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β-Adrenoceptor-Mediated Down-regulation of M_2 Muscarinic Receptors: Role of Cyclic Adenosine 5′-monophosphate-Dependent Protein Kinase and Protein Kinase C

JONATHAN ROUSELL, EL-BDAOUI HADDAD, JUDITH C. W. MAK, BENJAMIN L. J. WEBB, MARK A. GIEMBYCZ, and PETER J. BARNES

Department of Thoracic Medicine, National Heart and Lung Institute, London, UK SW3 6LY

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SUMMARY

Stimulation of β_2-adrenoceptors with the selective β_2 agonist procaterol caused a biphasic decrease in cell surface M_2 muscarinic receptor number in human embryonic lung 299 cells when measured with the hydrophilic antagonist [^{3}H]*N*-methylscopolamine. In contrast, total muscarinic receptor number, measured with the lipophilic antagonist [^{3}H]quinuclidinylbenzilate, decreased after only 24-hr treatments with procaterol. The loss in receptor number at 24 hr was mimicked with the use of forskolin and the cAMP analogue 8-bromo-cAMP, indicating a cAMP-mediated mechanism. Northern blot analysis showed a small and transient increase in m2-receptor mRNA levels up to 2 hr but no long term (24 hr) effect. Chronic (24-hr) treatment with 8-bromo-cAMP also had no effect on m2 muscarinic receptor mRNA, whereas forskolin caused a 50% reduction in the steady state levels of m2 mRNA that could be only partially blocked by the cAMP-dependent protein kinase inhibitor H-8 and the protein kinase C inhibitor GF 109203X. Procaterol-induced down-regulation of M_2 receptors was fully blocked by *N*-[2-(methylamino)ethyl]-5′-isoquinoline-sulfonamide and 2-[1-(3-dimethylaminopropyl)-inol-3-yl]-3-(indol-3-yl)maleimide, implicating both of these kinases in the M_2 muscarinic receptor down-regulation. Conversely, the forskolin- and 8-bromo-cAMP-induced down-regulation was only partially inhibited and unaffected by these inhibitors, respectively. In control cells and those treated with procaterol for ≤2 hr, cAMP generation was significantly inhibited by carbachol. The inhibitory effect of carbachol was, however, lost after 24-hr exposure to procaterol. This desensitization was partially reversed by preincubations with H-8 and GF 109203X. Collectively, these results suggest that transregulation of M_2 muscarinic receptors by β_2-adrenoceptor stimulation can be demonstrated at the protein level in human embryonic lung 299 cells. Furthermore, a role is suggested for cAMP-dependent kinase and PKC in M_2 muscarinic receptor down-regulation and their functional desensitization.

The muscarinic acetylcholine receptors belong to the larger superfamily of receptors that couple to G proteins. Five muscarinic receptor subtypes (m1–m5) are known to exist within the human genome and are preferentially coupled to distinct signal transduction pathways. Stimulation of m1, m3, and m5 muscarinic receptors results in PI hydrolysis, whereas stimulation of m2 and m4 muscarinic receptors causes the inhibition of adenylyl cyclase, along with a weaker stimulation of PI breakdown (1–3).

Cross-talk between G protein-coupled receptors has been demonstrated in a number of systems (4). For example, stimulation of β_2-adrenoceptors in DDTI MF-2 vas deferens smooth muscle cells alters α_1-adrenoceptor mRNA and protein levels through changes in gene transcription (5). In HEL 299 cells, we previously demonstrated that activation of PKC stimulates muscarinic receptor down-regulation and reduced m2 receptor gene transcription (6). Heterologous regulation has also been demonstrated between muscarinic and adrenoceptors cotransfected into Chinese hamster ovary cells, where agonist stimulation of the β_2-adrenoceptors resulted in a PKA-mediated desensitization, internalization, and down-regulation of the M_1 muscarinic receptors in these cells (7). Transregulation has also been described between adenylyl cyclase-coupled receptors. For example, in rat ventricular myocytes, carbachol promoted a time- and concentration-dependent decrease in cell surface β_1-adrenoceptors (8). Similarly, muscarinic and β-adrenoceptor transregulation has been also demonstrated at the functional level. Thus, in ileal and tracheal smooth muscles, antagonism of M_2 muscarinic receptors leads to an increase in the relaxant potency of β agonists (9–11).

In HEL 299 cells, a primary cell line derived from human

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ABBREVIATIONS: PKA, cAMP-dependent protein kinase; PKC, protein kinase C; [^{3}H]NMS, [^{3}H]*N*-methylscopolamine; [^{3}H]QNB, [^{3}H]quinuclidinylbenzilate; [^{125}I]IPIN, [^{125}I]iodopindolol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMSO, dimethylsulfoxide; PI, phosphoinositide; HEL, human embryonic lung; PG, prostaglandin; H-8, *N*-[2-(methylamino)ethyl]-5′-isoquinoline-sulfonamide; GF 109203X, 2-[1-(3-dimethylaminopropyl)-inol-3-yl]-3-(indol-3-yl)maleimide.

embryonic lung, functional M_2 muscarinic, and β_2-adrenoceptors has been demonstrated (6)[1] and therefore represents a useful system to investigate cross-talk between these receptors. In this study, we investigated cross-regulation between M_2 muscarinic and β_2-adrenoceptors at the gene and protein levels in an attempt to explain molecular mechanisms for the cross-talk described functionally between these receptors.

Materials and Methods

Cell culture. HEL 299 cells (code CCL 137; American Type Culture Collection, Rockville, MD) were maintained in culture as previously described (6). The medium was replaced every 3–4 days, and on reaching confluence, cells were subcultured through detachment of the monolayer with 0.05% trypsin/1 mM EDTA. Treatments were carried out so that cells could be harvested simultaneously at preconfluence.

Cells were exposed to one or more of the following: procaterol (5 μM), forskolin (50 μM), 8-bromo-cAMP (5 mM), H-8 (30 μM), GF 109203X (1 μM), cycloheximide (10 μg/ml), org 20241 (30 μM), and carbachol (100 μM). Control cells were incubated with an equivalent dilution of DMSO where appropriate. All reagents except H-8 and GF 109203X (CalBiochem-NovaBiochem, Nottingham, UK) were obtained from Sigma (Poole, Dorset, UK).

