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Functional expression of 5-HT_{1c} receptor cDNA in COS 7 cells and its influence on protein kinase C

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Two subtypes of receptors for serotonin (5-hydroxytryptamine; 5-HT) are known to stimulate inositol (1,4,5)-trisphosphate production, the 5-HT_{1c} and 5-HT_2 receptors. In this study we investigated the ability of 5-HT_{1c} receptors, transiently expressed in COS 7 cells, to functionally interact with protein kinase C-α, the indigenous (phorbol ester-responsive) isoform of the enzyme in those cells. Serotonin caused translocation of the [^{3}H]phorbol 12,13-dibutyrate (PDBu) binding site of PKC-α from the cytosolic to the membrane fraction in a Ca^{2+}-dependent manner which was prevented by the 5-HT_{1c} receptor antagonist mianserin. The lipid activators of PKC, PDBu and 1,2-dioctanoyl-*sn*-glycerol (DOG) also caused translocation, but through a mechanism apparently quite independent of Ca^{2+}.

Serotonin; 5-HT_{1c} receptor; Expression; Protein kinase C

1. INTRODUCTION

The G7 family of receptors is a large group consisting of proteins with seven transmembrane spanning regions. Their actions are mediated through G proteins which stimulate or inhibit a number of second messenger pathways or ion channels. Serotonin, or 5-hydroxytryptamine (5-HT), is the ligand for at least five members of this family [1]. One of these, the 5-HT_{1c} receptor, is known to stimulate production of inositol 1,4,5 trisphosphate [2] which causes release of intracellular Ca^{2+} [3]. This receptor has been identified in neural tissue [4–6] but is most highly expressed in choroid plexus [4,7]. Recently the receptor gene was isolated by expressing a rat choroid plexus cDNA library in *Xenopus* oocytes [8]. Within this system the expressed receptor and a partial clone [9] affect Ca^{2+} dependent Cl^- channels [10] and also K^+ channels [11]. When the receptor was expressed in rat fibroblasts, Ca^{2+} was released from intracellular stores [12], indicating that a similar mechanism of action was involved there to that operating endogenously and in transfected oocytes. The aim of this study was to use transient expression of the receptor in transfected COS 7 cells to investigate receptor-mediated stimulation of PKC, and in particular whether the 5-HT_{1c} receptor can functionally activate the α isoform of PKC.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Tissue culture media were obtained from Life Technologies, Paisley, UK. Standard laboratory chemicals of Analar grade were obtained from BDH Chemicals Ltd, Poole, UK, [20-^{3}H(N)]PDBu (spec.act. = 19.1 Ci/mmol) and ATP-γ-^{35}S (spec.act. = 1256 Ci/mmol) which were purchased from Du Pont, Dreieich, Germany. DEAE dextran was obtained from NBL (Cramlington, UK) or Promega Ltd (Southampton, UK). All other reagents and chemicals were obtained from Sigma Chemical Co Ltd, Poole, UK, unless otherwise indicated.

2.2. *Cell culture*

COS 7 cells (gift of Dr Janet Allen) were grown in DMEM supplemented with 10% newborn calf serum and 100 U/ml each of penicillin and streptomycin, in a humidified atmosphere of 95% air/5% CO_2 at a constant temperature of 37°C, and were passaged every 3–4 days. Cells for transfection were trypsinised the day before the experiment and plated at a density of approximately 50–60% in 75 cm^2 flasks.

