Acute desensitization of phospholipase C-coupled muscarinic M3 receptors but not gonadotropin-releasing hormone receptors co-expressed in αT3-1 cells: implications for mechanisms of rapid desensitization

Gary B. WILLARS*, Craig A. McARDLE† and Stefan R. NAHORSKI*

*Department of Cell Physiology and Pharmacology, University of Leicester, Medical Sciences Building, P.O. Box 138, University Road, Leicester LE1 9HN, U.K., and †Department of Medicine, University of Bristol, Bristol Royal Infirmary, Marlborough Street, Bristol BS2 8HW, U.K.

In the present study we have expressed the muscarinic M3 receptor in an immortalized mouse pituitary cell line (αT3-1), which expresses an endogenous gonadotropin-releasing hormone (GnRH) receptor, to examine potential differences in acute receptor regulation. Both of these receptors couple to the activation of phosphoinositide-specific phospholipase C (PLC) in these cells and we demonstrate that, despite expression in the same cell background, acute desensitization is a feature of muscarinic M3 receptors but not of GnRH receptors. We show that, when the concentrations of GnRH and methacholine are matched to give approximately equivalent maximal elevations of Ins(1,4,5)P$_3$, the GnRH receptor is able to sustain PLC activity at the initial rate, whereas the muscarinic M3 receptor cannot. Thus PLC-activating G-protein-coupled receptors are able to undergo rapid desensitization in this cell line, indicating that the desensitization profile is receptor-specific rather than cell-specific. This argues strongly that post-receptor regulatory features do not have a prominent role in mediating rapid desensitization in these cells. Furthermore GnRH receptor-mediated PLC activity is sustained despite a marked and persistent depletion in the steady-state level of PtdIns(4,5)P$_2$. In contrast, activation of muscarinic receptors is not sustained despite only a transient decrease in PtdIns(4,5)P$_2$ concentration. Thus, whereas the contribution of PtdIns(4,5)P$_2$ depletion to the temporal profile of receptor-mediated PLC signalling has been difficult to assess, the present results demonstrate that this is unlikely to be of importance in these cells. We suggest that unique structural features of the GnRH receptor result in a lack of appropriate regulatory phospho-acceptor sites and that the absence of agonist-dependent phosphorylation might underlie the lack of acute regulation.

INTRODUCTION

In recent years, evidence has accumulated indicating that, in common with G-protein-coupled receptors linked to adenylate cyclase, virtually all phosphoinositide-specific phospholipase C (PLC)-coupled receptors show decreased responses in the face of repetitive challenges with agonist: a process of desensitization. Studies on a number of these PLC-coupled receptors, in which the accumulation of total $^3$H-inositol phosphates ($[^3$H]InsP$_x$) has been measured against a Li$^+$-block of inositol monophosphatase activity, have demonstrated that desensitization can occur within seconds of the first exposure to agonist [1–6]. This desensitization is reflected by an initial (less than 1 min) rapid accumulation of $[^3$H]InsP$_2$ followed by either a decrease in, or an absence of, further accumulation. In reality it is likely that the majority of PLC-coupled receptors are subject to such acute regulation [7], making this a key event in altering cellular sensitivity and responsiveness. Despite this, the precise mechanism(s) involved remain to be elucidated.

Because the sequestration of plasma membrane receptors occurs too slowly to account for the observed speed of desensitization, other mechanisms have been sought [2,7–9]. By analogy with the phosphorylation and subsequent desensitization of the β$_2$-adrenoceptor [10], phosphorylation of PLC-linked receptors has been suggested as a possible mechanism. Indeed the rapid, agonist-dependent phosphorylation of a number of PLC-linked receptors has been demonstrated and implicated in desensitization [11]. However, many other components of the signalling pathway are subject to alteration and regulation during activation of the signal transduction pathway [2,7–9] and it is possible that such post-receptor mechanisms contribute to, or underlie, rapid desensitization. One outstanding possibility is that the availability of PtdIns(4,5)P$_2$ becomes rate-limiting in the face of an increased demand by activated PLC [2,7–9]. PtdIns(4,5)P$_2$ constitutes a minor proportion of the total cellular phosphoinositides [12] and has been demonstrated to undergo a rapid, marked and sustained depletion in a variety of cell types after the activation of vasopressin receptors [13], thrombin receptors [14], muscarinic M1 receptors [15] or muscarinic M3 receptors [5,16]. Such changes in the cellular concentration of PtdIns(4,5)P$_2$ and the subsequent limitation of its resupply clearly have the potential to cause, or contribute significantly to, the rapid desensitization of PLC-coupled receptors. This possibility and indeed the role of other post-receptor mechanisms has, however, been difficult to assess.