Binding studies. Radioligand binding experiments were performed on crude cell homogenates at 4°. Cells (~5–10 × 10^6 for each binding reaction) were washed twice with ice-cold Tris·HCl buffer (25 mM, pH 7.4), harvested by cell scraping, and homogenized with an Ultra-Turax homogenizer (one 30-sec burst). The crude membrane homogenates were isolated by centrifugation at 40,000 × *g* for 20 min and resuspended in an appropriate volume of Tris buffer. The protein concentration was measured according to the method of Lowry *et al.* (12).

[^{3}H]NMS, [^{3}H]QNB, and [^{125}I]IPIN (specific activity, 80.4, 49, and 2200 Ci/mmol, respectively; New England Nuclear, Stevenage, UK) saturation curves were elucidated with the use of concentrations of 0.06–2 nM, 0.02–1 nM, and 3–400 pM, respectively. Nonspecific binding was measured in the presence of 1 μM atropine for [^{3}H]NMS and [^{3}H]QNB binding and of 200 μM (−)-isoproterenol for [^{125}I]IPIN binding. Incubations were performed for 2 hr at 30° for [^{3}H]NMS, 3 hr at 30° for [^{3}H]QNB, and 30 min at 37° for [^{125}I]IPIN in 25 mM Tris·HCl buffer (supplemented with 0.1 mg/ml ascorbic acid for [^{125}I]IPIN). The reaction was terminated by rapid vacuum filtration over 0.2% polyethyleneimine pretreated Whatman GF/C glass fiber filters with the use of a Brandel cell harvester. Filters were washed three times with 4 ml of ice-cold Tris buffer and placed into vials with 4 ml of scintillation cocktail (Filtron X; National Diagnostics, Manville, NJ), and cells were counted with a Packard β counter (model 2200 CA) for tritiated ligands and with a Packard γ counter (model Cobra II) for [^{125}I]IPIN. Binding data were analyzed with use of the computerized iterative nonlinear regression program LIGAND (13).

Northern blot analysis. Cells were washed twice with Hanks' balanced salt solution and harvested by detachment of the monolayer with trypsin/EDTA. Total cellular RNA was prepared as described previously (6) before isolation of Poly(A)$^+$ RNA with use of an mRNA extraction kit (Promega) according to the manufacturer's instructions. Northern blots to nylon N$^+$ membranes (Amersham) were prepared subsequently to size fractionation by gel electrophoresis of the denatured mRNA on 1% agarose/formaldehyde gels containing 20 mM morpholinosulfonic acid, 5 mM sodium acetate, and 1 mM EDTA, pH 7.0. A cloned Hm2 *Eco*RI/*Pst*I fragment of human muscarinic Hm2 cDNA (a gift from Dr. N. J. Buckley, Wellcome Laboratory for Molecular Pharmacology, University College, London, UK) and a 1272-base pair *Pst*I fragment specific to rat GAPDH mRNA were used as probes for the Northern blot analyses.

Prehybridizations and hybridizations were carried out at 42° with the probes labeled to ~1.5 × 10^6 cpm/ml in a buffer containing 50% formamide, 50 mM Tris·HCl, pH 7.5, 5× Denhardt's solution, 0.1% sodium dodecyl sulfate, 5 mM EDTA, and 250 μg/ml denatured salmon sperm DNA. After hybridization, the blots were washed to a stringency of 0.1× standard saline citrate/0.1% sodium dodecyl sulfate at 65° before exposure to Kodak XAR-5 film. After suitable exposure times, autoradiographs were analyzed with the use of laser densitometry (PDI).

cAMP measurements. After stimulation, cells were washed with Hanks' balanced salt solution, and the phosphodiesterase inhibitor Org 20241 (30 μM) was added to fresh media for 15 min. cAMP levels were then measured in the presence or absence of carbachol (100 μM for 15 min). Cells were harvested by the addition of 1 ml of boiling water directly to each well followed by cell scraping before being boiled for an additional 2 min. After centrifugation at full speed in a microcentrifuge at 4° for 10 min, the supernatant was collected and stored at −20° before being assayed by radioimmunoassay as described by Dent *et al.* (14). Protein was estimated with a Bio-Rad protein assay (Hemel, Hempstead, UK), according to the manufacturer's instructions.

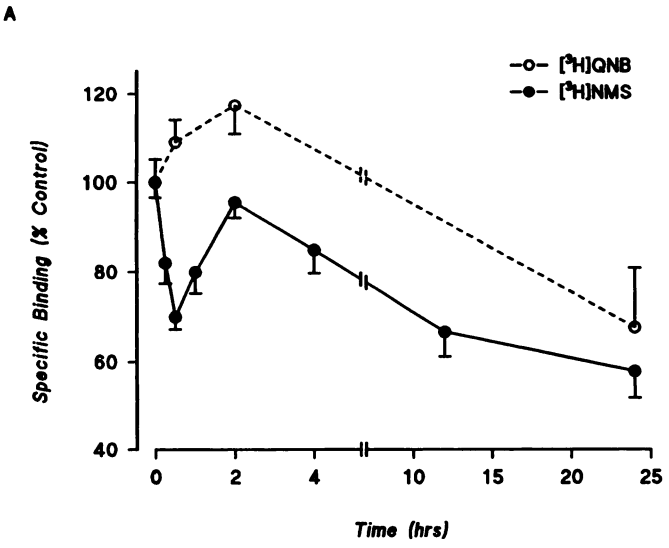
Results

Radioligand binding studies. Receptor binding studies were performed to determine muscarinic and β-adrenoceptor protein levels after cell stimulation with the β_2-adrenergic agonist procaterol and the muscarinic agonist carbachol, respectively. All receptor binding studies were performed on cells at ~70% confluence because a significant loss in the number of receptor binding sites was observed when the cells were allowed to reach confluence (data not shown). Saturation studies performed with the nonselective muscarinic receptor antagonists [^{3}H]NMS and [^{3}H]QNB revealed a single class of binding site (B_{max}, 452 ± 23.4 and 453 ± 23.1 fmol/mg protein, respectively) with equilibrium dissociation constants (K_D) of 0.20 ± 0.03 and 0.02 ± 0.01 nM, respectively.

