

Desensitization of the Y1 Cell Adrenocorticotropin Receptor

EVIDENCE FOR A RESTRICTED HETEROLOGOUS MECHANISM IMPLYING A ROLE FOR RECEPTOR-EFFECTOR COMPLEXES*

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Receptor desensitization provides a potential mechanism for the regulation of adrenocortical adrenocorticotropin (ACTH) responsiveness. Using the mouse adrenocortical Y1 cell line we demonstrate that ACTH effectively desensitizes the cAMP response of its own receptor, the melanocortin 2 receptor (MC2R), in these cells with a maximal effect between 30 and 60 min. Neither forskolin nor isoproterenol (in Y1 cells stably transfected with the β_2 -adrenergic receptor) desensitize this ACTH response. ACTH desensitizes its receptor at concentrations at which only a fraction of receptors are occupied, implying that this mechanism acts on agonist-unoccupied receptors. Y1 cells express G protein-coupled receptor kinase (GRK) 2 and 5, but stable expression of a dominant negative GRK2 (K220W) only marginally reduces the desensitization by ACTH. The protein kinase A (PKA) inhibitor, H89, extinguishes almost the entire desensitization response over the initial 30-min period at all concentrations of ACTH. A mutant MC2R in which the single consensus PKA phosphorylation site has been mutated (S208A) when expressed in MC2R-negative Y6 cells is also unable to desensitize. These data imply a heterologous, PKA-dependent, mode of desensitization, which is restricted to agonist-occupied and -unoccupied MC2R, possibly as a consequence of receptor/effector complexes that functionally compartmentalize this receptor.

The pituitary hormone, ACTH¹ has a primary endocrine function in the stimulation and release of glucocorticoids by the adrenal cortex and is the major determinant of physiological circadian variation and stress-related fluctuations of this ste-

roid. The physiological effect of ACTH in steroidogenesis is mediated by the ACTH receptor, also known as the melanocortin 2 receptor (MC2R) (1–3). This receptor is expressed primarily in the adrenal cortex (3–5) with lower expression in the adipocyte (6, 7). The MC2R is homologous to the other four cloned melanocortin receptors (8, 9), which together form a distinct subfamily of the seven transmembrane domain G protein-coupled receptors (GPCR). It has long been known that the binding of ACTH to its receptor stimulates adenylate cyclase, elevating cAMP (10) leading to activation of PKA and consequently stimulation of expression of steroidogenic enzymes.

Sensitivity of the adrenal cortex to the actions of ACTH is therefore dependent to a large extent on the expression or function of the MC2R. There is evidence that high concentrations of ACTH may regulate MC2R gene expression (11–13). However it seems more likely that short term changes in sensitivity are determined at a receptor level by the extent of its coupling to the adenylate cyclase pathways.

Previous studies have shown that adenylate cyclase activity and cAMP levels in the mouse adrenocortical Y1 cell line, which expresses a functional MC2R, decrease after repeated ACTH stimulation (14, 15) although the molecular mechanisms underlying this observation have remained obscure. Many other GPCRs exhibit the phenomenon of desensitization, which is traditionally described as being either heterologous or homologous in nature. In heterologous desensitization activation of several different receptor types may desensitize the receptor in question, and this is typically mediated by phosphorylation by nonspecific cellular kinases such as PKA or protein kinase C (16–18). In homologous desensitization only the agonist-occupied receptor is able to desensitize itself by a process involving activation of a GRK, which phosphorylates serine/threonine residues in the receptor, leading to its binding to an arrestin molecule with consequent interference in G protein coupling (16–18).

In this report we have characterized the nature of the desensitization process of the endogenous MC2R in the mouse Y1 cell. We find that desensitization has some characteristics of a homologous process, yet is clearly PKA-dependent.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents were purchased from Sigma-Aldrich (Dorset, UK) and Life Technologies, Inc. (Paisley, Scotland, UK). ACTH (1–24) and norleucine⁴ D-phenylalanine⁷-melanocyte-stimulating hormone (NDP-MSH) was purchased from Peninsula Laboratories (St. Helen's, Merseyside, UK) and H89 (*N*-[2-(*p*-bromocinnamyl)amino]ethyl]-5-isoquinolinesulphonamide, 2HCl) was from Calbiochem (Nottingham, UK).

