Identification of Mel_{1a} melatonin receptors in the human embryonic kidney cell line HEK293: evidence of G protein-coupled melatonin receptors which do not mediate the inhibition of stimulated cyclic AMP levels

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Abstract Binding assays using 2-[^{125}I]iodomelatonin revealed high-affinity, guanosine 5′-*O*-(3-thiotriphosphate) sensitive, melatonin binding sites (B_{max} 1.1 fmol/mg protein) in the human embryonic kidney cell line HEK293. Competition studies using the selective melatonin receptor antagonist luzindole and RT-PCR techniques identified these sites as human Mel_{1a} melatonin receptors. Challenge of HEK293 cells with 1 μM melatonin had no effect on forskolin stimulated cyclic AMP levels, whereas in HEK293 cells engineered to stably over-express the human Mel_{1a} melatonin receptor ($B_{max} > 400$ fmol/mg protein) melatonin dose-dependently inhibited stimulated cyclic AMP levels (IC_{50} 7.7 pM). These data may indicate that certain tissues, expressing low levels of G protein-coupled melatonin receptors, do not display melatonin mediated inhibition of cAMP.

Key words: Melatonin receptor; 2-[^{125}I]Iodomelatonin binding; Human kidney HEK293 cell; Cyclic AMP inhibition

1. Introduction

Melatonin has been shown to play a role in the control and regulation of several mammalian biological systems. The best characterised of these are the regulation of circadian rhythms [1], and the control of aspects of reproductive state in seasonally breeding mammals [2]. Melatonin acts on these systems by stimulating cell surface melatonin receptors found in the hypothalamic suprachiasmatic nucleus, and the pars tuberalis of the pituitary respectively [3,4]. Melatonin receptors were localised on these tissues by ligand binding experiments and in vitro autoradiography using the high-affinity radioligand ^{125}I-Mel [5]. Many other tissues, both central and peripheral, have also had melatonin receptors identified using ^{125}I-Mel (review [6]), but the possible effects of melatonin on the cellular regulation of these tissues remains poorly characterised.

One tissue which displays guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) sensitive ^{125}I-Mel binding, indicative of G-protein-coupled melatonin receptors, is the mammalian kidney. Recently melatonin receptors have been identified in human adult kidney [7], human fetal kidney [8] and adult guinea pig kidney [9,10]. Several reports identify circadian and melatonin mediated changes on mammalian kidney function [11,12] suggesting the possible direct action of melatonin on mammalian kidney via the identified melatonin receptors. Additional evidence of direct melatonin action on vertebrate kidney is provided by the identification of a melatonin mediated inhibition of forskolin stimulated cyclic AMP (cAMP) levels in explants of chicken kidney [13].

Investigations on possible melatonin mediated effects on human kidney are hampered by the lack of available tissue, however human embryonic kidney tissue has been transformed using adenovirus type 5 DNA to generate the cell line HEK293 [14] which may provide a suitable model for investigations to be performed. Here we report the identification of GTPγS sensitive human Mel_{1a} melatonin receptors in HEK293 cells and investigate the melatonin mediated effect on forskolin stimulated cAMP levels. To our knowledge HEK293 is the first mammalian (human) cell line shown to express melatonin receptors.

2. Materials and methods

2.1. Materials

HEK293 cells were provided by the European Collection of Animal Cell Cultures. Tissue culture media, supplements and sera were obtained from Life Technologies Inc. ^{125}I-Mel (2200 Ci/mmol) was purchased from NEN DuPont. Melatonin and other drugs were purchased from Sigma, except 2-iodomelatonin was obtained from Research Biochemicals Inc. All other reagents used in this study were purchased from Sigma.

2.2. Culture of HEK293 cells

HEK293 cells were cultured as monolayers in Eagle's minimum essential medium (EMEM) supplemented with 10% (v/v) fetal calf serum and 1% (v/v) antibiotic/antimycotic solution, in 5% (v/v) CO_2 at 37°C. When required for ligand binding experiments confluent plates were washed twice with PBS (pH 7.4) and harvested by gentle scraping. Cells were pelleted by centrifugation (13 000 × *g*, 1 min) and stored in aliquots at −80°C.