Pretreatment of HEL 299 cells with procaterol caused a biphasic decrease in [^{3}H]NMS binding. A 40% loss in the number of receptor binding sites at 30 min was followed by a recovery to control levels at 2 hr before a slower reduction to 50% after exposure to procaterol for 24 hr (Fig. 1A). Binding experiments with [^{3}H]QNB, however, showed a different profile of binding, although a loss in receptor number was seen after treatment with procaterol for 24 hr, suggesting sequestration of the M_2 muscarinic receptors followed by recycling back to the cell surface (Fig. 1A). Procaterol did not alter K_D values for either ligand. Further evidence to support this conclusion was obtained from studies with the protein synthesis inhibitor cycloheximide. The recovery in receptor number to control levels after 2 hr of procaterol treatment could only be partially blocked by pretreatment of HEL 299 cells with cycloheximide (data not shown). Collectively, the data suggests that two distinct mechanisms are involved in the down-regulation process: an initial sequestration and cycling of receptors, followed by down-regulation after chronic stimulation with procaterol.

HEL 299 cells were also exposed to other agents that elevate cellular cAMP levels (Fig. 1B). Forskolin (50 μM), which we have shown previously to elevate cAMP levels in these cells (6), and, after treatment for 24 hr, the stable

[1] J. C. W. Mak, J. Rousell, E.-B. Haddad, and P.J. Barnes, unpublished observations.



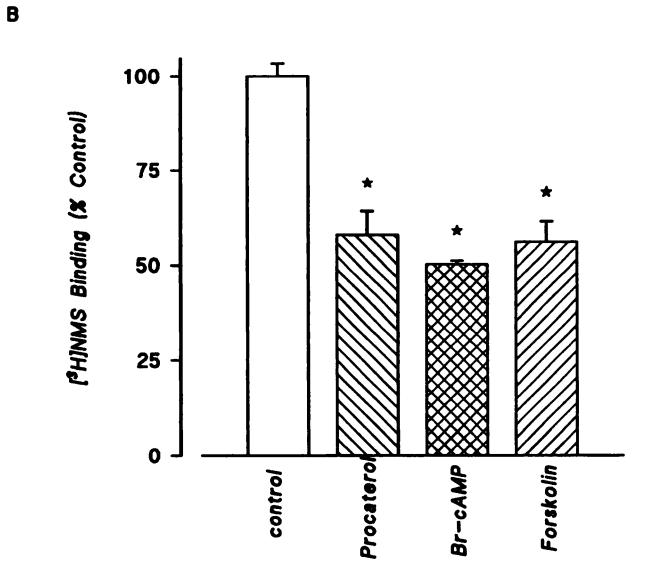


Fig. 1. Effects of cAMP elevation on M_2-receptor binding. A, HEL 299 cells were exposed to procaterol for the times indicated, and [^{3}H]NMS and [^{3}H]QNB binding assays were performed as described in Materials and Methods. B, Effects of treatment for 24 hr with procaterol (5 μM), 8-bromo-cAMP (5 mM), and forskolin (50 μM) on [^{3}H]NMS binding. All data are the mean ± standard error of four to six independent experiments. *, Significant at $p < 0.01$ with Student's t test.

cAMP analogue 8-bromo-cAMP (5 mM) caused down-regulation of the M_2 receptors to an extent similar to that seen with procaterol (Fig. 1B), suggesting a link between the elevated cAMP levels and M_2 muscarinic receptor down-regulation.

Functional β_2-adrenoceptors were demonstrated in these cells. The β-adrenoceptor antagonist [^{125}I]IPIN identified a single population of binding sites with K_D value and binding capacity of 20.9 ± 1.7 pM and 62 ± 18 fmol/mg protein, respectively. These binding sites display characteristic properties of the β_2-adrenoceptor subtype with regard to ligand binding, functional response (cAMP accumulation), and mRNA expression.[2] Saturation binding studies were performed with the β-adrenoceptor antagonist [^{125}I]IPIN to measure β_2-adrenoceptor number. Chronic stimulation of M_2 receptors with carbachol (100 μM for 24 hr) had no effect on [^{125}I]IPIN binding characteristics (B_{max} = 59.6 ± 18.0 fmol/mg protein after carbachol treatment). Although carbachol had no effect on β-adrenoceptor density, procaterol treatments (24 hr) resulted in a significant decrease in β-adrenoceptor density (40% of control, $p < 0.05$) without any significant change in K_D, indicating homogeneous down-regulation of the receptor.

[2] J. C. W. Mak, unpublished observations.

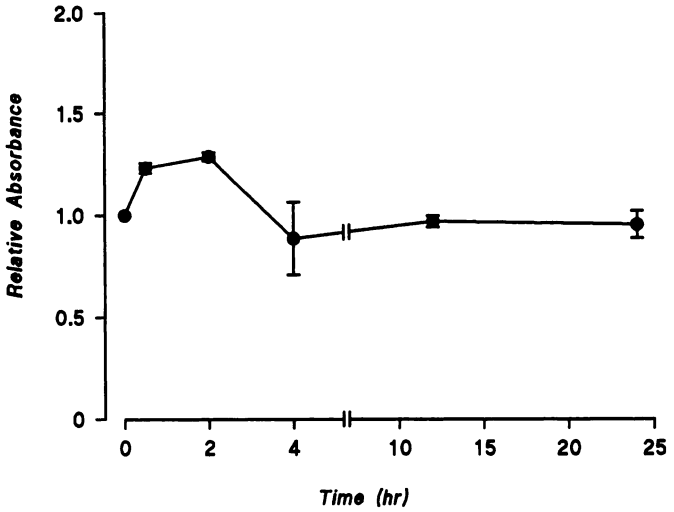


Fig. 2. Time-dependent effects of procaterol on the steady state levels of m2 receptor mRNA. Northern blot analyses were performed with ^{32}P-labeled cDNA probes specific to m2 receptor and GAPDH on isolated mRNA as described in Materials and Methods. Shown are m2 receptor mRNA levels relative to GAPDH after procaterol treatment for the times indicated after assessment by laser densitometry. Data are the mean ± standard error of four independent experiments.

