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Short communication

Tyr-D-Arg^2-Phe-sarcosine^4 activates phospholipase C-coupled μ_2-opioid receptors in SH-SY5Y cells

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Abstract

The dermorphin analogue Tyr-D-Arg^2-Phe-sarcosine^4 acts as a μ_1-opioid receptor agonist, but as a μ_2-opioid receptor antagonist, in vivo, yet the biochemical effects of Tyr-D-Arg^2-Phe-sarcosine^4 are unknown. Therefore, we characterized the effects of Tyr-D-Arg^2-Phe-sarcosine^4 on the μ-opioid receptor-mediated stimulation of inositol(1,4,5)trisphosphate, and inhibition of cAMP, in SH-SY5Y cells. We report here for the first time that Tyr-D-Arg^2-Phe-sarcosine^4 has no effect on basal cAMP or inositol(1,4,5)trisphosphate formation, but reversed the effects of fentanyl on these second messengers, consistent with Tyr-D-Arg^2-Phe-sarcosine^4 acting as a μ_2-opioid receptor antagonist, and confirming that the μ-opioid receptors in SH-SY5Y cells are of the μ_2 subtype.

Keywords: μ-Opioid receptor; SH-SY5Y cell; Inositol(1,4,5)trisphosphate; Phospholipase C; Tyr-D-Arg^2-Phe-sarcosine^4 (TAPS)

1. Introduction

There is strong biochemical and pharmacological evidence supporting the existence of μ-opioid receptor subtypes (Pasternak, 1993; Elliott et al., 1994). The major subdivision of μ-opioid receptors, into μ_1 and μ_2 subtypes, is based on the binding of the μ-opioid receptor antagonist naloxonazine, which binds irreversibly to μ_1-, but reversibly to μ_2-, opioid receptors (Pasternak, 1993; Elliott et al., 1994). Furthermore, use of naloxonazine in vivo suggests that these receptor subtypes have different physiological roles, with μ_1-opioid receptors mediating supraspinal analgesia, whilst μ_2-opioid receptors mediate respiratory depression (Pasternak, 1993).

SH-SY5Y human neuroblastoma cells express endogenous μ-opioid receptors (Smart et al., 1994), which we have demonstrated bind naloxonazine reversibly, indicating that these receptors are of the μ_2-opioid receptor subtype (Elliott et al., 1994). We have also recently reported that occupation of these receptors opens L-type voltage-sensitive Ca^{2+} channels via a pertussis toxin-sensitive G-protein, thus, allowing Ca^{2+} influx to activate phospholipase C, and stimulate inositol(1,4,5)trisphosphate formation (Smart et al., 1994, 1995; Smart and Lambert, 1995). Others have reported that δ-opioid receptor agonists also activate L-type voltage-sensitive Ca^{2+} channels via a pertussis toxin-sensitive G-protein in NG108-15 (Jin et al., 1992) and ND8-47 (Tang et al., 1994) cells, but whether this involves depolarization of the cell remains controversial.

Tyr-D-Arg^2-Phe-sarcosine^4 is a dermorphin analogue with a high selectivity for μ-opioid receptors, which causes naloxone-reversible analgesia, whilst inhibiting opiate-induced respiratory depression in rats, suggesting Tyr-D-Arg^2-Phe-sarcosine^4 acts as an agonist at μ_1-, but as an antagonist at μ_2-, opioid receptors (Paakkari et al., 1993). However, no studies of the biochemical effects of Tyr-D-Arg^2-Phe-sarcosine^4 have been reported.

Therefore, the present study was undertaken to characterize the effect of Tyr-D-Arg^2-Phe-sarcosine^4 on the μ-opioid receptor-mediated stimulation of inositol(1,4,5)trisphosphate, and inhibition of cAMP, formation in SH-SY5Y cells. In addition, we also examined the role of depolarization in the μ-opioid-induced inositol(1,4,5)trisphosphate response. We report here that Tyr-D-Arg^2-Phe-sarcosine^4 had no effect on basal inositol(1,4,5)trisphosphate or cAMP formation, but reverses the effects of fentanyl on these second messengers in SH-SY5Y cells, providing further evidence that these receptors are of the μ_2-opioid receptor subtype, and representing the first use of Tyr-D-Arg^2-Phe-sarcosine^4 in biochemical studies.

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2. Materials and methods

2.1. Cell culture and harvesting

SH-SY5Y human neuroblastoma cells (passages 70–85) were cultured in minimum essential medium with Earle's salts, supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone and 10% foetal calf serum (GIBCO, UK).