2.3. Stable transfection of HEK293 cells

A cloning methodology involving the screening a human genomic DNA library (Clontech) and PCR of human genomic DNA was used to isolate sequences encoding human Mel_{1a} [15] and human Mel_{1b} [16]

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Abbreviations: G protein, heterotrimeric guanine nucleotide-binding protein; RT-PCR, reverse-transcription polymerase chain reaction; PBS, phosphate buffered saline; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; PEG 8000, polyethylene glycol (average molecular weight 8000); IBMX, 3-isobutyl-1-methylxanthine; TCA, trichloroacetic acid; M-MLV, Moloney leukaemia virus; DTT, dithiothreitol; dNTP, deoxyribose nucleotide 5′-triphosphate

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melatonin receptors. The full coding sequence of each receptor was subcloned into pcDNA 3 (Invitrogen) and confirmed by DNA sequencing. Recombinant plasmids were introduced into HEK293 cells using the lipid transfection reagent DOTAP (Sigma). Cells were cultured in supplemented EMEM containing G-418 (0.5 mg/ml) until resistant clones were apparent. Individual clones were tested for ^{125}I-Mel binding and positive clones, expressing > 100 fmol of receptor/mg protein, were maintained for subsequent experiments.

2.4. ^{125}I-Mel equilibrium ligand binding

Equilibrium ^{125}I-Mel binding experiments were performed in glass test tubes in a final reaction volume of 200 µl of 'ligand binding buffer' (10 mM Tris-HCl pH 7.5, 1 mM EGTA), for 2 h at 37°C. For experiments using untransfected HEK293 cells 200–500 µg of cell protein per tube was used, while binding experiments using human Mel_{1a} or human Mel_{1b} stably transfected HEK293 cells typically had 2–4 µg of cell protein per tube. Protein determinations were performed in duplicate by the method of Bradford [17]. Binding reactions were terminated by rapid cooling on ice, followed by addition of 0.8 ml 0.1% (w/v) sheep γ-globulin and 1.0 ml of 24% (w/v) PEG 8000 (both dissolved in ice-cold ligand binding buffer). Samples were vortex mixed and the precipitated ^{125}I-Mel bound protein fraction was recovered by centrifugation (1800×*g*, 30 min at 4°C). The supernatant was poured off and the protein pellet resuspended by vigorous vortexing in 2 ml of 12% (w/v) PEG 8000, 0.05% (w/v) sheep γ-globulin and recovered by centrifugation as above. The supernatant was poured off and the pellet allowed to air dry before being counted on a Packard Cobra γ-counter. All determinations were performed in duplicate or triplicate and all experiments repeated at least 3 times. Analysis of ligand binding was performed using the GRAFIT software package (Sigma).

2.5. RT-PCR of HEK293 cell mRNA

Messenger RNA was isolated from HEK293 cells using a Message Maker mRNA isolation kit (R & D Systems) according to the manufacturer's instructions. First strand cDNA was synthesised from 1 µg of mRNA in 1×transcription buffer using 0.5 µg of random primers and 200 U of M-MLV reverse transcriptase in the presence of 0.8 mM DTT, 25 U of RNA guard and 1 mM dNTPs. PCR was performed on 2 µl aliquots of the 20 µl first strand cDNA reaction using human Mel_{1a} or human Mel_{1b} specific oligo primers (see Fig. 2a). The primer pairs used were designed to give amplification between exon 1 and exon 2 of the melatonin receptor gene thus eliminating amplification of genomic DNA. Hot start PCR was performed using 100 pmol of each primer, 2.5 U of AmpliTaq in the presence of 200 µM dNTPs and 1.5 mM $MgCl_2$. The PCR reaction cycles consisted of 94°C for 1 min, 58°C for 1.5 min and 72°C for 1.5 min for 35 cycles. Control reactions were performed with 1 µg of HEK293 mRNAs as above after RNase or DNase treatment. To ensure that solutions used for reverse transcription were free of contaminating sequences control reactions were processed without the addition of mRNA.

The PCR products were electrophoresed through 1% agarose gels and Southern blotted onto GeneScreen Nylon filters (NEN Dupont) using a pressure blotter (Stratagene). A human Mel_{1a} oligonucleotide, designed to hybridise to a site internal to the human Mel_{1a} specific PCR primers, was end labelled with ^{32}P dCTP (Amersham) by standard procedures utilising terminal deoxynucleotidyl transferase. The filters were stripped and reprobed with a human Mel_{1b} specific oligonucleotide probe internal to the human Mel_{1b} specific PCR primers. Hybridisation was performed at 65°C in QuikHyb (Stratagene) according to the manufacturers instructions. The filters were washed to a final stringency of 1×SSC at 60°C. PCR products giving a positive hybridisation signal were subcloned using a pGEM-T vector system (Promega) and DNA was prepared for sequencing to confirm the identity of the PCR products.

