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Rapid Desensitization of Muscarinic m3 Receptor-Stimulated Polyphosphoinositide Responses

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SUMMARY

Chinese hamster ovary (CHO) cells transfected with human m3 muscarinic receptor cDNA (B_{max}, 1343 ± 46.8 fmol/mg of protein) were used to investigate agonist-mediated muscarinic receptor desensitization. Stimulation of CHO-m3 cells with a maximal dose of carbachol resulted in a biphasic production of mass inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3], measured by radioreceptor binding assay. The first phase comprises a rapid 8–10-fold increase in Ins(1,4,5)P_3 that peaks after 10 sec and falls to levels 3–4-fold over basal within 1 min Ins(1,4,5)P_3 rises again over the next 20 min to approximately 8–10-fold above basal, where levels are sustained for at least 2 hr. This later phase is, therefore, considered to be a desensitization-resistant component of m3 receptor activation. A 5-min pre-exposure of CHO-m3 cells to carbachol resulted in attenuation of the initial peak Ins(1,4,5)P_3 response to a subsequent application of agonist. The attenuation of the Ins(1,4,5)P_3 response was reversible with a $t_{½}$ of ~7.5 min. Desensitization and recovery of the peak Ins(1,4,5)P_3 response correlated with a decrease and subsequent recovery of m3 receptor-mediated mobilization of intracellular calcium stores, suggesting that the consequence of peak Ins(1,4,5)P_3 desensitization is a reduced calcium mobilization response. *N*-[^{3}H]Methylscopolamine binding to intact cells revealed that there was no change in cell surface m3 receptors during the 5-min pre-exposure to agonist, indicating that the mechanism of muscarinic receptor desensitization described here is not sequestration or internalization of receptors.

Agonist-induced receptor desensitization appears to be a fundamental process by which receptor responsiveness at the plasma membrane can be regulated. A generalized mechanism, based on studies of the β-adrenoceptor (1, 2), proposes that homologous desensitization of G protein-linked receptors involves three distinct sequential steps, (i) rapid (minutes) uncoupling of the receptor from its effector enzyme, (ii) sequestration of the receptor from the plasma membrane, and (iii) receptor internalization and proteolytic degradation. The mechanism underlying desensitization of receptors coupled to the hydrolysis of phosphoinositides (PIC-linked receptors) probably cannot be described in such generalized terms. There is, for example, considerable variation in the susceptibility of PIC-linked receptors to desensitization. Muscarinic receptors coupled to phosphoinositide hydrolysis appear to be relatively insensitive to desensitization, because extended periods of agonist pre-exposure are generally necessary before diminished receptor responsiveness is observed (3–5). This is in marked contrast to the phosphoinositide response mediated by substance P receptors in parotid acinar cells (6) and histamine H_1 receptors in human astrocytoma cells (7), which appear to desensitize within minutes of agonist exposure. Similarly, a 10-min pre-exposure of rabbit platelets to platelet-activating factor almost completely abolishes the ability of a subsequent dose of platelet-activating factor to elevate [^{3}H]Ins(1,4,5)P_3 (8).

There is, however, some inconsistency between reports on muscarinic PIC-linked receptor susceptibility to desensitization. A ~50% reduction in muscarinic receptor-mediated inositol phosphate accumulation is observed after 1 hr of agonist pre-exposure in cultured cerebellar granule cells (9) and in the human neuroblastoma cell line SK-N-SH (10). Shorter pre-exposure times ranging from 5 to 40 min have been reported to result in a 50% loss of muscarinic receptor responsiveness in astrocytes (11), primary corticostriatal neurons (12), and the rat pancreatoma cell line AR4-2J (13). At the other end of the spectrum, Hu *et al.* (5), working on CHO cells transfected with m1 and m3 receptor cDNA, and Masters *et al.* (4), studying the astrocytoma cell line 1321N1, which expresses predominantly m3 receptors, reported a significant loss of muscarinic receptor phosphoinositide response only after several hours of pre-exposure to agonist.