Muscarinic receptor gene expression. Northern blot analyses of isolated mRNAs revealed the presence of a 6.1-kilobase transcript corresponding to the m2 receptor. Short incubations (≤2 hr) of HEL 299 cells with procaterol resulted in a small (25%) increase in the steady state levels of m2 receptor mRNA (Fig. 2), which resolved with longer incubation periods (≤24 hr; Fig. 2). Although 24-hr procaterol and 8-bromo-cAMP treatments had no effect on m2 receptor mRNA levels, forskolin significantly reduced the steady state levels of m2 receptor mRNA after 24 hr of incubation by 50% (Fig. 3).

Role of PKA and PKC in M_2 muscarinic receptor and m2 receptor mRNA down-regulation. Experiments with the PKA inhibitor H-8 and the PKC inhibitor GF 109203X were performed to address the potential involvement of PKA and PKC in β-adrenoceptor-, forskolin-, and 8-bromo-cAMP-mediated receptor down-regulation of M_2 receptors. The data from these experiments are summarized in Figs. 4 and 5. Both H-8 and GF 109203X completely inhibited the down-regulation observed with procaterol (95 ± 4.9% and 1056 ± 7.0% of control, respectively) without having an effect alone, suggesting that both of these kinases are involved in the down-regulation process. Although the procaterol-induced down-regulation was blocked completely by these enzyme inhibitors, the forskolin effect was only partially blocked by both inhibitors (81% and 80% of control for H-8 and GF 109203X, respectively; $p < 0.05$ for H-8, $p > 0.05$ for GF 109203X). Furthermore, preincubations with these inhibitors

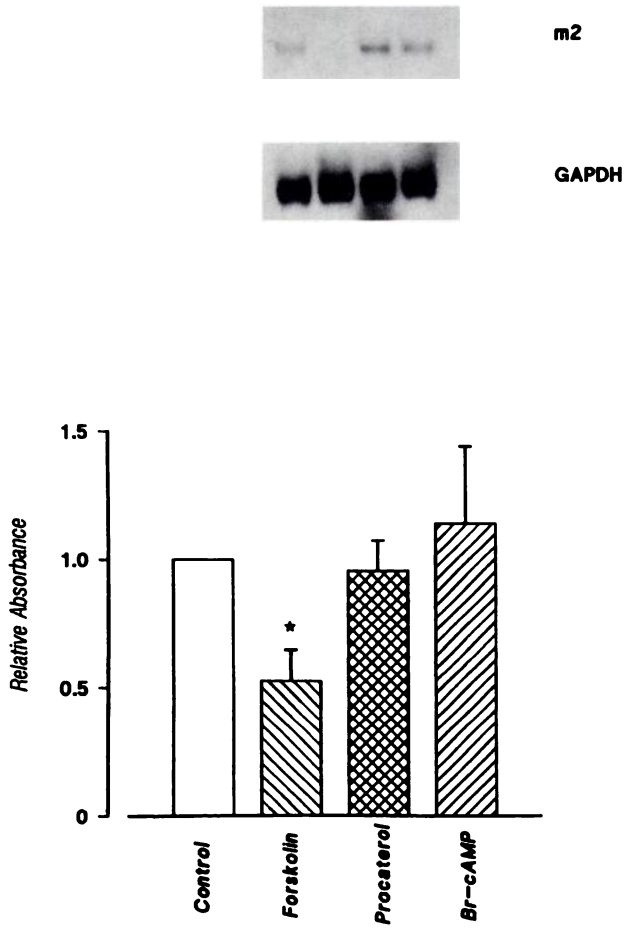


Fig. 3. Effect of agents that elevate cellular cAMP on steady state levels of m2 receptor mRNA. Northern blot analyses were performed on isolated mRNA from cells treated with procaterol (5 μM), 8-bromo-cAMP (5 mM), and forskolin (50 μM). *Top*, representative Northern blot for these treatments. *Bottom*, m2 receptor mRNA levels relative to GAPDH after the treatments indicated after assessment by laser densitometry. Data are the mean ± standard error of five independent experiments. *, Significant at $p < 0.05$ with Student's *t* test.

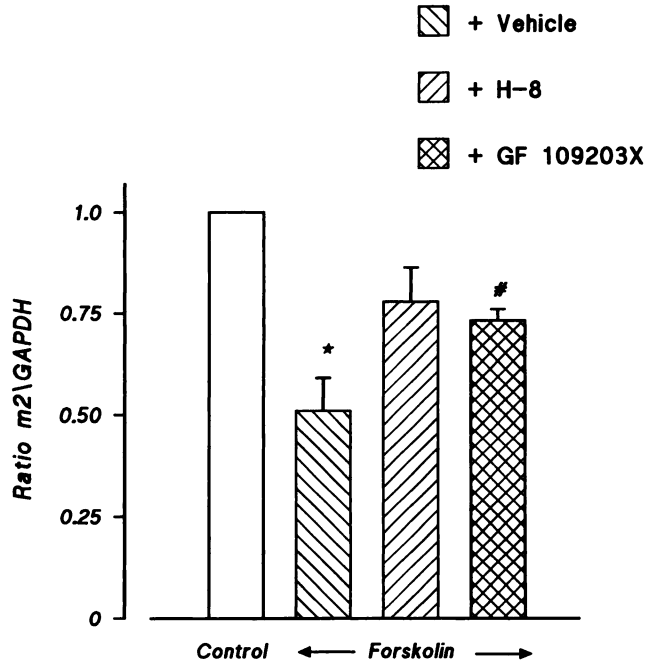


Fig. 4. Involvement of PKA and PKC in the down-regulation of m2 mRNA in HEL 299 cells. Cells were treated with vehicle (*Control*) or 50 μM forskolin (*Forskolin*) in the presence of DMSO, H-8, or GF 109203X for 24 hr before mRNA was extracted, and Northern blots were performed as described in Materials and Methods. The graph represents m2 mRNA levels relative to GAPDH after assessment by laser densitometry. Data are the mean ± standard error of four separate experiments. *, Significantly different than control at $p < 0.05$ with Student's *t* test. #, Significantly different than 24-hr forskolin at $p < 0.05$ with Student's *t* test.

had no effect on the 8-bromo-cAMP-mediated down-regulation. The inhibitory effects of H-8 and GF 109203X on the forskolin-induced down-regulation at the receptor level were mirrored at the mRNA level (Fig. 5). Both inhibitors attenuated the forskolin effect to a similar degree as seen in the binding experiments (78% and 75% of control for H-8 and GF 109203X, respectively). Thus, the effects of forskolin (and the inhibitory effects of H-8 and GF 109203X) may be attributed to changes at the mRNA level.