2.6. DNA sequencing

DNA was prepared for sequencing using a Wizard 373A DNA purification kit (Promega) according to the manufacturer's instructions. DNA sequencing was performed by using the Prism Dye-Deoxy terminator sequencing kit (ABI) and samples run and analysed using an Applied Biosystems model 373 DNA sequencer.

2.7. cAMP determinations of HEK293

Untransfected or stably transfected HEK293 cells were seeded into poly-D-lysine coated 24 well tissue culture plates at 2×10^5 cells per well. Following incubation for 24 h the growth medium was aspirated and the cells washed once with 0.5 ml of supplement free EMEM. The wash medium was removed and 0.5 ml EMEM (containing 10^{-3} M IBMX) and appropriate concentrations of drugs added for 30 min at 37°C. Routinely cAMP levels were stimulated with 10^{-5} M forskolin and melatonin effects were analysed over the range of 10^{-13}–10^{-6} M melatonin. Reactions were stopped by removal of the assay medium and addition of 0.4 ml 5% (w/v) TCA solution. cAMP determinations were performed on the TCA extract by radioimmunoassay as previously described [18]. Each experimental treatment was performed on 3 individual wells, with each well assayed in duplicate. Experiments were repeated at least 3 times.

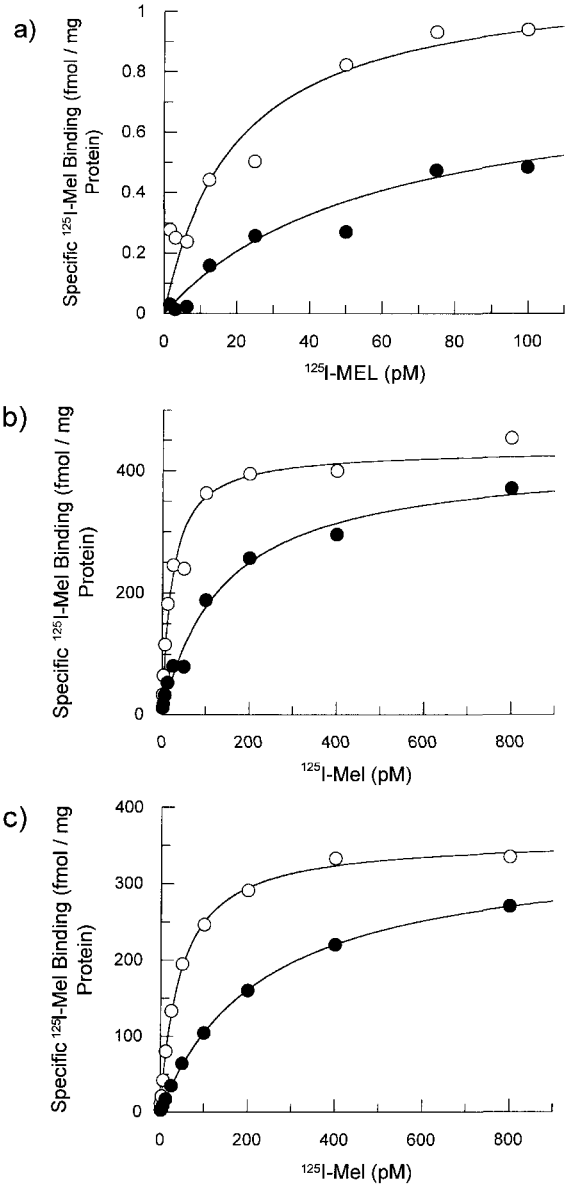


Fig. 1. ^{125}I-Mel saturation binding. Saturation curves in the absence (open circles) or presence (filled circles) of 10^{-4} M GTPγS. a: HEK293; b: HEK293 stably transfected with human Mel_{1a} melatonin receptor; c: HEK293 stably transfected with human Mel_{1b} melatonin receptor. Data shown are from a single experiment using mean values of triplicate determinations, and are representative of 3 or 4 similar experiments. All data are fitted with single-site saturation curves calculated using the GRAFIT software. Calculated K_d and B_{max} values ± S.E.M. are listed in Section 3.