In the present study we have addressed this issue by using an immortalized mouse pituitary cell line (αT3-1), which endogenously expresses a gonadotropin-releasing hormone (GnRH) receptor coupled to the activation of PLC. This receptor seems to be resistant to rapid desensitization because challenge with GnRH results in a linear accumulation of $[^3$H]InsP$_2$ against a Li$^+$-block [17]. At present this is the only cell line in which the activity of an endogenously expressed PLC-coupled receptor is not rapidly desensitized, indicating that the cellular environment does not participate in the rapid desensitization of phosphoinositide responses in these cells. This presents a unique op-

Abbreviations used: [Ca$^{2+}$], intracellular Ca$^{2+}$ concentration; CHO, Chinese hamster ovary; GnRH, gonadotropin-releasing hormone; GroPlIns, glycerophosphoinositol; GroPlIns$_4$P, glycerophosphoinositol 4-phosphate; GroPlIns(4,5)P$_2$, glycerophosphoinositol 4,5-bisphosphate; $[^3$H]InsP$_x$, total $^3$H-inositol phosphates; $[^3$H]NMS, 1-[N-methyl-$^3$H]scopolamine methyl chloride; PLC, phosphoinositide-specific phospholipase C.

1 To whom correspondence should be addressed (e-mail gbw2@leicester.ac.uk).
portunity to examine whether the acute regulation common to other receptor/cell combinations is a consequence of post-receptor regulatory mechanisms or a feature of the receptors themselves. In the present study we have therefore expressed the muscarinic M3 receptor in zT3-1 cells to determine whether or not the rapid desensitization of this receptor seen in a number of other cell types [2,3,5,6] is truly a receptor-dependent phenomenon.

EXPERIMENTAL

Materials

Reagents of analytical grade were obtained from suppliers listed previously [6,15,18] unless stated otherwise, or alternatively from Sigma (Poole, Dorset, U.K.). 1-[N-methyl-3H]Scopolamine methyl chloride ([3H]NMS) was from Amersham International (Little Chalfont, Bucks., U.K.). [3H]Ins(1,4,5)P3 (15–30 Ci/mmol) was from DuPont–NEN (Stevenage, Herts., U.K.). The [3H]-buserelin was kindly donated by J. Sandow and W. Von Rechenberg (Hoechst, Frankfurt, Germany).

Cell culture

The zT3-1 gonadotroph cell line was originally a gift from Dr. P. Mellon (University of California, San Diego, CA, U.S.A.). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 50 i.u./ml penicillin, 50 µg/ml streptomycin, 2 mM glutamine and 10% (v/v) fetal calf serum. Cultures were maintained at 37°C in humidified air/CO2 (19:1) and passed weekly. For experiments, cells were harvested with 10 mM Hepes/154 mM NaCl/0.54 mM EDTA (pH 7.4) and, except for experiments in which the intracellular Ca2+ concentration ([Ca2+]) was measured (see below), were reseeded at an approximately equivalent density into 24-well multidishes for use 1–2 days later. Cells were always maintained and the experimental manipulations performed at 37°C.

Transfection of zT3-1 cells with the cDNA encoding the human muscarinic M3 receptor

With the use of a standard calcium phosphate method, zT3-1 cells were transfected with cDNA encoding the human muscarinic M3 receptor that had been cloned (BamH1/EcoRI) into the pcDNA3 plasmid (Invitrogen, San Diego, CA, U.S.A.). Cells were selected with neomycin (300 µg/ml) and screened by binding of the muscarinic receptor antagonist [3H]NMS (see below).

Determination of receptor density

Muscarinic receptor density was determined by the binding of the muscarinic antagonist [3H]NMS (84 Ci/mmol) to intact cells. Cells in 24-well multidishes were washed and incubated for 1 h at 37°C in 1 ml of Krebs/Heps buffer [pH 7.4, composition (mM): Hepes 10, NaHCO3 4.2, glucose 11.7, MgSO4 1.2, KH2PO4 1.2, KCl 4.7, NaCl 118 and CaCl2 1.3] containing 0.3 nM [3H]NMS at the muscarinic M3 receptor of 0.3 nM [19]). Non-specific binding was determined in the presence of 10 µM atropine. Cells were washed rapidly with two 1 ml volumes of ice-cold buffer and digested with 0.5 ml of 0.1 M NaOH. This was neutralized with 0.5 ml of 0.1 M HCl samples mixed and centrifuged (3000 g, 10 min). A 1 ml aliquot of the upper phase was neutralized to pH 7 by the addition of NaHCO3 and applied to a Dowex (AG1X8) formate anion-exchange column. [3H]GroPIns and [3H]GroPIns(4,5)P2 were then eluted as described elsewhere [15] and quantified by liquid-scintillation spectrometry.