Cells were harvested with 10 mM HEPES-buffered saline/0.02% EDTA, pH 7.4, washed twice with, and then resuspended in, Krebs/HEPES buffer, pH 7.4, of the following composition, in mM, unless stated otherwise below: Na^+, 143.3; K^+, 4.7; Ca^{2+}, 2.5; Mg^{2+}, 1.2; Cl^-, 125.6; $H_2PO_4^-$, 1.2; SO_4^{2-}, 1.2; glucose, 11.7; and HEPES, 10. For Na^+-free experiments, Na^+ was replaced with choline.

2.2. Measurement of inositol(1,4,5)trisphosphate

Whole-cell suspensions (final volume 0.3 ml) were preincubated at 37°C in Na^+-containing or Na^+-free buffer, with or without Tyr-D-Arg^2-Phe-sarcosine^4 (1 nM–10 μM) for 15 min. The cells were then incubated with fentanyl (0.1 μM) or Tyr-D-Arg^2-Phe-sarcosine^4 (1 μM) for 0–300 s. Reactions were terminated by the addition of 0.3 ml 1 M trichloroacetic acid.

Inositol(1,4,5)trisphosphate was extracted with Freon/octylamine (1 : 1, v/v) and neutralized with 25 mM $NaCO_3$. Inositol(1,4,5)trisphosphate was assayed, as described previously (Smart et al., 1994), using a bovine adrenocortical binding protein and [^{3}H]inositol(1,4,5)trisphosphate (37 Ci/mol; Amersham, UK) at 4°C. Authentic inositol(1,4,5)trisphosphate (0.036–12 pmol; Siemat, UK) in buffer, taken through an identical extraction process, was used as a standard. Non-specific binding was defined in the presence of excess inositol(1,4,5)trisphosphate (0.3 nmol).

2.3. Measurement of cAMP

Whole-cell suspensions (0.3 ml) were preincubated at 37°C in the presence of isobutylmethylxanthine (1 mM), with or without Tyr-D-Arg^2-Phe-sarcosine^4 (1 μM), for 15 min. The cells were then incubated with fentanyl (1 μM) or Tyr-D-Arg^2-Phe-sarcosine^4 (1 μM) for 0–300 s. Reactions were terminated by the addition of 20 μl HCl (10 M), 20 μl NaOH (10 M) and 180 μl Tris (1 M, pH 7.5). The concentration of cAMP was measured in the supernatants using a specific radioreceptor assay, as described previously (Smart et al., 1995).

2.4. Data analysis

All data are given as mean ± S.E.M. unless otherwise stated. Half-maximal inhibition (IC_{50}) values were obtained by computer-assisted curve-fitting (non-linear regression model) using GRAPHPAD-PRISM. Statistical comparisons were made where appropriate by analysis of variance (ANOVA) and/or Student's *t*-test, and considered significant when $P < 0.05$.

3. Results

Fentanyl (0.1 μM) caused a monophasic increase in inositol(1,4,5)trisphosphate formation, which peaked (24.9 ± 1.9 pmol/mg protein, $n = 5$) at 15 s, and returned to basal levels (8.2 ± 0.9 pmol/mg protein, $n = 5$) between 1 and 2 min (Fig. 1). In contrast, Tyr-D-Arg^2-Phe-sarcosine^4 (1 μM) had no effect on basal inositol(1,4,5)trisphosphate formation (Fig. 1), but dose-dependently inhibited the fentanyl-induced inositol(1,4,5)trisphosphate response (Fig. 1, lower panel), with an IC_{50} of 16 nM. The fentanyl (0.1 μM)-induced inositol(1,4,5)trisphosphate response did not involve depolarization of the cell, as replacement of Na^+