Cell Culture—Y1 mouse adrenal tumor cells (ATCC, Manassas, VA) and the derived mutant cell line Y6 (a gift from B. Schimmer) were maintained in Dulbecco's modified Eagle's medium supplemented with 15% horse serum and 2.5% fetal bovine serum and 1% penicillin/strep-

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¹ The abbreviations used are: ACTH, adrenocorticotropin; MC2R, melanocortin 2 receptor; GRK, G protein-coupled receptor kinase; PKA, protein kinase A; GPCR, G protein-coupled receptor; NDP-MSH, norleucine⁴ D-phenylalanine⁷-melanocyte-stimulating hormone; IBMX, 3-isobutyl-1-methylxanthine; AKAP, protein kinase A-anchoring protein; RT-PCR, reverse transcriptase-polymerase chain reaction.

tomycin. The cells were incubated in a humidified atmosphere at 5% CO₂ at 37 °C.

cAMP Measurement—Cells were seeded in 6-well plates 24–48 h prior to stimulation with ACTH-(1–24) and incubated in serum-free medium for at least 1 h prior to stimulation. Cells were stimulated in the presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX) for 30 min at 37 °C. After incubation, cells were heated at 100 °C for 5 min, centrifuged to precipitate cell debris, and the supernatant was stored for assay. cAMP was determined by a competitive binding assay (19). Protein content in the samples was determined using the Bio-Rad protein assay.

Desensitization Studies—Cells were stimulated first with either ACTH (10⁻⁸ M) or in some experiments with isoproterenol (10⁻⁵ M) or forskolin (10⁻⁶ M) for varying lengths of time. The cells were then washed with serum-free medium and restimulated with ACTH (10⁻⁸ M) in the presence of IBMX (1 mM) for 30 min in this collection phase.

Site-directed Mutagenesis—Serine 208 on the third intracellular loop was changed to an alanine using the QuikChange protocol (Stratagene, Amsterdam, Netherlands). The mutation was confirmed by DNA sequencing.

Transfection—Cells were grown in 9-cm dishes to 50% confluence and transfected with 10–20 µg of DNA per plate using the calcium phosphate co-precipitation method (total amount of plasmid was adjusted by vector DNA). After 48 h, cells were selected in Geneticin (300 µg/ml). Medium was changed every 3–4 days. Resistant colonies appear after about 2 weeks, and these were isolated by ring cloning and expanded to produce stable cell lines.

RT-PCR—RNA was extracted using RNazol™ B (Biogenesis, Poole, UK). The reverse transcription reaction was performed as follows: 10 µg of total RNA was mixed with oligo(dT) (5 µM) and heated to 75 °C for 5 min and then chilled on ice for 5 min. After this, 5× RT buffer, dNTPs (0.4 mM), and Moloney murine leukemia virus reverse transcriptase (200 units/µl) were added and incubation was at 37 °C for 1 h. PCR was carried out on 2 µl of cDNA in a 50-µl reaction using *Taq* polymerase. The primers were designed based on the rat GRK2, -3, -5, and -6 to obtain products of different sizes (primer sequences available on request). The fragments were visualized after electrophoresis on a 1% agarose gel.

Immunoblotting—Y1 cells were washed twice in phosphate-buffered saline and scraped into ice-cold phosphate-buffered saline. The samples were spun at 6500 × *g* for 5 min at 4 °C, the supernatant was aspirated, and the pellet was resuspended in 100 µl of SDS sample buffer. Prior to use the total cell lysate was heated to 85 °C for 10 min and reconstituted using a fine needle attached to a syringe. Proteins were resolved by 8% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (PerkinElmer Life Science Products, Boston, MA), and then probed with either GRK2 or GRK5 primary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Chemiluminescent detection of proteins was performed with ECL reagents (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Experimental Design and Statistics—All data presented are the means (± S.E.) of three or more independent experiments with all points determined in duplicate or triplicate within experiments and each assayed in duplicate. Dose-response curves were analyzed by GraphPad Prism (GraphPAD Software, San Diego, CA). Where appropriate, statistical significance was determined by Student's *t* test or by two-way analysis of variance using MS Excel.