Measurement of total PLC activity

As an index of total PLC activity the agonist-induced accumulation of [3H]InsP2 was determined in cells prelabelled with myo-[3H]inositol in which inositol monophosphatase activity had been blocked with Li+ [20]. Cells were prelabelled with 3 µCi/ml myo-[3H]inositol (117 Ci/mmol) for 48 h in 24-well multidishes. Medium was then removed and the cell monolayers were washed and incubated for 10 min in 1 ml of Krebs/Heps buffer containing 10 mM Li+ and 0.1 M HCl. Buffer was then removed and 200 µl of buffer containing Li+ and agonist was added. Reactions were performed in duplicate and terminated with an equal volume of ice-cold 1 M trichloroacetic acid. After 15 min on ice the aqueous phase was removed and 10 µl of 10 mM EDTA was added. After being vortex-mixed with 0.5 ml of a 1:1 (v/v) freshly prepared mixture of tri-n-octylamine and 1,1,2-trichlorotrifluoroethane, 20 µl of 250 mM NaHCO3 was added to a 300 µl aliquot of the aqueous phase. This was applied to a Dowex AG1X8 formate column, which was then washed with 20 ml of water and 10 ml of 25 mM ammonium formate. Inositol phosphates were eluted with 10 ml of 1 M ammonium formate/0.1 M formic acid [15]. A 2 ml aliquot was then counted by liquid-scintillation spectrometry in 8 ml of Floscit IV.

Measurement of agonist-induced changes in [3H]InsP2 and [3H]-phosphoinositides

Cells were prelabelled with myo-[3H]inositol and challenged with agonist exactly as described above with the exception that the buffer contained 10 mM myo-inositol but no Li+. The [3H]InsP2 were extracted and determined as described above. [3H]Glycerophosphoinositol ([3H]GroPIns) and the glycerophosphoinositol-phosphates [3H]GroPIns(4,5)P2 and [3H]GroPIns(4,5)P3, as indices of PtdIns, PtdIns4P and PtdIns(4,5)P2 respectively, were then prepared from the remaining cell monolayers on the basis of previously described methods [15]. In brief, lipids were extracted into 0.94 ml of chloroform/methanol/10 M HCl (40:80:1, by vol.). Chloroform (0.31 ml) and 0.1 M HCl (0.56 ml) were then added to induce phase partition. A sample (400 µl) of the lower phase was removed, dried in a stream of N2 and stored at —20°C before further processing. These samples were dissolved in 1 ml of chloroform and 0.2 ml of methanol, then hydrolysed by the addition of 0.4 ml of 0.5 M NaOH in methanol/water (19:1, v/v). Samples were vortex-mixed at regular intervals during a 20 min incubation at room temperature. Chloroform (1 ml), methanol (0.6 ml) and water (0.6 ml) were then added, the samples mixed and centrifuged (3000 g, 10 min). A 1 ml aliquot of the upper phase was neutralized by using Dowex-50 (H+ form) columns (1 ml bed volume), which were washed twice with 2 ml of water. The pooled eluate was brought to pH 7 by the addition of NaHCO3 and applied to a Dowex (AG1X8) formate anion-exchange column. [3H]GroPIns, [3H]GroPIns4P and [3H]GroPIns(4,5)P2 were then eluted as described elsewhere [15] and quantified by liquid-scintillation spectrometry.

Measurement of Ins(1,4,5)P3

Cell monolayers were preincubated in Krebs/Heps buffer and challenged; the reaction was terminated as described above. A 160 µl aliquot of the acidified aqueous phase was removed and processed, and Ins(1,4,5)P3 was assayed exactly as described.
Measurement of $[Ca^{2+}]_i$.

Video imaging of fura 2-loaded cells was performed as described previously [23]. In brief, cells grown on glass coverslips were loaded with the acetoxymethyl ester of fura 2 (2 μM) for 30 min at 37°C in 1 ml of buffer [pH 7.4, composition (mM): NaCl 127, CaCl$_2$ 1.8, KCl 5, MgCl$_2$ 2, NaH$_2$PO$_4$ 0.5, NaHCO$_3$ 5, glucose 10, Heps 10, with 0.1% BSA]. Cells were then washed several times and placed within a heated (37°C) perfusion stage of a Nikon Diaphot inverted microscope. Image capture was performed with MagiCal hardware after excitation alternately at 340 and 380 nm with emission recorded at 510 nm. Values were subtracted before ratios were calculated. The ratio of fluorescence at 340 and 380 nm with emission recorded at 510 nm. Values were performed with MagiCal hardware after excitation alternately at 340 and 380 nm with emission recorded at 510 nm. Values were averaged from 16 or 32 video frames; background fluorescence was subtracted before ratios were calculated. The ratio of fluorescence at 340 and 380 nm was calculated on a pixel-by-pixel basis for 20–50 cells per experiment by using maximum and minimum values defined by treatment with 5 μM ionomycin in medium with 10 mM CaCl$_2$ or 10 mM EGTA respectively and assuming a dissociation constant of 225 nM for fura 2 and Ca$^{2+}$ at 37°C, as described [23].

