Control of CCK gene transcription by PACAP in STC-1 cells

DAMIAN G. DEAVAL,1 RAKTIMA RAYCHOWDHURY,2 GRAHAM J. DOCKRAY,1 AND ROD DIMALINE1
1Physiological Laboratory, University of Liverpool, Liverpool L69 3BX, United Kingdom; and 2Massachusetts General Hospital, Boston, Massachusetts 02114

Received 4 January 2000; accepted in final form 2 April 2000

Deavall, Damian G., Raktima Raychowdhury, Graham J. Dockray, and Rod Dimaline. Control of CCK gene transcription by PACAP in STC-1 cells. Am J Physiol Gastrointest Liver Physiol 279: G605–G612, 2000.—The mechanisms by which neuroendocrine stimulants regulate CCK gene transcription are unclear. We examined promoter activation by pituitary adenylate cyclase-activating polypeptide (PACAP), a known CCK secretagogue, in the enteroendocrine cell line STC-1. The promoter region from −70 to −87 bp, relative to the transcriptional start site, contains a composite calcium/cyclic AMP response element (CRE)/activator protein 1 (AP1) site that may bind CRE binding protein (CREB) and AP1. PACAP (with IBMX) stimulated expression of an 87-bp construct 3.35 ± 0.36-fold but had no effect on a −70 construct. The effect was blocked by the protein kinase A inhibitor H-89 and by a dominant-negative CREB plasmid. Mutation of the CRE/AP1 site to a canonical CRE site did not affect the response to PACAP, but mutation to a canonical AP1 site prevented it. CREB phosphorylation was increased after PACAP treatment. Electrophoretic mobility shift assay and supershift analysis revealed that CREB and not AP1 bound to the CRE/AP1 site and that PACAP increased the proportion of phosphorylated CREB that was bound. We conclude that PACAP increases CCK gene expression via a cAMP-mediated pathway involving CREB phosphorylation by protein kinase A and activation of a composite CRE/AP1 site.

cholecystokinin; pituitary adenylate cyclase-activating polypeptide; calcium/cyclic AMP response element; calcium/3',5'-monophosphate response element binding protein; adenosine 3',5'-cyclic monophosphate

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: R. Dimaline, Physiological Laboratory, Univ. of Liverpool, Crown St., Liverpool L69 3BX, UK (E-mail: r.dimaline@liverpool.ac.uk).
CREB or AP1, representing convergence of separate signal transduction pathways. In PC12 cells, cAMP-mediated transcriptional responses of the dopamine β-hydroxylase promoter are mediated by AP1 proteins acting through a composite CRE/AP1 site (33). In SK-N-MC cells, basic fibroblast growth factor increases CCK gene transcription via binding of CREB to the composite CRE/AP1 site (8). The CRE/AP1 site in the CCK promoter lies downstream of an E box binding domain for the basic helix-loop-helix family of transcription factors, and interaction between these two sites in cAMP-stimulated activity has also recently been described (28). We hypothesized that the CCK secretagogue PACAP may also increase CCK gene transcription, and in the present study we describe cis-regulatory elements within the promoter and trans-activating STC-1 cell nuclear proteins mediating the response. Our data suggest that PACAP-induced CCK gene expression is achieved by CREB family transcription factors acting at the CRE/AP1 site.

MATERIALS AND METHODS

Materials. Ovine PACAP-38 was purchased from Calbiochem-Novabiochem (Nottingham, UK). Forskolin and IBMX were obtained from Sigma (Poole, UK). Tissue culture media, supplements, and plasticware were obtained from Life Technologies (Paisley, UK). Luciferase assay reagents were obtained from Promega (Southampton, UK).

Tissue culture. STC-1 cells were maintained in DMEM supplemented with 10% horse serum, 5% fetal bovine serum, 100 μg/ml streptomycin, 100 μg/ml penicillin, and 5 μg/ml ascorbate in a humidified incubator at 37°C under 5% CO₂-95% O₂.