The source of such wide variation is likely, in part, to be due

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; NMS, *N*-methylscopolamine; Ins(1,4,5)P_3, inositol-1,4,5-trisphosphate; CHO, Chinese hamster ovary; PIC, phosphoinositidase C; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PKC, protein kinase C; $[Ca^{2+}]_i$, intracellular calcium concentration; PIP_2, phosphoinositide-4,5-bisphosphate; AM, acetoxymethyl ester.

to heterogeneity of muscarinic receptor populations found in various tissues and cultured cells. There are five cloned cholinergic muscarinic receptor subtypes; three (m1, m3, and m5) are efficiently coupled to phosphoinositide hydrolysis, via a pertussis toxin-insensitive G protein (possibly G_q protein) (14). Stimulation of these receptors results in the production of the second messengers Ins(1,4,5)P_3 and diacylglycerol, which mediate mobilization of intracellular calcium stores and activation of PKC, respectively (15–17). m2 and m4 muscarinic receptor subtypes are primarily coupled to the inhibition of adenylate cyclase via G_i protein (14), although there is some evidence that m2 receptors expressed at high enough levels can activate PIC via a pertussis toxin-sensitive G protein (18). It is also possible that by decreasing intracellular cAMP m2 and m4 receptors could indirectly affect the responses of muscarinic receptors linked to phosphoinositide hydrolysis, by "cross-talk" between receptor subtypes. Therefore, the muscarinic receptor subtypes present in a particular cell type or tissue may contribute directly (via m1, m3, or m5) or possibly indirectly (via m2 or m4) to the inositol phosphate response observed upon agonist application. If individual muscarinic receptor subtypes have different susceptibilities to desensitization then the overall desensitization response in a tissue expressing more than one muscarinic receptor will depend on the proportion of receptor subtypes present and their relative susceptibilities to desensitization. To avoid the complexities that arise when the characteristics of an individual receptor subtype are investigated in preparations that contain a mixture of muscarinic receptors, this study makes use of a CHO cell line that has been stably transfected with recombinant human m3 receptor cDNA (CHO-m3) and, therefore, contains an entirely homogeneous population of m3 muscarinic receptors (19).

Previous studies point to the mechanism for diminished muscarinic receptor responsiveness being a loss of receptor number at the plasma membrane (3, 9–11). The sequence of events is thought to be similar to that of the β-adrenoceptor, in that agonist occupation of PIC-linked muscarinic receptors results in a sequestration of the receptors from the plasma membrane followed, in the longer term, by receptor degradation (20). However, an uncoupling of the receptor from PIC that occurs independently of a loss in cell surface receptors, in a way analogous to the very rapid desensitization of the β-adrenoceptor, has not so far been reported for muscarinic receptors linked to phosphoinositide hydrolysis. Because previous studies have generally analyzed the accumulation of [^{3}H] inositol phosphates trapped by lithium inhibition of inositol monophosphatase, it is possible that a rapid desensitization event that occurs exclusively within the first few seconds of receptor activation could have gone undetected (see Discussion). To ensure that such an event is not overlooked here we assayed the early elevation of mass Ins(1,4,5)P_3 as an indicator of m3 receptor activation and we show that the peak Ins(1,4,5)P_3 response seen after 10 sec is susceptible to rapid desensitization and that this process is not associated with a loss of cell surface receptors.

Experimental Procedures

Materials and chemicals. α-Minimum essential medium, newborn calf serum, penicillin, streptomycin, fungizone, and tissue culture flasks were obtained from GIBCO. Fura-2/AM was from Calbiochem (La Jolla, CA). Atropine, carbachol, and tri-*n*-octylamine were from Sigma Chemical Co. D-[^{3}H]Ins(1,4,5)P_3 (17 Ci/mmol) was a gift from NEN DuPont. Freon (1,1,2-trichloro-1,2,2-trifluoroethane) was from Aldrich Chemicals. [^{3}H]NMS (85 Ci/mmol) and D-Ins(1,4,5)P_3 was obtained from Amersham International UK.

Cell culture. CHO-m3 stock cultures (a kind gift from Dr. N. J. Buckley, National Institute for Medical Research, London, UK) were routinely maintained in α-minimum essential medium supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml), and newborn calf serum (10%, v/v). Cultures were seeded into 24-well dishes containing 0.5 ml of supplemented medium/well and were allowed to reach confluence at 37° in 5% CO_2/95% humidified air. Cultures used for analysis of free intracellular calcium were seeded onto glass coverslips and maintained as described above.

