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The paired activation of the two components of the muscarinic M3 receptor dimer is required for induction of ERK1/2 phosphorylation

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Running title: Stimulation of ERK1/2 phosphorylation by M3 dimers

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Summary

Muscarinic M_3 receptors stimulate ERK1/2, the mitogen activated protein kinase pathway. The phosphorylation of the M_3 receptor by casein kinase 1 on sites in the third intracellular loop contributes to the mechanism of receptor activation of ERK1/2 by working in concert with the diacylglycerol/PKC arm of the phospholipase C signalling pathway. A mutant of the muscarinic M_3 receptor, in which most of the third intracellular (i3) loop had been deleted (M_3-short), completely lost the ability to stimulate the ERK1/2 phosphorylation in COS-7 cells. This loss was evident despite the fact that the receptor was able to couple efficiently to the phospholipase C second messenger pathway. In co-transfected cells, M_3-short greatly reduced the ability of M_3 to activate ERK1/2. In another set of experiments we tested the ability of a mutant M_3/M_2(16aa) receptor, in which the first 16 aminoacids of the i3 loop of the M_3 receptor were replaced with the corresponding segment of the muscarinic M_2 receptor to stimulate ERK1/2 phosphorylation. This mutant is not coupled to $G_\alpha q$ but it is weakly coupled to $G_\alpha i$. In spite of its coupling modification, this receptor was able to stimulate ERK1/2 phosphorylation. Again, M_3-short greatly reduced the ability of M_3/M_2(16aa) to activate ERK1/2 in co-transfected cells. Similar results were obtained in stable transfected CHO cells lines. In CHO-M_3 cells carbachol induced a biphasic increase of ERK1/2 phophorylation: a first increase at doses as low as 0.1 μM and a second increase starting from 10 μM. In CHO-M_3-short and in double transfected CHO-M_3/M_3-short cells we observed only the lower doses increase of ERK1/2 phophorylation, no further increase was observed up to 1 mM carbachol. This suggests that in double transfected CHO cells M_3-short is preventing the effect of the higher doses of carbachol on the M_3 receptor. In a final experiment we tested the ability of co-transfected chimeric α_2/M_3 and M_3/α_2 receptors to activate the ERK1/2 pathway. These two receptors were constructed in a way that both the chimeras retained the third cytoplasmic loop of M_3. When given alone, carbachol and, to a lesser extent, clonidine, stimulated the coupling of the co-transfected chimeric receptors to the phospholipase C second messenger pathway but they were unable to stimulate ERK1/2 phosphorylation. On the contrary, a strong stimulation of ERK1/2

phosphorylation was observed when the two agonists were given together, despite the fact that the overall increase in phosphatidylinositole hydrolysis was not dissimilar from that observed in cells treated with carbachol alone. Our data suggest that the activation of the ERK1/2 pathway requires the coincident activation of the two components of a receptor dimer.

Introduction

Over the past several years, it has become increasingly apparent that G-protein coupled receptors (GPCRs) interact with one another to form dimers (or heterodimers) or larger oligomeric complexes (for reviews see: 1 and 2). Despite the large number of studies that have been published on receptor dimerization it is not obvious whether the paired activation of the two halves (receptor monomers) of the dimer is required or not for a specific receptor response.

In most cases, it seems that the individual ability of a given receptor to couple with selective G-proteins is not impaired by heterodimerization and a single agonist is often sufficient to give a full receptor response (3-7), even though the concomitant presence of two agonists can eventually modify this response. This suggests that the paired activation of the two receptors constituting the homo- or heterodimers is not strictly required for proper G-protein coupling. This concept can be also substantiated by the fact that the activity of non-functional GPCRs can be rescued by truncated receptors. For instance, a non-functional chimeric M_3/M_2 muscarinic receptor, in which the corresponding region of M_2 has replaced the N-terminal i3 loop, can be re-activated by co-transfection with a truncated muscarinic M_3 receptor bearing the correct N-terminal i3 loop sequence. In this case, the dimer is formed by a full-length receptor and a *per se* inactive fragment, suggesting that only one half of the dimer is sufficient for the G-protein coupling (8).

Mitogenic signals mediated by mitogen-activated protein kinases (MAPK), ERK-1 and ERK-2, can be initiated by both receptor-tyrosine kinases and by GPCRs. Recent data suggest that, besides their well characterized roles in GPCR desensitization and sequestration, β-arrestins may contribute to GPCR activation of MAPK by functioning as adaptors or scaffolding for the recruitment of these signalling molecules into a complex with agonist occupied receptors (for a review see 9). The first evidence of this is reported in the work of Luttrell et al. (1999) (10), who demonstrated that agonist stimulation of β_2 adrenergic receptor phosphorylation leads to a rapid recruitment of β-arrestin1 that carries with it to the receptor activated c-Src. Subsequent reports showed that β-arrestins can also interact directly with component kinases of the ERK1/2 and c-Jun N-terminal kinase 3 mitogen-activated protein kinase cascades. β-arrestins have been shown to form complexes with

angiotensin II type 1A receptor, cRaf-1 and ERK (11, 12), with protease-activated receptor type 2, Raf-1 and ERK1/2 (13), and with neurokinin-1 receptor, c-Src and ERK1/2 (14).

In a recent study, Budd et al. (2001) (15) have shown that phosphorylation of the muscarinic M_3 receptor by casein kinase 1α on sites in the third intracellular loop contributes to the mechanism of receptor activation of ERK1/2 by working in concert with the diacylglycerol/PKC arm of the phospholipase C signalling pathway. Recruitment of β-arrestin1 to the phosphorylated third intracellular loop of the muscarinic M_3 receptor is essential in this signalling process (16)

M3 muscarinic receptors, like most of the GPCRs, have been shown to form dimers (17); in this work we investigated to see whether the paired activation of both of the receptor components of the M3 dimer plays a role in the cascade of events leading to ERK1/2 phosphorylation.

Experimental procedures

Materials

Reagents. N-[^{3}H]methylscopolamine (83 Ci/mmol), [^{3}H]MK-912 (81 Ci/mmol) and [^{3}H]Adenine (32 Ci/mmol) was from DuPont-New England Nuclear. *Myo*-[3H]inositol (23 Ci/mmol) was from Amersham. Tissue culture media and sera were from Sigma and Celbio. Forskolin, pertussis toxin, carbachol, clonidine, atropine and yohimbine were from Sigma. Ro-20-1724 was from Calbiochem.

Eukaryotic Expression Vectors

We used human M_2 and rat M_3 muscarinic receptors inserted into a pCD plasmid (18). The construction of the M_3-short, M_3/M_2(16aa) and the chimeric adrenergic/muscarinic α_2/M_3 and M_3/α_2 receptors, has been described elsewhere (8, 19, 20). In particular, the M_3-short was obtained by cutting out 196 amino acids from the third cytoplasmic (i3) loop of M_3 (the remaining i3 loop was 43 amino acids long). In M_3/M_2(16aa), the first 16 amino acids of the i3 loop of the M_3 receptor were replaced with the corresponding segment of the M_2 muscarinic receptor. The chimeric adrenergic/muscarinic α_2/M_3 and M_3/α_2 receptors were obtained by exchanging the last two transmembrane regions between the adrenergic α_{2C} and the muscarinic M_3 receptors. In both chimeras, the third cytoplasmic loop was from the M_3 receptor. A schematic representation of the receptor mutants used is shown in **Fig. 1**. For co-immunoprecipitation experiments, the chimeric M_3/α_2 receptor was tagged at its N-terminus with the HA antigen (HA-M_3/α_2).

Cell cultures and transfection

COS-7 cells were incubated at +37°C in a humidified atmosphere (containing 5% CO_2) and grown in Eagle's medium as modified by Dulbecco, which was supplemented with 5% foetal bovine serum, 100 units/ml penicillin, and 100 ¼g/ml streptomycin. Cells were seeded at a density of 7.5 x 10^5 per 100-mm dish and, 24 h later, they were transiently transfected with the plasmid DNA by

the DEAE-dextran chloroquine method (21). The total amount of DNA used for each transfection was brought to 4 μg by adding an appropriate amount of vector DNA. Generation of stable CHO cell lines expressing the muscarinic M_3 and M_3-short receptors was described previously (Maggio et al. 1996). To establish the double-transfected CHO-M_3/M_3-short cell line, we co-transfected the M_3-short receptor in CHO-M_3. Since originally the CHO-M_3 cells were selected with the neomycin analogue G-418, the M_3-short receptor was subcloned in the pcDNA3.1/ hygro(–) vector (Invitrogen) and the selection of the CHO-M_3/M_3-short cells was done with hygromycin. After several round of clonal selection, several colonies were isolated with the cloning ring and tested for the presence of the M_3-short by PCR amplification of the i3 loop. Double transfected CHO-M_3/M_3-short cells were then tested in binding assay to evaluate the increase in binding respect to CHO-M_3. The ultimate modification in the amount of M_3 after selection was quantified by immunoblot with anti-M_3 antibody directed against the third loop.

Membrane preparation and binding assay

On day one, the COS-7 cells were transfected with the plasmid(s) of interest. Three days after transfection, confluent plates of cells were lysed by replacing the medium with ice-cold hypotonic buffer (1 mM Na-HEPES, 2 mM EDTA). After 20 min, the cells were scraped off the plate and centrifuged at 17,000 *rpm* for 20 min at +4°C. The lysed cell pellet was homogenised with a Polytron homogeniser in ice-cold binding buffer (50 mM Tris HCl pH 7.4, 155 mM NaCl, 0.01 mg/ml bovine serum albumin). Binding was carried out at +30°C in a final volume of 1 ml, and atropine 1 μM or noradrenaline 100 μM was used to define non-specific binding. The bound ligand was separated from the unbound ligand using glass-fiber filters (Whatmann, GF/B) with a Brandel Cell Harvester, and the filters were counted with a scintillation β-counter.

Detection of phosphorylated MAP kinase isoforms (ERK1/2)

On day one, the COS-7 cells were transfected with the plasmid(s) of interest. On day three, the cells were exposed to serum free medium until the day of the assay. On day four, cells were treated

for the indicated times with agonists or antagonists at +37 °C. Cells were then lysed in a buffer containing 50 mM Tris (pH 7.5), 200 mM NaCl, 1 mM EDTA, 0.1% NIAPROOF, 1.5 μM pepstatine, 4 μM leupeptin, 300 nM aprotinin 0.1 mM PMSF. The samples were then incubated for 30 min at +4°C and centrifuged for 15 min at 17,000 *rpm*. The pellet was discharged and protein concentration was measured in the supernatant with a BIO-RAD Protein Assay Kit. Proteins were run on a 15% SDS PAGE and the extent of phosphorylation of (ERK1/2) MAP kinase was determined by immuno-blotting with antiphosphoERK (Sigma). The blots were then stripped and reblotted with the antiERK1/2 (Sigma) to control the total amount of kinases loaded. Data from separate experiments were digitized on a flatbed scanner and analysed with a Kodak scientific imaging system software.

Immuno-precipitation and Western blotting of M_3, M_3M_2(16aa) and M_3-short

For immuno-precipitation, a N-terminal hemagglutinin tagged M_3-short (HA-M_3-short) was used. Three days after transfection, confluent plates of cells were washed twice with sterile 0.9% NaCl and scraped with 300 μl of buffer solution A (0.2% digitonin, EDTA 1 mM in PBS) containing a cocktail of protease inhibitors (1.5 μM pepstatin, 4 μM leupeptin, 0.01 M aprotinin and 500 μM PMSF).

Lysed cells were incubated on ice for 20 minutes and then centrifuged at 6000 *rpm* for 10 min at +4°C. The supernatant was removed and the pellet was re-suspended in 200 μl of buffer solution B (1% digitonin, 0.5% deoxycholate, 1 mM EDTA in PBS) containing the protease inhibitor cocktail and incubated at +4°C for 1 hour. Cells were centrifuged at 17000 *rpm* for 20 min at +4°C and then the supernatant was recovered for immuno-precipitation.

In order to eliminate proteins that could non-specifically bind magnetic beads, the cell extract was pre-exposed to 25 μl of protein G magnetic beads for 1 hour at +4°C. A magnetic field was applied to the side of the tube and the supernatant was recovered and transferred to a clean 1.5 ml micro-centrifuge tube. For immuno-precipitation, 5 μg of an anti-M_3 antibody directed against the i3-loop (Santa Cruz Biotechnology, Inc) was added to the tube and the suspension was incubated at +4°C for 1 hour. At the end of the incubation, 25 μl of protein G magnetic beads were added and incubated at +4°C for an additional hour. A magnetic field was applied to pull beads to the side of

the tube and the supernatant was carefully removed.