Receptor coupling studies. Stimulation of HEL 299 cells with procaterol resulted in a rapid accumulation of cAMP, which decreased afterward in a time-dependent manner (Fig. 6A). cAMP levels remained significantly above basal levels after treatment with procaterol for 24 hr (0.18 ± 0.01 at base-line; 8.59 ± 0.569 pmol/mg protein at 24 hr). In cells treated with vehicle or procaterol (≤2 hr), cAMP accumulation was significantly inhibited by carbachol (~50%). The inhibitory effect of carbachol was, however, lost after 24 hr of treatment with procaterol, suggesting M_2 muscarinic receptor uncoupling (Fig. 6B). This was confirmed by restimulation of HEL 299 cells with PGE_2 (Fig. 6C). In untreated cells and in cells coincubated with procaterol and PGE_2 for 15 min, cAMP accumulation was significantly inhibited (30% and 25%, respectively; $p < 0.05$) by carbachol, but this inhibitory effect was lost in cells treated with procaterol for 24 hr, even though restimulation of adenylyl cyclase was possible. Co-treatments with procaterol and PGE_2 resulted in a greater accumulation of cAMP, indicating that β-adrenoceptors and PGE_2 receptors do not couple exclusively to the same pool of adenylyl cyclase. The uncoupling of M_2 receptors elicited with procaterol could be reversed by preincubations with the PKA and PKC inhibitors H-8 and GF 109203X (31%, $p < 0.05$ for H-8; 29%, $p < 0.05$ for GF 109203X), implicating a role for both of these kinases in the receptor desensitization process (Fig. 6D).

Discussion

These results demonstrate transregulation of M_2 muscarinic receptors by β_2-adrenoceptor stimulation in HEL 299 cells and suggest the involvement of PKA and PKC in the desensitization and down-regulation processes.

Specific [^{3}H]NMS and [^{3}H]QNB binding to muscarinic receptors in HEL 299 cell membranes was saturable and best described by interaction of the radioligands with a single population of high affinity binding sites. In these cells, muscarinic receptors can be equally recognized by tertiary and quaternary antagonists, in agreement with data obtained in other cell lines (15, 16). Stimulation of β_2-adrenoceptors resulted in a biphasic decrease of cell surface receptors as measured with the hydrophilic ligand [^{3}H]NMS, whereas saturation binding experiments with the lipophilic ligand

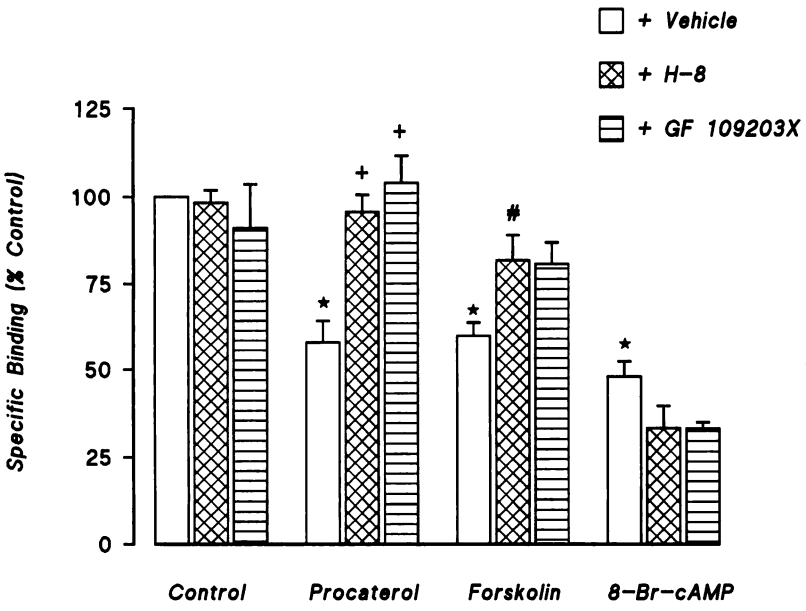


Fig. 5. Involvement of PKA and PKC in the down-regulation of M_2 receptors in HEL 299 cells. Cells were treated in the presence of vehicle, 30 μM of the PKA inhibitor H-8, or 1 μM of the PKC inhibitor GF 109203X in the absence (*Control*) or presence of procaterol (*Procaterol*), forskolin (*Forskolin*), or 8-bromo-cAMP (*8-Br-cAMP*) for 24 hr before preparation of membranes. Saturation binding assays were performed with [^{3}H]NMS as described in Materials and Methods. Data are the mean ± standard error of three to six independent experiments. *, Significantly different than control at $p < 0.01$ with Student's *t* test. +, Significantly different than 24-hr procaterol at $p < 0.05$ with Student's *t* test. #, Significantly different than 24-hr forskolin at $p < 0.05$ with Student's *t* test.

[^{3}H]QNB revealed a different profile, in which long but not short treatments with procaterol resulted in a loss of receptor number. Together, these data suggest that short term treatment of HEL 299 cells with procaterol induces sequestration of the muscarinic receptors followed by their recycling to the cell surface before subsequent down-regulation, a process reminiscent of that seen on agonist occupancy of muscarinic receptors (17–19). This conclusion was further substantiated with the use of cycloheximide. Preincubation of the cells with this protein synthesis inhibitor did not prevent receptor recovery at 2 hr, thus excluding the synthetic origin of the new receptors (data not shown). Although stimulation of β_2-adrenoceptors resulted in a loss of M_2 receptor binding sites, carbachol (100 μM) stimulation had no effect on the levels of β_2-adrenoceptor number, indicating that cross-talk was unidirectional. This is in contrast to previous studies (8) showing carbachol-induced down-regulation of β-adrenoceptors in rat cardiac myocytes. Our data, however, are consistent with a number of other studies. Lee *et al.* (7) described cross-talk between β-adrenoceptors and muscarinic receptors cotransfected into Chinese hamster ovary cells, where β_2-adrenoceptor stimulation resulted in sequestration of M_1 receptors. Our results also agree with data reported in both rat heart and lung, where chronic β antagonist treatment (6 days) resulted in a significant down-regulation of M_2 muscarinic and A_1 adenosine receptors (20). Although carbachol did not elicit any heterologous regulation of β-adrenoceptor receptor density, we have previously shown that the same treatments result in a significant loss in M_2 muscarinic binding sites in these cells (21). Similarly, homogeneous regulation of β_2-adrenoceptors occurred in HEL 299 cells where prolonged β agonist treatment resulted in a significant reduction in β_2-adrenoceptor density. This is in accordance with published data from a number of other systems (for a review, see Ref. 22).