Data presentation

For all quantitative determinations of inositol phosphates and phosphoinositides, cell incubations were performed in duplicate. Values from each duplicate were then averaged to give a single value representing one experiment, with the exception of samples for the determination of $[^{3}H]$phosphoinositides. For these, duplicate stimulations were combined and the $[^{3}H]$phosphoinositides extracted to give single determinations for each experimental point. All results are presented as means ± S.E.M.; the numbers of experiments are given as n.

RESULTS

Wild-type (untransfected) αT3-1 cells express a relatively small number of muscarinic receptors of unknown subtype ($B_{max}$ 42 ± 9 fmol/mg of total cell protein by $[^{3}H]$NMS binding; n = 3). Although at least some of these probably couple to the phosphoinoside signal transduction pathway given the minor elevations of $[Ca^{2+}]_i$, evoked by the muscarinic agonist methacholine (less than 50 nM increase; results not shown), phosphoinositide responses were essentially absent (see Figure 2; also results not shown). Therefore to examine the pattern of muscarinic receptor-mediated phosphoinositide signalling in the same cellular background as the GnRH receptor it was necessary to increase the expression of PLC-coupled muscarinic receptors in these cells. Transfection of αT3-1 cells with the cDNA encoding the human muscarinic M3 receptor produced a number of clones in which the binding of $[^{3}H]$NMS was greater than that in wild-type cells. A number of these clones were selected for further experimentation; here we present results predominantly from a clone (Clone 72) that had a $B_{max}$ for $[^{3}H]$NMS binding of 376 ± 21 fmol/mg of total cell protein (n = 3). This closely resembles the level of expression of endogenous receptors in, for example, cells of the human neuroblastoma cell line SH-SY5Y [5], with which we have much experience of the characteristics of muscarinic M3 receptor-mediated phosphoinositide and Ca$^{2+}$ signalling [3,6,16]. GnRH receptor expression in wild-type cells and cells of Clone 72 were 1.57 ± 0.10 and 1.42 ± 0.10 pmol/mg of total cell protein respectively, which is comparable to that of pituitary gonadotrophs [24].

Inositol (poly)phosphate responses

Challenging Clone 72 with a maximal concentration of GnRH (1 μM) [23,25] evoked a rapid accumulation of Ins(1,4,5)P$_3$ that was maximal by 60 s and remained at this level for the remainder of the experiment (an additional 240 s) (Figure 1). In contrast, challenging this clone with 1 mM methacholine evoked a biphasic accumulation of Ins(1,4,5)P$_3$ that consisted of a transient peak (at 20 s) followed by a decline to levels greater than basal at 5 min (Figure 1). These agonist-mediated accumulations of Ins(1,4,5)P$_3$ were concentration-dependent as shown by EC$_{50}$ values (log$_{10}$ M) of $-7.59 ± 0.01$ (n = 3) (26 nM) for GnRH at 60 s and $-5.37 ± 0.07$ (n = 3) (4 μM) for methacholine at 20 s.

Neither the magnitude nor the profile of Ins(1,4,5)P$_3$ responses to maximal concentrations of GnRH or methacholine was affected by pretreating the cells with pertussis toxin (100 ng/ml, 24 h) (results not shown). Measurement of cAMP [19] after challenge of cells with a maximal concentration of either GnRH or methacholine indicated that neither agonist elevated levels of cAMP over a 5 min period (results not shown). These results illustrate a lack of involvement of either Gs or G-proteins or cAMP accumulation in signalling differences between GnRH receptor activation and muscarinic receptor activation.

To allow more direct comparisons of other aspects of phosphoinositide metabolism, a concentration of GnRH was selected (100 nM) that produced a maximal Ins(1,4,5)P$_3$ accumulation, approximately equivalent to the peak response evoked by a maximally effective concentration of methacholine (1 mM) (Figure 2c). Although of lower magnitude, the profile of accumulation of Ins(1,4,5)P$_3$ in response to this submaximal concentration of GnRH was identical with that in response to a maximal concentration. Furthermore Ins(1,4,5)P$_3$ responses to this submaximal concentration of GnRH were identical in wild-type cells, in cells of Clone 72 and in cells of other clones selected for the expression of different densities of muscarinic receptors after transfection (Figure 2c, and results not shown). This indicates that the sensitivity and responsiveness of the GnRH-mediated phospho-
Figure 2 GnRH- or methacholine-mediated Ins(1,4,5)P$_3$ accumulation in wild-type αT3-1 cells or αT3-1 cells expressing differing levels of recombinant human muscarinic M3 receptors

Time course of Ins(1,4,5)P$_3$ accumulation in response to a submaximal concentration of GnRH (100 nM; ■) or a maximal concentration of methacholine (1 mM; *■) in αT3-1 cells: wild-type (a); Clone 33 (b); Clone 72 (c). $B_{	ext{max}}$ values for muscarinic receptor antagonist ([3H]NMS) binding were 42 ± 9, 154 ± 16 and 376 ± 21 fmol/mg of total cell protein respectively (all $n = 3$). Agonists were added at zero time. Data are means ± S.E.M., $n = 3$.

