 37° for 60 min in Krebs/HEPES buffer pH 7.4 of the following composition (mM): Na^+ (143.3), K^+ (4.7), Ca^{2+} (2.5), Mg^{2+} (1.2), Cl^- (125.6), HCO_3^- (25), $H_2PO_4^{2-}$ (1.2), SO_4^{2-} (1.2), glucose (11.7) and HEPES (10) [19]. Non-specific binding was defined in the presence of 10 μM naloxone. Bound and free [^{3}H]DPN were separated by vacuum filtration using a Brandell cell harvester onto Whatman GF/B filters and washed with 2×4 mL aliquots of ice-cold buffer. Saturation studies (0.01–4 nM [^{3}H]DPN) and displacement studies (0.3–0.5 nM [^{3}H]DPN) were performed in 1 mL assay volumes.

Measurement of cAMP formation. Whole cell suspensions (approx. 200 μg) were incubated in microfuge tubes using Krebs/HEPES buffer containing isobutylmethylxanthine (1 mM), forskolin (10 μM) and opioids in various combinations. Incubations were performed at 37° for 15 min. The reaction was terminated by addition of 20 μL HCl (10 M), 20 μL NaOH (10 M) and 180 μL Tris (1 M) pH 7.4. cAMP in the supernatant (after centrifugation, 13,000 rpm/2 min, MSE microcentaur) was determined by a radioreceptor method [20] using a binding protein prepared from bovine adrenal cortex.

Measurement of [^{3}H]*noradrenaline release.* [^{3}H]-Noradrenaline release was measured using a modification of the method described by Murphy *et al.* [17]. Briefly, SH-SY5Y cells grown in six well multitrays were loaded with 0.5 mL [^{3}H]-noradrenaline (48 nM) for 1 hr at 37° in Krebs/HEPES buffer supplemented with pargyline (0.2 mM) and ascorbic acid (0.2 mM). After this loading period excess [^{3}H]noradrenaline not taken up was removed by 3×15 min $\times$ 1 mL washes with fresh buffer. Cells were then exposed to 1 mL high potassium buffer (100 mM with Na^+ adjustment to maintain tonicity) for 3 min. Opioids were preincubated by inclusion in the second and third wash period and were present during stimulation. Naloxone was added from the first wash onwards. [^{3}H]Noradrenaline released was removed and that remaining in the monolayer was extracted with perchloric acid (0.4 M). [^{3}H]Noradrenaline release was expressed as percentage of total content.

Data analysis. Saturation isotherms were analysed according to Scatchard [21] to yield the equilibrium dissociation constant (K_d) and the maximum binding capacity (B_{max}). IC_{50} values (half maximum displacement) were obtained by computer-assisted curve fitting using ALLFIT [22]. IC_{50} values were corrected for competing mass of ligand to yield K_{50} values according to Cheng and Prusoff [23]. Full dose–response curves were analysed statistically by ANOVA and where appropriate IC_{50}/K_{50} values and degrees of inhibition were then compared using Student's unpaired *t*-test and considered significant when $P < 0.05$.