RESULTS

cAMP is produced in a dose-responsive manner after a single stimulation of Y1 cells with ACTH giving an EC₅₀ of 1.3 × 10⁻⁸ M in the presence of IBMX (Fig. 1a). Following a single stimulus, the production and accumulation of cAMP increases steadily with time until 120 min, after which it reaches a plateau. In the absence of IBMX, cAMP does not accumulate and is undetectable (data not shown). The existence of other melanocortin receptors in these cells was excluded by demonstrating that NDP-MSH, a superagonist peptide that stimulates all of the melanocortin receptors except the MC2R, failed to generate a cAMP response. Thus in Y1 cells, ACTH signals exclusively through the MC2R.

Desensitization of the MC2R can be detected following only 5 min of pre-exposure to ACTH, and the extent of desensitization increased gradually for periods of time up to 6 h (Fig. 1c and data not shown). We wished to determine whether this desen-

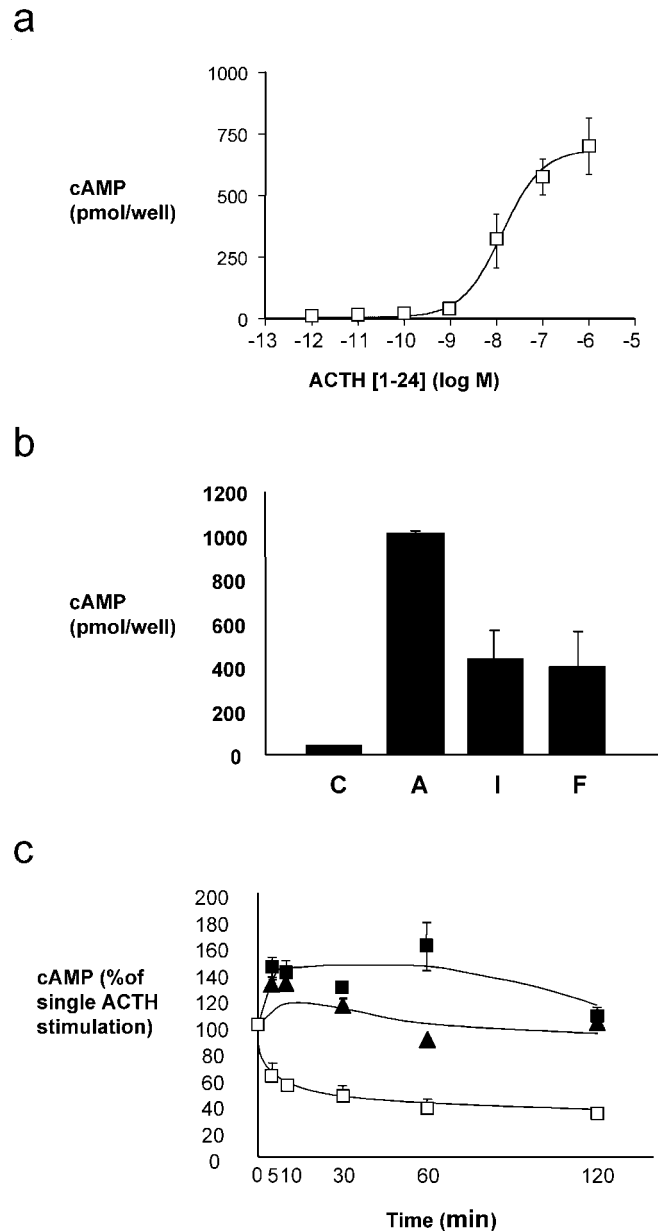
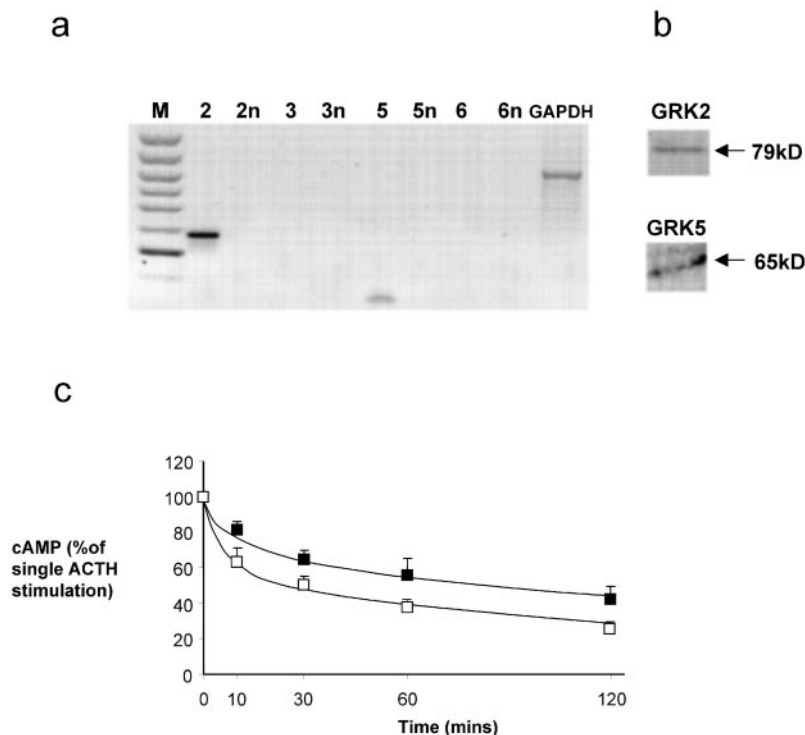