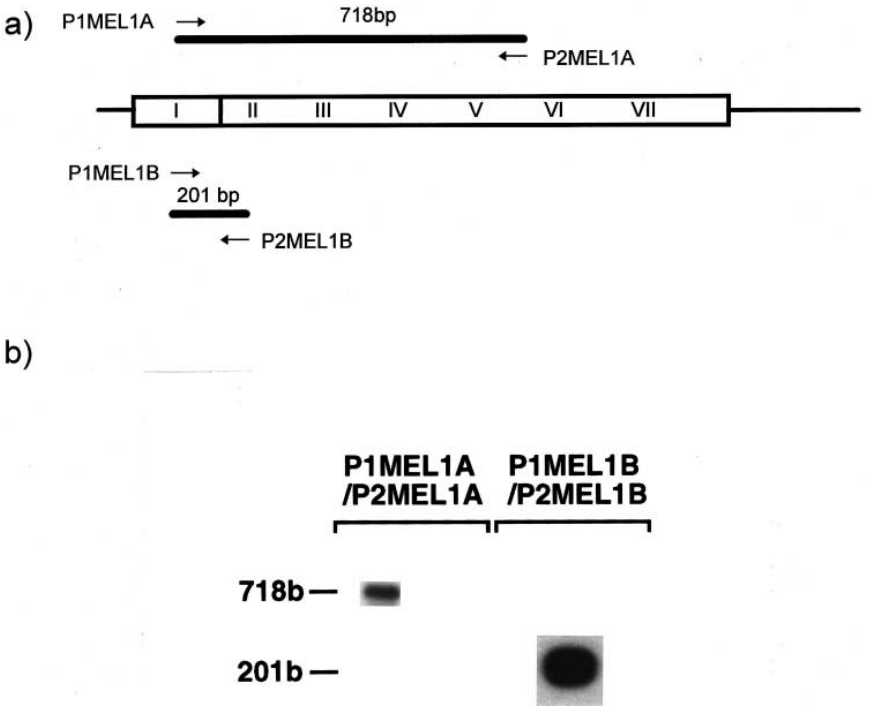


Fig. 2. a: Schematic diagram of melatonin receptor cDNA showing location of primer sites. The primer pair shown above the diagram of the melatonin receptor cDNA are specific for the human Mel_{1a} receptor and consist of P1MEL1A (5′-CTGGCCTGCGTCCTCATCTTCACCATCGTG-3′), positions 88–117 and P2MEL1A (5′-CCATGCTGGCGGGGTCAGAGGC-3′), positions 784–805. The primer pair shown below the diagram are specific for the human Mel_{1b} receptor and consist of P1MEL1B (5′-CGCTGTCCGCGGTGCTCATCGTCACCACC-3′), positions 125–153 and P2MEL1B (5′-CCAGGGCCCAGCCGTCATAGAAGATG-3′), positions 300–325. The size of the PCR product obtained with each primer pair is indicated. The splice site between exon 1 and exon 2 of melatonin receptor genes is indicated by the vertical line between transmembrane domains I and II in the diagram. b: RT-PCR for melatonin receptor gene expression in HEK293 cells. A Mel_{1a} specific ^{32}P-radiolabeled oligonucleotide probe (5′-TGCGTTCCTGAGCTTCTTGTTCCGATACAC-3′, positions 154–183) and a Mel_{1b} specific ^{32}P-radiolabeled oligonucleotide probe (5′-TGCGTTCCGGAGCTTGCGGTTCCTGAGCAC-3′, positions 193–222) internal to the PCR primers showed hybridisation to products of the appropriate size using the PCR primers indicated. These RT-PCR products were subcloned and confirmed by DNA sequencing.