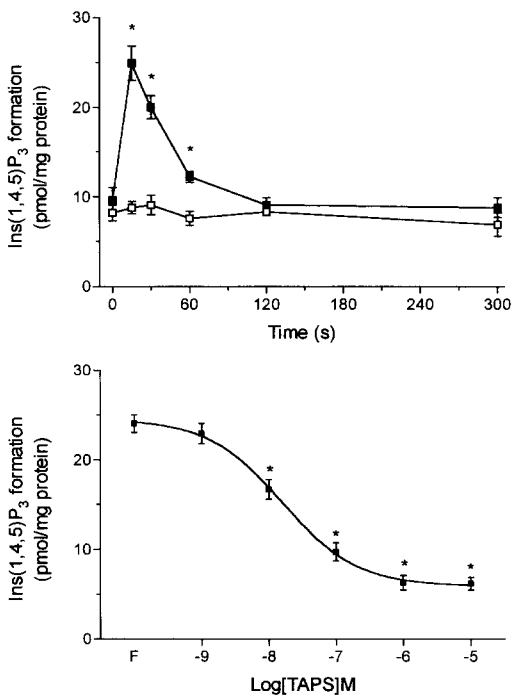

Fig. 1. Fentanyl, but not Tyr-D-Arg^2-Phe-sarcosine^4, stimulates inositol(1,4,5)trisphosphate formation in SH-SY5Y cells. Upper panel depicts inositol(1,4,5)trisphosphate formation following fentanyl (■) or Tyr-D-Arg^2-Phe-sarcosine^4 (□). Whole-cell suspensions (0.3 ml) were preincubated at 37°C for 15 min, and then incubated with fentanyl (0.1 μM) or Tyr-D-Arg^2-Phe-sarcosine^4 (1 μM) for 0–300 s. Data are mean ± S.E.M., where $n = 4$–5. All fentanyl time courses are $P < 0.05$ by ANOVA. * $P < 0.05$ increased compared with basal. Lower panel depicts the dose-dependent inhibition of fentanyl-induced inositol(1,4,5)trisphosphate formation with Tyr-D-Arg^2-Phe-sarcosine^4. Whole-cell suspensions (0.3 ml) were preincubated at 37°C with Tyr-D-Arg^2-Phe-sarcosine^4 (1 nM–10 μM) for 15 min, and then incubated with fentanyl (0.1 μM) for 15 s. Data are mean ± S.E.M., where $n = 5$. Dose-response curve is $P < 0.05$ by ANOVA. * $P < 0.05$ decreased compared to fentanyl alone.

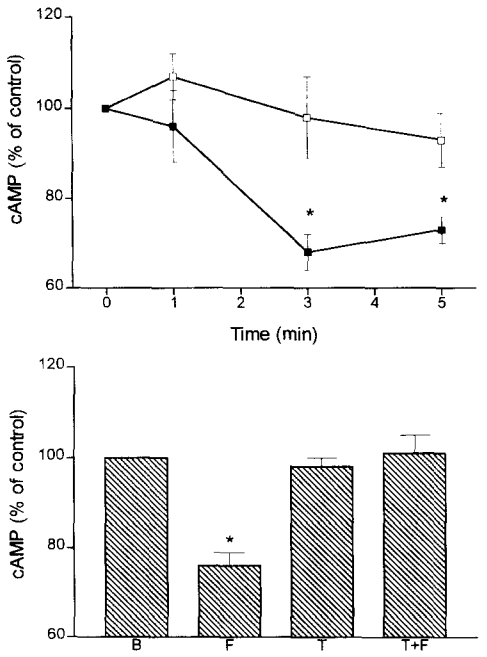

Fig. 2. The effect of Tyr-D-Arg^2-Phe-sarcosine^4 and fentanyl on cAMP formation in SH-SY5Y cells. Upper panel depicts basal cAMP formation following fentanyl (■) or Tyr-D-Arg^2-Phe-sarcosine^4 (□). Lower panel depicts effects of fentanyl (F), Tyr-D-Arg^2-Phe-sarcosine^4 (T), and fentanyl + Tyr-D-Arg^2-Phe-sarcosine^4 (T + F) on basal (B) cAMP formation at 5 min. Whole-cell suspensions (0.3 ml) were preincubated with or without Tyr-D-Arg^2-Phe-sarcosine^4 (1 μM) at 37°C for 15 min and then incubated with fentanyl (1 μM) or Tyr-D-Arg^2-Phe-sarcosine^4 (1 μM) for 0–300 s. Data are normalized to paired basal (= 100%) and are given as mean ± S.E.M., where $n = 4$. * $P < 0.05$ decreased compared to basal.

with choline was without effect on the peak response (with $Na^+ = 24.1 \pm 2.6$, without $Na^+ = 23.6 \pm 1.9$ pmol/mg protein, $n = 4$).