2.3. *Preparation of cDNA*

The coding region for the rat 5-HT_{1c} receptor was included in a 3.0 kbp cDNA insert in the *Eco*RI site of the plasmid, pMV7SR1c, a gift from Dr D. Julius [8]. The *Eco*RI insert was excised from a low melting point gel, purified with Geneclean II Sephaglass resin (Stratech, Luton, UK) and the ends filled in using T_4 DNA polymerase (Boehringer Mannheim, Lewes, UK) and a mixture of deoxynucleotides (USB, Cambridge, UK) [13]. The cDNA was then ligated to *Bst*XI linkers (British Biotechnology Ltd, Cowley, UK) and subcloned into the *Bst*XI site of the expression vector pCDM8; a gift from Dr B. Seed, [14]. The subsequent clones were restricted with various endonucleases to verify which clone had the receptor cDNA in the proper orientation. The selected clone was grown up in 5 ml overnight culture using Luria broth supplemented with 7.5 μg/ml tetracycline and 12.5 μg/ml ampicillin and extracted according to the alkaline lysis miniprep method [13]. After a further precipitation step the plasmid was then used to transfect cells. The cDNA inserted in the opposite orientation was used as one of the controls.

2.4. *Transfection of cells*

Cells were washed two times with OptiMEM supplemented with 100

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U/ml each of streptomycin and penicillin at 37°C prior to exposure to transfecting medium for 4 h. The transfecting medium consisted of OptiMEM/penicillin/streptomycin, 400 μg/ml DEAE dextran, 100 μM chloroquine phosphate and approximately 1/5 of an overnight culture miniprep/flask. This was replaced with 10% DMSO in DMEM/penicillin/streptomycin for 2 min, then DMEM/10% newborn calf serum/penicillin/streptomycin or DMEM/2% UltraSerG/penicillin/streptomycin. Cells were harvested 72 h later. The efficiency of transfection was assessed using a pCH110 β-galactosidase gene construct and indicated that approximately 10% of the cells expressed β-galactosidase activity (data not shown).

2.5. *Phorbol binding assay*

Cytosolic [^{3}H]PDBu binding was performed in a method similar to that described by Leach et al. [15]. After stimulation for 15 min in culture flasks, the medium was aspirated and cells were scraped into 0.5 ml of ice-cold 'Kuo' buffer (20 mM Tris-HCl (pH 7.5) 50 mM 2-mercaptoethanol, 2 mM EDTA and 1 mM phenylmethylsulphonyl fluoride) containing 0.01% leupeptin + 20 μM transepoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) and homogenised. Samples were centrifuged (38,000 × *g*, 20 min, 4°C). The supernatant and pellet were carefully separated and the pellet was re-homogenised in 0.5 ml 'Tris-BSA' (50 mM Tris-HCl) (pH 7.4), 4 mg/ml essential fatty acid-free bovine serum albumin. While membrane binding was carried out in Tris-BSA, cytosolic binding assays additionally contained 1 mM $CaCl_2$ and 75 mM magnesium acetate and 1.25 mg/ml sonicated phosphatidylserine, sodium salt (Lipid Products Ltd, Nutfield, UK). Assays were conducted in a total volume of 250 μl (30 min, 37°C), with 5 nM [^{3}H]PDBu (approximately 0.03 μCi per tube) and dimethylformamide (0.5% final) or 10 μM PDBu in dimethylformamide for total and non-specific binding measurements, respectively. Tissue samples gave a total binding of less than 10% of total radioactivity present. Protein was precipitated at 4°C by the addition of 100 μl of 12 mg/ml bovine γ-globulin and 300 μl of 24% polyethyleneglycol 8000 in 50 mM Tris-HCl (pH 7.4). After 20 min, assay tubes were centrifuged (12,000 × *g*, 5 min, 4°C), the supernatant aspirated and the ^{3}H radioactivity in each pellet determined after solubilisation.