3. Results

3.1. ^{125}I-Mel saturation binding

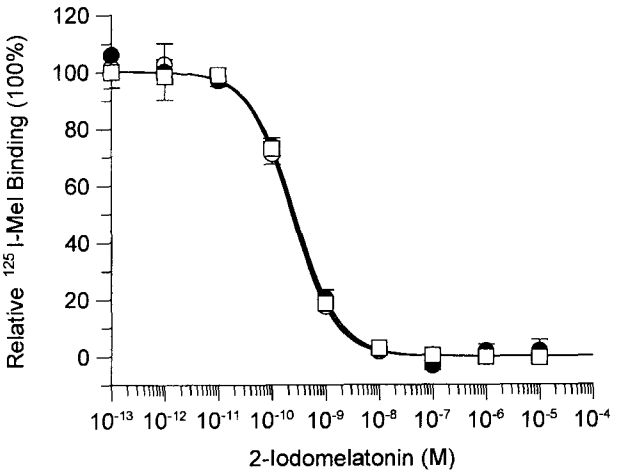
Specific ^{125}I-Mel binding was observed in HEK 293 cells (K_d 23.5 ± 2.8 pM, B_{max} 1.11 ± 0.02 fmol/mg protein; mean ± S.E.M., $n = 4$) (Fig. 1a). The binding had a similar K_d value to stably transfected HEK293 cells over-expressing either the human Mel_{1a} melatonin receptor (K_d 22.8 ± 3.3 pM, B_{max} 567.7 ± 96.4 fmol/mg protein; mean ± S.E.M., $n = 3$) (Fig. 1b), or the human Mel_{1b} melatonin receptor (K_d 80.3 ± 18.7 pM, B_{max} 432.0 ± 84.6 fmol/mg protein; mean ± S.E.M., $n = 3$) (Fig. 1c). The ^{125}I-Mel binding in HEK293 cells was sensitive to 10^{-4} M GTPγS (K_d 38.5 ± 3.4 pM, B_{max} 0.72 ± 0.06 fmol/mg protein; mean ± S.E.M., $n = 4$) (Fig. 1a), as was the binding observed in the HEK293 clones stably expressing the human Mel_{1a} melatonin receptor (K_d 161.1 ± 24.4 pM, B_{max} 536.2 ± 74.2 fmol/mg protein; mean ± S.E.M., $n = 3$) (Fig. 1b), or the human Mel_{1b} receptor (K_d 298.6 ± 25.6 pM, B_{max} 369.3 ± 52.1 fmol/mg protein; mean ± S.E.M., $n = 3$) (Fig. 1c). Therefore it would seem that HEK293 cells express high affinity G protein-coupled melatonin receptors.

3.2. RT-PCR of human Mel_{1a} and Mel_{1b} melatonin receptor transcripts

To confirm if HEK293 cells possess the genes encoding for either of the two cloned human melatonin receptors, RT-PCR was performed on HEK293 mRNA with either human Mel_{1a} or human Mel_{1b} specific primers (Fig. 2a). Amplified products were resolved by agarose gel and Southern blotted, using specific human Mel_{1a} and human Mel_{1b} oligonucleotide probes, internal to the amplification primers. Both human Mel_{1a} and human Mel_{1b} specific products were identified on a Southern blot (Fig. 2b) and confirmed by subcloning and DNA sequencing. DNase treatment of HEK293 mRNAs prior to RT-PCR did not eliminate amplification of PCR products hybridising to Mel_{1a} and Mel_{1b} specific oligo probes. No hybridising PCR products were observed in reagent controls and RT-PCR products were eliminated in samples treated with RNase prior to RT-PCR. These data suggest that HEK293 contains both human Mel_{1a} and human Mel_{1b} mRNA transcripts. This experiment however was not quantitative and therefore no indication of the relative abundance of each receptor transcript could be inferred.

3.3. Competitive displacement of ^{125}I-Mel binding

To characterise the HEK293 melatonin receptor competitive displacement of ^{125}I-Mel binding (100 pM ^{125}I-Mel concentration) was performed with 5 drugs: 2-iodomelatonin, melatonin, 6-hydroxymelatonin, *N*-acetylserotonin and luzindole. Parallel experiments were also performed on the stably transfected HEK293 cells over-expressing either the human Mel_{1a} or human Mel_{1b} melatonin receptor to allow direct comparison of results. Four of these drugs: 2-iodomelatonin,


Relative ^{125}I-Mel Binding (100%)
120
100
80
60
40
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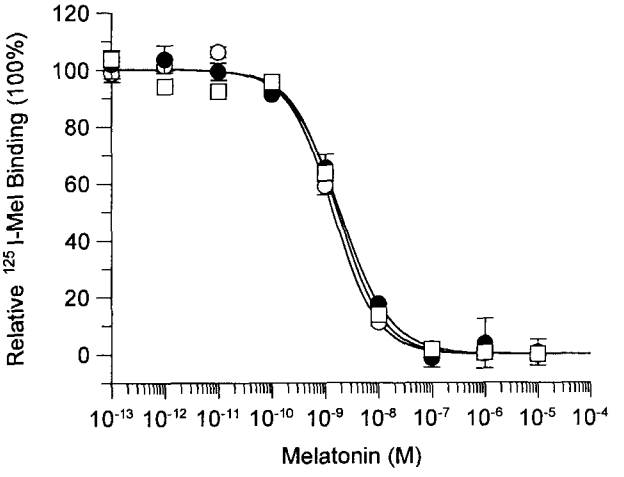
0
10^{-13} 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
2-Iodomelatonin (M)