FIG. 1. Characterization of desensitization of the endogenous MC2R in Y1 cells. *a*, cAMP dose response curve to ACTH-(1–24) stimulation in the presence of IBMX (1 mM) for 30 min. After this time, cells were harvested and prepared for the cAMP assay. *n* = 3, mean ± S.E. *b*, cAMP response of Y1 cells to stimulation for 30 min with medium alone (C), ACTH (10⁻⁸ M) (A), isoproterenol (10⁻⁵ M) in Y1 cells stably transfected with the β₂-adrenergic receptor (I) and forskolin (10⁻⁵ M) (F). *c*, cAMP response to ACTH (10⁻⁸ M) stimulation for 30 min following an initial stimulus with ACTH 10⁻⁸ M (□), isoproterenol 10⁻⁵ M (■), and forskolin 10⁻⁵ M (▲) for the periods of time shown.

sensitization was of a homologous or heterologous nature. Because cAMP responses to several agonists other than ACTH could not readily be identified in the Y1 cell, the rat β₂-adrenergic receptor was stably transfected into the Y1 cell line. Previous studies have shown that the transfection of this receptor into Y1 cells results in a fully functional adenylate cyclase-coupled receptor that is able to produce the morphological changes that accompany cAMP production (20). Clonal Y1-β₂-AR cells that responded to both isoproterenol and ACTH were selected for further study. The extent of the cAMP response to a single stimulus with isoproterenol (10⁻⁵ M) or forskolin (10⁻⁵ M) is shown in Fig. 1b.

Desensitization of the MC2R was investigated after preincu-

FIG. 2. GRKs in the Y1 cell line. *a*, RT-PCR using primers designed to GRK2, -3, -5, and -6 with cDNA from Y1 cells. Control reactions with the n suffix indicate reverse transcriptase reactions lacking the enzyme to control for genomic or laboratory contamination. GAPDH primers were used to confirm the quality of the cDNA. *b*, immunoblot to demonstrate the presence of expressed GRK2 and GRK5 proteins in Y1 cell extracts using enhanced chemiluminescence (ECL). *c*, the effect of dominant negative GRK2 on desensitization of Y1 cells. Y1 control cells (\square) and Y1 cells stably expressing GRK2-K220W (\blacksquare) were stimulated with 10^{-8} M ACTH for varying times as shown, then restimulated in the presence of IBMX for 30 min to produce a desensitization time course.



bation with isoproterenol (10^{-5} M) for varying times. As shown in Fig. 1c this treatment clearly did not desensitize the MC2R and if anything appeared to enhance the response to ACTH (10^{-8} M). A similar absence of desensitization was also seen after preincubation of untransfected Y1 cells with forskolin (10^{-5} M) (Fig. 1c).