Measurement of D-Ins(1,4,5)P_3 mass. Intact CHO-m3 cells grown in 24-well dishes were washed in 250 μl of Krebs bicarbonate buffer (composition, in mM: NaCl, 118; KCl, 4.3; $MgSO_4 \cdot 7H_2O$, 1.17; KH_2PO_4, 1.17; $CaCl_2 \cdot 2H_2O$, 1.30; $NaHCO_3$, 25; glucose, 11.7) and allowed to stabilize for 10 min at 37° in 5% CO_2/95% humidified air. The buffer was then replaced with 100 μl of Krebs bicarbonate buffer with or without carbachol (1 mM) for the indicated time periods. The reaction was stopped with an equal volume of trichloroacetic acid (0.5 M). Ins(1,4,5)P_3 was extracted from the supernatant with Freon/tri-*n*-octylamine (1:1, v/v) and neutralized with $NaHCO_3$ (25 mM). Ins(1,4,5)P_3 concentration was then determined by a radioreceptor assay described previously (21). In desensitization protocols cells were challenged with a desensitizing concentration of carbachol (1 mM) for 5 min at 37°, after which they were rapidly washed (three times) in warm buffer and incubated in carbachol-free buffer for the desired time period (i.e., 2–15 min). The buffer was then replaced with buffer with or without carbachol (1 mM), and the stimulation of Ins(1,4,5)P_3 production was analyzed as described above.

Measurement of $[Ca^{2+}]_i$. Intracellular calcium measurements in small groups of cells (four to six cells) were made by standard epifluoresence microscopy. Monolayers of CHO-m3 cells grown on glass coverslips were incubated with 4 μM fura-2/AM for 30 min in supplemented tissue culture medium. The loaded cells were then postincubated at room temperature for an additional 30 min in Krebs/50 mM HEPES (pH 7.4) buffer, to enable ester hydrolysis.

$[Ca^{2+}]_i$ was measured using a Photon Technology International Data scan system set at 340 and 380 nm. Cells were constantly perfused at ~0.6 ml/min and at 37°. All drugs were added via local flow pipette. Raw 340/380 traces, from which autofluoresence (measured with unloaded cells) had been subtracted, are presented throughout. Typically, loaded cells gave a fluorescence signal approximately double the background.

Radioligand binding experiments. Intact CHO-m3 cells grown in 24-well dishes were washed with Krebs bicarbonate buffer and allowed to stabilize for 10 min at 37°. The buffer was replaced with 250 μl of buffer containing carbachol (1 mM) and the incubation was continued at 37° for the appropriate time (0–120 min) in 5% CO_2/95% humidified air. The cells were washed (three times) in ice-cold Krebs bicarbonate buffer containing 50 mM HEPES (pH 7.4). [^{3}H]NMS binding was performed in a total volume of 1 ml at 4° for 16–20 hr, by which time equilibrium had been reached. Bound and free ligand were separated by rapid washing in ice-cold buffer. Cells were dissolved in 0.1 N NaOH and the radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined in the presence of 5 μM atropine. Saturation analysis was performed over a range of [^{3}H]NMS concentrations (1.6–0.001 nM), and total receptor binding was measured at saturating concentrations of [^{3}H]NMS (~2 nM).

Data analysis. Saturation isotherms were analyzed by computer-assisted curve fitting to obtain equilibrium dissociation constants (K_d) and maximum binding capacity (B_{max}). Results are expressed as means ± standard errors of at least three determinations. Where appropriate, statistical significance was assessed by Students *t* test and was consid-

ered significant when p was <0.05. Protein concentrations were assayed by the method of Bradford (22).

Results

Mass Ins(1,4,5)P_3 levels in CHO-m3 cells stimulated by carbachol. The application of a maximal dose of carbachol (1 mM) to intact CHO-m3 cells resulted in a biphasic elevation of mass Ins(1,4,5)P_3 (Fig. 1). Initially, Ins(1,4,5)P_3 levels elevated rapidly upon agonist application, from basal levels of 37.5 ± 12.8 pmol/mg of protein to 403.2 ± 86.5 pmol/mg of protein within 10 sec (Fig. 1). Over the next 50 sec levels of Ins(1,4,5)P_3 fell to 141.4 ± 30.3 pmol/mg of protein, where they were then maintained for at least 5 min. A further rise in Ins(1,4,5)P_3 then followed and constituted the second component of the biphasic response. After 20 min of agonist stimulation Ins(1,4,5)P_3 levels were elevated to a new steady state that persisted in the presence of agonist for 2 hr and hence represents a desensitization-resistant phase of Ins(1,4,5)P_3 production. Dose-response curves conducted at 10 sec and 60 min of carbachol stimulation revealed that the early and late phases of Ins(1,4,5)P_3 production had identical EC_{50} values of 10 μM (data not shown). The muscarinic antagonist atropine (5 μM) totally inhibited the carbachol response (data not shown).