The magnetic beads were washed with 500 µl of immuno-precipitation buffer (0.1% niaproof, 0.2 M NaCl, 1 mM EDTA, 50 mM Tris, pH 7.5) including the protease inhibitor cocktail. Bead pellets were recovered by applying a magnetic field and they were re-suspended in 30 µl of Laemli buffer (plus 150 mM dithiotreitol) and incubated at +70°C for 5 min.

A magnetic field was applied to the sample and the supernatant was recovered and loaded on 15% SDS-polyacrylamide gel. Proteins were run at 90 V for 2-3 hours and transferred to the nitrocellulose membrane for 1 hour at 100V. The co-immunoprecipitated HA-M_3-short protein was determined by immunoblotting with an anti-HA antibody from (Sigma). Data from separate experiments were digitized on a flatbed scanner and analysed with a Kodak scientific imaging system software.

Immuno-precipitation of [^{3}H]NMS and [^{3}H]MK-912 labelled HA-M_3/α_2 and α_2/M_3 receptors

COS-7 cells were transfected with the chimeric HA-M_3/α_2 and the α_2/M_3 receptors. Three days after transfection, confluent plates of cells were washed twice with sterile 0,9% NaCl and scraped off the plate into ice-cold buffer (Buffer C) containing: 20 mM KH_2PO_4, 20 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 0.02% (w/v) sodium azide, 1 mM benzamide, 2 µg/ml pepstatin A, 0.2 µg/ml leupeptin, 200 µg/ml bacitracin, pH 7.6. They were homogenised in a Polytron homogeniser for 30 seconds and spun down in a centrifuge for 40 min at +4°C and 17000 *rpm*. This step was then repeated in a slightly different buffer (Buffer D): 20 mM KH_2PO_4, 20 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, pH 7.4. Detergents were added in Buffer D to a final concentration of 1% digitonin and 0.06% sodium cholate. The suspension was shaken on a horizontal shaker for 40 min at +4°C and then centrifuged for 40 min at +4°C and 17000 *rpm*. Protein content was assessed in the supernatant fraction and the relative concentration was adjusted to 20 mg of protein/ml. Binding was carried out in a final volume of 55 µl: 5 µl of soluble receptor preparation and 50 ¼l of [^{3}H]NMS or [^{3}H]MK912 in Buffer E (20 mM KH_2PO_4, 20 mM NaCl, 1 mM EDTA, 5 mM $MgSO_4$, 0.1 mM PMSF, pH 7.4) containing 0.3% digitonin and 0.02% sodium cholate. The reaction was carried out at

+30°C in Eppendorf microfuge tubes. Nonspecific binding was calculated in the presence of 1 μM atropine or 100 μM noradrenaline.

At the end of the incubation period, each sample was immunoprecipitated with the magnetic beads as described in the previous section. Immunoprecipitation of the α_2/M_3 receptor was perfomed with 20 μg of an anti-M_3 antibody directed against the carboxyl terminus (Santa Cruz Biotechnology, Inc), while immunoprecipitation of HA-M_3/α_2 was performed with 20 μg of the anti-HA antibody.

Bead pellets containing the labelled HA-M_3/α_2 and α_2/M_3 receptors were counted with a scintillation β-counter.

Phosphatidylinositol Breakdown Assay

Transfected COS-7 cells were incubated with *myo*-[^{3}H]inositol (3 μCi/ml) for 48 h. Immediately prior to the experiment, the cells were washed twice with phosphate-buffered saline and incubated for 15 min in Eagle's medium containing 10 mM LiCl and 20 mM HEPES. The medium was then replaced by 0.25 ml of the same medium containing the experimental agents. After a one-hour incubation at +25°C, the reaction was arrested by the addition of 0.75 ml of 7.5% (w/v) ice-cold trichloroacetic acid, followed by a 30-min incubation on ice. The trichloroacetic acid was extracted with water-saturated diethyl ether (3 x 4 ml), and levels of IP_1 were determined by anion exchange chromatography (22).

Adenyl cyclase assay

COS-7 cells were transfected with the plasmid(s) containing the receptor of interest plus the adenyl cyclase V. Twenty-four hours after transfection, the cells were trypsinized and recultured in 24-well plates, and after an additional 24 h, they were assayed for adenyl cyclase activity. The assay was performed in triplicate as described by Avidor-Reiss et al., (1995) (23). In brief, the cells in the 24-well plates were incubated for 2 h with 0.25 ml/well of fresh growth medium containing 5 μCi/ml [^{3}H]adenine, and this medium was replaced with 0.5 ml/well of DMEM containing 20 mM HEPES, pH 7.4, 0.1 mg bovine serum albumin, and the phosphodiesterase inhibitors 1-methyl-3-

isobutylxanthine (0.5 mM) and RO-20-1724 (0.5 mM). AC activity was stimulated by the addition of 1 μM forskolin in the presence or absence of carbachol. After 10 min of incubation at +30°C, the medium was removed and the reaction terminated by the addition of perchloric acid containing 0.1 mM unlabelled cAMP, followed by neutralization with KOH. The amount of [^{3}H]cAMP formed was determined by a two-step column separation procedure, as described by Avidor-Reiss et al., (1995) (23).

Results

In a first set of experiments we characterized the ability of the muscarinic M_3 receptor to stimulate the phosphorylation of ERK1/2 in COS-7 cells. As shown in **Fig. 2a,** carbachol at a concentration of 0.1 mM increases the fraction of ERK1/2 phosphorylated in COS-7 cells transiently transfected with the muscarinic M_3 receptor. This effect was already evident after 1 min, it reached the maximum at 5 min and vanished after 20 min. The dose-response curve of ERK1/2 phosphorylation was characterized by a sudden change in the response to carbachol between 10 and 100 μM (**Fig. 2b**). This was completely different form the dose-response curve of M_3-induced phosphatidylinositol hydrolysis, in which a gradual increase in IP_1 accumulation with the increase of the concentration of carbachol was observed (**Fig. 4a**).

Since Budd et al. (2001) (15) have already shown that phosphorylation of the muscarinic M_3 receptor on sites in the third intracellular loop by casein kinase 1α contributes to the mechanism of receptor activation of ERK1/2, we tested to see whether a muscarinic M_3 receptor in which 196 aminoacids of the i3 loop were deleted, named M_3-short, was able to stimulate ERK1/2 phosphorylation. In agreement with the data reported by Budd et al. (2001) (15), the M_3-short receptor was unable to stimulate ERK1/2 phosphorylation (**Fig. 3a**), despite the fact that this receptor was efficiently coupled to $G_{\alpha}q$ as shown by the increase in phosphatidylinositol hydrolysis (**Fig. 4a**).

In order to see if the M_3-short receptor can interfere with the ability of the wild type muscarinic M_3 receptor to stimulate ERK1/2 phosphorylation, we co-transfected these two receptors together. As shown in **Fig. 3a**, the M_3-short completely blocks the ability of the wild type M_3 receptor to induce ERK1/2 phosphorylation. It is interesting to note that the co-transfection of these two receptors together did not modify the extent of the IP_1 accumulation in phosphatidylinositol breakdown assays (**Fig. 4a**).

In another set of experiments we tested another mutant muscarinic M_3 receptor in which 16

aminoacids of the N-terminal i3 loops were exchanged with 16 aminoacids of the M_2 [M_3/M_2(16aa)]. While the binding affinity of this receptor for muscarinic ligands remained unchanged, its coupling selectivity was altered. As reported by Wess et al. (1990) (20), this receptor loses the ability to stimulate the phosphatidyl inositol hydrolysis (**Fig. 4a**) and acquires the ability to inhibit the accumulation of cAMP (**Fig. 4b**). Regardless of its change in coupling selectivity, this receptor efficiently stimulated ERK1/2 phosphorylation (**Fig. 3b**).

Pertussis toxin at a concentration of 130 ng/ml given 24 h before the assay reduced but did not abolish the ability of carbachol to stimulate ERK1/2 phosphorylation (**Fig. 3b**). The magnitude of the basal phosphorylation of ERK1/2 was reduced, and after the correction for the change in the basal level, the stimulation was on average 3.29-fold in cells treated with pertussis toxin and 4.14-fold in cells untreated. This difference was statistically significant at the 0.05 level (Two tailed Paired Student's *t*-test).

In order to see if the M_3-short receptor was able to inhibit the effect of the M_3/M_2(16aa) to stimulate ERK1/2 phosphorylation, we co-transfected these two receptors together. Similar to what has been observed with the wild type M_3 receptor, the M_3-short completely abolished the M_3/M_2(16aa) induction of ERK1/2 phosphorylation. Remarkably, the ability of the two receptors expressed together to activate the phosphatidyl inositol hydrolysis (**Fig. 4a**) and to inhibit the accumulation of cAMP (**Fig. 4b**) was not altered, indicating that the co-expression of these two receptors does not interfere with G-protein coupling.

In a control experiment, we co-transfected the M_3/M_2(16aa) with the wild type M_3 receptor. In this condition carbachol was able to activate ERK1/2 phosphorylation (**Fig. 3c**); furthermore, there was no modification in the ability of the two receptors to induce the phosphatidylinositol hydrolysis (**Fig. 4a**) and to inhibit the accumulation of cAMP (**Fig. 4b**).

An alternative explanation for the inhibition of ERK1/2 phosphorilation by M_3-short could be that it sequesters some proteins needed for the activation of ERK1/2. To exclude this possibility, in a control experiment, we co-transfected M_3-short with the adrenergic α_{1B} receptor. Stimulation of M_3-short with carbachol (100 μM) did not interfere with the induction of ERK1/2 phosphorylation

by the noradrenaline stimulated adrenergic α_{1B} receptor (unpublished result).

We extended our experimental observations to stable transfected CHO cell lines. CHO-M_3 and CHO-M_3-short cells were generated previously (19); double transfected CHO-M_3/M_3-short cells, were generated by transfecting the M_3-short receptor in CHO-M_3 cells. The relative amount of the M_3 receptor in CHO-M_3/M_3-short cells (detected with an antibody against the third cytoplasmic loop) was slightly decreased compared to CHO-M_3 cells (**Table 1**).

By normalizing for the B_{max} obtained in CHO-M_3 cells, we calculated that the absolute amount of M_3 in CHO-M_3/M_3-short cells was 636 fmol/mg of proteins (753 * 84.5/100). Subtracting this value from the total B_{max} we obtained that the amount of the M_3-short receptor was 1190 fmol/mg of proteins (1826 - 636).

In contrast to transiently transfected COS-7 cells, the basal level of ERK1/2 in serum starved CHO-M_3 cells was very low or undetectable. The dose response-curve of carbachol induced ERK1/2 phosphorylation in CHO-M_3 cells was biphasic, with an initial increase at lower doses of the agonist (0.1 μM) and a second increase starting at 10 μM (**Fig. 5**). In contrast in CHO-M_3-short cells the dose response-curve of carbachol induced ERK1/2 phosphorylation was monophasic. Low doses of agonist increased ERK1/2 phosphorylation and no further increase was observed up to 1 mM carbachol (**Fig. 5**). Carbachol induction of ERK1/2 phosphorylation in double transfected CHO-M_3/M_3-short cells, was similar to CHO-M_3-short cells; low doses of carbachol increased ERK1/2 phosphorylation and no further increase was observed with higher doses of the agonist (**Fig. 5**). Carbachol up to 1 mM did not induce ERK1/2 phosphorylation in untransfected CHO cells, indicating that the effect of the agonist in stable transfected CHO cells was receptor mediated (unpublished result).

We have shown in the past that the co-transfection of chimeric adrenergic/muscarinic α_2/M_3 and M_3/α_2 receptors results in the rescue of the binding of muscarinic and adrenergic ligands. Furthermore, we have shown that carbachol efficiently stimulates phosphatidylinositol hydrolysis in COS-7 cells co-transfected with these two chimeric receptors (8). In both of these chimeric

receptors, the third cytoplasmic loop is from the muscaric M_3 receptor; therefore it is likely that the activation of the adrenergic half of the reconstituted heterodimer results in the stimulation of phosphatidylinositol hydrolysis. As predicted, the adrenergic agonist clonidine was able to stimulate the phosphatidylinositol hydrolysis, even though the extent of the stimulation was markedly less than that of carbachol (**Fig. 6a**). When carbachol was given in the presence of a 100 μM concentration of clonidine, the shape of the curve was not substantially different from that observed with carbachol alone, except for the fact that the curve began at 30% over the basal, due to the effect of clonidine, and the extent of stimulation at high concentrations was roughly the same (**Fig. 6a**). This suggest that clonidine did not modify the affinity of carbachol for the reconstituted M_3 receptor; in accord, the shape of the [^{3}H]NMS displacement curves did not change substantially in the presence of 100 μM clonidine, suggesting that the two receptors work independently (**Fig. 6b**).