Inositol responses were unaffected by the transfection and selection protocol. However, the magnitude of methacholine-induced Ins(1,4,5)P$_3$ responses was related to the level of muscarinic receptor expression, with higher expressing clones giving larger responses, although in all of the selected clones ($n = 4$) responses were biphasic (Figure 2, and results not shown). Challenge of wild-type cells with methacholine provoked an accumulation of Ins(1,4,5)P$_3$ that was similar to the exchange of buffer alone (Figure 2a, and results not shown).

Figure 3 GnRH- or methacholine-mediated [3H]InsP$_3$ accumulation in αT3-1 cells expressing recombinant human muscarinic M3 receptors

Time course of [3H]InsP$_3$ accumulation in response to 100 nM GnRH (a, b; ■) or 1 mM methacholine (a, c; ■) in [3H]inositol-prelabelled αT3-1 cells of Clone 72 in which inositol monophosphatase activity had been blocked with 10 mM Li$^+$. Agonists were added at zero time and time-matched basal values were subtracted from each point. Data are means ± S.E.M., $n = 4$. The solid lines from 0 to 60 s represent the linear regression lines of best fit; the broken lines are the extrapolation of these lines over the subsequent 240 s.

Activation of the muscarinic receptors of Clone 72 resulted in a biphasic accumulation of [3H]InsP$_3$ under Li$^+$-block (Figures 3a and 3c). This consisted of an initial phase lasting approx. 1 min, followed by a lower sustained phase. The rate of [3H]InsP$_3$ accumulation between 2 and 5 min was 41 ± 8 % (n = 4) of the accumulation rate over the first min of agonist stimulation, consistent with a rapid desensitization of phosphoinositide signalling. In contrast, the accumulation of [3H]InsP$_3$ in response to challenge with GnRH was approximately linear for up to 5 min after agonist addition (Figure 3b). The rate of [3H]InsP$_3$
Receptor signalling in αT3-1 cells

Effects of combined stimulation with GnRH and methacholine on Ins(1,4,5)P₃ signalling in αT3-1 cells expressing recombinant human muscarinic M3 receptors

Cells of Clone 72 were challenged with 1 µM GnRH (left bar of each group), 1 mM methacholine (middle bar of each group) or the two in combination (right bar of each group) for 20, 60 or 300 s. Results are means ± S.E.M., n = 4. Statistical analysis was by one-way analysis of variance followed, where P < 0.05, by Duncan’s multiple range test at *P < 0.05.

accumulation between 2 and 5 min was 87 ± 4% (n = 4) of the accumulation rate over the first minute of agonist stimulation. In wild-type cells the rate of GnRH-stimulated [³H]InsP₃ accumulation between 2 and 5 min was 113 ± 19% (n = 3) of the accumulation rate over the first minute (results not shown). These results indicate a lack of acute regulation of the GnRH receptor-mediated phosphoinositide response. In the continued presence of GnRH and under a Li⁺-block, the accumulation of [³H]InsP₃ reached a plateau approx. 10 min after the addition of agonist (Figure 3a). In the presence of Li⁺ there was also a slowing in the rate of [³H]InsP₃ accumulation between 10 and 15 min under muscarinic receptor stimulation (Figure 3a). In the absence of Li⁺ the [³H]InsP₃ accumulation in response to activation of either receptor type was, however, sustained for at least 30 min (results not shown), indicating that these later (more than 5–10 min) deviations from linearity are unlikely to represent receptor inactivation. Instead they are likely to be the consequence of depletion of radiolabelled PtdIns(4,5)P₂ caused by the Li⁺ block of [³H]inositol reincorporation into the phosphoinositides [20]. This suggests that αT3-1 cells have a relatively small intracellular pool of inositol, similar to that of Chinese hamster ovary (CHO) cells, for example, but in contrast with the relatively large pool found in cell lines such as the SH-SYSY neuroblastoma [26].

Effects of combined stimulation with GnRH and methacholine on Ins(1,4,5)P₃ signalling

Simultaneous stimulation of Clone 72 with maximal concentrations of GnRH (1 µM) and methacholine (1 mM) resulted in accumulations of Ins(1,4,5)P₃ that were similar to those evoked by GnRH alone (Figure 4), indicating a lack of additivity between the two agonists.