Relative ^{125}I-Mel Binding (100%)
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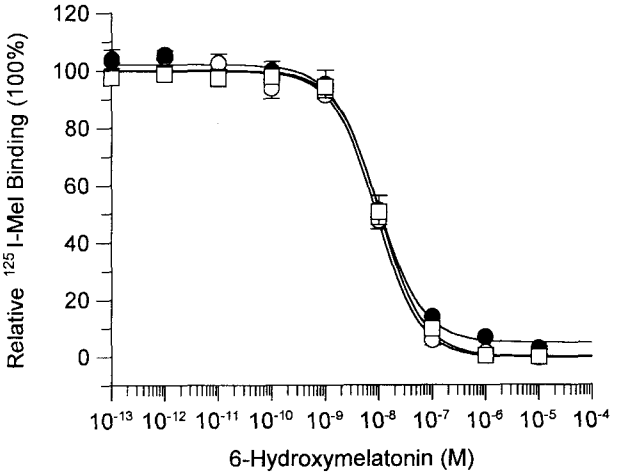
Melatonin (M)


Relative ^{125}I-Mel Binding (100%)
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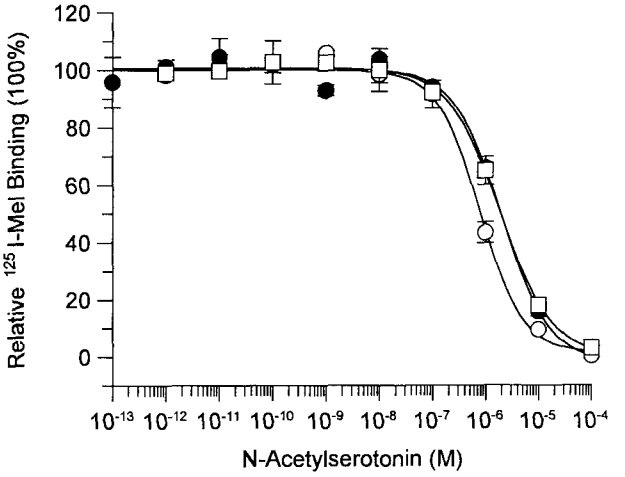
6-Hydroxymelatonin (M)


Relative ^{125}I-Mel Binding (100%)
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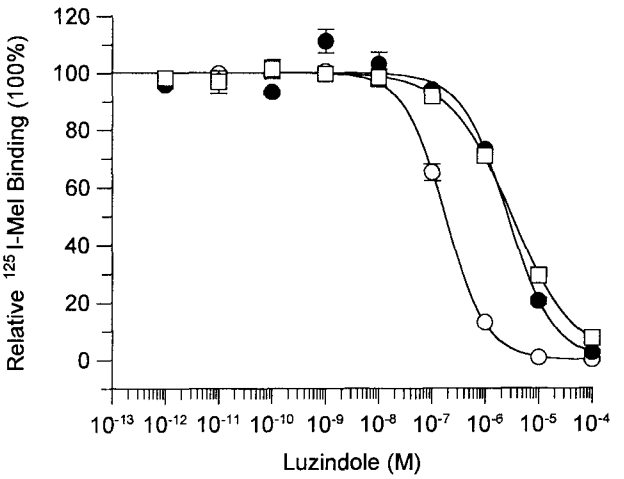
N-Acetylserotonin (M)


Relative ^{125}I-Mel Binding (100%)
120
100
80
60
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20
0
10^{-13} 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
Luzindole (M)