COS-7 cells co-transfected with chimeric α_2/M_3 and M_3/α_2 receptors are a good model for testing whether the paired stimulation of a dimer is required for ERK1/2 stimulation; in fact, the only form compatible with a function in this case is a dimer, and only inactive monomers are eventually present in the cells. We started to test whether carbachol or clonidine given alone at a concentration of 100 μM were able to stimulate ERK1/2 activation. Surprisingly, neither of the agonists stimulated ERK1/2 phosphorylation, (**Fig. 7**). Conversely, carbachol and clonidine given together at a concentration of 100 μM clearly activated ERK1/2 phosphorylation (**Fig. 7**).

In order to make clear whether the two agonists given together stimulate ERK1/2 phosphorylation by a specific activation of the chimeric muscarinic/adrenergic heterodimer, we tested the ability of the muscarinic and the adrenergic antagonists, atropine and yohimbine, respectively, to prevent the effect of the paired carbachol/clonidine ERK1/2 stimulation. As shown in **Fig. 8a** and **b,** both antagonists effectively prevented the effect of the paired carbachol/clonidine stimulation.

We also tested whether antibodies against each of the α_2/M_3 and M_3/α_2 chimeric receptor were able to immunoprecipitate cholinergic ([^{3}H]NMS) and adrenergic ([^{3}H]MK912) ligands. To this purpose the M_3/α_2 receptor was tagged in its N-terminus with the HA-antigen and immunoprecipitated with anti-HA antibodies, while the α_2/M_3 receptor was immunoprecipitated

with an antibody against the C-terminal of the M_3 receptor. As shown in **Table 2a** when the two receptors were transfected alone, no radiolabelled HA-M_3/α_2 or α_2/M_3 receptors were detected in the immunoprecipitate. Conversely when the two receptors were transfected together both antibodies were able to immunoprecipitate labelled [^{3}H]NMS and [^{3}H]MK912 receptors. Again, binding analysis carried out on cell membranes showed that both antagonists bound to the α_2/M_3 and M_3/α_2 receptors only when they were co-transfected (**Table 2b**). The affinities of [^{3}H]NMS and [^{3}H]MK912 for the co-transfected α_2/M_3 and M_3/α_2 receptors were not significantly different from that for the wild type M_3 and /α_{2C} receptors.

In a final set of experiments we tested the ability of M_3-short to physically interact with the wild type M_3 and the mutant M_3/M_2(16aa) receptors. We immuno-precipitated the M_3 or the M_3/M_2(16aa) receptor with an antibody directed against the i3 loop, and we blotted the membrane with an anti-HA antibody. As shown in **Fig. 9a** and **b**, the HA-M_3-short co-immunoprecipitate with both M_3 and M_3/M_2(16aa). For unknown reasons, we were not able to perform the reverse experiment; that is, co-immuno-precipitating with the HA-M_3-short and blotting with anti-M_3 antibody. Furthermore, all the tagged M_3 receptors that we tested gave very weak signals and were ineffective in co-immunoprecipitation experiments of M_3 and M_3/M_2(16aa). For this reason, we tried to quantify indirectly the amount of M_3 receptor in complex with M_3-short.

In cells co-transfected with 1 μg M_3 and 2 μg HA-M_3-short we determine the fraction of M_3 receptor in the cell lysates before and after immunoprecipitation with anti-HA antibodies. As shown in **Fig. 9c** the fraction of M_3 receptor remaining in the cell lysates after immunoprecipitaion was on average 23.4%, therefore up to 76.6% was co-immunoprecipitated with HA-M_3-short. Conversely, the fraction of HA-M3 short receptor in the cell lysates after immunoprecipitation with the anti-M3 antibodies was 62.8% therefore up to 37.2% was co-immunoprecipitated with M_3. If we consider that the amount of HA-M_3-short receptor transfected was twice as much that of the

M_3, these data make sense.

Discussion

It is now well established that G-protein coupled receptors form homo- and heterodimers. While the mechanism(s) of dimerization still remains to be elucidated, it is becoming increasingly apparent that this phenomenon is important for several receptor functions. One of the critical points in receptor homo- and heterodimerization is determining whether the paired activation of the dimer is required or not for specific receptor functions.

Most of the reports dealing with receptor dimerization indicate that the individual stimulation of one of the two interacting receptors in co-transfected cells is often sufficient for G-protein activation (3-7). On the other hand, the simultaneous activation of both receptors with selective agonists can change the pharmacology of one or both of the two receptors (6). These data indicate that heterodimerization by itself does not necessarily interfere with G-protein coupling. This conclusion is supported also by the fact that heterodimerization between receptors that bind distinct G-proteins often leaves the coupling selectivity of each receptor unaltered. For instance, β_2 adrenergic receptors that couple to stimulatory G-proteins, or δ and κ opioid receptors that couple to inhibitory G-proteins, form heteromeric complexes, but heterodimerization does not significantly alter the ligand binding or the coupling properties of these receptors (24).

Consistently with these data, we observed that the co-transfection of a mutant muscarinic M_3 receptor, which couples preferentially to $G_\alpha i$, the M_3/M_2(16aa), with an other mutant M_3 receptor M_3-short that couples to $G_\alpha q$, leaves the coupling selectivity of these two receptors unaltered, despite the fact that they form dimers. It is interesting to note that the binding properties of these two receptors were identical (19) but that the potency of carbachol in the phosphatidyl inositol breakdown assay and in the cAMP assay were quite different, indicating that in spite of their interaction these two receptors are rather independent in their G-protein coupling properties.

Certainly, we must consider that only a fraction of the entire population of receptors in the membrane forms M_3-short/M_3/M_2(16aa) heterodimers; it is possible, therefore, that the residual monomers (or homodimers) are responsible for the observed responses. This consideration obviously applies to the above mentioned adrenergic/opioid heterodimer as well.

More direct evidence that heterodimerization can leave the coupling property of each receptor

unaltered comes from our experiment with chimeric adrenergic/muscarinic receptors (8, 19). In the past we have shown that chimeric α_2/M_3 and M_3/α_2 receptors, when co-transfected together, reconstitute the binding activity of the wild type adrenergic and muscarinic receptors, while neither of the two chimeras was able to bind adrenergic or muscarinic ligands. These results imply that in cells co-transfected with the two chimeras the only active receptor is the heterodimer α_2/M_3-M_3/α_2. In the present work we proved this belief by immunoprecipitating the α_2/M_3 and HA-M_3/α_2 receptors labelled with cholinergic and adrenergic ligands. Only when the α_2/M_3 and HA-M_3/α_2 receptors were transfected together, selective antibodies immunoprecipitated receptor bound cholinergic and adrenergic ligands, confirming that the only active receptor is the heterodimer α_2/M_3-M_3/α_2.

Since in both chimeras the third cytoplasmic loop was from the muscarinic M_3 receptor, it was not surprising that the stimulation of both the muscarinic and the adrenergic counterparts of this heterodimer led to the increase in the phosphatidyl inositol hydrolysis. Yet we observed that the binding affinity of carbachol in the presence of clonidine was not significantly modified. These data support the view that the single activation of part of a heterodimer is sufficient for G-protein activation, and that the occupancy of both sites of the heterodimer can perhaps only modify the coupling efficiency.

Besides G-protein coupling, a G-protein coupled receptor can activate mitogenic signals mediated by mitogen-activated protein kinases (MAPK), ERK-1 and ERK-2 (25). We analysed the activation of this pathway to see if it requires the paired activation of a receptor dimer. For this purpose we used the muscarinic M_3 receptor that has been shown to form dimers (17) and to stimulate MAPK. In a recent study, Budd et al. (2001) (15) have shown that phosphorylation of the muscarinic M_3 receptor by casein kinase 1α on sites in the third intracellular loop contributes to the mechanism of receptor activation of ERK1/2 by working in concert with the diacylglycerol/PKC arm of the phospholipase C signalling pathway. They came to this conclusion by using a modified M_3 receptor in which a large part of the i3 loop was removed.

In our study, using an analogous mutant of the muscarinic M_3 receptor, M_3-short, we confirmed

the importance of the i3 loop in the stimulation of ERK1/2 phosphorylation. Since the recruitment of β-arrestin1 to the phosphorylated third intracellular loop of the muscarinic M_3 receptor is essential in this signalling process (16), and in the light of the fact that β-arrestins may contribute to GPCR activation of MAPK, by functioning as adaptors or scaffolding for the recruitment of these signalling molecules into a complex with agonist occupied receptors (for a review see 9), it is very likely that this segment of the receptor concurs to the activation of ERK1/2 by binding β-arrestin 1, which in turn functions as an adaptor or scaffolding for the recruitment and the activation of c-Src.

As we mentioned above, Budd et al. (2001) (15) in their work have demonstrated that both the diacylglycerol/PKC arm of the phospholipase C signalling pathway and the phosphorylation of the i3 loop are essential for the activation of ERK1/2. In contrast with these data, in COS-7 cells a mutant M_3/M_2(16aa) receptor (20) virtually unable to couple to $G_\alpha q$ though, with a weak coupling selectivity for $G_\alpha i/o$, efficiently stimulated the ERK1/2 pathway in a manner similar to that observed with the wild type M_3 receptor. Pre-treatment with pertussis toxin did reduce, however, the ability of this mutant M_3/M_2(16aa) to stimulate ERK1/2 phosphorylation. Furthermore, pertussis toxin reduced the basal phosphorylation of ERK1/2 in these cells. The reason why this receptor remains active despite the alteration in G-protein coupling is beyond the scope of this study; nevertheless, this receptor was used as an additional control to study the interference of M_3-short in the stimulation of ERK1/2 phosphorylation.

When we co-transfected the M_3-short receptor with the wild type M_3 receptor, the carbachol stimulation of ERK1/2 phosphorylation in COS-7 cells was greatly subdued. This inhibition was not due to a shift in the dose-response curve to carbachol since doses as high as 1 mM were ineffective. Indirectly, by measuring the fraction of the M_3 receptor in the cell lysates after co-immunoprecipitation, we calculated that the amount of M_3 receptor co-immunoprecipitated with M_3-short was as much as 76.6% of the total M_3 receptor expressed in the membrane. This suggests that most of the M_3 receptor forms heterodimers.

It remained to be established whether the effect of the M_3-short was due to its lack of the i3 loop or to an induced alteration of the functional activity of the M_3. Since both of these receptors are

coupled to $G_{\alpha}q$, and there is no difference in binding and coupling efficiency, we addressed this question by using the mutant M_3/M_2(16aa) mentioned above. Similarly to what has been observed with the wild type M_3 receptor, the M_3-short also prevented the ability of M_3/M_2(16aa) to stimulate ERK1/2 phosphorylation. Despite this effect on ERK1/2, as mentioned above, the M_3-short did not modify the coupling efficacy of M_3/M_2(16aa), indicating that the effect on ERK1/2 was not due to a functional alteration of the receptor. To further support the lack of the i3 loop being cause of the M_3-short inhibition of M_3 and M_3/M_2(16aa) stimulated ERK1/2 phosphorylation, we demonstrated that co-transfection of M_3/M_2(16aa) with M_3 did not reciprocally alter their effect on ERK1/2 phosphorylation.

Results similar to those in COS-7 cells were obtained in stable transfected CHO-M_3, CHO-M_3-short and CHO-M_3/M_3-short cells. Low doses of carbachol stimulated the phosphorylation of ERK1/2 in all the three cell lines. A second increase of ERK1/2 phosphorylation was observed in CHO-M_3 cells starting from 10 μM carbachol. No further increase on the contrary was observed in CHO-M_3-short and CHO-M_3/M_3-short cells up to 1 mM carbachol. While the first component of this increase is most probably linked to the activation of $G_{\alpha}q$, the second component in CHO-M_3 cells is very likely to be correlated to the recruitment of β-arrestin to the phosphorylated third intracellular loop of the muscarinic M_3 receptor.

A likely explanation for these results is that, for an efficient activation of the ERK1/2 pathway to take place, both of the receptors in the dimer have to bear a full-length i3 loop. Since, as mentioned before, recruitment of β-arrestin1 to the phosphorylated third intracellular loop of the muscarinic M_3 receptor is essential for ERK1/2 activation, it is possible that β-arrestin binding requires the phosphorylation of two i3 loops.