These findings support the notion that MC2R desensitization is homologous in nature and consequently likely to be mediated by one or more of the six recognized GRKs. GRK 1 and 4 are expressed predominantly in the retina and testis (21). The expression of GRKs 2, 3, 5, and 6 was investigated by RT-PCR analysis of total RNA in Y1 cells and revealed that only GRK 2 and 5 are expressed in this cell line (Fig. 2a). Immunoblotting confirms these findings (Fig. 2b).

The possible function of these kinases in MC2R desensitization was tested using a dominant negative GRK (22, 23). Stable Y1 cell lines expressing the K220W GRK2 mutant were created, and the pattern of desensitization was investigated. The results show that there is a degree of inhibition of desensitization at all time points with the dominant negative GRK, although these differences do not achieve statistical significance (Fig. 2c).

As this result suggests that other kinases might be involved, the role of PKA was investigated. H89 used in concentrations that are specific for PKA inhibition (10^{-7} M) (24) was highly effective in blocking the initial phase of MC2R desensitization (Fig. 3a). Because the mechanism of desensitization of this receptor might depend on the degree of receptor occupancy and hence the desensitizing concentration of ACTH, a desensitization dose response curve was constructed with or without H89. Fig. 3b shows that in the absence of H89 50% maximal desensitization was achieved at 8.5×10^{-10} M ACTH. The inhibitory effect of H89 on desensitization was complete at all concentrations of ACTH studied.

The third intracellular loop of the MC2R contains a single putative PKA consensus phosphorylation site (25) in the MC2R (RKIST) centered around Ser-208. This site is conserved in the MC2R across species. This residue was changed to an alanine by site-directed mutagenesis, and the mutated

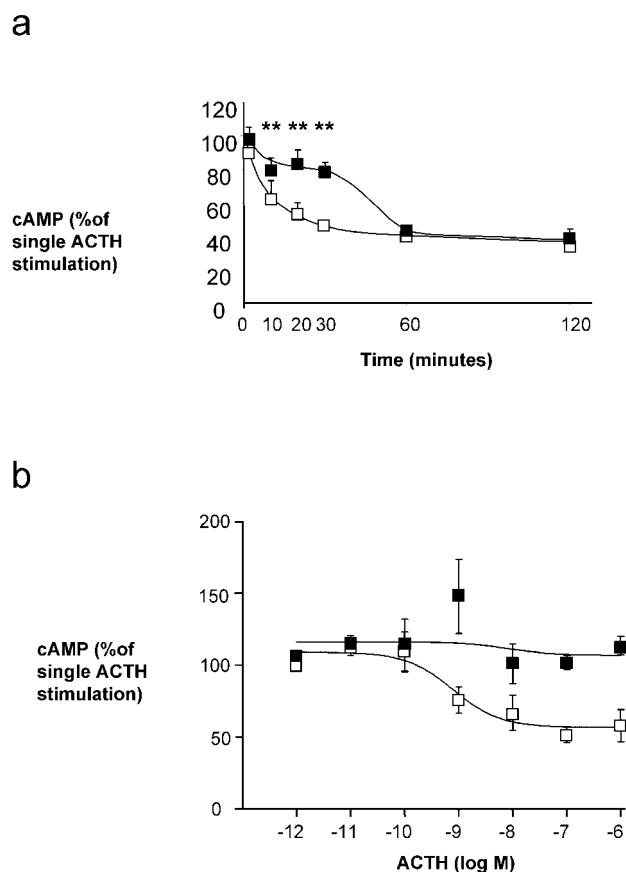


FIG. 3. Influence of the PKA antagonist, H89, on ACTH-induced desensitization. *a*, Y1 cells preincubated for 30 min and incubated with vehicle alone (\square) or H89 (10^{-7} M) (\blacksquare) were stimulated with 10^{-8} M ACTH for the times shown, then restimulated with the same concentration of ACTH (+ IBMX) for 30 min. **, $p < 0.01$. *b*, ACTH dose response of ACTH-induced desensitization determined by a 30-min preincubation with ACTH at the concentration shown, followed by a 30-min incubation with ACTH (10^{-8} M) in the absence (\square) or presence of H89 (10^{-7} M) (\blacksquare).