Fig. 3. Competitive displacement of ^{125}I-Mel binding in HEK293. Competitive displacement of ^{125}I-Mel binding using 5 drugs was performed on, HEK293 (filled circles), HEK293 stably transfected with human Mel_{1a} melatonin receptor (open squares) and HEK293 stably transfected with human Mel_{1b} melatonin receptor (open circles). Each set of data was normalised to percentage relative binding and plotted on individual graphs. Data shown are means of duplicate determinations from a single experiment ± S.E.M., and are representative of 3 or 4 similar experiments. Each set of data is fitted with a 4-parameter logistic competition curve calculated by the GRAFIT software. K_i values (and slope factors) for HEK293 (mean ± S.E.M., $n=3$); 2-iodomelatonin, $4.28 \pm 0.23 \times 10^{-11}$ M (1.00 ± 0.02); melatonin, $2.81 \pm 0.23 \times 10^{-10}$ M (0.89 ± 0.03); 6-hydroxymelatonin, $1.89 \pm 0.03 \times 10^{-9}$ M (1.10 ± 0.07); *N*-acetylserotonin, $4.06 \pm 0.54 \times 10^{-7}$ M (1.00 ± 0.02); luzindole, $5.29 \pm 0.09 \times 10^{-7}$ M (1.02 ± 0.01).
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melatonin, 6-hydroxymelatonin and *N*-acetylserotonin gave essentially similar results for HEK293 and both stably transfected clones (Fig. 3). These values were consistent with those originally identified for human Mel_{1a} and human Mel_{1b} melatonin receptors [15,16] and support the RT-PCR data that the identified melatonin receptors in HEK293 are human Mel_{1a} and/or human Mel_{1b}. The fifth drug, luzindole [19], was selective for human Mel_{1b} (IC_{50} $1.84 \pm 0.03 \times 10^{-7}$ M, mean ± S.E.M., $n=3$) over human Mel_{1a} (IC_{50} $3.35 \pm 0.13 \times 10^{-6}$ M, mean ± S.E.M., $n=4$) by a factor of 18.2, consistent with previous reports [20]. HEK293 cells gave an IC_{50} value for luzindole of $2.78 \pm 0.05 \times 10^{-6}$ M, (mean ± S.E.M., $n=3$) very similar to that obtained for the stably transfected human Mel_{1a} clone (Fig. 3). These data identify the expression of only human Mel_{1a} melatonin receptors in HEK293.

3.4. *Effect of melatonin on cAMP levels in HEK293*

HEK293 cells did not display a melatonin mediated effect on 10^{-5} M forskolin stimulated cAMP levels (Fig. 4a), whereas stably transfected HEK293 cells over-expressing human Mel_{1a} or human Mel_{1b} melatonin receptors displayed almost complete inhibition of cAMP at physiological melatonin concentrations, with IC_{50} values of 7.7 pM and 117 pM respectively (Fig. 4b,c).

4. Discussion

^{125}I-Mel saturation binding identifies HEK293 as the first mammalian (human) cell line in which high-affinity G-protein-coupled melatonin receptors have been detected. This is consistent with the previous identification of melatonin receptors in fetal human kidney [8]. RT-PCR suggests that HEK293 cells have transcripts encoding for both human Mel_{1a} and human Mel_{1b} subtypes, but competitive displacement of ^{125}I-Mel binding by luzindole revealed that only the Mel_{1a} receptor was expressed at detectable levels. This is again consistent with fetal human kidney in which Mel_{1a} and Mel_{1b} receptor transcripts were identified by RT-PCR but only Mel_{1a} was identified by in situ hybridisation [8]. Therefore Mel_{1a} transcripts appear to be present at levels above those of Mel_{1b} transcripts in human fetal kidney and suggest that Mel_{1a} receptors are preferentially expressed. Such preferential expression would explain our finding of only Mel_{1a} receptors in HEK293, although Mel_{1b} receptors may be expressed at very low undetectable levels. The significance of this differential expression of melatonin receptor subtypes remains unclear. Note: During the preparation of this manuscript Song et al. [10] discussed initial findings of ^{125}I-Mel binding in HEK293 and reported the identification of a 37 kDa protein immunoreactive to peptide derived anti-Mel_{1a} antibodies. This would appear consistent with our findings.