The decrease in muscarinic receptor binding sites seen on chronic stimulation with procaterol was mimicked with 8-bromo-cAMP, a stable cAMP analogue, and forskolin, an agent that directly stimulates adenylyl cyclase. These data strongly implicate a cAMP-dependent process in the M_2 receptor down-regulation.

We also evaluated the steady state levels of m2 muscarinic receptor mRNAs after procaterol treatment. Northern blot analyses of cellular mRNA revealed no overall changes in the steady state levels of m2 mRNA, although a small increase was seen after shorter incubation periods (≤2 hr). Unlike the data obtained with procaterol and 8-bromo-cAMP, 24-hr forskolin treatments resulted in a significant reduction in the steady state levels of m2 muscarinic receptor mRNA. The reasons for the discrepancy in the results obtained with these compounds are not clear but may reflect the differential sensitivity of the adenylyl cyclase isoforms (of which at least eight have been identified) to different stimuli (23). Forskolin is also known to elicit several other effects that are not adenylyl cyclase dependent (24). Preincubation with PKA inhibitor H-8 (25) was able to partially but not significantly attenuate the reduction in m2 receptor mRNA, suggesting such a nonselective effect of forskolin. Furthermore, preincubations with the PKC inhibitor GF 109203X also inhibited the forskolin-induced down-regulation. This may indicate a role for this kinase in the changes in m2 mRNA as we have previously described a role for PKC in the down-regulation of m2 mRNA in these cells (6).

To assess the roles of PKA and PKC in the M_2 receptor down-regulation, H-8 and GF 109203X (26) were used in binding studies. Both compounds attenuated the loss of [^{3}H]NMS binding sites in HEL 299 cells treated for 24 hr with procaterol, implicating a role for both of these kinases in the down-regulation process. PKA is known to phosphorylate muscarinic receptors (27, 28), and similar results were reported by Lee *et al.* (7), including reduction in the down-regulation of M_1 receptors through stimulation of β_2-receptors in Chinese hamster ovary cells with H-8. The involvement of PKC, however, was more surprising. A variety of studies provide evidence to support cross-talk between the PLC and cAMP signal transduction pathways (4), and PKC is known to phosphorylate muscarinic receptors (29, 30). Stimulation of PKA has also been shown to enhance