2.6. *PKC activity assay*

After stimulation, cells were harvested and initially treated as for the [^{3}H]PDBu binding assay. The membrane fraction from the 38,000 × *g* centrifugation was resuspended in 'Kuo' buffer with peptidase inhibitors, then both it and the cytosolic fraction were made up to 0.1% Triton X-100 and stirred for 1 h at 4°C to solubilise the membrane-associated PKCs. Samples were again centrifuged at 38,000 × *g* for 20 min at 4°C. The PKCs present in the supernatants were partially purified by loading onto a diethylaminoethyl cellulose, DE52 (Whatman Biosystems Ltd, Maidstone, UK) (pH 7.5)-loaded Bio-Rad Poly-Prep Chromatography column (Bio-Rad Laboratories, Richmond, CA, USA) prewashed with 9 column volumes of 'Kuo' buffer + 0.01% leupeptin + 20 μM E64. The sample was allowed to run through before washing with a further 9 column volumes of 'Kuo' buffer + 0.01% leupeptin + 20 μM E64. The partially purified PKCs were eluted with 3 column volumes of 'Kuo' buffer + 0.01% leupeptin + 20 μM E64 supplemented with 150 mM NaCl.

Phosphatidylserine vesicles were prepared by sonication in ice-cold 'Tris-EGTA' (20 mM Tris-HCl (pH 7.5) + 0.5 mM EGTA) at a stock concentration of 400 μg/ml. The suspension had 0.16% Nonidet P-40 (Calbiochem, Nottingham, UK) added and was vortexed for 2 min before allowing to settle at room temperature for a minimum of 15 min.

All assay dilutions were performed in Tris-EGTA. Partially purified cytosol (150 mM NaCl eluate) was added to eppendorf tubes containing (final concentrations): 1.25 mM $MgCl_2$, 100 μg/ml phosphatidylserine + 0.04% Nonidet P-40, 1.25 mg/ml lysine-rich histone III-S as substrate, 100 μM ATP-γ-S containing ATP-γ-^{35}S (approximately 0.18 μCi/tube). Assay tubes contained PDBu or 1,2-dioctanoyl-*sn*-glycerol (Sigma) DOG dissolved with dimethylformamide (0.05% final) in Tris-EGTA (or dimethylformamide in Tris-EGTA alone for control measurements), 600 μM $CaCl_2$ (100 μM final free Ca^{2+}) or 5 mM EGTA (< 3 nM final free Ca^{2+}), kinase inhibitors or control solvent where appropriate. Cytosol was added to pre-warmed (30°C) tubes (final assay volume = 100 μl) and reactions were started by brief centrifugation in a bench-top centrifuge and incubated for 15 min at 30°C (a time at which enzyme kinetics were determined to be linear). Reactions were stopped by addition of 20 μl of ice-cold 0.1 M ATP in 0.1 M EDTA (pH 7.0) followed by being placed on ice for 20 min then spun (16,000 × *g*, 15 min, 4°C) 50 μl of each stopped sample was spotted onto a 4 cm^2 Whatman P81 cellulose phosphate ion-exchange chromatography paper (Whatman) and washed (3 × 2 min, room temperature) in 10 ml of 75 mM H_3PO_4. Papers were dried overnight in a fume hood before counting in liquid scintillation fluid for ^{35}S radioactivity.

3. RESULTS

The 5-HT_{1c} receptor cDNA was inserted into the pCDM8 vector and transiently expressed in COS 7 cells. After 72 h these cells were assayed for functional receptors by stimulating with serotonin and determining the relative number of phorbol binding sites which had translocated from cytosol into the membrane, as compared to basal levels. Table Ia shows that 20 μM serotonin induced only a modest translocation of specific [^{3}H]PDBu binding sites into the membrane (42% compared to 32% in controls). The effect of 300 nM PDBu was much greater, with 79% of sites appearing in the membrane fraction after PDBu treatment. Cells which were not exposed to serotonin had an unexpectedly large proportion (32%) of the [^{3}H]PDBu binding sites within the membrane fraction. Treatment with the 5-HT_{1c} antagonist, mianserin, reduced this basal migration of [^{3}H]PDBu binding sites into the membrane fraction. It has been reported [16] that serum supplemented-medium contains sufficient serotonin to partially activate the 5-HT_{1c} receptor in transfected fibroblasts. Thus, in subsequent experiments transfected cells were no longer cultured in medium supplemented with newborn calf serum, instead the synthetic serum substitute UltroSerG was used. Table Ib shows that serotonin also appeared to cause a small translocation of PKC enzyme activity into the membrane fraction in comparison with control or mianserin-treated cells. COS 7 cell extracts contain very high basal histone kinase activity (which is in part sensitive to the selective PKC inhibitor [17] Ro 31–8220; data not shown). This may be due to the presence of the constitutively-active cleaved catalytic domain of PKC, resulting from the action of calpain-like activity in the cells and indeed considerable amounts of low molecular weight fragments of PKC δ, ε and ζ were detected when they were expressed in COS cells [18]. Alternatively, the presence of PKC ζ [19] which is activated by phosphatidylserine with no requirement for phorbol esters [20] may be responsible. Due to the much better signal to noise ratio, only the translocation of [^{3}H]PDBu binding sites was pursued in further experiments as an index of receptor-mediated influence on PKC.