Similar conclusions can be drawn by analysing the recent work by Lavoie et al. (2002) (5). These authors demonstrated that co-expression of the β_1AR completely inhibited β_2AR stimulated ERK1/2 MAP kinase activity in HEK 293 cells, although β_1AR and β_2AR alone or together led to similar increases in the agonist stimulated adenyl cyclase activity. As shown by Lutrell et al. (1999) (10),

the activation of the β_2AR leads to a rapid recruitment of β-arrestin 1 that carries with it to the receptor activated c-Src. Conversely, the β_1AR interact weakly with β-arrestins (26), therefore it is likely that its interaction with the β_2AR in the heterodimer prevents β-arrestin binding and consequently ERK1/2 activation. This led us to speculate that for an efficient coupling of the heterodimer to β-arrestins both of the receptors must bind this protein efficiently.

This idea is also based on a recent study on the organization of rhodopsin in native membranes (27). Arrestin, the cognate β-arrestins in the visual system, has a bipartite structure of two structurally homologous seven-stranded β-sandwiches forming two putative rhodopsin-binding groves that are separated by 3.8 nm (28, 29). The positive charge arrangement of the surface of the rhodopsin dimer matches the negative charges on arrestin, thus, one arrestin monomer is likely to bind one rhodopsin dimer.

The evidence reported above indicate that dimers rather than monomers are required for the cascade of events that lead to ERK1/2 phosphorylation, but this does not answer the question of whether or not the two receptors constituting the dimer have to be activated at the same time. In order to address this issue, we performed experiments with chimeric muscarinic/adrenergic α_2/M_3 and M_3/α_2 receptors. As mentioned above, these two receptors exist only as heterodimers, and they were constructed in a way that both chimeras carried the i3 loop of the muscarinic M_3 receptor. We tested to see whether the single muscarinic or adrenergic agonist (carbachol or clonidine) was able to stimulate ERK1/2 phosphorylation. Surprisingly, and in contrast to what has been observed with G-protein coupling, both agonists given alone were unable to induce ERK1/2 phosphorylation. Conversely, when the two agonists were given together there was a clear activation of ERK1/2. This effect was specific, as demonstrated by the fact that both the adrenergic and muscarinic antagonists prevented ERK1/2 phosphorylation induced by the paired stimulation of carbachol and clonidine. These results demonstrated that, in order to have an activation of ERK1/2 by this heterodimer, both the adrenergic and muscarinic receptor components must be activated.

In conclusion the data in this paper raise the possibility that in certain conditions the stimulation of ERK1/2 phosphorylation requires the paired activation of a receptor dimer.

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TABLE I

Characterization of stable transfected CHO-M_3, CHO-M_3-short and CHO-M_3/M_3-short cell lines

CHO cells were stable transfected with M_3, M_3-short and with both receptors. The double transfected CHO-M_3/M_3-short cell line was established by transfecting the M_3-short in CHO-M_3 cells. Since the two receptors are indistinguishable in binding assay, we measured the relative amount of M_3 in CHO-M_3/M_3-short cells with respect to CHO-M_3 cells on western blot (W.B.), using an anti-M_3 antibody against the third cytoplasmic loop. By normalizing for the B_{max} obtained in CHO-M_3 cells we calculated that the absolute amount of M_3 in CHO-M_3/M_3-short cells was 636 fmol/mg of proteins (753 * 84.5/100). Subtracting this value from the total B_{max} of CHO-M_3/M_3-short cells we obtained that the amount of the M_3-short receptor was 1190 fmol/mg of proteins (1826 - 636).

	B_{max} (fmol/mg protein)	K_d	% of M_3 receptor in W.B.
CHO-M_3	753 ± 86	86.3 ± 11.5	100 ± 12.7
CHO-M_3/M_3-short	1826 ± 205	84.5 ± 8.51	84.5 ± 13.3
CHO-M_3-short	949 ± 101	92.3 ± 7.81	—

TABLE II

Characterization of α_2/M_3 and M_3/α_2 receptor binding in membranes and solubilized receptors

(**A**) Immunoprecipitation of [^{3}H]NMS and [^{3}H]MK912 labelled α_2/M_3 and HA-M_3/α_2 receptors with anti-HA and anti-C-terminal M_3 antibodies (Anti-M_3). The anti-C-terminal M_3 antibody recognizes only the α_2/M_3 chimeric receptor. The values of fmol/mg of proteins in the immunoprecipitation experiments were calculated in the membrane extracts before immunoprecipitation. (**B**) Binding parameters of co-transfected α_2/M_3 and M_3/α_2 receptors in COS-7 cell membranes compared to wild type M_3 and α_{2C} receptors. N.D. = non detectable.

A	α_2/M_3 (DNA transfected 4 µg)		α_2/M_3 + HA-M_3/α_2 (DNA transfected 2 µg + 2 µg)		HA-M_3/α_2 (DNA transfected 4 µg)	
I.P.	[^{3}H]NMS (fmol/mg)	[^{3}H]MK912 (fmol/mg)	[^{3}H]NMS (fmol/mg)	[^{3}H]MK912 (fmol/mg)	[^{3}H]NMS (fmol/mg)	[^{3}H]MK912 (fmol/mg)
Anti-HA	N.D.	N.D.	14.9 ± 3.91	17.8 ± 4.62	N.D.	N.D.
Anti-M_3	N.D.	N.D.	16.7 ± 4.43	19.4 ± 5.37	N.D.	N.D.
B	M_3 (DNA transfected 4 µg)		α_2/M_3 + M_3/α_2 (DNA transfected 2 µg + 2 µg)		α_{2C} (DNA transfected 4 µg)	
Ligand	K_D (pM)	B_{max} (fmol/mg)	K_D (pM)	B_{max} (fmol/mg)	K_D (pM)	B_{max} (fmol/mg)
[^{3}H]NMS	84.6 ± 5.31	1126 ± 85.4	81.3 ± 15.7	39.3 ± 3.18	—	—
[^{3}H]MK912	—	—	87.3 ± 13.6	42.4 ± 4.25	77.1 ± 8.12	1259 ± 117

Figure legends

Fig. 1 *Schematic representation of wild type muscarinic M_3 receptor and the derived receptor mutants.* The M_3–short has been obtained by cutting out 196 aminoacids from the i3 loop. In the M_3/M_2(16aa), the first 16 amino of the i3 loop of the M_3 receptor have been replaced with the corresponding segment of the M_2 muscarinic receptor. The α_2/M_3 and M_3/α_2 chimeras were obtained by exchanging the last two transmembrane domains between the muscarinic M_3 and the adrenergic α_{2C} receptor. In both chimeras, the i3 loop was from the M_3 receptor.

Fig. 2 *Time and concentration-dependent increase of ERK1/2 phosphorylation in COS-7 cells transfected with 1 μg DNA of the muscarinic M_3 receptor* (**a**) Confluent monolayers of COS-7 cells were stimulated with carbachol (100 μM) for the time indicated, (**b**) or with different concentrations of carbachol for 5 min. Incubation was terminated, cell lysates prepared and ERK1/2 phosphorylation detected as described in the Methods section. ERK1/2 activation, shown in the upper part of each figure, was measured by anti-phospho ERK1/2 antibody and normalized for total ERK1/2. The blots are representative of three (**a**) and four (**b**) experiments. Bar graph shows the means ± s.e. of all the experiments. *Significantly different from basal (Paired Student's two-tailed *t*-test, $P<0.05$)

Fig. 3 *Inhibition of carbachol induced ERK1/2 phosphorylation in (**a**) COS-7 cells co-transfected with 1 μg DNA of M_3 and 2 μg DNA of the M_3-short receptors (**b**) COS-7 cells co-transfected with 1 μg DNA of the M_3/M_2(16aa) and 2 μg DNA of the M_3-short receptors and (**c**) COS-7 cells co-transfected with 1 μg DNA of the M_3 and 2 μg DNA of the M_3/M_2(16aa) receptors.* Confluent monolayers of COS-7 cells were stimulated with carbachol (100 μM) for 5 min. Incubation was terminated, cell lysates prepared and ERK1/2 phosphorylation detected as described in the Methods section. ERK1/2 activation, shown in the upper part of each figure, was measured by anti-phospho ERK1/2 antibody and normalized for total ERK1/2. In (**b**), where indicated, cells were exposed to pertussis toxin 130 ng/ml 24 h before the assay. The blots are

representative of four (**a** and **b**) and two (**c**) experiments. Bar graphs are the means ± s.e. of all the experiments. *Significantly different from basal (**a**, **b** and **c**), **significantly different from carbachol stimulated M_3 (**a**) or M_3M_2(16aa) (**b**) and #significantly different from basal M_3/M_2(16aa) (**b**) (Paired Student's two-tailed *t*-test, P<0.05).

Fig. 4 *Carbachol stimulation of inositol phosphate hydrolysis (**a**) or inhibition of cAMP accumulation (**b**) in COS-7 cells separately transfected or co-transfected with 1 μg DNA of M_3, 1 μg DNA of M_3/M_2(16aa) (2 μg when co-transfected with M_3) and 2 μg DNA M_3-short receptors.* For inositol phosphate breakdown assay, cells were pre-labelled for 48 h with 3 μCi/ml [^{3}H]inositol and, immediately before the assay, incubated for 15 min in Eagle's medium containing 10 mM LiCl and 20 mM HEPES. The medium was then replaced by the same medium containing increasing concentrations of carbachol, and the cells were then incubated for 1 h at +25°C. For the adenyl cyclase assay, cells were pre-labelled for 2 h with 5 μCi/ml [^{3}H]adenine, then adenyl cyclase activity was stimulated for 10 min at +30°C by the addition of 1 μM forskolin in the presence of increasing concentrations of carbachol. The graphs are representative of two experiments, each performed in triplicate (**a**) or in quadruplicate (**b**).

Fig. 5 *Concentration-dependent increase of ERK1/2 phosphorylation in CHO cells stable transfected with the muscarinic M_3, M_3-short and M_3/M_3-short receptors*. Serum starved confluent monolayers of CHO cells were stimulated with different concentrations of carbachol for 5 min. Incubation was terminated, cell lysates prepared and ERK1/2 phosphorylation detected as described in the Methods section. ERK1/2 activation, shown in the upper part of the figure, was measured by anti-phospho ERK1/2 antibody and normalized for total ERK1/2. The blots are representative of three experiments. Bar graph shows the means ± s.e. of all the experiments. *Significantly different from carbachol 0.l and 1 μM (Paired Student's two-tailed *t*-test, P<0.05)

Fig. 6 *Carbachol and clonidine stimulation of inositol phosphate hydrolysis (**a**) and carbachol displacement of [^{3}H]NMS binding (**b**) in COS-7 cells co-transfected with the chimeric*

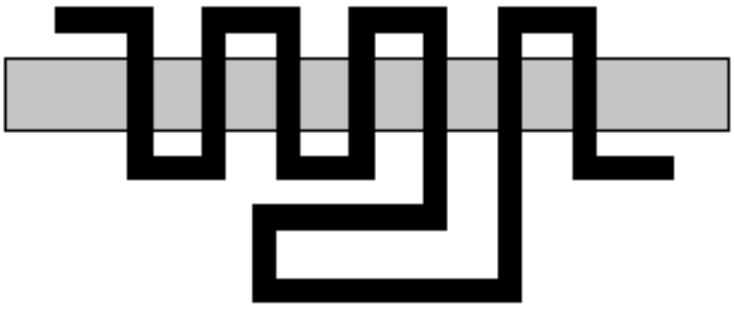
muscarinic/adrenergic α_2/M_3 and M_3/α_2 receptors (1 μg DNA each). For the inositol phosphate breakdown assay, cells were pre-labelled for 48 h with 3 μCi/ml [^{3}H]inositol and, immediately before the assay, incubated for 15 min in Eagle's medium containing 10 mM LiCl and 20 mM HEPES. The medium was then replaced by the same medium containing increasing concentrations of clonidine or carbachol (in the presence or absence of 100 μM of clonidine) and the cells incubated for 1 h at +25°C. Carbachol displacement of [^{3}H]NMS binding was performed at +30°C for 3 h. The graphs are representative of three (**a**) or two (**b**) experiments, each performed in triplicate.

Fig. 7 *Paired stimulation with carbachol and clonidine induced ERK1/2 phosphorylation in COS-7 cells co-transfected with the chimeric muscarinic/adrenergic α_2/M_3 and M_3/α_2 receptors (1 μg DNA each).* Confluent monolayers of COS-7 cells were stimulated with carbachol (100 μM), clonidine (100 μM) or a mixture of the two at the same concentration for 5 min. Incubation was terminated, cell lysates prepared and ERK1/2 phosphorylation detected as described in the Methods section. ERK1/2 activation, shown in the upper part of the figure, was measured by anti-phospho ERK1/2 antibody and normalized for total ERK1/2. The blot is representative of six experiments. Bar graphs are the means ± s.e. of all six experiments. *Significantly different from basal (Paired Student's two-tailed *t*-test, $P<0.05$).