CCK-luciferase plasmids. The promoter fragment of the C-1089 construct was created from rat genomic DNA by PCR. The forward primer was directed between −1089 and −1061 bases upstream of the published major transcriptional start site (10), and a reverse primer was directed between +38 and +55 of exon 1 (which is common to all constructs). C-87, C-125, and C-70 were created by a similar strategy using C-1089 as a template. C-87CRE, C-87AP, C-87m1, C-87m2, C-87m3, and C-87m4 were generated using C-87 as a template. The primers used to generate constructs are shown in Table 1. PCR products were purified from agarose gels and cloned into pCRII (Invitrogen, Groningen, The Netherlands) before insertion as Hind III/Xho I fragments into pGL3-Basic luciferase reporter vector (Promega). Integrity of the constructs was confirmed by automated sequencing.

Transient transfection. In initial experiments, cells were transfected by electroporation, and 5 × 10⁶ cells were incubated on ice for 5 min in DMEM with 20 μg CCK-luciferase (CCK-LUC) and 2.5 μg pRL-TK (Promega) as an internal control. The pRL-TK vector contains a gene encoding sea pansy luciferase driven by the thymidine kinase promoter. Cells were pulsed at 200 mV/1,070 μF and plated into 60-mm poly-L-lysine-coated dishes containing Ultraculture media (BioWhittaker, Wokingham, UK). Cells were maintained for 18 h before stimulation and harvested at 24 h after transfection. For all subsequent experiments, lipofection was used. Cells (1 × 10⁶ cells per well) were plated into six-well plates 24 h before transfection. Cells were transfected with Trans FAST reagent (Promega) using 0.7 μg CCK-LUC, 0.2 μg of either dominant-negative plasmid, empty vector, or pBlue-script II SK– (Stratagene, LaJolla, CA) and 0.1 μg pRL-TK, according to the manufacturer’s protocol. Cells were then maintained in full media for 18 h before stimulation and harvested after 24 h. Luciferase activity was determined by luminometry (Turner Designs TD20–20) using the dual luciferase assay system (Promega). Transfection efficiency was assessed following transfection of cells (1 × 10⁶ cells per well), with 1 μg of the green fluorescent protein (GFP) expression vector pEGFP-C1 (Clontech, Basingstoke, UK). GFP-expressing cells were visualized 24 h later by fluorescence microscopy, and the number present in four visual fields for each of five wells was counted. Transfection efficiency was determined to be 5.58 ± 0.28% (mean ± SE; n = 5).

Northern blot analysis. Cells were plated (1 × 10⁶ cells per well) in six-well plates 18 h before use and then treated with PACAP/IBMX (10⁻⁷ M/0.5 mM) or vehicle for 6, 24, or 48 h. Total RNA was extracted with TRIzol (Life Technologies), and Northern blot analysis was performed as previously described (7) using a cRNA probe to rat CCK.

Western blot analysis. Immunoblotting of STC-1 cell protein was performed using a PhosphoPlus CREB (Ser133) antibody kit (New England Biolabs, Beverly, MA) according to the manufacturer’s protocol. Briefly, STC-1 cells were plated at 10⁶ cells per well on six-well plates and harvested into SDS-sample buffer 48 h later. Whole cell extracts were run on 10% SDS-PAGE and immunoblotted with primary antibodies for phosphorylated and total CREB. After incubation with horseradish peroxidase-conjugated secondary anti-