Table I

Translocation of PKC in COS 7 cells transfected with the cDNA for the 5-HT_{1c} receptor and cultured in 10% newborn calf serum

(a)	[^{3}H]PDBu binding (dpm per assay tube)		
Treatment	Cytosol	Membrane	% in membrane
Control	1705 ± 349	805 ± 155	32 ± 7
Serotonin (20 μM)	1194 ± 295	854 ± 108	42 ± 7
Mianserin (100 μM)	2278 ± 295	301 ± 88	12 ± 4
PDBu (300 nM)	463 ± 210	1701 ± 186	79 ± 8

(b)	PDBu-induced kinase activity (dpm per assay tube)		
Treatment	Cytosol	Membrane	% in membrane
Control	1420 ± 205	137 ± 74	9 ± 6
Serotonin (20 μM)	988 ± 164	246 ± 106	20 ± 9
Mianserin (100 μM)	1322 ± 199	97 ± 65	7 ± 5

Part **a** shows specific equilibrium binding of [^{3}H]PDBu as dpm per assay tube in cytosol and membrane fractions prepared from transfected COS 7 cells treated as indicated for 15 min immediately prior to homogenisation. Values are means ± S.E.M. from 3 separate determinations carried out in duplicate. Typical values for control total and non-specific binding were ~ 950 dpm per tube respectively for cytosol and ~ 1900 and ~ 700 respectively for membranes. Part **b** shows the Ca^{2+}-dependent PDBu (1 μM)-evoked [^{35}S]thiophosphorylation of histone III-s as determined in a mixed-micelle, PS-dependent assay for partially purified PKC. Values are means ± S.E.M. dpm incorporated per assay tube from three separate determinations carried in duplicate. Typical control values in the absence of PDBu were ~ 4000 and ~ 500 dpm per assay tube, respectively, for cytosol and membrane fractions.

Table II shows that in serum-free conditions the basal proportion of [^{3}H]PDBu binding sites within the membrane fraction is low (12%) and not significantly different from transfected cells incubated with mianserin alone. When exposed to serotonin, a clear translocation was recorded with 35% of the [^{3}H]PDBu binding sites within the membrane. This response was prevented by mianserin. It is not known whether higher concentrations of serotonin would have elicited a greater response. Cells which were transfected with receptor cDNA inserted in the antisense orientation (Table IIb) and cells which were exposed to transfection without receptor cDNA (Table IIc) were not responsive to serotonin. In all of these experiments, translocation was observed after treatment with PDBu (300 nM) or DOG (200 μM) indicating that the cells would in principle have been capable of responding to any serotonin-elicited signal. Interestingly, DOG (200 μM) always caused much less translocation of [^{3}H]PDBu binding sites than did PDBu (300 nM), even though these concentrations produce equivalent effects in a model of PKC-mediated facilitation of Ca^{2+} channels [21].

In order to assess whether the influence of the 5-HT_{1c} receptor on PKC was dependent on calcium, experiments were carried out in medium depleted of Ca^{2+} (to ~100 nM) by the addition of EGTA (3 mM) to the normal medium (1.8 mM Ca^{2+}). Table III indicates that receptor-elicited translocation of [^{3}H]PDBu binding sites does indeed show a dependence on calcium levels but in clear contrast that responses elicited by PDBu or DOG are unaffected by Ca^{2+}-depletion.