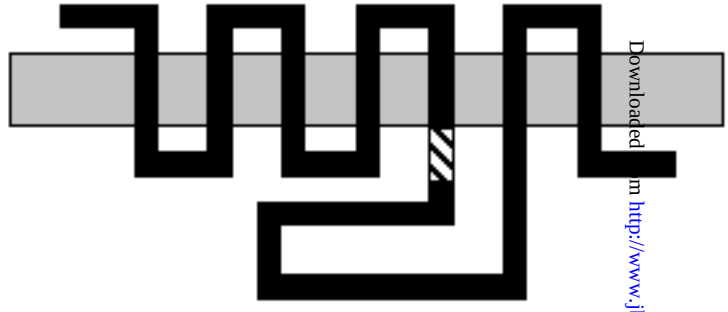
Fig. 8 *Inhibition by atropine (**a**) or yohimbine (**b**) of carbachol + clonidine induced ERK1/2 phosphorylation in COS-7 cells co-transfected with the chimeric muscarinic/adrenergic α_2/M_3 and M_3/α_2 receptors (1 μg DNA each).* Confluent monolayers of COS-7 cells were stimulated with a mixture of carbachol (100 μM) and clonidine (100 μM) for 5 min in the presence or absence of atropine (**a**) or yohimbine (**b**) at a concentration of 10 μM. Incubation was terminated, cell lysates prepared and ERK1/2 phosphorylation detected as described in the Methods section. ERK1/2 activation, shown in the upper part of the figure, was measured by anti-phospho ERK1/2 antibody and normalized for total ERK1/2. The blots are representative of four experiments. Bar graphs are the means ± s.e. of all four experiments. *Significantly different from basal (Paired Student's two-tailed *t*-test, $P<0.05$).

Fig. 9 *Co-immunoprecipitation of M_3-short with M_3 (**a**) and M_3/M_2(16aa) (**b**).* 2 μg DNA of HA-tagged M_3-short receptor was transfected either alone or together with 1 μg DNA of M_3 or 1 μg DNA of M_3/M_2(16aa) receptors into COS-7 cells. Complexes were immuno-precipitated (IP) with anti-M_3 antibodies directed against the third cytoplasmic loop and detected (IB) with anti-HA antibodies. Lanes 3 in blots **a** and **b** just show the position of authentic HA-M_3-short. In these lines it is possible to see that most of the HA-M_3-short receptor is in the homodimeric form. Monomers and dimers are represented in the blots respectively by * and **. Blots are representative of four (**a**) and two (**b**) experiments. Receptors were indicated as follow: HA-M_3-short = M_3-S; M_3/M_2(16aa) = M_3/M_2; M_3 wild type = M_3; (**c**) Calculation of the amount of HA-M_3-short and M3 in the cell lysates of transiently co-transfected COS-7 cells, before and after immunoprecipitation with anti-M3 and anti-HA antibodies.

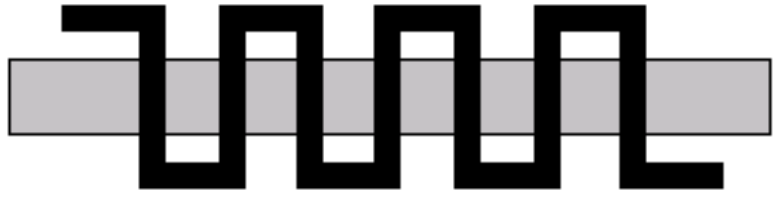
Figure 1



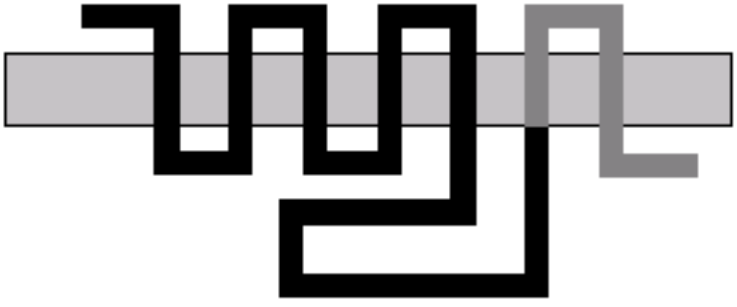
M_3



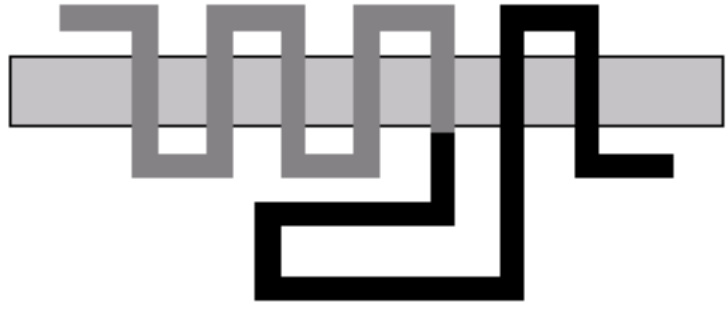
$M_3/M_2(16aa)$



M_3-short



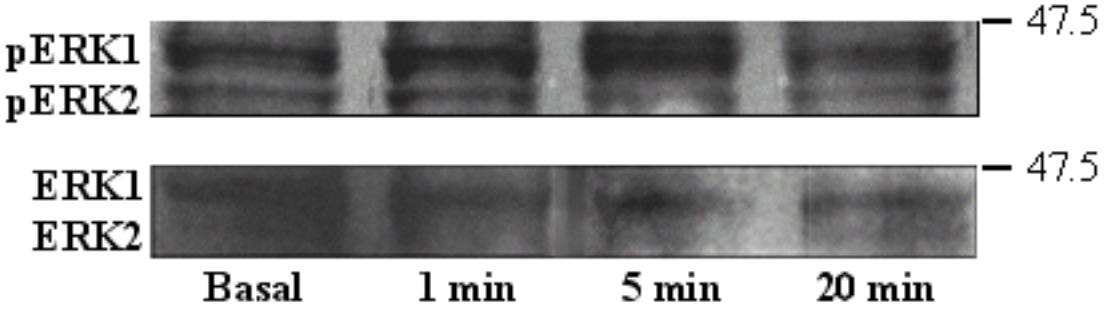
M_3/α_2



α_2/M_3

Figure 2

a



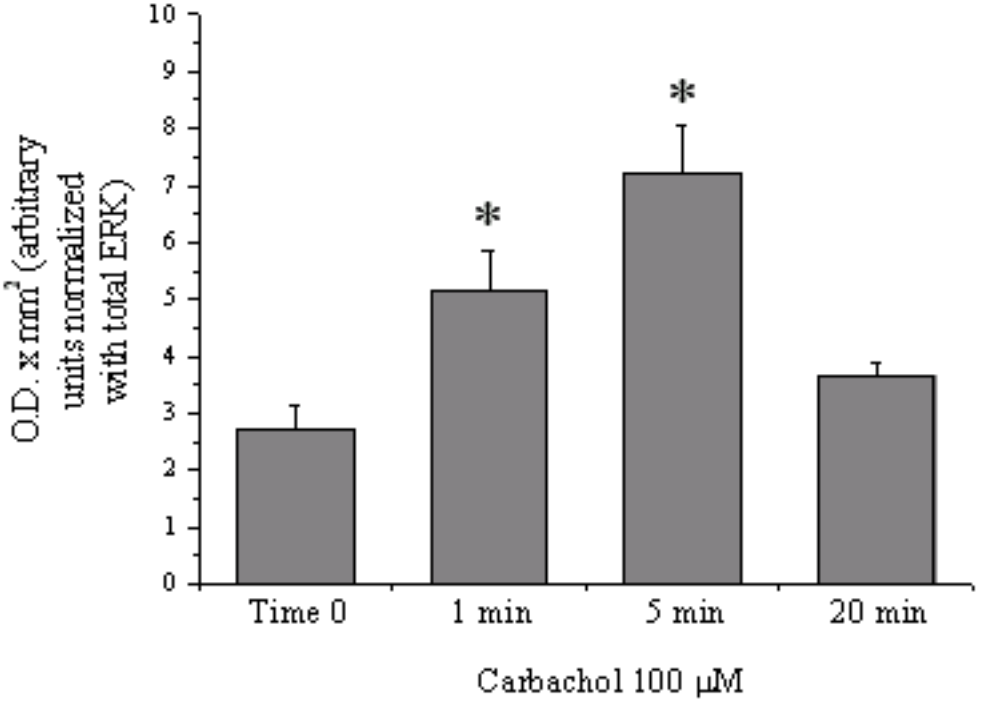
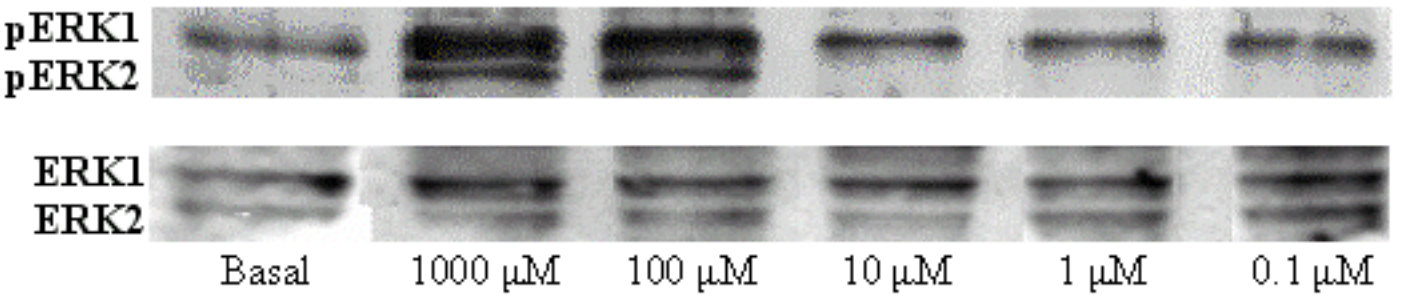


Figure 2

b



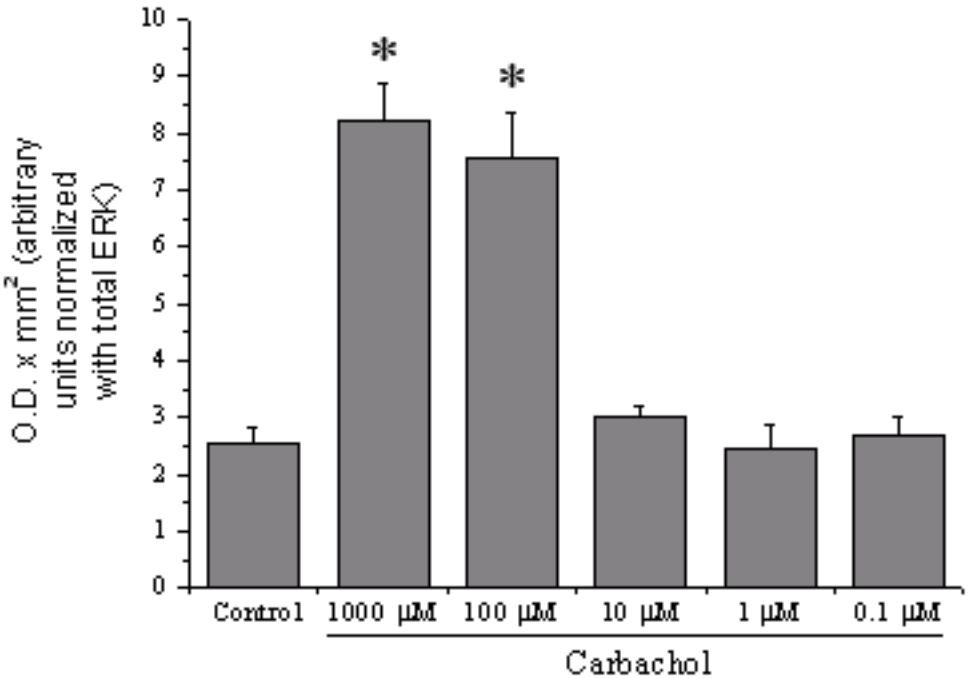
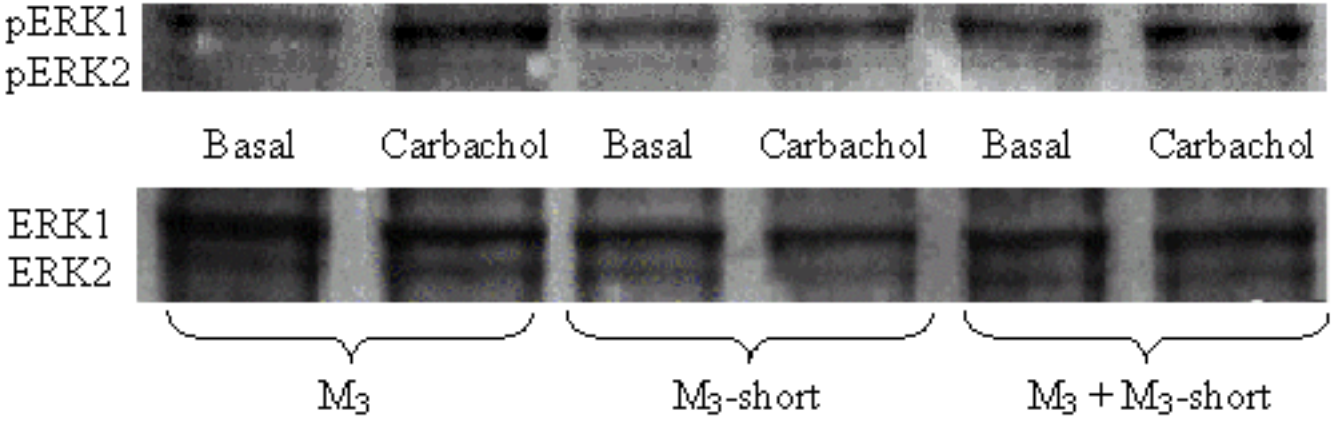