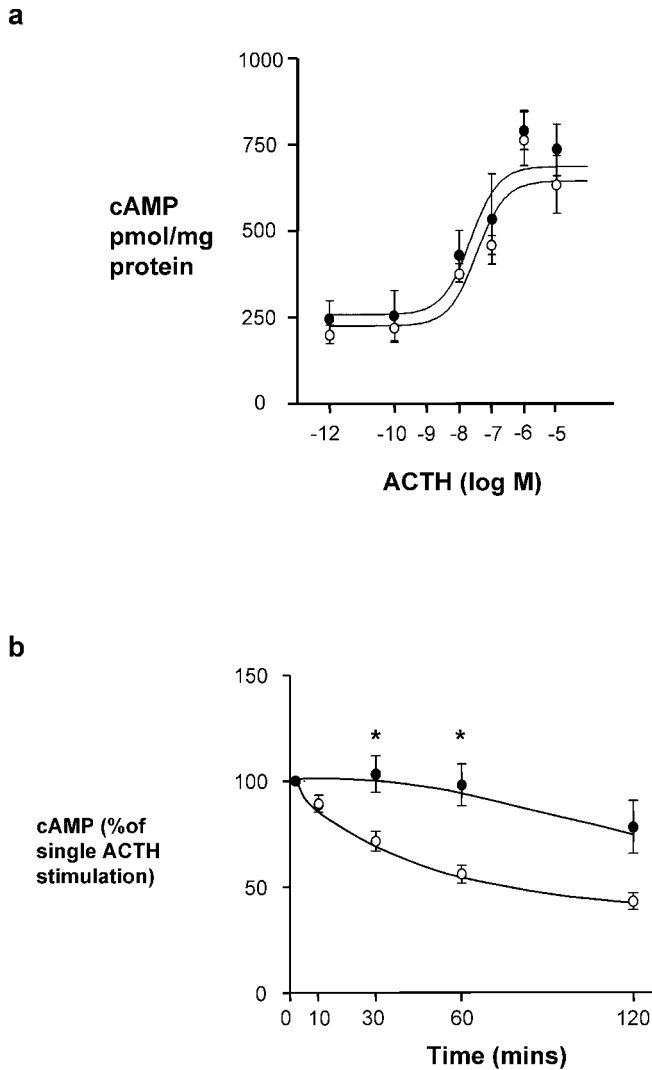


FIG. 4. **Functional response of the mutant MC2R, S208A, to ACTH stimulation and desensitization.** *a*, dose response curves to a single ACTH stimulation of Y6 cells stably expressing either the wild type (○) or S208A mutant MC2R (●). *b*, desensitization of wild type (○) and S208A mutant MC2R (●) in Y6 cells. Cells were stimulated with 10^{-6} M ACTH for varying times as shown, then restimulated with the same dose of ACTH for 30 min. *, $p < 0.05$.

gene (S208A) was stably expressed in Y6 cells. The Y6 cell is a cell line derived from the Y1 cell that fails to express the endogenous MC2R. It can be successfully used for exploring the function of transfected MC2Rs (26, 27). Stable cell lines expressing the wild type and S208A receptors were selected. These receptors expressed in Y6 cells have highly comparable function with very similar EC_{50} values (1.8×10^{-7} M (wild type); 2.1×10^{-7} M (S208A) although these values are about an order of magnitude greater than that of the endogenous MC2R expressed by Y1 cells (Figs. 1*a* and 4*a*). In addition, the basal cAMP values in these cell lines are greater than those in Y1 cells reflecting an intrinsic difference in the Y6 cell, as compared with the Y1 cell, under these conditions. In accordance with the result obtained using H89, desensitization is absent for the first 30–60 min in cells expressing S208A in comparison with the wild type MC2R-expressing cells (Fig. 4*b*). This finding supports the view that PKA is the principal kinase involved in MC2R desensitization and that its actions are mediated through a single consensus phosphorylation site.