<table>
<thead>
<tr>
<th>Name</th>
<th>Use</th>
<th>Primer Sequence, 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1089</td>
<td>PCR</td>
<td>CAT TTA ATC TGC TGC TGT TTT</td>
</tr>
<tr>
<td>C-87</td>
<td>PCR</td>
<td>CCC GGG ACT GGC TCA GCA CTG</td>
</tr>
<tr>
<td>C-70</td>
<td>PCR</td>
<td>CCC GGA TAA ACA GAT GAC TGG</td>
</tr>
<tr>
<td>C-87m1</td>
<td>PCR</td>
<td>TTC CCG GGC ATG CGT CAG GAC</td>
</tr>
<tr>
<td>C-87m2</td>
<td>PCR</td>
<td>TTC CCG GAC TGT ATC AGC ACT GGG T</td>
</tr>
<tr>
<td>C-87m3</td>
<td>PCR</td>
<td>TTC CCG GAC TGC TTC ACT GGG TAA ACA GAT</td>
</tr>
<tr>
<td>C-87m4</td>
<td>PCR</td>
<td>TTC CCG GAC TGC TTC ACT GGG TAA ACA GAT</td>
</tr>
<tr>
<td>C-87mAP</td>
<td>PCR</td>
<td>TTC CCG GCA CTG AGT AAC GAG TGT</td>
</tr>
<tr>
<td>C-87mCRE</td>
<td>PCR</td>
<td>TTC CCG GCA CTG AGC TCG GCA CTG GGT</td>
</tr>
<tr>
<td>Common reverse</td>
<td>PCR</td>
<td>CTT CCG ATG GCG AGT CA</td>
</tr>
<tr>
<td>CRE/AP1wt</td>
<td>EMSA</td>
<td>GATCCCGGGAGACTCGTGCTAGCGACTG</td>
</tr>
<tr>
<td>CRE/AP1mut</td>
<td>EMSA</td>
<td>TCCGGGACTGTGACTCGGACTG</td>
</tr>
<tr>
<td>CRE</td>
<td>EMSA</td>
<td>TCCGGGACTGTGACTCGGACTG</td>
</tr>
<tr>
<td>AP1</td>
<td>EMSA</td>
<td>TCCGGGACTGTGACTCGGACTG</td>
</tr>
</tbody>
</table>

EMSA, electrophoretic mobility shift assay; AP, activator protein; CRE, calcium/cyclic AMP response element; wt, wild type; mut, mutant.
body and the chemiluminescence reaction, the signal was detected by exposure to HyperFilm (Amersham Biotech, Little Chalfont, UK).

Electrophoretic mobility shift assay. Crude nuclear extracts from STC-1 cells were made as previously described (26). Double-stranded oligonucleotides were radiolabeled with [\(\alpha\]32P]dCTP and incubated with 4 \(\mu\)g of nuclear extract in a reaction containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 1 mM EDTA, 1 \(\mu\)g poly(dA-dT), and 10% glycerol. Reactions containing 10 fmol of double-stranded oligonucleotide probe were incubated at room temperature for 20 min before electrophoresis at 30 mA on 6% nondenaturing polyacrylamide gels containing 0.25× Tris borate/EDTA. Gels were then dried and exposed to a Phosphor screen before image analysis using ImageQuant (Molecular Dynamics), or gels were exposed to HyperFilm (Amersham Biotech) at room temperature for up to 72 h. For competition experiments, extracts were incubated with 200-fold excess of competitor oligonucleotide for 10 min at room temperature before addition of the labeled probe. For supershift band analysis, extracts were incubated with antibody for 10 min at room temperature followed by 20 min on ice before addition of the probe for 10 min at room temperature. The anti-P-CREB antibody used in supershift analyses recognizes p43-phosphorylated CREB (Upstate Biotechnology, Lake Placid, NY), the anti-CREB ATF-1 antibody recognizes CREB-1 p43, ATF-1 p35, and cyclic AMP response element modulator (CREM)-1 (sc-270 X, Santa Cruz Biotechnology, Santa Cruz, CA), and the anti-Jun/AP1 antibody recognizes c-Jun, Jun B, and Jun D p39 proteins (sc-44-G X, Santa Cruz Biotechnology).

Statistical analysis. All results are expressed as means ± SE. Statistical difference was determined by Student’s t-test or one-way ANOVA.

RESULTS

Basal activity. The pattern of basal activity of the CCK promoter in STC-1 cells was established by performing 5’ deletional analysis. Deletion from C-1089 to C-125 resulted in significantly elevated activity, but further deletion to C-87 reduced basal activity to a level similar to the C-1089 construct. Further deletion to C-70 decreased basal activity 14.02 ± 0.02-fold compared with C-125 (Fig. 1), indicating the importance of the region between −125 and −70.