Fentanyl (1 μM) inhibited basal cAMP formation (Fig. 2), as previously reported (Smart et al., 1995), but it is worth noting that basal cAMP formation varied considerably (21.0–45.8 pmol/mg protein) between batches of cells, and therefore the data have been expressed relative to the appropriate paired basal. The inhibition of basal cAMP by fentanyl was maximal (32%) within 3 min, and was maintained as long as the agonist remained on the receptor (Fig. 2). However, Tyr-D-Arg^2-Phe-sarcosine^4 (1 μM) had no effect on basal cAMP formation, but abolished the fentanyl-induced inhibition (Fig. 2).

4. Discussion

We describe here for the first time the biochemical effects of Tyr-D-Arg^2-Phe-sarcosine^4, consistent with this agent acting as a μ_2-opioid receptor antagonist. Fentanyl caused a transient, monophasic increase in inositol(1,4,5)trisphosphate formation, as previously reported (Smart et al., 1994, 1995; Smart and Lambert, 1995). However, Tyr-D-Arg^2-Phe-sarcosine^4 had no effect on basal, whilst dose-dependently inhibiting fentanyl-induced, inositol(1,4,5)trisphosphate formation. Similarly, fentanyl inhibited basal cAMP formation, as previously reported (Smart et al., 1995), whilst Tyr-D-Arg^2-Phe-sarcosine^4 had no effect on basal, but antagonized the fentanyl-induced inhibition of, adenylyl cyclase. We have previously demonstrated that both the fentanyl-induced activation of phospholipase C (Smart et al., 1994) and inhibition of cAMP formation (Lambert et al., 1993) are μ-opioid receptor mediated, and others have reported that Tyr-D-Arg^2-Phe-sarcosine^4 acts as an antagonist at μ_2-, but as an agonist at μ_1-, opioid receptors (Paakkari et al., 1993; Kim and Cox, 1993). Therefore, the current data suggest that SH-SY5Y cells express μ_2-opioid receptors. Indeed, we have previously reported that the μ-opioid receptors in these cells reversibly bind naloxonazine (Elliott et al., 1994), again consistent with μ_2-opioid receptor subtype expression. Therefore, it appears that Tyr-D-Arg^2-Phe-sarcosine^4, as well as naloxonazine (Pasternak, 1993), can differentiate between μ_1- and μ_2-opioid receptors, and so is both a useful pharmacological tool, and may be of potential clinical interest, particularly with reference to the reversal of opioid-induced respiratory depression.

The μ-opioid receptor has been cloned from rats and humans, but only one μ-opioid receptor clone has been identified for each species (Knapp et al., 1995). This clone apparently corresponds to the μ_1-opioid receptor subtype, based on its affinity for naloxonazine (Raynor et al., 1995), although it should be emphasized that in these studies reversability of the binding was not examined. These data suggest that the different μ-opioid receptor subtypes do not arise from separate genes, but may occur via alternative splicing of the same gene or gene product. Indeed, alternative splice variants of the μ-opioid clone do occur (Bare et al., 1994; Rossi et al., 1995), including one which has recently been putatively identified as the μ_2-opioid receptor subtype (Rossi et al., 1995). Moreover, in SH-SY5Y's parent cell-line, SK-N-SH, two splice variants of the μ-opioid receptor are expressed (Bare et al., 1994), and opioids have a dual excitatory/inhibitory effect on noradrenaline release in these cells (Keren et al., 1994). Nevertheless, in SH-SY5Y cells fentanyl has no direct effect on noradrenaline release (Smart et al., 1994).

The fentanyl-induced inositol(1,4,5)trisphosphate response is dependent on the μ-opioid receptor-mediated opening of L-type voltage-sensitive Ca^{2+} channels, which allows Ca^{2+} influx to activate phospholipase C in SH-SY5Y cells (Smart et al., 1995; Smart and Lambert, 1995), suggesting that μ-opioids may depolarize the cell. Indeed, in NG108-15 cells δ-opioids open L-type voltage-sensitive Ca^{2+} channels by depolarizing the cell (Jin et al., 1992). However, removal of extracellular Na^+ (tonicity was maintained using choline) had no effect on the fentanyl-induced activation of phospholipase C in the present study, indicating that depolarization is not the mechanism by which μ-opioid receptor agonists open L-type voltage-sen-

sitive Ca^{2+} channels in SH-SY5Y cells. Similarly, others have reported that δ-opioid receptor agonists open L-type voltage-sensitive Ca^{2+} channels in ND8-47 dorsal root ganglion-neuroblastoma hybrid cells without depolarizing the cell (Tang et al., 1994). We have previously shown that the μ-opioid receptor-mediated activation of phospholipase C is pertussis toxin-sensitive (Smart et al., 1994), indicating that the μ-opioid receptors open L-type voltage-sensitive Ca^{2+} channels via G_i/G_o.