It has been demonstrated that PKA phosphorylation of the β_2 -adrenergic receptor leads to a switch in G protein coupling from G_{α_s} to G_{α_i} (26). Furthermore, it has been proposed that the opposing action of G_{α_i} leads to a reversal and apparent desensitization of this receptor (25). To test whether a similar mechanism might be involved with the MC2R the desensitization of the MC2R in the presence of pertussis toxin to inhibit G_{α_i} was investigated. Data shown in Fig. 5 suggests that signaling is mediated solely by G_{α_s} and that there is very little ACTH-dependent G_{α_i} activity in these cells.

DISCUSSION

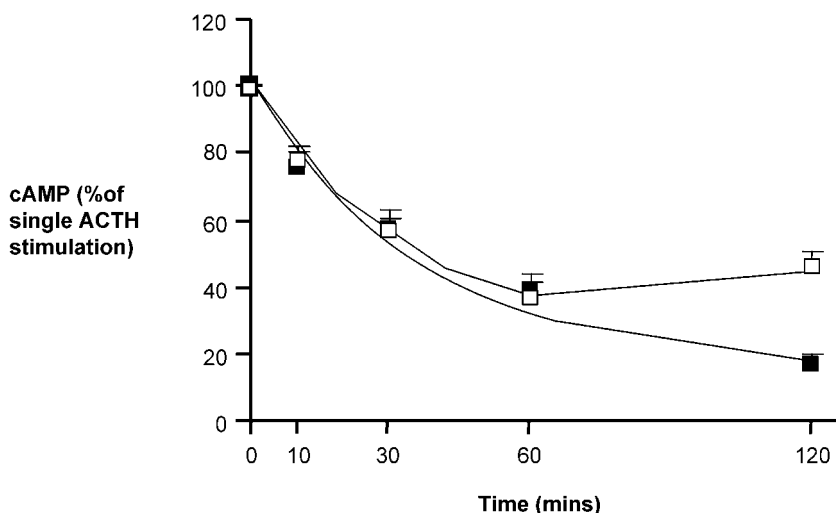
The adrenal response to ACTH stimulation is of considerable importance in physiological and pathophysiological circumstances. Although there is evidence for the modulation of the MC2R at the level of gene expression (12, 13), this is not extensive or rapid, and it seems more probable that short term responsiveness is modulated at the receptor level by the processes of desensitization, sequestration, and down-regulation. Earlier studies have shown that the desensitization of ACTH-stimulated adenylate cyclase activity is readily detectable in Y1 cells and tends to be a more prominent phenomenon than the loss of cell surface binding observed after prolonged ACTH stimulation (14, 15). We have attempted to characterize the nature of MC2R desensitization using the mouse Y1 cell, which is the only cell line in which endogenous MC2R is normally expressed in sufficient numbers to make such a study feasible.

In keeping with earlier data, we show that the MC2R desensitizes in response to ACTH relatively rapidly (given the limitations of the assay system). Treatment of cells with the β_2 -agonist, isoproterenol or with forskolin results in elevations of cAMP comparable to those achieved with ACTH, yet neither treatment desensitizes the MC2R. These characteristics are suggestive of homologous mechanisms of desensitization and imply the involvement of a GRK in this process. However the dominant negative GRK2 only weakly influences the normal pattern of desensitization. This result should be treated with caution because the inhibitory action of the mutant GRK2 requires there to be sufficient mutant protein to saturate potential GRK phosphorylation sites. Wild type GRK2 protein is fairly easily detected in Y1 cells and may be in adequate quantity to phosphorylate agonist-occupied MC2Rs despite the presence of the dominant negative enzyme. Furthermore, the inhibitory activity of GRK2 K220W on GRK5 phosphorylation is not clear, although the immunoblots suggest that the quantities of GRK5 may be less than those of GRK2 in this cell line.