RESULTS

The specific binding of [^{3}H]DPN to whole SH-

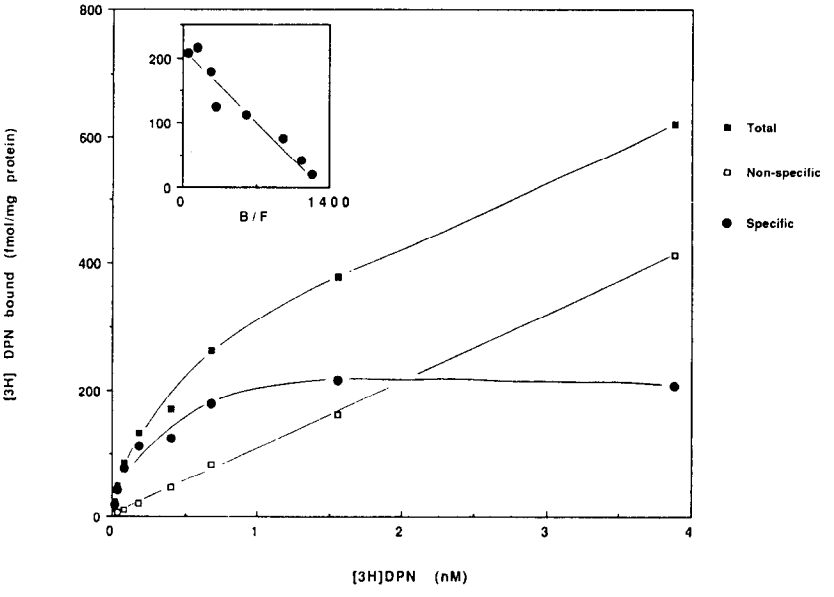

Fig. 1. Dose-dependent and saturable binding of [^{3}H]DPN to whole SH-SY5Y cells. Insert shows Scatchard transformation. Studies were performed at 37° in 1 mL vol. for 60 min. Non-specific binding was defined in the presence of 10 μM naloxone. Data are from a single representative experiment from N = 4.

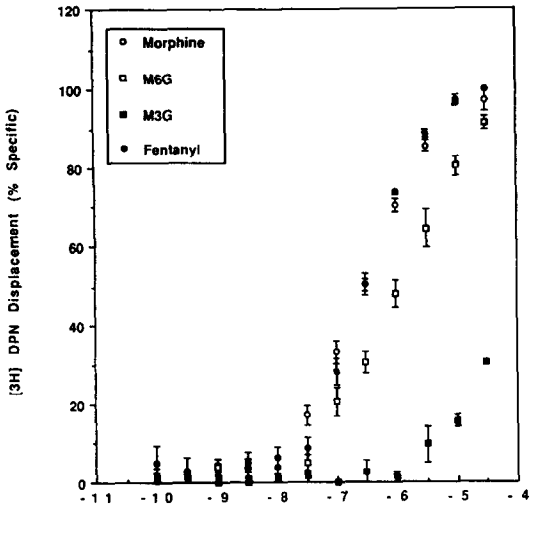

Fig. 2. Inhibition of specific [^{3}H]DPN binding to whole SH-SY5Y cells by morphine, M6G, M3G and fentanyl. Studies were performed at 37° in 1 mL vol. for 60 min. Non-specific binding was defined in the presence of 10 μM naloxone. Data are means ± SEM N = 5, except M3G where N = 3. Computer analysis of the curves to yield K_{50} is shown in Table 1.

SY5Y cell suspensions was dose-related and saturable (Fig. 1). Scatchard analysis revealed B_{max} and K_d values of 167 ± 26 fmol/mg protein and 0.17 ± 0.01 nM (N = 4), respectively. Nonspecific binding at the radioligand K_d was < 25% of the specific bound ligand. [^{3}H]DPN binding was displaced dose-dependently by morphine, M6G and fentanyl ($P < 0.05$, ANOVA); significant displacement by M3G was not observed until the concentration exceeded 10 μM (Fig. 2). The rank order potency (K_{50}) for displacement was: morphine = fentanyl > M6G >>> M3G (Table 1). Binding slope factors were 0.71 ± 0.05, 0.94 ± 0.05 and 0.69 ± 0.03 for morphine, fentanyl and M6G, respectively.

Basal and forskolin (10 μM)-stimulated cAMP levels in whole cell suspensions were 10.6 ± 2.8 and 177 ± 12.7 pmol/mg protein, respectively, and represented a 17-fold increase over basal. Forskolin-stimulated cAMP formation was inhibited dose-dependently by morphine, M6G and fentanyl ($P < 0.05$, ANOVA); M3G was inactive up to 10 μM (Fig. 3). The rank order potency (IC_{50}) for cAMP inhibition was fentanyl > morphine ≈ M6G >>> M3G (Table 1). Fentanyl also produced a greater maximum inhibition (approx. 2-fold) than either morphine or M6G (Table 1).