---

Figure 4 Effects of combined stimulation with GnRH and methacholine on Ins(1,4,5)P₃ signalling in αT3-1 cells expressing recombinant human muscarinic M3 receptors

Figure 5 GnRH- or methacholine-mediated effects on inositol phosphates and inositol phospholipids in αT3-1 cells expressing recombinant human muscarinic M3 receptors

Time course of 100 nM GnRH-mediated (شرك) or 1 mM methacholine-mediated (シャーク) changes in [³H]InsP₃ (a), [³H]GroPIns (b), [³H]GroPIns4P (c) and [³H]GroPIns(4,5)P₂ (d) in αT3-1 cells expressing the recombinant human muscarinic M3 receptor (Clone 72). Cells were prelabelled with [³H]inositol and challenged with agonist (added at zero time) in the absence of Li⁺. Results are means ± S.E.M., σ = 3.
and 2.5% evoked rapid and sustained accumulations of [Ca\textsuperscript{2+}] with either 100 nM GnRH or 1 mM methacholine continued accumulation of methacholine (1 mM) had comparable biphasic effects on [Ca\textsuperscript{2+}] (Figure 5c). The second stimulus (submaximal concentrations of agonist) were performed in the absence of Li\textsuperscript{+} to assess the contribution of store release only. Responses in cells prechallenged with a maximal concentration of agonist (○) were compared with responses in cells that had not been previously challenged with agonist (●). Only the responses of the cells to the submaximal concentrations of agonist are shown. Results are means ± S.E.M. for three experiments, each consisting of averaged data from approx. 20–50 cells.

**Figure 6** GnRH- or methacholine-mediated changes in [Ca\textsuperscript{2+}], in α3T-1 cells expressing recombinant human muscarinic M3 receptors

Comparison of GnRH- and methacholine-evoked changes in inositol phosphates and inositol phospholipids were made by using Clone 72 prelabelled with [\textsuperscript{3}H]inositol and challenged in the absence of Li\textsuperscript{+} to determine steady-state levels resulting from the net effect of both generation and metabolism. In this cell line under basal (non-stimulated) conditions, [\textsuperscript{3}H]PtdIns, [\textsuperscript{3}H]PtdIns(4)\textsubscript{P}, and [\textsuperscript{3}H]PtdIns(4,5)\textsubscript{P}_{2} represented 94.4%, 3.1% and 2.5% respectively of the total inositol phospholipid pool.

**Figure 7** Susceptibility of GnRH- or methacholine-mediated [Ca\textsuperscript{2+}], signalling to homologous desensitization in α3T-1 cells expressing recombinant human muscarinic M3 receptors

To assess whether or not the [Ca\textsuperscript{2+}] responses to either GnRH or methacholine underwent rapid desensitization, cells were challenged with a maximal concentration of either GnRH (100 nM) (a) or methacholine (1 mM) (b) for 5 min and then, after being washed for 5 min, rechallenged with the same agonist at a concentration approximating to its EC\textsubscript{50} (5 nM GnRH or 1 µM methacholine). The first stimulus and the wash were in normal Ca\textsuperscript{2+}-containing buffer, whereas the second stimulus (submaximal concentrations of agonist) were performed in the absence of extracellular Ca\textsuperscript{2+} to assess the contribution of store release only. Responses in cells prechallenged with a maximal concentration of agonist (○) were compared with responses in cells that had not been previously challenged with agonist (●). Only the responses of the cells to the submaximal concentrations of agonist are shown. Results are means ± S.E.M. for three experiments, each consisting of averaged data from approx. 20–50 cells.

**[Ca\textsuperscript{2+}], signalling**

In wild-type α3T-1 cells, maximally effective concentrations of GnRH (10 nM to 1 µM) cause a robust biphasic (spike–plateau) increase in [Ca\textsuperscript{2+}], [23,25]; a similar response was observed in four separate clones of M3 receptor transfected cells (results not shown). Methacholine also had a modest effect on [Ca\textsuperscript{2+}], in wild-type α3T-1 cells but much more pronounced effects in four separate clones of M3 receptor transfected α3T-1 cells (results not shown). Maximal concentrations of GnRH (100 nM) and methacholine (1 mM) had comparable biphasic effects on [Ca\textsuperscript{2+}], in Clone 72 cells (Figure 6), with GnRH increasing the basal [Ca\textsuperscript{2+}], of 27 ± 7 nM (n = 3) to spike and plateau values of 232 ± 27 (n = 3) and 141 ± 16 nM (n = 3) respectively and methacholine increasing the basal [Ca\textsuperscript{2+}], of 35 ± 4 nM (n = 3) to spike and plateau values of 262 ± 66 (n = 3) and 115 ± 25 nM (n = 3) respectively. It should be noted that agonist potency was greater for the [Ca\textsuperscript{2+}] response, as often occurs in this signalling pathway [6]. Thus, whereas 100 nM GnRH represents a maximally effective concentration for elicitation of [Ca\textsuperscript{2+}], signalling to homologous desensitization in α3T-1 cells expressing recombinant human muscarinic M3 receptors.