Figure 3

a


pERK1
pERK2
Basal
Carbachol
Basal
Carbachol
Basal
Carbachol
ERK1
ERK2
M3
M3-short
M3 + M3-short

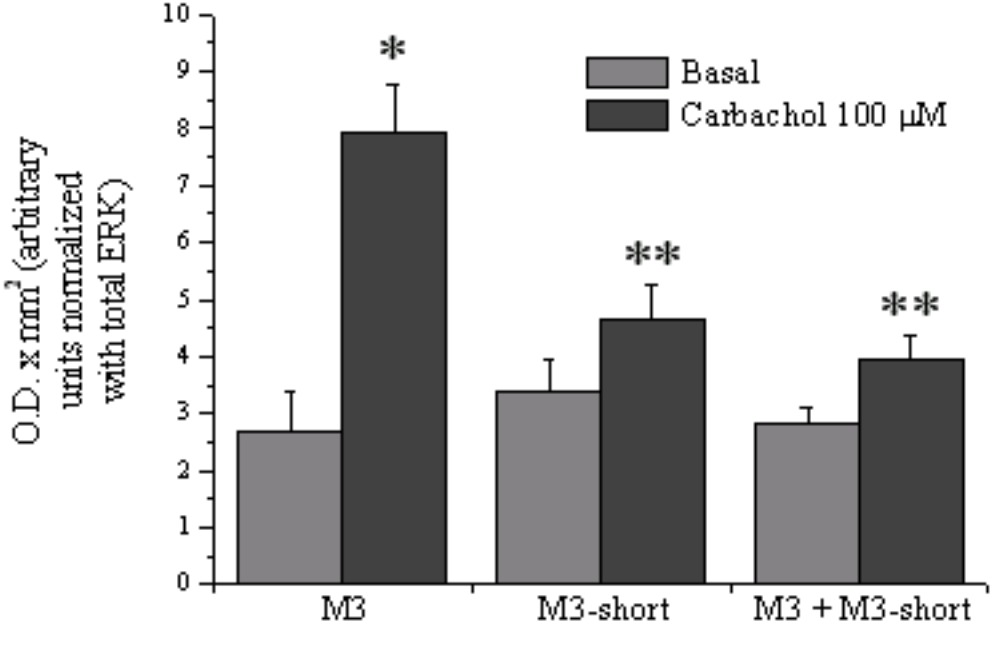

O.D. x mm² (arbitrary units normalized with total ERK)
Basal
Carbachol 100 µM
*
**
**
M3
M3-short
M3 + M3-short

Figure 3

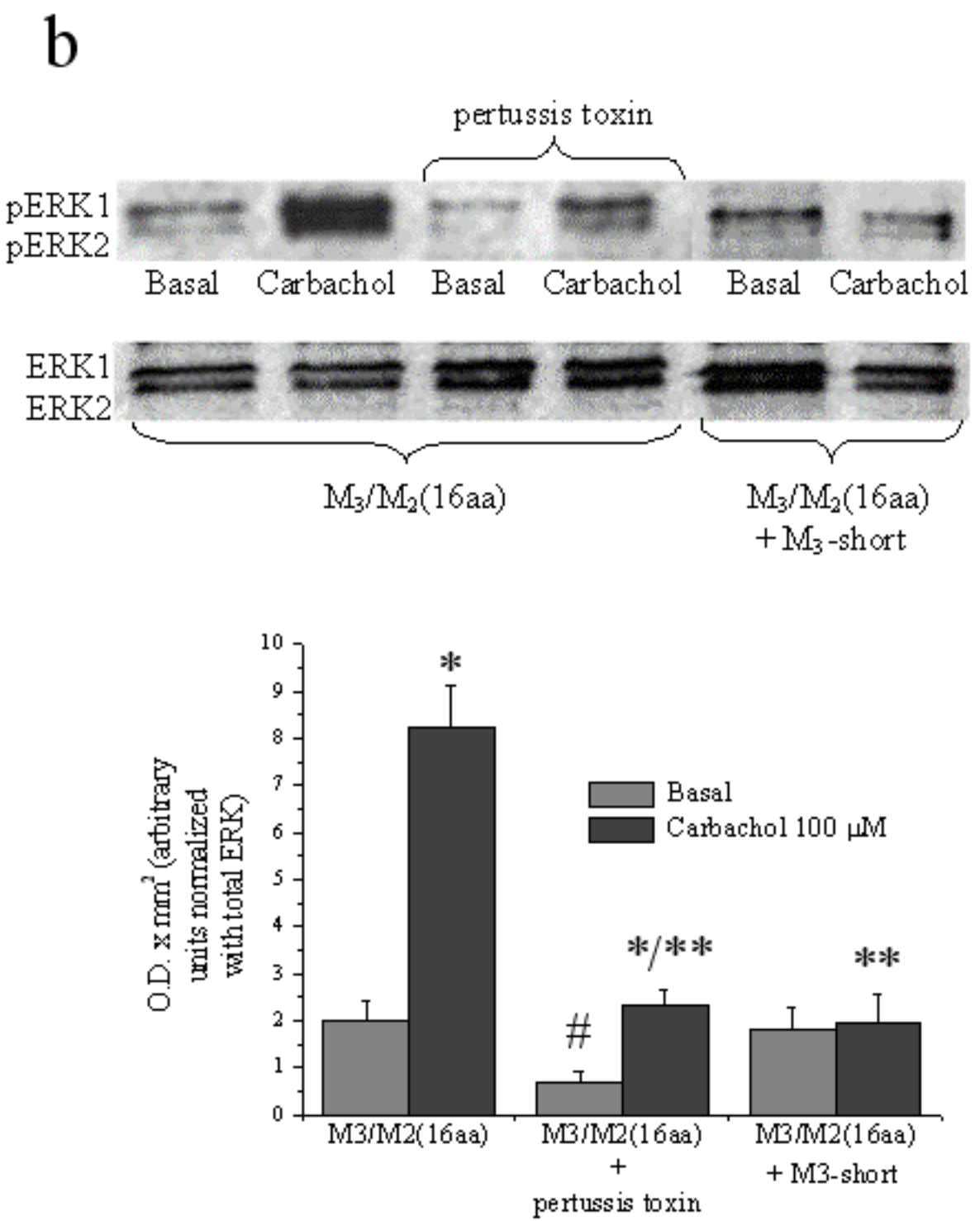
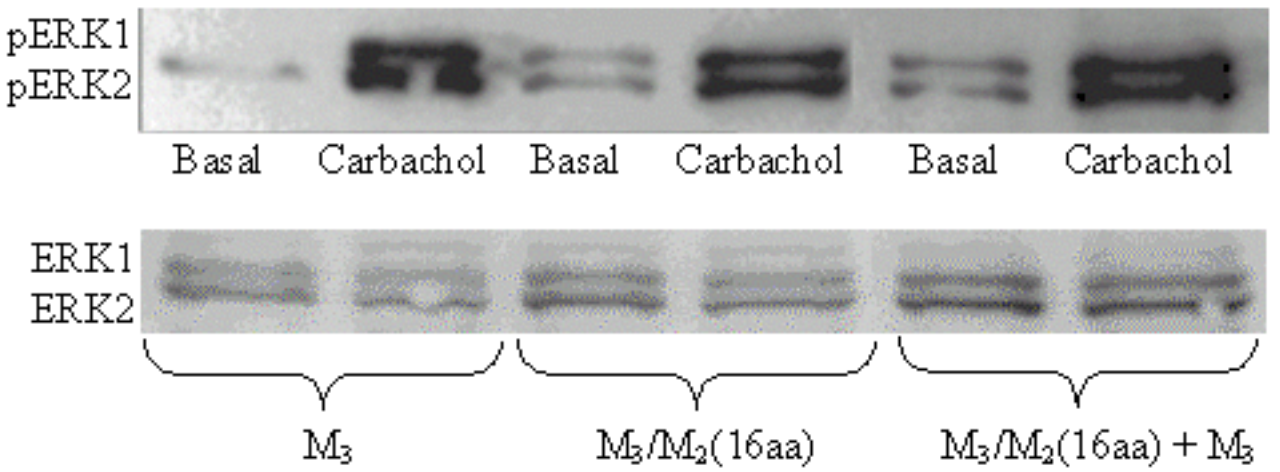


Figure 3

C



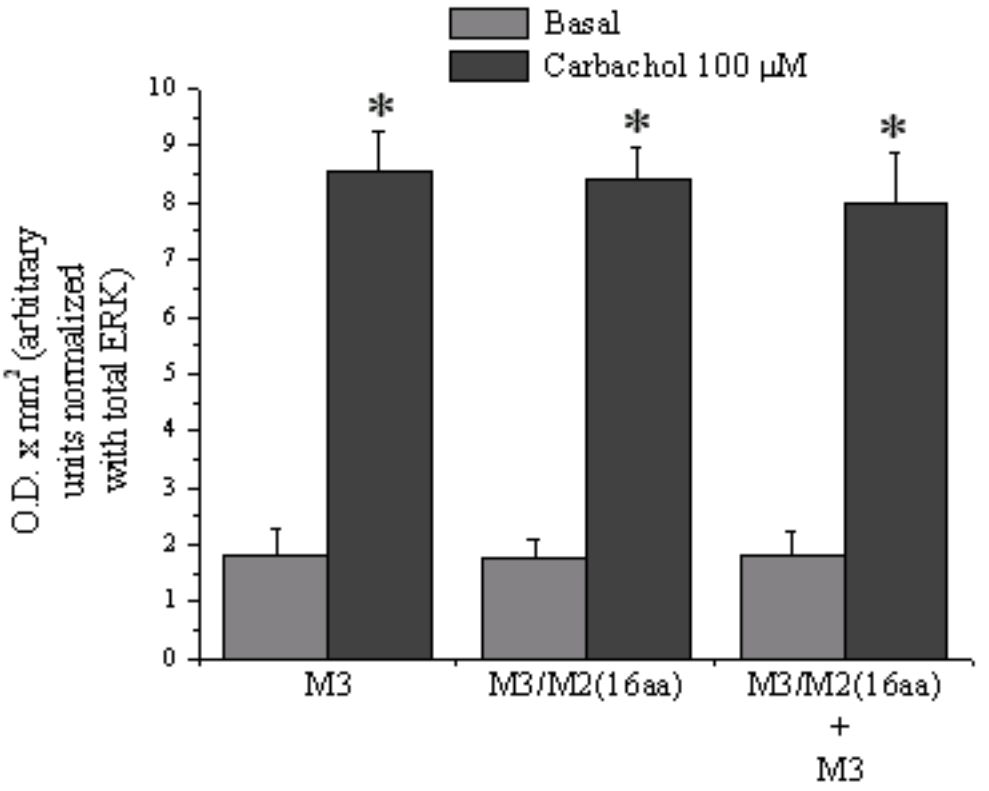
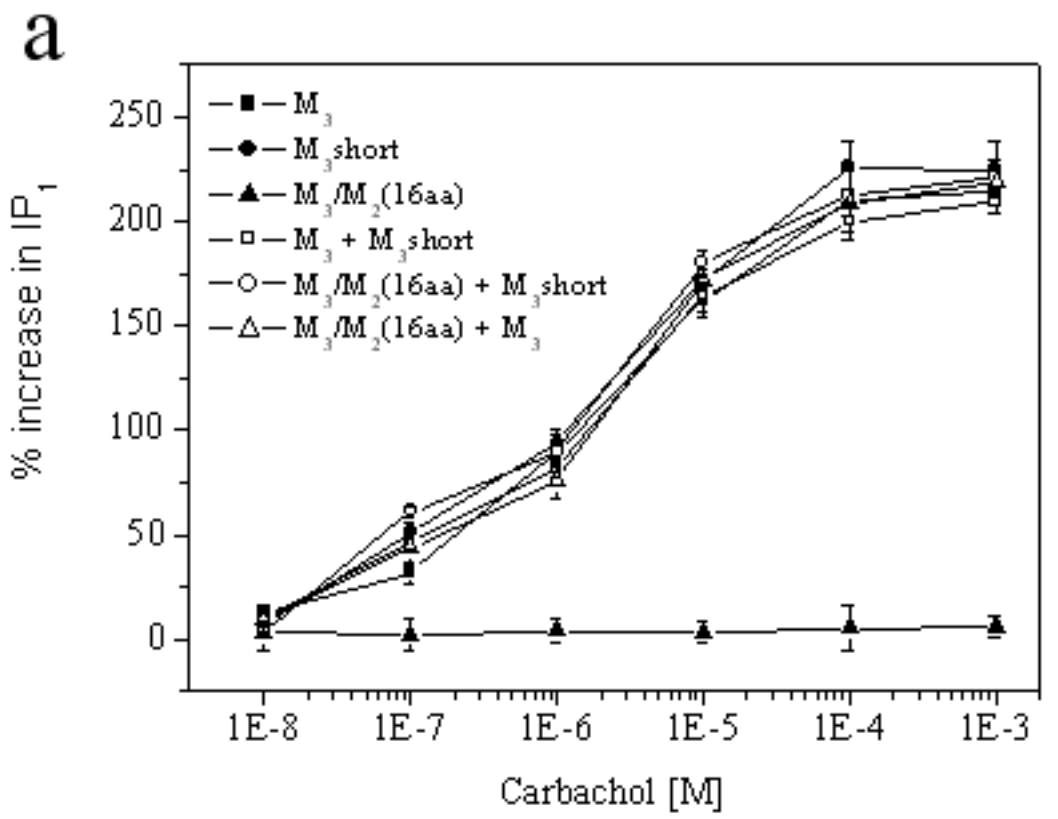