Agonist-mediated desensitization of Ins(1,4,5)P_3 response. A 5-min pre-exposure of CHO-m3 cells to carbachol (1 mM) followed by a 2-min wash period dramatically changed the profile of the Ins(1,4,5)P_3 response to a subsequent application of agonist. There was still a rapid rise in Ins(1,4,5)P_3 but the levels attained after 10 sec were reduced from 8-fold above basal to 4.5-fold above basal (basal levels, 45.3 ± 9.3 pmol/mg of protein) (Fig. 2). In contrast, pre-exposure had no effect on Ins(1,4,5)P_3 levels obtained after extended periods of agonist stimulation (i.e., >5 min; data not shown).

By increasing the wash period after agonist pre-exposure from 2 min to 10 min, the Ins(1,4,5)P_3 response to a second application of carbachol was partially restored (Fig. 3B). The response was, however, totally restored after a 15-min wash

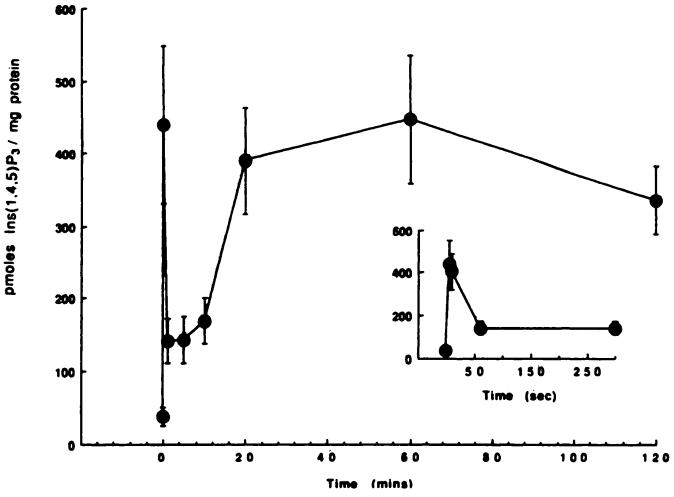

Fig. 1. Mass Ins(1,4,5)P_3 in CHO-m3 cells stimulated with carbachol. CHO-m3 cells were incubated in the presence of carbachol (1 mM) for the indicated time periods. The reaction was stopped using trichloroacetic acid (0.5 M) and the Ins(1,4,5)P_3 was assayed as described in the text. *Inset*, first five time points on a different x-axis scale. Basal levels of Ins(1,4,5)P_3 were 37.5 ± 12.8 pmol/mg of protein. Data represent the mean ± standard error of three experiments.

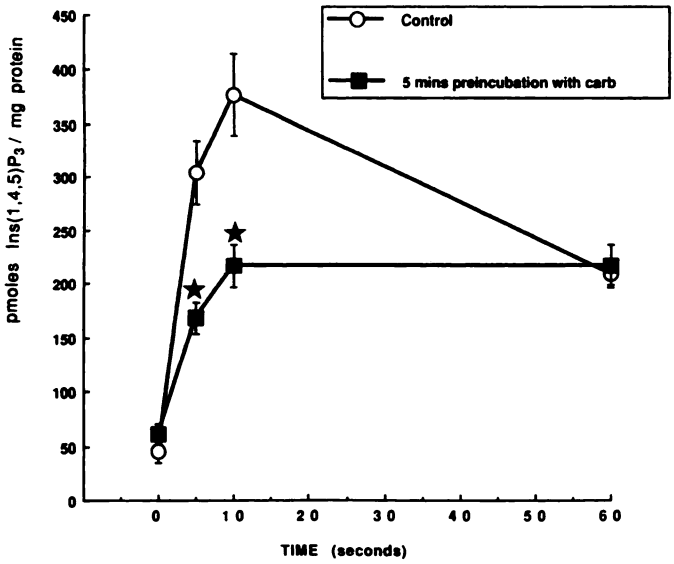

Fig. 2. Desensitization of peak Ins(1,4,5)P_3 elevated levels. CHO-m3 cells were preincubated for 5 min in the presence (■) or absence (○) of carbachol (1 mM), followed by a 2-min wash period. Ins(1,4,5)P_3 levels stimulated by a subsequent application of carbachol (1 mM) were then analyzed. Data represent the mean ± standard error of three experiments. ★, $p < 0.05$, compared with control values.