Given the similarity of the profiles of [Ca\textsuperscript{2+}], signalling to GnRH and methacholine, in order to assess whether or not the [Ca\textsuperscript{2+}], responses to either GnRH or methacholine underwent rapid desensitization a protocol was designed in which cells were
first challenged with a maximal concentration of either GnRH (100 nM) or methacholine (1 mM) and then, after washing, rechallenged with the same agonist at a submaximal concentration close to its EC$_{50}$ (5 nM GnRH; 1 μM methacholine). The first stimulations (maximally effective concentrations) were performed in normal Ca$^{2+}$-containing medium, whereas the second stimulations (submaximal concentrations) were conducted in the absence of extracellular Ca$^{2+}$ to assess the contribution of store release only. The cells were washed extensively (six changes of medium over 5 min) between these stimulations in Ca$^{2+}$-containing buffer to allow refilling of the agonist-sensitive Ca$^{2+}$ pool, which refills with a half-time of 5–20 s in these cells [22]. Rechallenge was performed with a submaximal rather than maximal agonist concentration on the basis that this would be a more sensitive method for detection of desensitization, which might manifest itself as a decrease in agonist potency before a decrease in the maximal response. The results from this protocol showed a clear desensitization of the muscarinic receptor-mediated response but no evidence of desensitization of the GnRH receptor-mediated response (Figure 7).

**DISCUSSION**

The present study demonstrates clearly that, despite expression in the same cell, GnRH receptors show little or no evidence of acute regulation, whereas muscarinic M3 receptors undergo a rapid desensitization. These observations provide strong evidence that the regulation of PLC signalling in these cells is receptor-specific and focuses attention on receptor structure and away from other aspects of the cellular environment.

Rapid desensitization of the muscarinic M3 receptor-mediated responses seen here in αT3-1 cells is entirely consistent with that observed for both the endogenously expressed receptor in, for example, SH-SY5Y cells [3,5,6] and for the receptor expressed as a recombinant protein in CHO cells [2]. Differences in acute desensitization between muscarinic M3 receptors and GnRH receptors in the present study were apparent at the level of [H]Ins 1,4,5P$_3$ accumulation against a Li$^+$-block, thereby demonstrating unequivocally the ability of GnRH receptors but not muscarinic M3 receptors to sustain PLC activation at the high rate seen immediately on the addition of agonist. Although the pattern of Ins(1,4,5)P$_3$ accumulation is a product of both generation and metabolism, we also emphasize that this difference in acute regulation was reflected at the level of Ins(1,4,5)P$_3$ with activation of the two receptor types resulting in markedly different patterns of accumulation. Despite this, the [Ca$^{2+}$], responses after receptor activation were identical, consisting of rapid transient peaks followed by lower sustained elevations. Previous studies on both receptor types have indicated that the initial peak is predominantly a consequence of Ca$^{2+}$ release from intracellular stores, whereas the sustained component is dependent on Ca$^{2+}$ entry [3,6,23,25]. The present study emphasizes that the profile of the [Ca$^{2+}$] response is not dictated by a rapid partial desensitization at the level of the plasma membrane receptor but by the extent of accessible intracellular Ca$^{2+}$ stores and the mechanisms designed to replenish them. Furthermore, despite the Ca$^{2+}$ facilitation of GnRH receptor-mediated and muscarinic receptor-mediated Ins(1,4,5)P$_3$ responses [6,3,23], these results demonstrate that differences in the temporal profile of [Ca$^{2+}$] signalling do not underlie the differences in acute regulation of these two receptor types. Despite the similarity in the temporal profiles of [Ca$^{2+}$] elevations, GnRH receptor-mediated and muscarinic receptor-mediated [Ca$^{2+}$] responses did display differences in acute desensitization when cells were rechallenged by agonist after a potentially desensitizing exposure to the same agonist. Thus, provided that the intracellular Ca$^{2+}$ stores were allowed to refill (t½ of 5–20 s) [23], the lack of acute regulation of the GnRH receptor allowed a full [Ca$^{2+}$] response on rechallenge, whereas the response to muscarinic receptor stimulation was blunted, fully supporting the evidence for desensitization of PLC activation.