4. DISCUSSION

The present results illustrate, by demonstrating coupling to a downstream signalling response, that the 5-HT_{1c} receptor cDNA can be successfully transfected and expressed in COS 7 cells in a functional state. Serotonin (5-HT) was able to elicit translocation/activation of PKC specifically in response to 5-HT in a manner sensitive to a 5-HT_{1c} receptor antagonist mianserin. We do not know which of the several agonist affinity states of the 5-HT_{1c} receptor expressed in fibroblasts [22,23] is functionally involved in the present response. Both the cell-permeant diglyceride, DOG and the synthetic activator of the diglyceride recognition site on PKC, PDBu also caused translocation of PKC in these cells.

Table II

Translocation of PKC in COS 7 cells transfected with the cDNA for the 5-HT_{1c} receptor or controls and cultured in serum-free conditions.

(a)	[^{3}H]PDBu specific binding (dpm per assay tube)		
Treatment	Cytosol	Membrane	% in membrane
Control	9260 ± 1030	1272 ± 270	12 ± 4
Serotonin (20 μM)	6291 ± 825*	3386 ± 482*	35 ± 6
Mianserin (100 μM)	10276 ± 1332	1651 ± 350	14 ± 5
Serotonin + mianserin	8703 ± 906**	1755 ± 355**	17 ± 4
DOG (200 μM)	6347 ± 980*	3294 ± 653*	34 ± 8
PDBu (300 nM)	2022 ± 627*	2935 ± 787*	59 ± 13
(b)			
Control	7203 ± 1408	1481 ± 567	17 ± 6
Serotonin (20 μM)	7860 ± 1294	1756 ± 601	18 ± 7
DOG (200 μM)	4484 ± 1830	3546 ± 1215*	44 ± 12
PDBu (300 nM)	2044 ± 1470*	5253 ± 897*	72 ± 13
(c)			
Control	6879 ± 1075	1680 ± 379	20 ± 5
Serotonin (20 μM)	6563 ± 988	1785 ± 616	21 ± 7
DOG (200 μM)	3839 ± 1379*	2669 ± 702	41 ± 11
PDBu (300 nM)	1588 ± 979*	3788 ± 655*	70 ± 12

The values represent specific binding of [^{3}H]PDBu to cytosolic or membrane PKC in cells transfected with (**a**) the 5-HT_{1c} receptor cDNA, (**b**) its antisense control or (**c**) transfecting medium alone. All results are the means ± S.E.M. from 4 separate experiments carried out in duplicate. The statistical significance of changes from control ($P < 0.05$ by Student's t-test) is indicated by * and from serotonin alone by **.

Table III

Ca^{2+}-dependence of PKC translocation in COS 7 cells transfected with the cDNA for the 5-HT_{1c} receptor

Treatment	Specific [^{3}H]PDBu binding (% in membrane fraction)	
	+ Ca^{2+}	− Ca^{2+}
Control	17 ± 3	19 ± 4
Serotonin (20 μM)	36 ± 5*	21 ± 6**
DOG (200 μM)	38 ± 5*	41 ± 5*
PDBu (300 nM)	83 ± 8*	79 ± 11*

The values represent the percentage of specific [^{3}H]PDBu binding recovered in the membrane fraction from experiments conducted as in Table II. Cells were cultured in serum-free conditions with either normal (1.8 mM) Ca^{2+} or the addition of 3 mM EGTA 10 min prior to and during the challenge with agonist. Results are the means ± S.E.M. from 5 experiments. Statistical significance of changes ($P < 0.05$, Man-Whitney U-test) is indicated by * (comparison with control unstimulated values) and ** (comparison with corresponding response in the presence of Ca^{2+}). Removal of Ca^{2+} had no effect on the absolute dpm per assay tube in control cells or those stimulated with either DOG or PDBu.