Figure 4



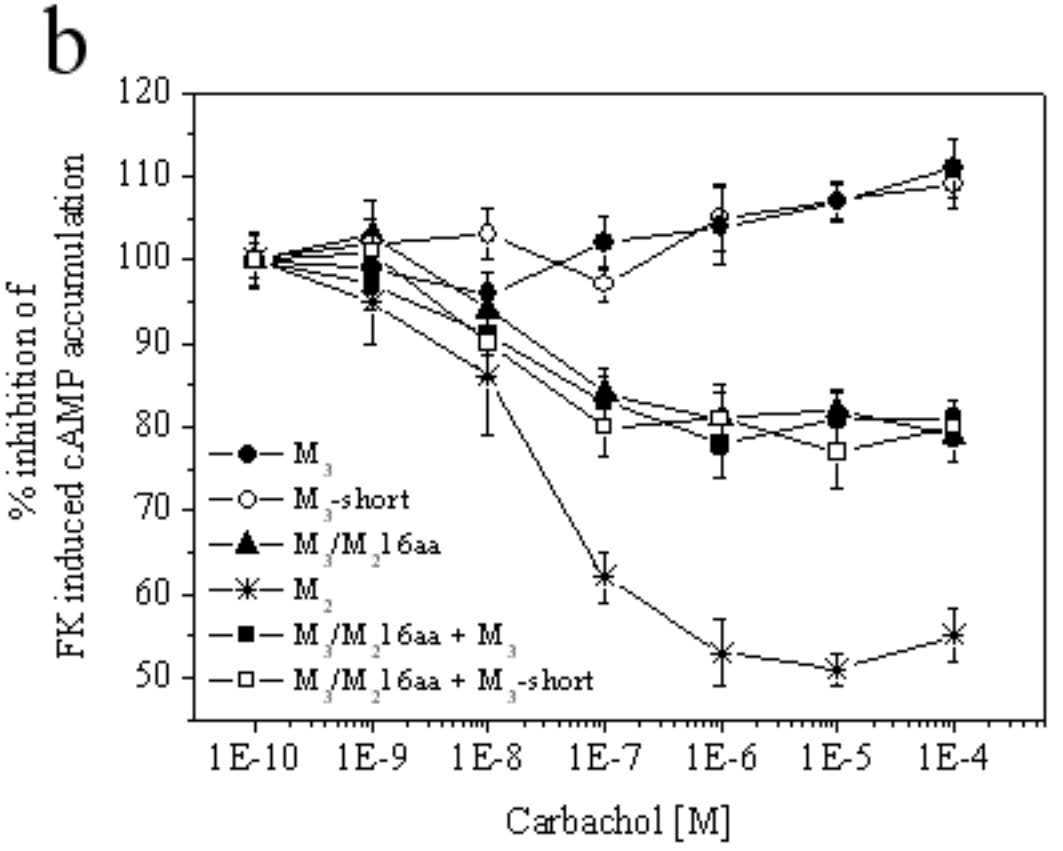
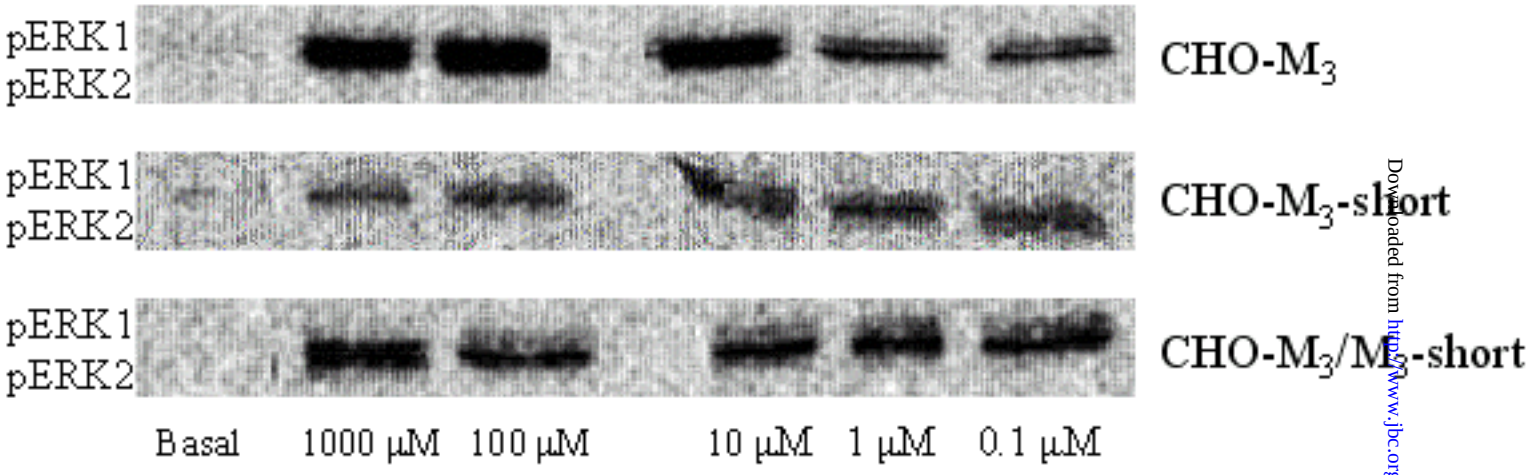


Figure 5



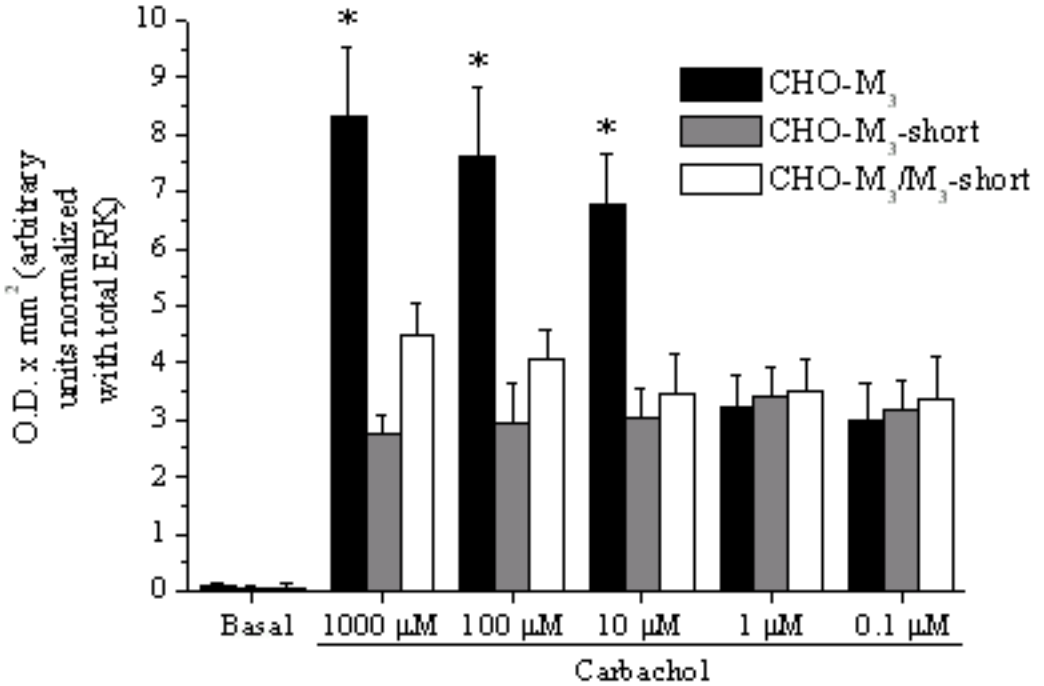


Figure 6

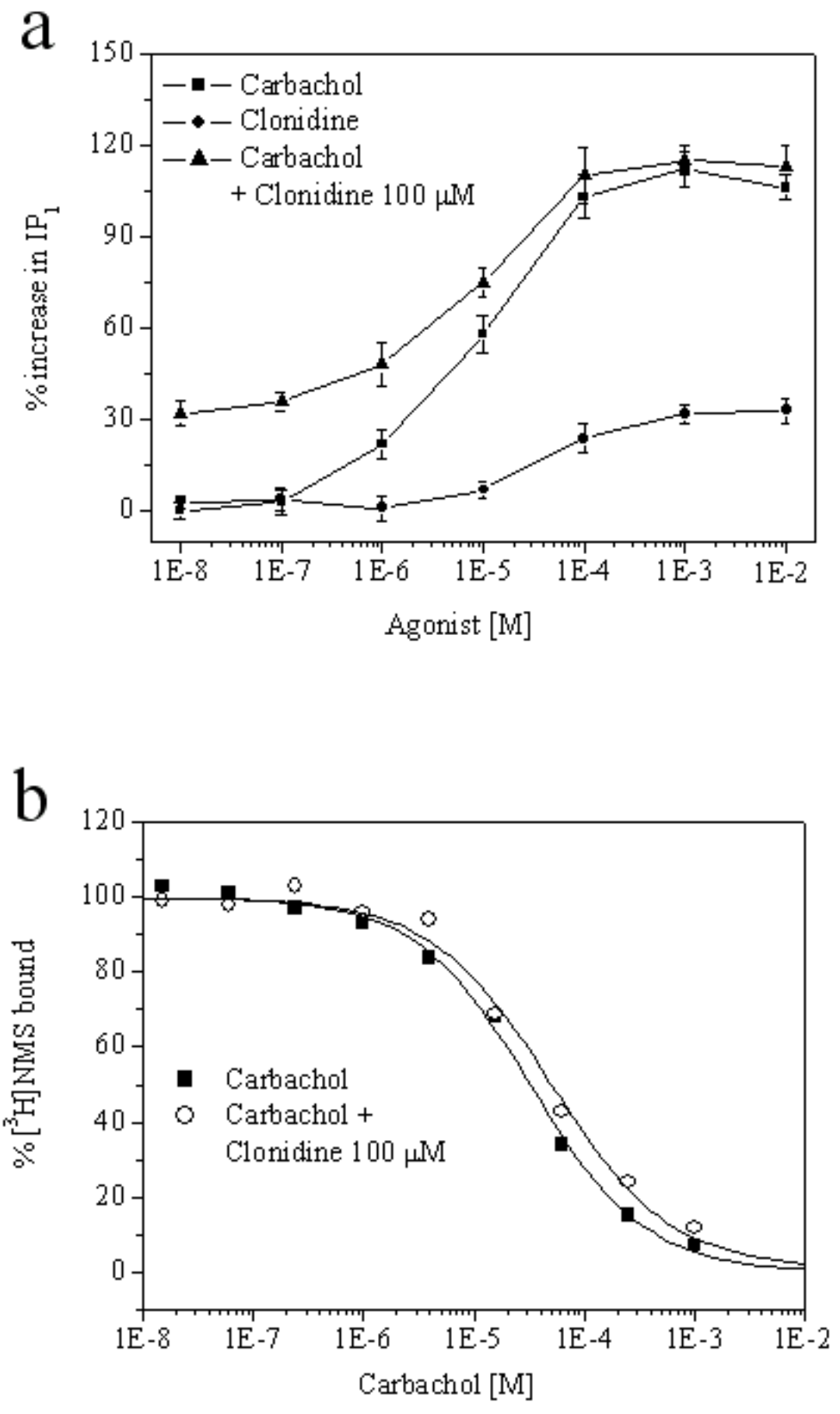
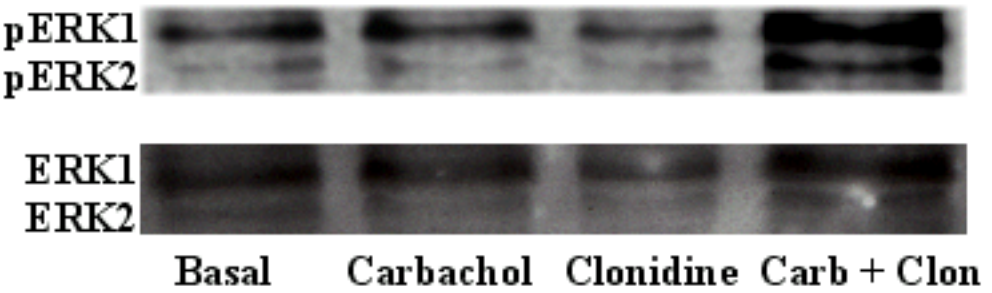