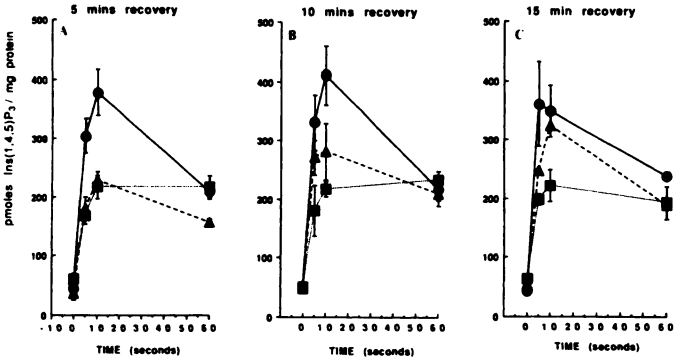

Fig. 3. Recovery of desensitized peak Ins(1,4,5)P_3 response. The wash period between the desensitizing pulse of carbachol and the subsequent stimulatory carbachol application was extended to 5 min (A), 10 min (B), or 15 min (C). ●, = Control values; ■, = desensitized values; ▲, = recovery values. Data represent the mean ± standard error of three experiments.

period, demonstrating that desensitization of the peak Ins(1,4,5)P_3 is reversible with a $t_{1/2}$ of ~7.5 min (Fig. 3).

Desensitization of carbachol-mediated calcium mobilization. Despite attenuation of the peak Ins(1,4,5)P_3 response at 10 sec, there was still a rapid increase in Ins(1,4,5)P_3 production, which may be sufficient to elicit a normal calcium mobilization response in desensitized cells. This was investigated further using fura-2/AM-loaded cells to monitor changes in Ca^{2+}_i by using standard epifluorescence microscopy with groups of 4–6 cells.

Addition of 1 mM carbachol to CHO-m3 cells resulted in a rapid rise in $[Ca^{2+}]_i$, which peaked within seconds of agonist application (Fig. 4). In the continued presence of agonist, $[Ca^{2+}]_i$ fell to basal levels within 5 min in ~70% of experiments (Fig. 4, A and B). In the remaining 30% of cases the peak rise in $[Ca^{2+}]_i$ was followed by a distinct plateau phase that was maintained for the full 5 min of agonist treatment (Fig. 4C).

In each of these experiments the initial 5-min exposure to

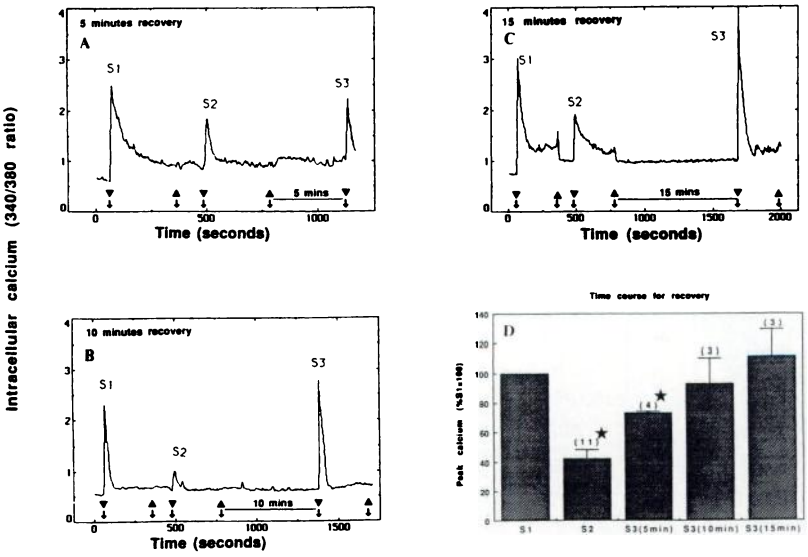

Fig. 4. Desensitization and recovery of intracellular Ca^{2+} mobilization response to 1 mM carbachol. Intracellular calcium mobilization response was measured in four to six CHO-m3 cells. An initial, desensitizing, 5-min application of carbachol (*S1*) was followed by a 2-min wash period and a subsequent 5-min application of carbachol (*S2*). The cells were then allowed to recover for 5 min (A), 10 min (B), or 15 min (C), after which the response to a third application of carbachol was assessed (*S3*). D, Accumulated data from the number of experiments indicated in parentheses. ▼, Carbachol on; ▲, carbachol off. A, B, and C, representative *traces* from at least three experiments. ★, $p < 0.05$, compared with S1.