In agreement with the previous study of Murphy *et al.* [17] SH-SY5Y cells are clearly capable of the uptake and release of [^{3}H]noradrenaline. After 60 min loading, uptake accounted for around 15% of the total added [^{3}H]noradrenaline. This is also consistent with our previous work examining the effects of fentanyl on [^{3}H]noradrenaline uptake [19]. A 3 min exposure of the monolayer to 100 mM K^+ increased basal release from 3.6 ± 0.2% (N = 21) to 11.7 ± 0.6% (N = 21) amounting to a 3-fold stimulation. Because of variation in maximum response observed all data are normalized to the K^+ challenge (= 100%). Only fentanyl (10 μM) produced a significant inhibition of [^{3}H]noradrenaline release but this response was not reversed by 10 μM naloxone (Fig. 4).

DISCUSSION

Metabolism of morphine *in vivo* produces two glucuronides: M3G and M6G [1], with levels of the former generally peaking before the latter and reaching higher peak concentrations [24]. Peak serum concentrations seen in healthy volunteers are increased in a variety of disease states [25–27] but are consistent with those used in this study. Plasma levels of fentanyl vary considerably [1] and values of up to 0.6 μM have been reported in cardiopulmonary bypass [28, 29].

Generally, drug metabolism (e.g. glucuronidation) is described as a means of termination of bioactivity [2]. However, M6G retains significant bioactivity as shown in this study. *In vivo* studies show, in general, that M6G is more potent or equipotent with morphine as an analgesic (differences appear related to the route of administration) and M3G is inactive [1, 3–6]. However, there is evidence to support an anti-analgesic role for this latter glucuronide, where

Table 1. Effects or morphine, M6G, M3G and fentanyl on opiate receptor binding and cAMP formation in undifferentiated SH-SY5Y cells

Opioid	Binding K_{50} (nM)	cAMP IC_{50} (nM)	cAMP Maximum inhibition (%)
Morphine	95.9 ± 5.2	192.7 ± 57.8	28.8 ± 4.1
M6G	393.3 ± 95.8*	112.8 ± 40.4	29.1 ± 2.7
M3G	>30,000	>30,000	—
Fentanyl	98.7 ± 5.2	26.9 ± 7.7†	56.3 ± 5.3‡

Data are means ± SEM, N = 5–8, except M3G binding, where N = 3.
$P < 0.05$, *weaker than morphine, †more potent than morphine or M6G, ‡greater than morphine or M6G.

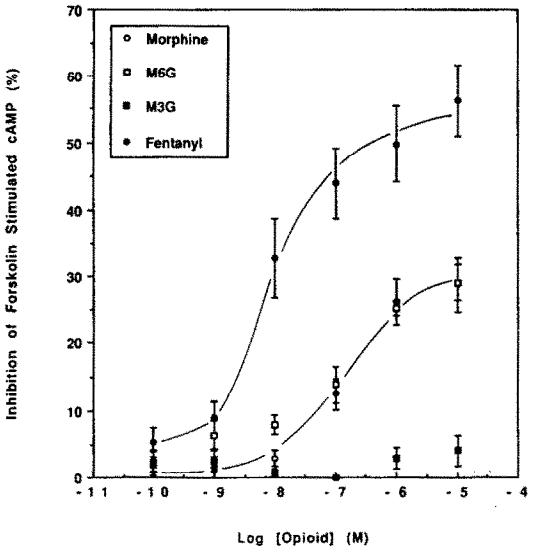

Fig. 3. Effects of morphine, M6G, M3G and fentanyl on forskolin-stimulated cAMP formation. Studies were performed at 37° in 0.3 mL vol. for 15 min containing forskolin (10 μM), isobutylmethylxanthine (1 mM) and opioids at the indicated concentrations. Data are means ± SEM N = 6–8. Computer analysis of the curves to yield IC_{50} and maximum inhibition are shown in Table 1.