However we also show that the PKA antagonist H89 is capable of almost completely eradicating the early phase of desensitization at all concentrations of ACTH used. Y6 cells were stably transfected with an MC2R in which the sole consensus PKA phosphorylation site, Ser-208, was replaced. S208A and wild type MC2R-expressing cell lines show highly comparable dose response curves, yet the mutant receptor shows a similar effect on early desensitization (Fig. 4*b*) as is seen when Y1 cells are treated with H89 (Fig. 3*a*). This suggests that Ser-208 has an essential role in the mechanism of MC2R desensitization by ACTH.

Thus desensitization of the endogenous Y1 cell MC2R exhibits a paradox. The mode of desensitization appears at first sight to be homologous in that agents other than ACTH that elevate intracellular cAMP do not desensitize the receptor. There may be other agonists that we have not studied that act through other signaling pathways that are capable of desensitizing this receptor, but it seems clear that elevation of cAMP should be able to activate existing heterologous desensitization mechanisms. Nevertheless there is good evidence for a major role for PKA mediation of this process.

FIG. 5. **Desensitization of the MC2R in the presence of pertussis toxin.** Y1 cells were starved overnight in the presence (■) or absence (□) of pertussis toxin (100 ng/ml) before determination of desensitization by incubation with ACTH (10^{-8} M) for the times shown, followed by a restimulation with ACTH (10^{-8} M) for 30 min. $n = 3$.



One possibility is that conformational changes in the MC2R following ligand binding result in Ser-208 only becoming accessible to PKA in the agonist-occupied state. In such a model activated PKA would be unable to phosphorylate the unliganded MC2R, and an apparent homologous form of desensitization will be observed. However the data in Fig. 3b indicates that the agonist concentration at which 50% of the maximum desensitization takes place, 8.5×10^{-10} M, is an order of magnitude lower than the EC_{50} for cAMP generation. This implies that receptor activation results in desensitization of both agonist-occupied and -unoccupied receptors, a feature typical of heterologous desensitization.

Evidence is accumulating that many GPCRs exist in a complex with other components of the receptor regulation and cell signaling system including PKA, protein kinase C, β -arrestin, and phosphatases complexed with a member of the protein kinase A anchoring proteins (AKAPs) (28–32). Such a complex may effectively compartmentalize the receptor-effector system in question and limit the accessibility of non-complexed PKA such as that activated by forskolin or the β_2 -adrenergic receptor. The influence of these AKAP complexes on desensitization of the adrenergic receptor has been investigated by suppression of their expression by antisense oligonucleotides and has revealed only an inhibition of receptor resensitization (33). However these studies only investigated desensitization by a single agonist, isoproterenol, so that any potential role of AKAPs in restricting PKA-mediated desensitizing mechanisms remains to be investigated.

The discordance between desensitization dose responsiveness and the EC_{50} argues against the model of a single receptor containing complex and is suggestive of complexes containing two or more molecules of receptor. This would enable the co-existence in a single complex of both agonist-occupied and -unoccupied MC2R. There is considerable evidence that many GPCRs exist as dimers or oligomers (34), although such evidence has not yet been obtained for the MC2R.

An alternative to this model is that rather than desensitizing, the receptor switches its coupling to the opposing $G\alpha_i$ signaling pathway. A precedent for this has been described in the case of the β_2 -adrenergic receptor in which Daaka *et al.* (35) demonstrated that agonist-induced PKA phosphorylation of the receptor switches its G protein coupling, enabling $G\alpha_i$ -coupled MAP kinase signaling and inhibition of adenylate cyclase to occur (35). Under these circumstances inhibition of cyclase has been proposed as an additional mechanism of apparent receptor desensitization (36). However we have been able to show in the case of the MC2R that pertussis toxin has

no influence on the pattern of desensitization, and hence this mechanism is unlikely to be relevant to this receptor.

In summary, we have demonstrated a major role for PKA in desensitization of the MC2R and that Ser-208 in the third intracellular loop is the probable target for PKA-mediated phosphorylation. However, in contrast to most PKA-mediated desensitization models, MC2R desensitization appears to be insensitive to PKA activation from heterologous sources.

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