carbachol served firstly as an internal control for agonist-mediated calcium mobilization and secondly as a desensitizing pulse of agonist. After a 2-min wash period the calcium mobilization of a subsequent 5-min application of 1 mM carbachol was reduced to 42.4 ± 5.8% (mean ± standard error, 11 experiments) of the initial (S1) response (Fig. 4). The desensitized calcium mobilization response was not restored by an additional 5-min wash period (Fig. 4A) but was completely restored by extending the wash period to 10 and 15 min (Fig. 4, B-D). Hence, the recovery of the desensitized calcium mobilization response more or less mirrors the recovery of the desensitized peak Ins(1,4,5)P_3 response (Figs. 3 and 4).

It is possible that the desensitization of agonist-mediated calcium mobilization is due to there being insufficient time to refill internal calcium stores between agonist applications (i.e., 2 min). This question was addressed by depleting intracellular calcium stores with a 5-min application of carbachol (1 mM) to CHO-m3 cells in a nominally calcium-free buffer. After a 15-min wash period in calcium-free buffer (sufficient time for "resensitization"), an additional application of carbachol elicited a dramatically reduced calcium mobilization response (Fig. 5A). This would be predicted if the internal calcium stores are empty and unable to be replenished (23). However, if during the 15-min wash period a 2-min pulse of calcium-containing buffer was applied (to refill internal calcium stores) then the calcium mobilization response to a subsequent application of carbachol was restored (Fig. 5B). This demonstrates, therefore, that the 2-min wash period used in the desensitization experiments (Fig. 4) was sufficient for intracellular stores of calcium to be refilled.

[^{3}H]NMS binding to intact desensitized CHO-m3 cells. Saturating concentrations (~2 nM) of the hydrophilic muscarinic antagonist [^{3}H]NMS were used to determine changes in cell surface m3 receptor number (24). Pre-exposure of intact CHO-m3 cells to carbachol (1 mM) for 5 or 10 min had no effect on [^{3}H]NMS binding carried out at 4° (Fig. 6). In contrast, a 15-min agonist preincubation did result in a small but significant 10% decrease in [^{3}H]NMS binding. For each of the aforementioned time points a 2-hr pre-exposure to agonist was run in parallel as a positive control for receptor down-regulation (3). Under these latter conditions there was no change in the