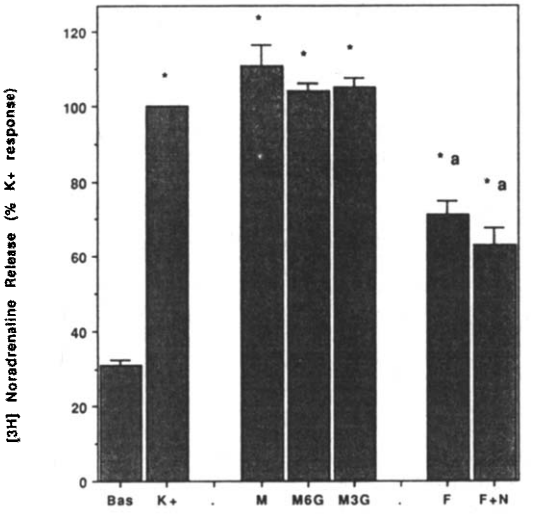

Fig. 4. Effects of morphine, M6G, M3G and fentanyl (F), all 10 μM, on K^+-evoked [^{3}H]noradrenaline release from SH-SY5Y cells. Cells were incubated for 3 min with either basal (Bas) buffer or 100 mM K^+ in the presence or absence of the opiates as indicated. N, 10 μM naloxone. Data are means ± SEM N = 4–21 and are normalized to the K^+ stimulus (=100%). Absolute values for basal and K^+-stimulated release were 3.6 ± 0.2% (range 1.4–5.3) and 11.6 ± 0.6% (range 6.2–16.2) of content, respectively. $P < 0.05$, *Increased compared with basal, [a]reduced compared with K^+.

M3G antagonized morphine- and M6G-induced analgesia (measured as tail flick latencies in rats) [6]. The *in vivo* characteristics of morphine and its glucuronides described above are somewhat contradictory to data from *in vitro* studies. Firstly, M3G is generally inactive at the μ and δ receptors [7, 9]. This inactivity implicates a non-opioid receptor site of action in the anti-analgesic effects reported for this glucuronide. Secondly, M6G varies from being less than 2-fold to a maximum of 7-fold more potent than morphine [4, 7–9]. In this study M3G was inactive and M6G was 4-fold weaker than morphine in binding experiments but roughly equipotent for cAMP inhibition. The use of [^{3}H]-DPN, a relatively non-selective ligand, would give little information regarding any μ and δ selective effects of the opiates used in this study. However, it is unlikely that a significant proportion of the binding would be to δ receptors since the small selectivity difference of DPN for μ over δ receptors (approx. 5-fold [30]) and the expression of 5-fold less δ than μ receptors implies that probably <10% of [^{3}H]DPN in displacement studies would be bound to δ receptors. Furthermore, morphine, M6G and fentanyl show 35-, 20- [7] and 118-fold [31] μ selectivity. In addition, the Hill slope of <1 for morphine and M6G could not be explained in terms of multiple receptor subtypes. A more likely explanation relates to the G-protein coupling in that the opiates used here are all agonists. Further experiments in membrane preparations will be required to determine the relative proportion of G-protein-coupled (high affinity) and free (low affinity) receptors.