In conclusion, the biochemical effects of Tyr-D-Arg^2-Phe-sarcosine^4 in SH-SY5Y cells are consistent with this compound acting as a μ_2-opioid receptor antagonist, thus, providing further evidence that these cells express μ_2-, but not μ_1-, opioid receptors. Furthermore, we have shown that the μ_2-opioid receptor-mediated activation of phospholipase C is not dependent on cell depolarization.

Acknowledgements

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References

Bare, L.A., E. Mansson and D. Yang, 1994, Expression of two variants of the human μ-opioid receptor mRNA in SK-N-SH cells and human brain, FEBS Lett. 354, 213.

Elliott, J., D. Smart, D.G. Lambert and J.R. Traynor, 1994, Characterization of mu-opioid receptors on SH-SY5Y cells using naloxonazine and beta-funaltrexamine, Eur. J. Pharmacol. Mol. Pharmacol. Sect. 268, 447.

Jin, W., N.M. Lee, H.H. Loh and S.A. Thayer, 1992, Dual excitatory and inhibitory effects of opioids on intracellular calcium in neuroblastoma ×glioma hybrid NG108-15 cells, Mol. Pharmacol. 42, 1083.

Keren, O., M. Garty and Y. Sarne, 1994, Dual regulation by opioids of ^{3}H-norepinephrine release in the human neuroblastoma cell-line SK-N-SH, Brain Res. 646, 319.

Kim, K.W. and B.M. Cox, 1993, Inhibition of norepinephrine release from rat cortex slices by opioids: differences among agonists in sensitivites to antagonists suggest receptor heterogeneity, J. Pharmacol. Exp. Ther. 267, 1153.

Knapp, R.J., E. Malatynska, N. Collins, L. Fang, J.Y. Wang, V.J. Hruby, W.R. Roeske and H.I. Yamamura, 1995, Molecular biology and pharmacology of cloned opioid receptors, FASEB J. 9, 516.

Lambert, D.G., R. Atcheson, R.A. Hirst and D.J. Rowbotham, 1993, Effects of morphine and its metabolites on opiate receptor binding, cAMP formation and [^{3}H]noradrenaline release from SH-SY5Y cells, Biochem. Pharmacol. 46, 1145.

Paakkari, P., I. Paakkari, S. Vonhof, G. Feuerstein and A.L. Siren, 1993, Dermorphin analog Tyr-D-Arg^2-Phe-sarcosine induced opioid analgesia and respiratory stimulation: the role of mu1-receptors, J. Pharmacol. Exp. Ther. 266, 544.

Pasternak, G.W., 1993, Pharmacological mechanisms of opioid analgesics, Clin. Neuropharmacol. 16, 1.

Raynor, K., H. Kong, A. Mestek, L.S. Bye, M. Tian, J. Liu, L. Yu and T. Reisine, 1995, Characterization of the cloned human mu opioid receptor, J. Pharmacol. Exp. Ther. 272, 423.

Rossi, G.C., Y.X. Pan, G.P. Brown and G.W. Pasternak, 1995, Antisense mapping the MOR1 opioid receptor: evidence for alternative splicing and a novel morphine-6β-glucuronide receptor, FEBS Lett. 369, 192.

Smart, D. and D.G. Lambert, 1995, Desensitization of the μ-opioid activation of phospholipase C in SH-SY5Y cells: the role of protein kinases C and A, and Ca^{2+}-activated K^+ currents, Br. J. Pharmacol. 116, 2655.

Smart, D., G. Smith and D.G. Lambert, 1994, μ-Opioid receptor stimulation of inositol(1,4,5)trisphosphate formation via a pertussis toxin-sensitive G-protein, J. Neurochem. 62, 1009.

Smart, D., G. Smith and D.G. Lambert, 1995, μ-Opioids activate phospholipase C in SH-SY5Y human neuroblastoma cells via calcium channel opening, Biochem. J. 305, 577.

Tang, T., J.G. Kiang and B.M. Cox, 1994, Opioids acting through delta receptors elicit a transient increase in the intracellular free calcium concentration in dorsal root ganglion-neuroblastoma hybrid ND8-47 cells, J. Pharmacol. Exp. Ther. 270, 40.