Thus the presence of the response to 5-HT may reflect generation of diglyceride as a consequence of agonist-stimulation of the receptor, most likely by activation of a phospho-inositidase C (PIC). It is not certain which isoform of PIC was involved in the response to 5-HT, especially since the α subunit of the G protein Gq (which has been reported to couple phospho-inositide-hydrolysing receptors to activation of PIC β_1 [24]) is not strongly expressed in COS 7 cells [25]. Nevertheless, other G α isoforms that could substitute for Gq α may be present allowing activation of PIC via the 5-HT_{1c} receptor to occur.

It has been thought for a number of years that COS 7 cells expressed only the α isoform of PKC [26] and although there is recent evidence that ζ PKC may also be present [19], this isoform appears to have little affinity for phorbol esters [20] and therefore should not contribute to the current results.

Both PDBu and DOG caused clear translocation of [^{3}H]PDBu binding sites in COS 7 cells, although the effect of DOG (at a concentration maximal in other PKC response models [21, 27] was much less than that of PDBu. This is entirely consistent with previous results which demonstrated that PKC α (predominant here in COS 7 cells) shows both a distinctively reduced binding affinity and potency of kinase activation with respect to short chain saturated diglycerides such as DOG but not phorbol esters [27].

The relative roles of diglyceride/phorbol ester and Ca^{2+} in the interaction of PKC with membranes remains somewhat unclear. It is clear that phorbol esters alone readily induce translocation of Ca^{2+}-dependent PKC isoforms and act to stabilise the membrane PKC complex with phospholipid and Ca^{2+} [28,29]. However, the initial interaction of enzyme with phospholipid membrane appears to be highly dependent on Ca^{2+} and requires levels which are certainly in excess of those encountered in the cytosol of resting cells [28–30]. Although one group has provided (indirect) evidence that PDBu increased the association of PKC with plasma membranes at a range of set Ca^{2+} concentrations, there was no response at all at free Ca^{2+} concentrations of 230 nM and below [30], such as would pertain in the cytosol of resting cells. However, since all these studies were carried out with mixtures of PKC isoforms, it is not clear that one could extrapolate directly to responses of PKC α, as recorded in the present experiments.

Whilst DOG and PDBu were able to cause translocation of PKC (presumably the α isoform) in a manner independent of Ca^{2+} (Table III), agonist activation of the 5-HT_{1c} receptor was only effective in this response in the presence of Ca^{2+}. It is conceivable that such Ca^{2+}-dependence would be apparent with the receptor agonist rather than the direct PKC activators because rather low levels of diglyceride (requiring some synergy with Ca^{2+} for an adequate effect) would be produced by receptor activation. Nevertheless, translocation to a very similar extent caused by addition of exogenous diglyceride showed no evidence whatsoever of Ca^{2+}-dependence. It is possible though that additional elevation of cytosolic calcium in these cells would have further enhanced any submaximal lipid-induced translocation response (such as that to DOG). In contrast, DOG-induced histone III-s thiophosphorylation by the α isoform of PKC is entirely Ca^{2+}-dependent [28]; indicating that translocation and enzymic activation of PKC α are mechanistically quite distinct. Interestingly, activation of PKC α by PDBu differs from that by DOG in that it can occur partially even in the absence of Ca^{2+} [27]. Thus, the presumed abilities of the 5-HT_{1c} receptor to generate diglyceride and also to elevate cytosolic Ca^{2+} both appear to play some role in its ability to translocate/activate PKC α.

Interestingly, expression of this receptor in NIH 3T3 (but not CCL 39) fibroblasts has been shown to cause mitogenic transformation [12, 16, 31], much in the same manner as observed in Rat 6 fibroblasts overexpressing protein kinase C βI [32]. It seems likely that at least in some circumstances and in certain cells, the ability of G7 receptors to activate PKC (as investigated here) may play an important role in their induction of cellular transformation [16].

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