The region between −87 and −70 of the rat CCK promoter confers PACAP responsiveness. Because the region between −87 and −70 includes the CRE/AP1 site, we asked whether this sequence conferred responsiveness to extracellular stimuli by administration of PACAP and the adenyl cyclase activator forskolin. The latter stimulated activity of C-1089, C-125, and C-87 by 1.65 ± 0.24-, 2.34 ± 0.18-, and 2.39 ± 0.19-fold, respectively (P < 0.05) but had no effect on C-70 (Fig. 1). The C-1089 and C-87 constructs, but not C-70, were also stimulated by PACAP (Fig. 2). Stimulation was both dose (Fig. 2) and time dependent (not shown), being maximal at 6 h with 10−7 M PACAP in the presence of 0.5 mM IBMX. We therefore established that PACAP responsiveness lay between −87 and −70 bp upstream of the reported transcriptional start site. Compatible with the idea that PACAP stimulates CCK gene transcription, we found using Northern blot that endogenous CCK mRNA abundance was also elevated when STC-1 cells were treated for 48 h with PACAP (10−7 M) in the presence of 0.5 mM IBMX (Fig. 2).

Inhibition of protein kinase A and overexpression of a dominant negative for CREB inhibits the response to PACAP. PACAP receptors may be linked exclusively to adenyl cyclase or to adenyl cyclase and phospholipase C (3). To examine the role of protein kinase A (PKA)-mediated phosphorylation in our system, we used the specific PKA inhibitor H-89. At a concentration of 20 \(\mu\)M, H-89 completely inhibited PACAP stimulation of C-87 (Fig. 3). We considered the possibility that PKA activated CREB, and to investigate this we
cotransfected a plasmid encoding a dominant negative for CREB (A-CREB). This plasmid possesses an acidic extension of the CREB leucine zipper domain that binds with the basic region of wild-type CREB to prevent the latter from interacting with the CRE (1). PACAP-induced activation of C-87 was decreased from 3.14 ± 0.09-fold to 1.36 ± 0.10-fold in the presence of A-CREB (Fig. 3), indicating a role for CREB in PACAP-stimulation of the CCK promoter.

PACAP and forskolin increase CREB phosphorylation. The data presented above are compatible with PACAP activation of CREB, and to determine whether the abundance of phosphorylated CREB was altered, we performed Western blot analysis on cells stimulated with PACAP or forskolin. The total CREB abundance (i.e., phosphorylated and unphosphorylated) did not change (Fig. 4). However, the abundance of both phosphorylated CREB and phosphorylated ATF was increased in response to both forskolin and PACAP (Fig. 4).

Electrophoretic mobility shift assay reveals complex formation with STC-1 nuclear extracts and a CRE/AP1 probe. To establish the nature of the protein/DNA interactions at the composite site, in vitro binding assays were performed using electrophoretic mobility shift assay (EMSA). Nuclear protein extracts from STC-1 cells formed a complex with a 24mer oligonucleotide probe that included the wild-type CCK promoter CRE/AP1 sequence (Fig. 5). Competition shift experiments revealed that complex formation was dependent on an

Fig. 2. Pituitary adenylate cyclase-activating polypeptide (PACAP)-stimulated activity of CCK-LUC promoter constructs. A: STC-1 cells were transfected by electroporation with CCK-LUC constructs that contained 87 or 70 bp of promoter. After transfection (18 h), cells were treated with PACAP (10^{-7} M for 6 h) in the presence of 0.5 mM IBMX or vehicle. Values are means ± SE; n = 3; *P < 0.05 by Student’s t-test. B: dose-response profile of C-87 to PACAP. STC-1 cells were transfected by electroporation with C-87, and after 18 h they were treated for 6 h with PACAP at the doses (M) indicated in the presence of 0.5 mM IBMX. Values are means ± SE; n = 3. C: Northern blot of STC-1 cell total RNA (2.5 μg per lane) after treatment for 48 h with PACAP (10^{-7} M) in the presence of 0.5 mM IBMX (P) or vehicle (V). Membranes were hybridized with a cRNA probe for rat CCK.