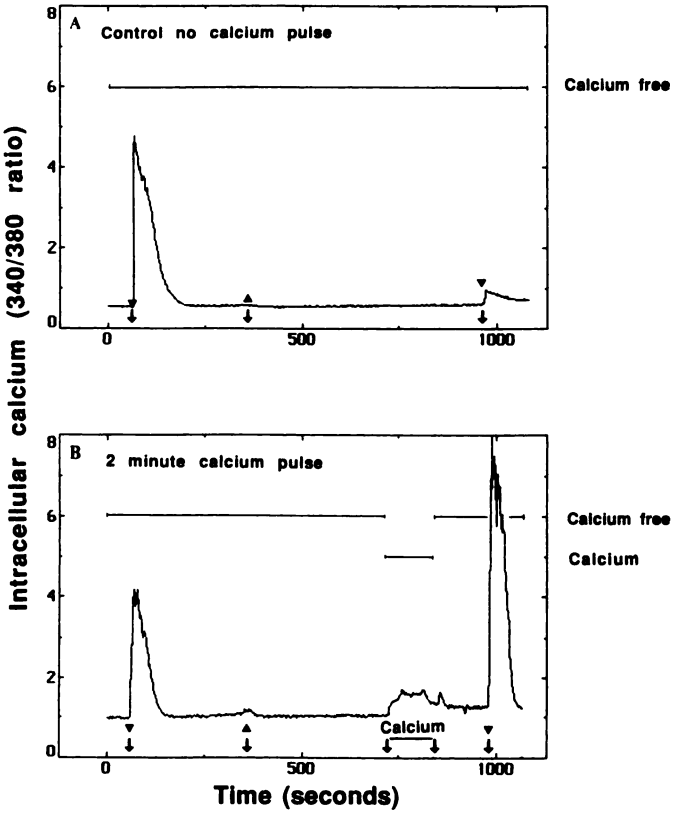

Fig. 5. Refilling of intracellular Ca^{2+} stores after m3 receptor activation. A, Five-minute stimulation with carbachol (1 mM) was followed by a 15-min wash period and a second carbachol challenge. The entire experiment was carried out in calcium-free buffer. B, The experiment was carried out in exactly the same way as in A except that a 2-min pulse of calcium-containing buffer was applied during the 15-min wash period. ▼, Carbachol on; ▲, carbachol off. The *traces* are representative of at least three experiments.

K_d of [^{3}H]NMS binding but a 38 ± 7.5% decrease in the B_{max} was observed, a result consistent with previous studies (3).

Discussion

The primary finding reported here is that the early phase of muscarinic m3 receptor-mediated phosphoinositide hydrolysis

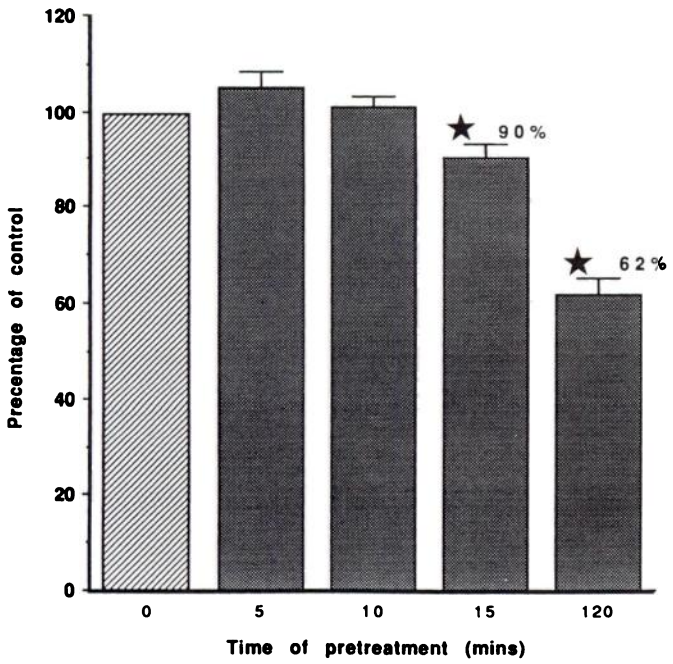

Fig. 6. [^{3}H]NMS binding to intact CHO-m3 cells after carbachol pretreatment. CHO-m3 cells were preincubated with carbachol (1 mM) at 37° for the indicated times. Cells were then immediately washed in ice-cold buffer and [^{3}H]NMS binding was performed at 4° for 16–20 hr. Data represent the mean ± standard error from three experiments. ★, $p < 0.05$, compared with the zero time point.

is susceptible to desensitization in CHO cells transfected with recombinant human m3 receptor cDNA. Stimulation of CHO-m3 cells with a maximal dose of muscarinic agonist results in a biphasic elevation of Ins(1,4,5)P_3. The first phase of this response comprises a rapid increase in mass Ins(1,4,5)P_3 that peaks within 10 sec of agonist application and then falls to levels 3–4-fold over basal. This profile is similar to that described previously for muscarinic receptor activation in the human neuroblastoma cell line SH-SY5Y (25–27) and for bombesin and cholecystokinin stimulation of the rat pancreatoma cell line AR4-2J (13). The initial peak of Ins(1,4,5)P_3 correlates, in this study, with a rapid rise in $[Ca^{2+}]_i$, a consequence presumably of Ins(1,4,5)P_3 acting at intracellular receptors to release vesicular Ins(1,4,5)P_3-sensitive calcium stores (16). Support for this action has been obtained with permeabilized CHO-m3 cells, which can release calcium from intracellular stores in response to Ins(1,4,5)P_3 or muscarinic agonists.[1]