Functionally HEK293 cells did not display melatonin medi-

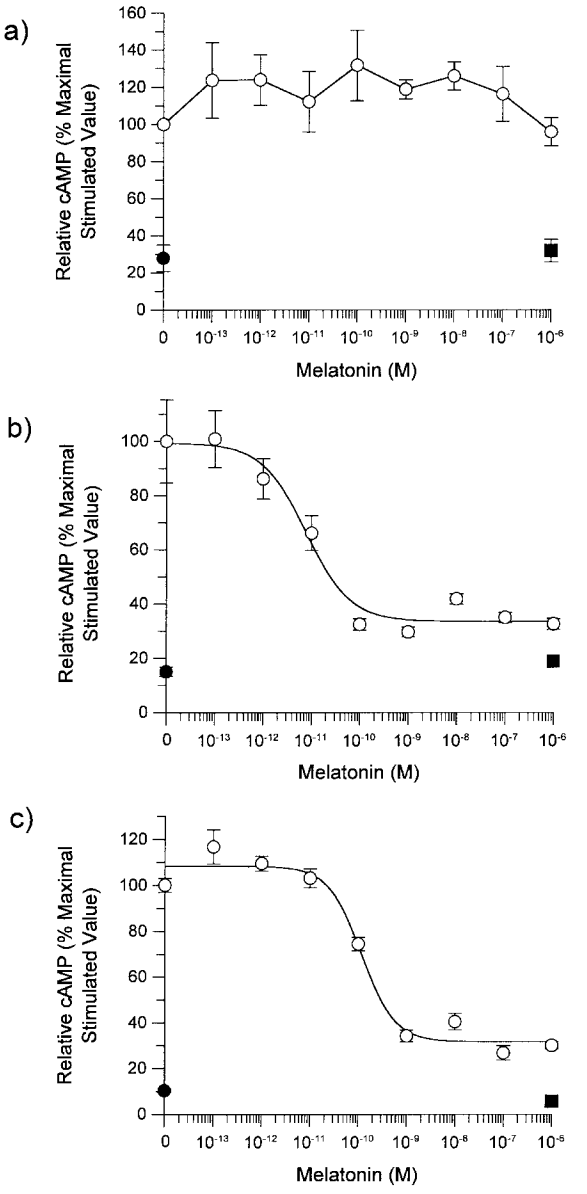

Fig. 4. Effect of melatonin of forskolin stimulated cAMP levels in HEK293. a: HEK293 (mean of 3 experiments ± S.E.M.); b: HEK293 stably transfected with human Mel_{1a} melatonin receptor (mean of single experiment with duplicate determinations of triplicate points ± S.E.M., repeated 3 times); c: HEK293 stably transfected with human Mel_{1b} melatonin receptor (mean of single experiment with duplicate determinations of triplicate points ± S.E.M., repeated 3 times). Basal cAMP levels (filled circles), incubation with 10^{-6} M melatonin alone (filled squares), incubation with 10^{-5} M forskolin ± melatonin (0, 10^{-13}–10^{-6} M) (open circles). IC_{50} values were determined by the GRAFIT software and were 7.67×10^{-12} M for Mel_{1a} receptor and 1.17×10^{-10} M for the Mel_{1b} receptor.

ated effects on forskolin stimulated cAMP levels, whereas HEK293 cells over-expressing either human Mel_{1a} or human Mel_{1b} melatonin receptors almost completely inhibited stimulated cAMP levels at physiological melatonin levels (see Fig. 4). The lack of effect in untransfected HEK293 is consistent with the previous report of Yung et al. [21] in which melatonin displayed no effect ($P > 0.9$) on hormone stimulated cAMP levels in HEK293. These authors did not know that HEK293 had a native melatonin receptor and performed this experiment as a control in their studies. As the human Mel_{1a} stably transfected HEK293 cells displayed an inhibition of cAMP there would appear to be a receptor concentration dependent phenomenon, i.e. HEK293 are unresponsive to melatonin if Mel_{1a} melatonin receptors are expressed at 1.1 fmol/mg protein but responsive at 400 fmol/mg protein. The large difference in these B_{max} values is not ideal, however it does illustrate that there may be a level below which melatonin receptors, despite being G-protein coupled, are not able to modulate cellular cAMP levels in HEK293. Interestingly there also appears to be a difference in the GTPγS effect on HEK293 compared to the stably transfected cells. HEK293 displays an approximately 2-fold reduction in K_d and a 35% reduction in B_{max} whereas the Mel_{1a} stably transfected cells show a 6–7-fold reduction in K_d but no change in B_{max} (see Fig. 1). Therefore it is possible that the nature of the G-protein-coupling is different in untransfected HEK293 compared to the Mel_{1a} transfected cells. As many mammalian tissues, including the kidney, have similar reported levels of ^{125}I-Mel binding to HEK293 (ca. 1 fmol/mg protein) this may indicate that melatonin does not have a significant effect on signalling through cAMP inhibition in these tissues. The identification of an ~40% inhibition of stimulated cAMP levels in explants of chicken kidney (receptor $B_{max} \approx 1.5$ fmol/mg protein) [13] appears to confound this proposal. However, localisation data on human fetal kidney [8] and guinea pig kidney [10] would appear to indicate that expression of melatonin receptors in the kidney is restricted to specific cell types, therefore the B_{max} reported in chicken kidney may be an under-representation of melatonin receptor density of these cells. These data could also indicate that tissues may display different responses to melatonin in a tissue and/or species dependent manner.

The identification of G protein-coupled Mel_{1a} melatonin receptors in HEK293 provides an accessible system for the study of melatonin mediated effects on human derived tissue. This cell line may provide valuable insights into melatonin receptor action which will be difficult to study on native human tissue and could reveal subtleties in melatonin mediated signal transduction pathways which may not be apparent by using transfected cell systems.

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