Fig. 3. Effects of H-89 and dominant-negative calcium response element binding protein (CREB) on PACAP-induced CCK promoter activity. A: STC-1 cells were transfected by lipofection with the CCK-LUC construct C-87, and the effect of the specific protein kinase A (PKA) inhibitor H-89 (20 μM) on PACAP responses was determined (see Fig. 2 legend for details). B: STC-1 cells were transfected by lipofection with C-87 and either a dominant negative CREB/ATF vector (A-CREB) or empty vector control. Values are means ± SE; n = 3; *P < 0.05 by Student’s t-test.
intact CRE/AP1 site since the wild-type oligonucleotide, but not an oligonucleotide containing a mutated CRE/AP1 site, effectively inhibited the binding reaction. In extracts of unstimulated cells, the consensus CRE-containing sequence, but not a consensus AP1 oligonucleotide, also successfully inhibited the binding reaction. The data indicate that CRE/ATF family proteins, but not AP1 family transcription factors, bind to the CRE/AP1 site. This was further confirmed by supershift band analysis, which showed that an antibody to CREB shifted the bound complex in both unstimulated and forskolin/PACAP stimulated cells (Fig. 5). However, supershifts were not seen using an antibody to the AP1 transcription factor (anti-Jun/AP1; Fig. 5). In the extracts from forskolin and PACAP-stimulated cells, but not unstimulated cells, there was an increase in the binding of phosphorylated CREB to the CRE/AP1 probe because anti-P-CREB produced a supershift of a proportion of the bound material (Fig. 5).

Mutagenesis of the region between −87 and −70 reveals the importance of an intact CRE in cis for PACAP responsiveness. We hypothesized that CREB activated CRE, and to establish the importance of an intact CRE in cis we performed mutagenesis of the region between −87 and −70 of the CCK promoter (Fig. 6). The unstimulated transcriptional activity of

Fig. 4. Western blot analysis of STC-1 cell protein extracts. Protein extracts from STC-1 cells treated with either forskolin (10⁻⁵ M), PACAP/IBMX (10⁻⁷ M/0.5 mM), or vehicle were subjected to PAGE. A: immunoblot with a broad-specificity antibody directed against members of the CREB/ATF transcription factor family (CREB-1, CREM, and ATF-1). B: immunoblot with an antibody recognizing only phosphorylated forms of CREB/ATF.

Fig. 5. Electrophoretic mobility shift assay of STC-1 nuclear extracts using a CRE/AP1 probe. A: nuclear protein extracts from unstimulated STC-1 cells were incubated with the CRE/AP1 probe (see Table 1) in the presence or absence of 200-fold excess unlabeled oligonucleotide (either wild-type, mutated to canonical CRE or canonical AP1, or having nonsense mutations; see Table 1). B: supershift assays were performed as indicated on nuclear extracts from STC-1 cells treated with forskolin (10⁻⁵ M), PACAP (10⁻⁷ M), or vehicle control.
C-87 was maintained by C-87mAP (89.9 ± 2.5% of C-87; n = 4), whereas C-87mCRE showed a reduced basal activity compared with the wild type (62.2 ± 1.5%; n = 4). Analysis of the mutants in response to PACAP revealed the importance of an intact CRE for maximal activity. Thus when the composite CRE/AP1 site was mutated randomly or to a pure AP1 site, there was a significantly decreased stimulation in response to PACAP compared with wild-type C-87. Mutations outside the CRE site or to a pure CRE consensus did not significantly diminish PACAP responsiveness (Fig. 6).

**DISCUSSION**

The main finding of the present study is that PACAP increased CCK gene transcription in STC-1 cells. Our data indicate that PACAP acts via PKA-mediated phosphorylation of CREB/ATF transcription factors at Ser133 to activate the CCK promoter. Increased phosphorylation of CREB-1 and ATF-1 was confirmed by Western blot analysis. CREB/ATF protein was shown to bind to a CRE/AP1-containing oligonucleotide probe in EMSAs, and the bound material was supershifted with a CREB/ATF antibody. Supershift band analysis using anti-P-CREB antibody delayed the migration of the binding complex in the forskolin- and PACAP-treated cells but not in control cells. An intact CRE site in cis was shown to be essential for increased transcription, because deletion or mutation of the composite CRE/AP1 site to a non-CRE-like sequence in the promoter-reporter constructs prevented PACAP-induced activation. The importance of CREB in extracts from unstimulated cells was further verified by competition EMSAs in which CREB- and not AP1-family transcription factors bound to the CRE/AP1 site.

The composite CRE/AP1 site potentially facilitates convergence of signal transduction pathways impinging on both the AP1 and CREB transcription factors. Previously, it has been demonstrated that the CCK promoter exhibited increased activation in response to overexpression of CREB and the AP1 dimerization partners, Fos and Jun, in SK-N-MC cells (25). More recently, promoter activation with basic fibroblast growth factor, via activation of CREB by mitogen-activated protein kinase and PKA, has been demonstrated in the SK-N-MC cell line. This has been proposed as a mechanism by which growth and neurotrophic factors, in concert with neuropeptides, may regulate expression of the CCK gene (10).