The above results suggest that it is unlikely that features of signalling common to both GnRH receptors and muscarinic M3 receptors at the post-receptor level are important in acute desensitization. Thus, despite the requirement for resynthesis of PtdIns(4,5)P$_2$ to enable sustained Ins(1,4,5)P$_3$ generation [5,27], the ability of GnRH but not methacholine to evoke a sustained phospholipid breakdown equivalent to the initial rate suggests that the availability of PtdIns(4,5)P$_2$ does not limit muscarinic receptor-mediated responses. Even under conditions in which the maximal accumulation of Ins(1,4,5)P$_3$ and the initial rates of [H]InsP$_3$ accumulation to the two agonists were approximately equivalent (100 nM GnRH and 1 mM methacholine), the muscarinic receptor-mediated response still displayed desensitization. At these levels of stimulation both receptors must have, at least initially, access to pools of PtdIns(4,5)P$_2$ of similar sizes to allow equivalent responses. Unless the activation of GnRH receptors is able to enhance the resupply of PtdIns(4,5)P$_2$ to a greater extent than the activation of muscarinic receptors, other mechanism(s) must underlie the rapid desensitization of muscarinic receptors. This latter conclusion is supported by measurements of agonist-evoked changes in the cellular concentration of PtdIns(4,5)P$_2$. These results demonstrate that, despite agonist concentrations matched for similar initial phosphoinositide responses, GnRH caused a sustained depletion of [H]PtdIns(4,5)P$_2$ compared with only a transient decrease in the continued presence of methacholine. Thus the initial rate of GnRH-stimulated [H]InsP$_3$ was maintained in spite of a pronounced decrease in PtdIns(4,5)P$_2$ concentration during the latter phase of the response (1–5 min), whereas the initial rate of methacholine-stimulated [H]InsP$_3$ accumulation was not maintained in spite of the recovery of PtdIns(4,5)P$_2$ levels. This suggests that substrate is not limiting muscarinic receptor-mediated responses and that rapid desensitization reflects instead the uncoupling of the receptor from the effector system. The lack of additivity of Ins(1,4,5)P$_3$ responses to simultaneous stimulation of αT3-1 cells with maximal concentrations of GnRH and methacholine does, however, indicate that a component of the signalling pathway common to both receptors might limit the maximal magnitude of the response, although it does not prove that the two receptor types use identical signalling components throughout the transduction pathway.

Most G-protein-coupled receptors, irrespective of the effector system to which they are linked, undergo rapid desensitization on exposure to agonist. This phenomenon has been extensively studied for G$_s$-coupled β$_2$-adrenergic receptor and it is accepted that receptor phosphorylation is responsible for receptor-G-protein uncoupling [10]. Agonist-dependent phosphorylation of a number of PLC-linked receptors has also been reported (reviewed in [11]) and linked to desensitization [11,28,29]. The site(s) of receptor phosphorylation, irrespective of the signal transduction pathway to which it is linked, seem to be primarily within the cytoplasmic C-terminal tail and/or regions of the third intracellular loop [11,30–32]. Indeed the phosphorylation of serine residues within the third intracellular loop might well underlie the rapid desensitization of the muscarinic M3 receptor [33]. In contrast, the GnRH receptor has essentially no predicted cytoplasmic C-terminal tail and a small predicted third intracellular loop with relatively few potential (serine/threonine) phosphorylation sites [34,35]. It has recently been indicated that
the GnRH receptor is not phosphorylated on exposure to agonist when transiently expressed in CHO cells [36]. However, and in marked contrast with the present results, that study also demonstrated a rapid and full desensitization of the GnRH receptor on exposure to agonist. Although this would seem to dissociate desensitization and phosphorylation, the interpretation of functional data in transient expression systems is difficult given that only a comparatively small proportion of cells will express the receptor and in these cells levels will most probably be supra-physiological. Thus other regulatory features perhaps not usually of importance (for example depletion of a key component of the signalling pathway) might come into play. We therefore believe that a lack of appropriate regulatory phosphorylation sites in the GnRH receptor is at present the most likely explanation for the lack of acute regulation. This would be consistent with a number of receptors linked preferentially to effector systems other than PLC. These include the G\_\text{\textalpha}{\text{c}}-coupled \(\beta_2\) adrenergic receptor [37], the G\_\text{\textalpha}{\text{c}}-coupled C4 adrenergic receptor [38] and the A\_\text{1} adenosine receptor [39], which are all resistant to acute desensitization and are not phosphorylated after exposure to agonist.

In summary, we have provided evidence that the GnRH receptor, unlike other PLC-linked receptors, does not undergo rapid desensitization following agonist stimulation. In contrast, the human muscarinic M3 receptor, when co-expressed in the same cell line, displays features of rapid desensitization characteristic of its activation profile in other cell lines. This suggests that rapid desensitization is therefore receptor-dependent rather than cell-dependent and is likely to reflect the action of receptor kinases and/or second messenger kinases rather than be a consequence of cellular factors such as a decreased supply of PtdIns(4,5)\(\text{P}_2\).

We thank Dr. A. B. Tobin for useful discussion during the preparation of this manuscript. This work was supported by grants 16895/1.5 and 051555/Z/97/Z/MP/RD/JF from the Wellcome Trust.

REFERENCES