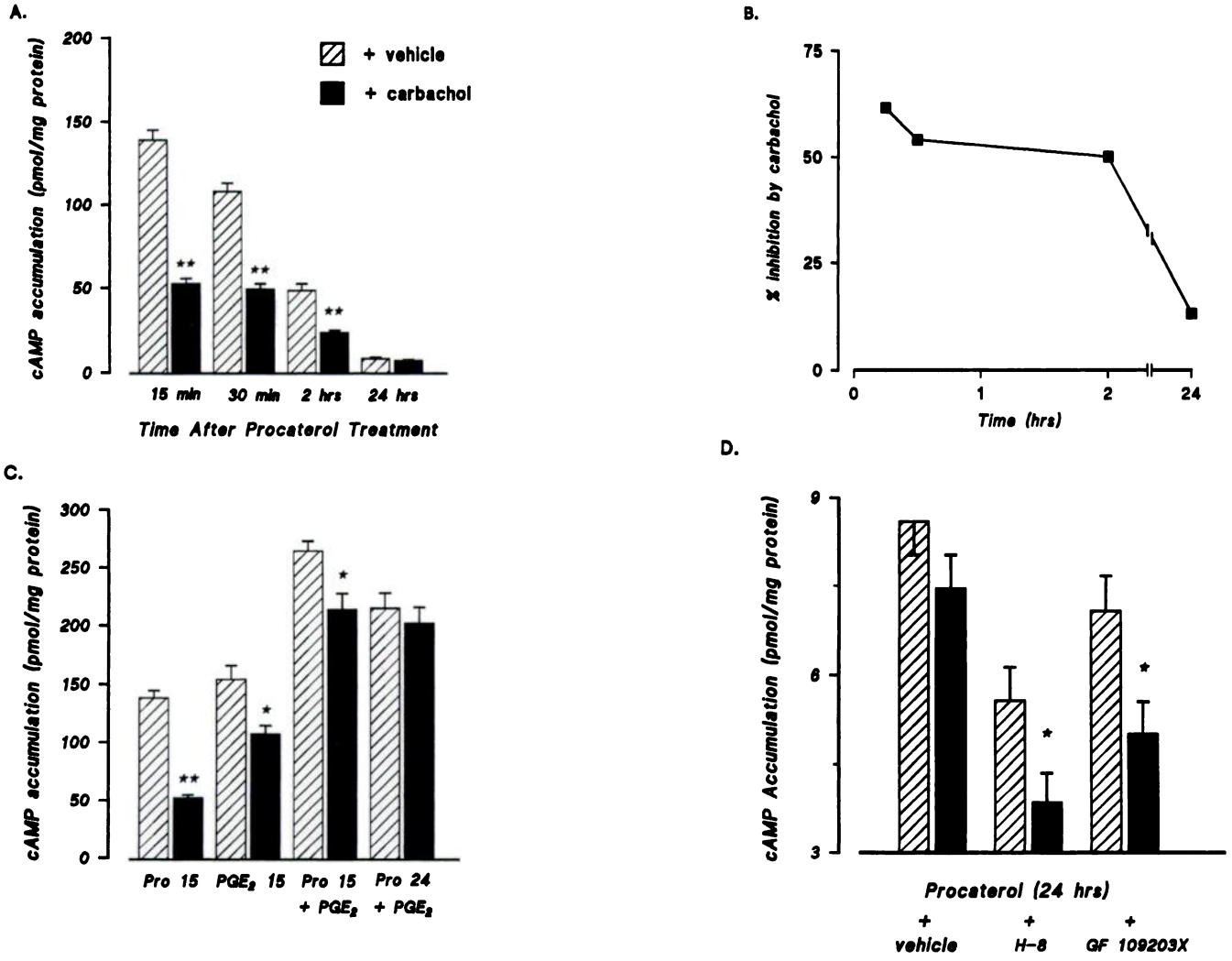


Fig. 6. Coupling studies in HEL 299 cells. Cells were treated for the times indicated before the addition of a phosphodiesterase inhibitor for 15 min. A, Cells were then treated with vehicle or 100 μM carbachol for an additional 15 min before cAMP was extracted. Accumulation of cAMP with procaterol could be significantly inhibited by carbachol for ≤2 hr but not after 24 hr. B, Percentage inhibition by carbachol for the times indicated. C, Accumulation of cAMP through stimulation of PGE_2 receptors in the presence and absence of carbachol before (*PGE_2*) and after procaterol treatments for 15 min (*Pro 15 + PGE_2*) and 24 hr (*Pro 24 + PGE_2*). D, Effect of pretreatment with the PKA and PKC inhibitors H-8 and GF 109203X on procaterol-stimulated cAMP levels. Data are the mean of at least three independent experiments performed in triplicate. **, Significant at $p < 0.01$ with Student's *t* test. *, Significant at $p < 0.05$ with Student's *t* test.

inositol-1,4,5-trisphosphate-mediated Ca^{++} mobilization (31–33), which may account for the activation of PKC. Non-selective inhibition of PKA by GF 109203X is unlikely because inhibition of PKA activity by GF 109203X in bovine airway smooth muscle indicates an IC_{50} value of >10 μM,[3] although the effect of H-8 on PKC is not known in these cells. Although preincubations with H-8 and GF 109203X were able to completely inhibit the procaterol-induced down-regulation of M_2 receptors, forskolin-induced down-regulation was only partially blocked by these inhibitors. This result is consistent with the Northern blot data suggesting that the effects of forskolin are a result of changes in the levels of m2 mRNA and not the result of any phosphorylation events at the receptor protein level. Finally, H-8 and GF 109203X did not inhibit the 8-bromo-cAMP-induced down-regulation, for reasons that are not clear. Although the reasons for the discrepancies in the action of these three adenylyl cyclase activators are not known, the experiments may illustrate subtle differences in the pathways activated by these compounds or selective inhibition of different isoforms of PKA or PKC by H-8 and GF 109203X.

Receptor coupling studies also revealed an involvement of PKA and PKC in M_2 muscarinic receptor desensitization through β_2-adrenoceptor stimulation. The accumulation of cAMP after short term procaterol treatment was inhibited by carbachol. This inhibition was lost after 24-hr treatment with procaterol, suggesting M_2 receptor uncoupling. Although acute PGE_2 (and forskolin) stimulated cAMP was inhibited by carbachol, prestimulation with procaterol for 24 hr attenuated this inhibition. Restimulation of the adenylyl cyclase system was possible after 24-hr procaterol treatment with PGE_2, indicating that the time-dependent reduction in the accumulation of cAMP probably occurred at the β-adrenoceptor level and is consistent with the loss in β-adrenoceptor number. The involvement of PKC and PKA in M_2 receptor desensitization was also demon-

[3] J. Rousell and B. Webb, unpublished observations.

strated as the procaterol-induced uncoupling could be partially inhibited by H8 and GF 109203X.

In summary, we described a molecular mechanism for the transregulation of muscarinic M_2 receptors by β_2-adrenoceptor stimulation that results in an uncoupling and down-regulation of the M_2 receptor. The down-regulation of M_2 receptors by procaterol did not seem to be the result of changes in the steady state levels of m2 receptor mRNA. Furthermore, the loss of muscarinic receptors and their functional uncoupling apparently involves PKA and PKC. Although the down-regulation of M_2 receptors could be mimicked by other agents that activate adenylyl cyclase, the biochemical pathways involved seem not to be shared.