In the STC-1 cell line, both CCK mRNA abundance and activity of an 800-bp CCK promoter construct were increased by peptone, which represents a luminal stimulus, although the transcriptional activation pathway was not reported (6). In addition, however, gene expression in enteroendocrine cells may be modulated by neurohumoral agents. Recently, PACAP was shown to be a potentially important modulator of the gastric enterochromaffin-like cell and the chromaffin cell (34, 36). Furthermore, PACAP has been shown to stimulate CCK secretion from the STC-1 cell line and from a mucosal I cell-enriched preparation from rat small intestine (3). Given its localization in central neurons and within the submucosal plexus of the small intestine, PACAP has itself been proposed as a candidate neuromodulator of CCK release in vivo (16). The mechanisms behind such secretogogue effects are unclear but are known to be associated with elevation of cAMP levels (3). PACAP is known to act through at least three receptor subtypes (9). The type II receptor VPAC₁ is activated by both PACAP and VIP and is coupled exclusively to adenylyl cyclase (12), whereas the type I PAC₁ receptor is not responsive to VIP and utilizes both cAMP and inositol 1,4,5-trisphosphate as second messengers (32). It has previously been reported that the effects of PACAP on STC-1 cells are mediated by the VPAC₁ receptor and thus involve only cAMP as the second messenger (3). The present data also suggest that cAMP is the second messenger involved in the PACAP signaling pathway, because responsiveness is lost in the presence of the PKA inhibitor H-89. Therefore, as well as being a candidate neuromodulator of secretion, PACAP may serve to regulate expression of the CCK gene via cAMP-dependent mechanisms.
It has been proposed that the CCK promoter CRE/AP1 site and the proenkephalin CRE-2 site preferentially bind CREB over AP1 because of the nonpalindromic nature of the site and its core C residue (TGCGTCA) (28). However, it has also been reported that increases in AP1 proteins may directly mediate cAMP-induced activation of the composite site in the dopamine β-hydroxylase promoter, which is also of the type TGCGTCA (33), implicating both families of transcription factors in cAMP-induced modulation of this site. Moreover, AP1-related proteins have been shown to represent the majority of protein binding to the proenkephalin promoter CRE-2 site in the adrenal chromaffin cell, which contrasts with the predominance of CREB binding to this site in the central nervous system (20). We did not observe an AP1-mediated element to the PACAP-induced activation of the CCK CRE/AP1 site within the STC-1 cell line. The dominant-negative A-CREB vector has previously been shown not to interfere with dimerization of members of the AP1 complex (1), and yet PACAP-induced activation is inhibited in the presence of A-CREB. Furthermore, mutation of the CRE/AP site to a consensus AP1 site in the promoter-reporter gene to a consensus AP1 site in the promoter-reporter gene greatly decreased the PACAP effect. The lack of a supershift with a Jun/AP1 antibody in the EMSA and the lack of competition for the binding reaction with the consensus AP1-containing oligonucleotide suggest that AP1 binding to the composite site is less important than CREB binding in the PACAP-stimulated or unstimulated STC-1 cells. We demonstrated, however, that the unstimulated activity of C-87 is fully maintained by C-87mAP and is slightly reduced by C-87mCRE. Thus AP1 proteins may directly activate C-87mAP in a manner not observed with the composite site, whereas CREB binding to the composite site may be of a higher affinity than binding to the C-87mCRE. In PACAP-stimulated cells, AP1 does not appear to play a role in increasing transcriptional activation of the CCK promoter. Thus the signaling pathway used by PACAP to activate the CCK promoter in this cell type appears to be the classic pattern of CREB/ATF activation by PKA, whereby phosphorylation at Ser\(^{133}\) stimulates association of the CREB binding protein required for trans-activation of the target gene.

A-CREB dominant-negative plasmid was a kind gift from Dr. D. D. Ginty (The Johns Hopkins University School of Medicine, Baltimore, MD). We are grateful to Dr. Tim Wang for helpful advice. This work was funded by the Wellcome Trust and the Medical Research Council.

REFERENCES