There have been a number of studies describing the effects of opiate peptides on cAMP formation in SH-SY5Y cells and the involvement of a pertussis toxin-sensitive G-protein (G_i/G_o) in this response has been demonstrated (Ref. 32 and Lambert DG, unpublished). In addition μ receptor occupation in SH-SY5Y cells produces greater inhibition of cAMP formation than δ receptor occupancy [16]. In a recent, elegant study, Laugwitz *et al.* [33] reported that activated μ and δ opioid receptors on SH-SY5Y cells coupled preferentially to separate pertussis toxin-sensitive G_i G-proteins, with the μ receptor coupling to G_{i3} and the δ receptor coupling to G_{i1}. Whether this differential coupling is the underlying basis of the differences in maximum inhibition of cAMP formation is unclear. However, it should also be remembered that SH-SY5Y cells express around 5-fold more μ than δ receptors [15, 16]. Also, in agreement with many other studies in a variety of other tissues, opiate receptor-mediated inhibition of cAMP rarely exceeded 50% [12]. However, the lower maximum inhibition of cAMP formation produced by morphine (and M6G) relative to fentanyl can be explained by the partial agonist nature of morphine (and presumably M6G) relative to fentanyl [31].

Inhibition of neurotransmitter release is thought to be the underlying cellular mechanism of analgesia [14]. Opiates inhibit the release of a variety of neurotransmitters including noradrenaline, dopamine and acetylcholine [34, 35]. Yet, in this study fentanyl (via the opiate receptor, i.e. naloxone

reversible), morphine and its metabolites were unable to inhibit K^+-evoked release of [^{3}H]-noradrenaline. K^+-evoked release of [^{3}H]noradrenaline in these cells is dependent on extracellular Ca^{2+} (Ref. 17 and Atcheson R, unpublished). Whilst the link between inhibition of cAMP formation and inhibition of neurotransmitter release is tenuous [36, 37], opiate receptor-mediated closing of voltage-sensitive Ca^{2+} channels has been demonstrated [11] and may be important in the control of neurotransmitter release. Indeed, in differentiated SH-SY5Y cells Seward *et al.* [38] showed a closing of N-type voltage-sensitive Ca^{2+} channels via a pertussis toxin-sensitive G-protein [38]. Interestingly and in parallel with the inhibition of cAMP formation, maximum inhibition of the Ca^{2+} current was less than 50%. This information makes our data more difficult to explain unless the opioid receptor and Ca^{2+} channel (opened by K^+) are poorly coupled, but this seems unlikely. Furthermore, in NG108-15 cells which express δ opioid receptors, an increase in intracellular Ca^{2+} has been reported in Indo-1-loaded cells. The δ agonist [D-Ala^5, D-Leu^5]-enkephalin produced a transient increase in Ca^{2+} that was dependent on extracellular Ca^{2+} and blocked by nitrendipine suggesting an excitatory effect of opiates on dihydropyridine-blockable voltage-sensitive Ca^{2+} channels in these cells [13].

In conclusion, we have examined the binding profiles for morphine, M6G, M3G and fentanyl, and have shown inhibition of cAMP formation with morphine, M6G and fentanyl, whose bioactivity is in agreement with the analgesic effects of these compounds. However, all of the opiates used were unable to reduce (via the opiate receptor) K^+-evoked [^{3}H]noradrenaline release. Whilst clearly not involved directly in [^{3}H]noradrenaline release in this preparation, alterations in neuronal cAMP formation, and the subsequent alterations in protein phosphorylation [39], may be an important facet of the cellular basis of analgesia.

Acknowledgement—R. Atcheson is a *British Journal of Anaesthesia* research fellow.