Figure 7



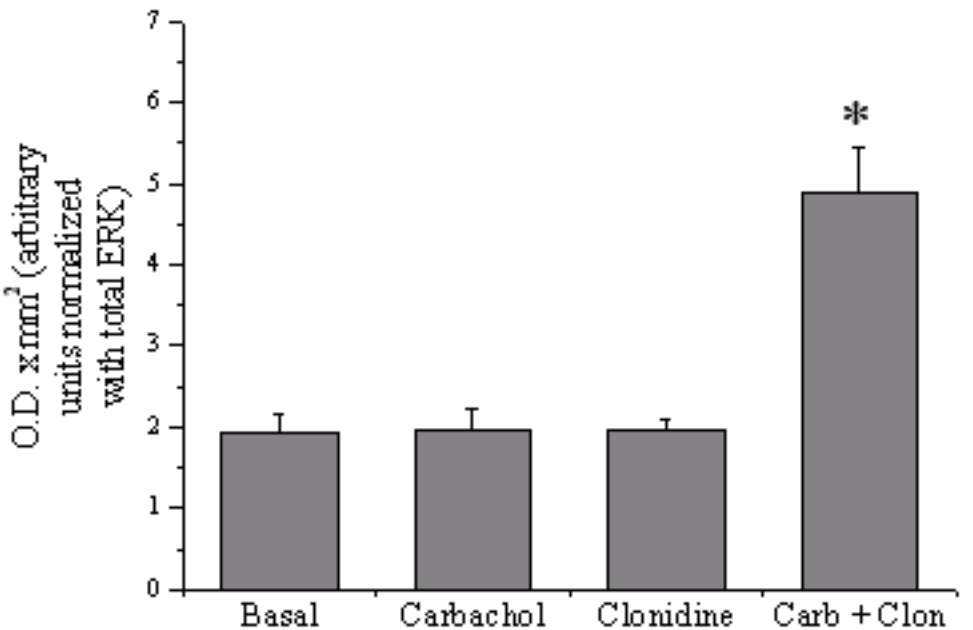
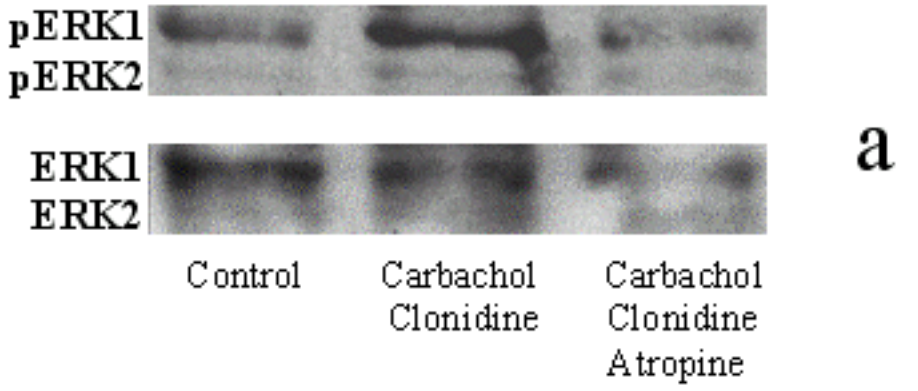
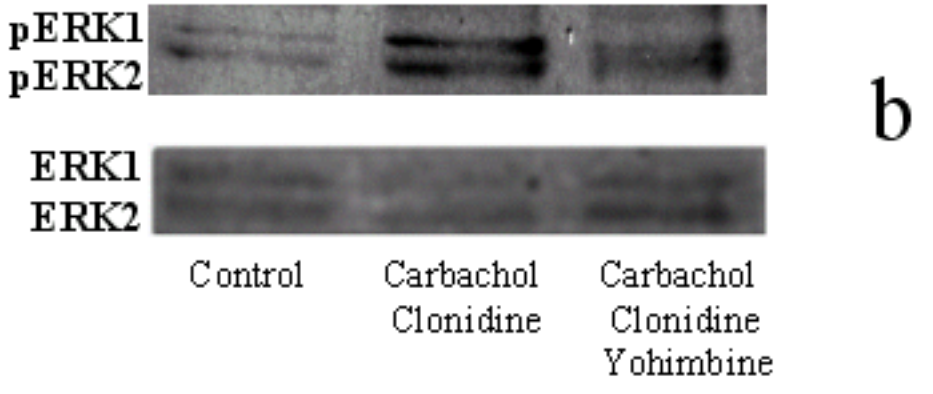


Figure 8





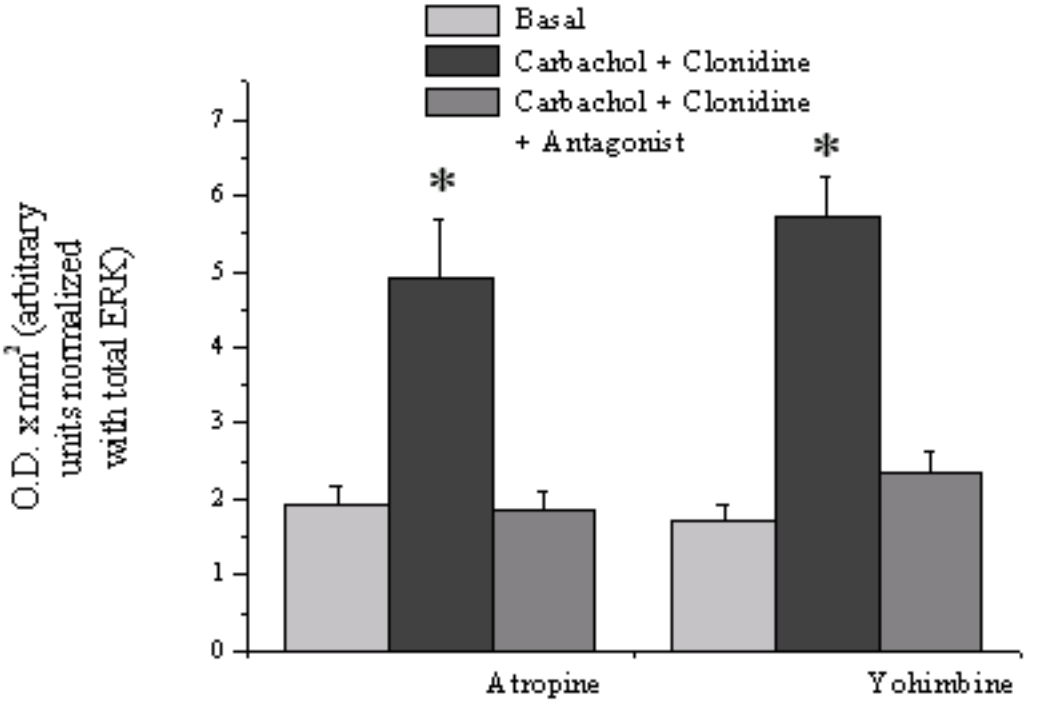
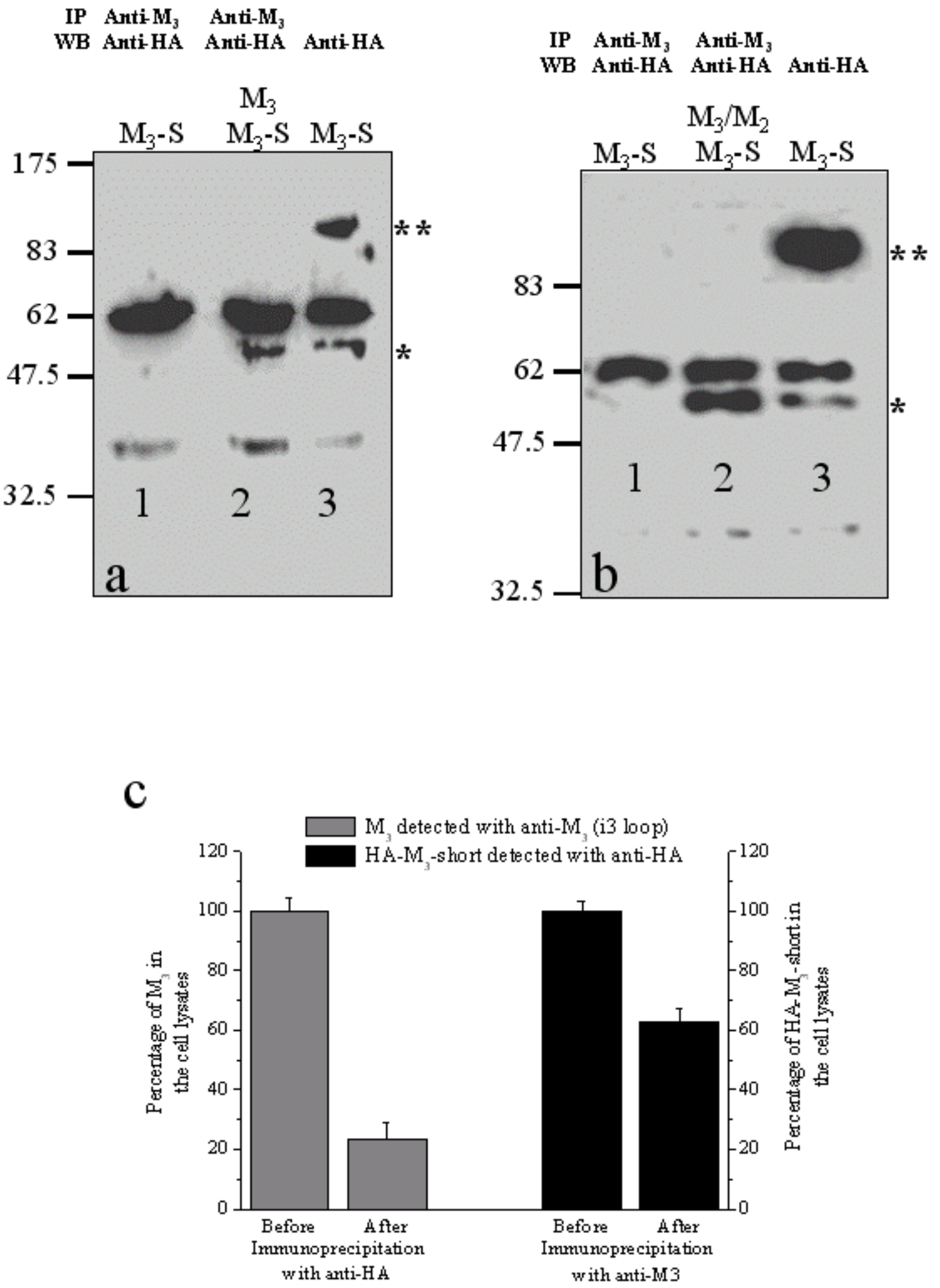


Figure 9



The paired activation of the two components of the muscarinic M3 receptor dimer is required for induction of ERK1/2 phosphorylation

Francesca Novi, Marco Scarselli, Giovanni U. Corsini and Roberto Maggio

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