References

1. Hulme, E. C., N. J. M. Birdsall, and N. J. Buckley. Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.* **30:**633–650 (1990).
2. Caulfield, M. P. Muscarinic receptors, characterization, coupling and function. *Pharmacol. Ther.* **58:**319–379 (1993).
3. Wess, J. Molecular basis of muscarinic acetylcholine receptor function. *Trends Pharmacol. Sci.* **14:**308–313 (1993).
4. Fisher, S. K. Homologous and heterologous regulation of receptor-stimulated phosphoinositide hydrolysis. *Eur. J. Pharmacol.* **288:**231–250 (1995).
5. Morris, G. M., J. R. Hadcock, and C. C. Malbon. Cross regulation between G-protein coupled receptors: activation of β_2-adrenoceptors increases α_1-adrenoceptor mRNA levels. *J. Biol. Chem.* **266:**2233–2238 (1991).
6. Rousell, J., E.-B. Haddad, J. C. W. Mak, and P. J. Barnes. Transcriptional down-regulation of m2 muscarinic receptor gene expression in human embryonic lung (HEL 299) cells by protein kinase C. *J. Biol. Chem.* **270:** 7213–7218 (1995).
7. Lee, N. H., J. Earle-Hughes, and C. M. Fraser. Agonist-mediated destabilization of m1 muscarinic acetylcholine receptor mRNA. *J. Biol. Chem.* **269:**4291–4298 (1994).
8. Paraschos, A., and J. S. Karliner. Receptor crosstalk: effects of prolonged carbachol exposure on β_1-adrenoceptors and adenylyl cyclase activity in neonatal rat ventricular myocytes. *Nuanyn-Schmiedeberg's Arch. Pharmacol.* **350:**267–276 (1994).
9. Fernandes, L. B., A. D. Fryer, and C. A. Hirshman. M_2 muscarinic receptors inhibit isoproterenol-induced relaxation of canine airway smooth muscle. *J. Pharmacol. Exp. Ther.* **262:**119–124 (1992).
10. Eglen, R. M., H. Reddy, N. Watson, and R. A. Challiss. Muscarinic acetylcholine receptor subtypes in smooth muscle. *Trends Pharmacol. Sci.* **15:** 114–119 (1994).
11. Thomas, E. A., Baker, S. A., and Ehlert, F. J. Functional role for the M_2 muscarinic receptor in smooth muscle of guinea pig ileum. *Mol. Pharmacol.* **44:**102–110 (1993).
12. Lowry, O. H., N. J. Roseborough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275 (1951).
13. Munson, P. J., and D. Rodbard. Ligand: a versatile computerized approach to characterization of ligand binding systems. *Anal. Biochem.* **107:**220–239 (1980).
14. Dent, G., M. A. Giembycz, P. M. Evans, K. F. Rabe, and P. J. Barnes. Suppression of human eosinophil respiratory burst and cyclic AMP hydrolysis by inhibitors of type IV phosphodiesterase: interaction with the *beta* adrenoceptor agonist albuterol. *J. Pharmacol. Exp. Ther.* **271:**1167–1174 (1994).
15. Fisher, S. K. Recognition of muscarinic cholinergic receptors in human SK-N-SH neuroblastoma cells by quaternary and tertiary ligands is dependent upon temperature, cell integrity, and the presence of agonists. *Mol. Pharmacol.* **33:**414–422 (1988).
16. Wei, H.-B., H. I. Yamamura, and W. R. Roeske. Down-regulation and desensitization of the muscarinic M_1 and M_2 receptors in transfected fibroblast B82 cells. *Eur. J. Pharmacol.* **268:**381–391 (1994).
17. Galper, J. B., L. C. Dziekan, D. S. O'Hara, and T. W. Smith. The biphasic response of muscarinic cholinergic receptors in cultured heart cells to agonists. *J. Biol. Chem.* **257:**10344–10356 (1982).
18. Feigenbaum, P., and E. E. El-Fakahany. Regulation of muscarinic cholinergic receptor density in neuroblastoma cells by brief exposure to agonist: possible involvement in desensitization of receptor function. *J. Pharmacol. Exp. Ther.* **233:**134–140 (1985).
19. Harden, T. K., L. A. Petch, S. F. Traynelis, and G. L. Waldo. Agonist-induced alteration in the membrane form of muscarinic cholinergic receptors. *J. Biol. Chem.* **260:**13060–13066 (1985).
20. Marquetant, R., B. Brehm, and R. H. Strasser. Chronic β-blockade transregulates inhibitory A_1-adenosine and muscarinic M_2 receptors of the adenylyl cyclase system. *J. Mol. Cell Cardiol.* **24:**535–548 (1991).
21. Haddad, E.-B., Rousell, J., Mak, J. C. W., and Barnes, P. J. Long term carbachol treatment-induced down-regulation of muscarinic M_2-receptors but not m2 mRNA in a human lung cell line. *Br. J. Pharmacol.* **116:**2027–2032 (1995).
22. Barnes, P. J. *Beta*-adrenergic receptors and their regulation. *Am. J. Respir. Crit. Care Med.* **152:**838–860 (1995).
23. Taussig, R., and A. G. Gilman. Mammalian membrane-bound adenylyl cyclases. *J. Biol. Chem.* **270:**1–4 (1995).
24. Laurenza, A., E. McHugh Sutkowski, and K. B. Seamon. Forskolin: a specific stimulator of adenylyl cyclase or a diterpine with multiple sites of action? *Trends Pharmacol. Sci.* **10:**442–447 (1989).
25. Hidaka, H., M. Inagaki, S. Kawamoto, and Y. Sasaki. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23:**5036–5041 (1984).
26. Toullec, D., P. Pianetti, H. Coste, P. Bellevergue, T. Grand-Perret, M. Ajakane, V. Baudet, P. Boissin, E. Boursier, F. Loriolle, L. Duhamel, D. Charon, and J. Kirilovsky. The bisindolymaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* **266:**15771–15781 (1991).
27. Ho, A. K. S., and J. H. Wang. Calmodulin regulation of cholinergic muscarinic receptor: effects of calcium and phosphorylation states. *Biophys. Biochem. Res. Commun.* **133:**1193–1200 (1985).
28. Rozenbaum, L. C., D. A. Malencik, S. R. Anderson, M. R. Tota, and M. I. Schimerlik. Phosphorylation of porcine atrial muscarinic acetylcholine receptor by cyclic AMP dependent protein kinase. *Biochemistry* **26:**8183–8188 (1987).
29. Liles W. C., D. D. Hunter, K. E. Meier, and N. M. Nathanson. Activation of protein kinase C induces rapid internalization and subsequent degradation of muscarinic acetylcholine receptors in neuroblastoma cells. *J. Biol. Chem.* **261:**5307–5313 (1986).
30. Hosey, M. M., J. L. Benovic, S. K. DeBurman, and R. M. Richarson. Multiple mechanisms involving protein phosphorylation are linked to desensitization of muscarinic receptors. *Life Sci.* **56:**951–955 (1995).
31. Enouf, J., F. Giraud, R. Bredoux, N. Bourdeau, and S. Levy-Toledano. Possible role for cAMP-dependent phosphorylation in the calcium release mediated by inositol 1,4,5-trisphosphate in human platelet membrane vesicles. *Biochim. Biophys. Acta* **928:**76–82 (1987).
32. Volpe, P., and B. H. Alderson-Lang. Regulation of inositol 1,4,5-triphosphate-induced Ca^{++} release. *Am. J. Physiol.* **258:**C1086–C1091 (1990).
33. Burgess, G. M., G. St. J. Bird, J. F. Obie, and J. W. Putney. The mechanism for synergism between phospholipase C- and adenylyl cyclase-linked hormones in liver. *J. Biol. Chem.* **266:**4772–4781 (1991).

Send reprint requests to: Dr. Peter J. Barnes, Department of Thoracic Medicine, National Heart and Lung Institute, Dovehouse Street, London, UK SW3 6LY. E-mail: p.j.barnes@ic.ac.uk