REFERENCES

1. Hug CC, Pharmacokinetics and dynamics of narcotic analgesics. In: *Pharmacokinetics of Anaesthesia* (Eds. Prys-Roberts C and Hug CC), pp. 187–234. Blackwell Scientific Publications, Oxford, 1984.
2. Mulder GJ, Pharmacological effects of drug conjugates: is morphine 6-glucuronide an exception? *Trends Pharmacol Sci* **13**: 302–304, 1992.
3. Hanna MH, Peat SJ, Woodham M, Knibb A and Fung C, Analgesic efficacy and CSF pharmacokinetics of intrathecal morphine-6-glucuronide: comparison with morphine. *Br J Anaesth* **64**: 547–550, 1990.
4. Abbott FV and Palmour RM, Morphine-6-glucuronide: analgesic effects and receptor binding profile in rats. *Life Sci* **43**: 1685–1695, 1988.
5. Gong Q-L, Hedner T, Hedner J, Bjorkman R and Nordberg G, Antinociceptive and ventilatory effects of the morphine metabolites: morphine-6-glucuronide and morphine-3-glucuronide. *Eur J Pharmacol* **193**: 47–56, 1991.
6. Smith MT, Watt JA and Cramond T, Morphine-3-glucuronide—a potent antagonist of morphine analgesia. *Life Sci* **47**: 579–585, 1990.
7. Pasternak GW, Bodnar RJ, Clark JA and Inturrisi CE, Morphine-6-glucuronide, a potent mu agonist. *Life Sci* **41**: 2845–2849, 1987.
8. Paul D, Standifer KM, Intirrisi CE and Pasternak GW, Pharmacological characterization of morphine-6β-glucuronide, a very potent morphine metabolite. *J Pharmacol Exp Ther* **251**: 447–483, 1989.
9. Christensen CB and Jorgensen LN, Morphine-6-glucuronide has high affinity for the opioid receptor. *Pharmacol Toxicol* **60**: 75–76, 1987.
10. North RA, Williams JT, Surprenant A and Christie MJ, Mu and delta receptors belong to a family of receptors that are coupled to potassium channels. *Proc Natl Acad Sci USA* **84**: 5487–5491, 1987.
11. Porzig H, Pharmacological modulation of voltage-dependent calcium channels in intact cells. *Rev Physiol Biochem Pharmacol* **114**: 209–262, 1990.
12. Childers SR, Opioid receptor-coupled second messenger systems. *Life Sci* **48**: 1991–2003, 1991.
13. Jin W, Lee NM, Loh HH and Thayer SA, Dual excitatory and inhibitory effects of opioids on intracellular calcium in neuroblastoma X glioma hybrid NG108-15 cells. *Mol Pharmacol* **42**: 1083–1089, 1993.
14. Leslie FM, Methods used for the study of opioid receptors. *Pharmacol Rev* **39**: 197–249, 1987.
15. Yu VC, Richards ML and Sadee W, A human neuroblastoma cell line expresses mu and delta opioid receptor sites. *J Biol Chem* **261**: 1065–1070, 1986.
16. Kazmi SMI and Mishra RK, Comparative pharmacological properties and functional coupling of mu and delta opioid receptor sites in human neuroblastoma SH-SY5Y cells. *Mol Pharmacol* **32**: 109–118, 1987.
17. Murphy NP, Ball SG and Vaughan PFT, Potassium and carbachol-evoked release of [^{3}H]noradrenaline from human neuroblastoma cells, SH-SY5Y. *J Neurochem* **56**: 1810–1815, 1991.
18. Lambert DG and Hirst RA, Effects of morphine and its metabolites on opiate receptor binding and cyclic adenosine-3′,5′-monophosphate (cAMP) formation in human neuroblastoma cells. *Br J Anaesth* **70**: 475P–476P, 1993.
19. Atcheson R, Rowbotham DJ and Lambert DG, Fentanyl inhibits the uptake of [^{3}H]noradrenaline in cultured neuronal cells. *Br J Anaesth* **71**: 540–543, 1993.
20. Brown BL, Albano JDM, Ekins RP and Sgherzi AM, A simple and sensitive saturation assay method for the measurement of adenosine 3′:5′-cyclic monophosphate. *Biochem J* **121**: 561–562, 1971.
21. Scatchard G, The attraction of proteins for small molecules and ions. *Ann NY Acad Sci* **51**: 660–672, 1949.
22. De Lean A, Munson PJ and Rodbard D, Simultaneous analysis of families of sigmoidal curves: application to bioassay radioligand assay and physiological dose-response curves. *J Physiol* **235**: E97–E102, 1978.
23. Cheng YC and Prusoff WH, Relationship between the inhibitor constant (K_i) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**: 3099–3108, 1973.
24. Osborne R, Joel S, Trew D and Slevin M, Morphine and metabolite behaviour after different routes of morphine administration: demonstration of the importance of the active metabolite morphine-6-glucuronide. *Clin Pharmacol Ther* **47**: 12–19, 1990.
25. Sawe J and Cederlof IO, Kinetics of morphine in patients with renal failure. *Eur J Clin Pharmacol* **32**: 377–382, 1987.
26. Sear JW, Hand CW, Moore RA and McQuay HJ,

Studies on morphine disposition: influence of renal failure on the kinetics of morphine and its metabolites. *Br J Anaesth* **62**: 28–32, 1989.
27. Sear JW, Hand CW, Moore RA and McQuay HJ, Studies on morphine disposition: influence of general anaesthesia on plasma concentrations of morphine and its metabolites. *Br J Anaesth* **62**: 22–27, 1989.
28. McClain DA and Hug CC, Intravenous fentanyl kinetics. *Clin Pharmacol Ther* **28**: 106–114, 1980.
29. Bovill JG and Sebel PS, Pharmacokinetics of high-dose fentanyl. A study in patients undergoing cardiac surgery. *Br J Anaesth* **52**: 795–801, 1980.
30. Traynor JR, Corbett AD and Kosterlitz HW, Diprenorphine has agonist activity at opioid k-receptors in the myenteric plexus of the guinea pig. *Eur J Pharmacol* **137**: 85–89, 1987.
31. Maloteaux JM, Octave JN, Laterre EC and Laduron PM, Down-regulation of ^{3}H-lofentanyl binding to opiate receptors in different cultured neuronal cells. *Naunyn Schmiedebergs Arch Pharmacol* **336**: 192–199, 1989.
32. Yu VC and Sadee W, Efficacy and tolerance of narcotic analgesics at the mu opioid receptor in differentiated human neuroblastoma cells. *J Pharmacol Exp Ther* **245**: 350–355, 1988.
33. Laugwitz K-L, Offermanns S, Spicher K and Schultz, Mu and delta opioid receptors differentially couple to G protein subtypes in membranes of human neuroblastoma SH-SY5Y cells. *Neuron* **10**: 233–242, 1993.
34. Schoffelmeer ANM, Rice KC, Jacobson AE, Van-Gelderen JG, Hogenboom F, Heijna MH and Mulder AH, Mu, delta and kappa opioid receptor-mediated inhibition of neurotransmitter release and adenylate cyclase activity in rat brain slices: studies with fentanyl isothiocyanate. *Eur J Pharmacol* **154**: 169–178, 1988.
35. Lapchak PA, Araujo DM and Collier B, Regulation of endogenous acetylcholine release from mammalian brain slices by opiate receptors: hippocampus, striatum and cerebral cortex of guinea pig and rat. *Neuroscience* **31**: 313–325, 1989.
36. Mulder AH, Schoffelmeer ANM and Stoof J, On the role of adenylate cyclase in presynaptic modulation of neurotransmitter release mediated by monoamine and opioid receptors in the brain. *Ann NY Acad Sci* **604**: 237–249, 1991.
37. Heijna MH, Hogenboom F, Mulder AH and Schoffelmeer ANM, Opioid receptor-mediated inhibition of ^{3}H-dopamine and ^{14}C-acetylcholine release from rat nucleus accumbens slices. *Naunyn Schmiedebergs Arch Pharmacol* **345**: 627–632, 1992.
38. Seward E, Hammond C and Henderson G, Mu-opioid-receptor-mediated inhibition of the N-type calcium-channel current. *Proc R Soc Lond [Biol]* **244**: 129–135, 1991.
39. Fleming LM, Ponjee G and Childers SR, Inhibition of protein phosphorylation by opioid-inhibited adenylyl cyclase in rat brain membrane. *J Pharmacol Exp Ther* **260**: 1416–1424, 1992.