In the majority (~70%) of our experiments peak $[Ca^{2+}]_i$ falls back to basal within a few minutes of agonist application despite elevated levels of Ins(1,4,5)P_3 (see Fig. 4). The apparent absence of a sustained elevation of $[Ca^{2+}]_i$ is in contrast to previous studies on muscarinic receptor stimulation in SH-SY5Y cells (26, 27) and in fibroblasts transfected with m3 receptor cDNA (28), which report a persistent rise in $[Ca^{2+}]_i$ attributable to an entry of extracellular calcium. Calcium entry may, however, still occur in CHO-m3 cells, not least because in 30% of experiments a persistent elevation of $[Ca^{2+}]_i$ is observed. Whether this represents entry of extracellular calcium has yet to be fully established, although stimulation of CHO-m3 cells in calcium-free medium results, in all cases, in a peak elevation of $[Ca^{2+}]_i$ but no sustained phase of elevated $[Ca^{2+}]_i$ (see Fig. 5). Furthermore, the second phase of the Ins(1,4,5)P_3 response, in which levels of Ins(1,4,5)P_3 rise to a new steady state level after 20 min of agonist exposure, is dependent on extracellular calcium.[2] This indicates that m3 receptors in CHO-m3 cells may mediate calcium entry, which in the continued presence of agonist maintains a long term activation of PIC in a way similar to that reported for SH-SY5Y cells (26). The possibility that calcium entry in the majority of our experiments is masked by calcium efflux is presently under investigation.

The second phase of Ins(1,4,5)P_3 response is a feature of m3 receptor activation that sets the process of second messenger generation by this receptor apart from that of the better understood β-adrenoceptor. Activation of β-adrenoceptors results in the rapid synthesis of cAMP, which is attenuated by homologous receptor desensitization within minutes of agonist application (29). In comparison, m3 receptor activation mediates a rapid peak of Ins(1,4,5)P_3 that is followed by a second, desensitization-resistant, phase of Ins(1,4,5)P_3 production that is maintained for at least 2 hr. The rapid rise and fall in Ins(1,4,5)P_3 levels may represent an uncoupling of receptor from PIC that is later overcome, to generate the second phase of Ins(1,4,5)P_3 production. Alternatively, the later response may reflect the ability of extracellular calcium to maintain PIC activity despite loss of coupling and indeed a loss of muscarinic receptors (see Fig. 6). Whatever the mechanism, the present data clearly reveal the susceptibility of the initial Ins(1,4,5)P_3 response to desensitization, by its loss after a brief 5-min pre-exposure to agonist.

Based on recent findings that Ins(1,4,5)P_3-mediated release of intracellular calcium pools is quantal in nature (30, 31), it might be anticipated that the physiological consequence of attenuated peak Ins(1,4,5)P_3 production would be a reduced efficacy of agonist-induced mobilization of intracellular calcium stores. This prediction is upheld in this study, where a 5-min pre-exposure to carbachol diminishes the $[Ca^{2+}]_i$ response to a subsequent application of agonist (see Fig. 4). Furthermore, the time course for recovery of the desensitized peak Ins(1,4,5)P_3 response ($t_{1/2}$ ~7.5 min) is very similar to that for the recovery of desensitized $[Ca^{2+}]_i$ response. These data strongly suggest that desensitization of the calcium mobilization response in CHO-m3 cells pre-exposed to carbachol is due to diminished peak Ins(1,4,5)P_3 production.

Previous studies have reported that desensitization of muscarinic receptor-mediated calcium mobilization in the astrocytoma cell line 1321N1 is due to depletion of intracellular stores of calcium (32). The possibility that this may account for the diminished calcium response in the present study was addressed by investigating refilling of calcium stores. It was found that, after agonist stimulation, a time of 2 min was sufficient to allow muscarinic receptor-sensitive intracellular calcium stores to be replenished. Because a 2-min wash period was used between the desensitizing agonist application (S1) and the stimulatory agonist application (S2), the diminished calcium response seen in S2 cannot be due to depleted calcium stores but is consistent with the reduced production of Ins(1,4,5)P_3.

Measurement of mass Ins(1,4,5)P_3 levels have allowed us to establish the early peak Ins(1,4,5)P_3 production as the component of the muscarinic receptor inositol phosphate response

[1] R. J. H. Wojcikewicz and S. R. Nahorski, unpublished observations.

[2] A. B. Tobin and S. R. Nahorski, Phosphoinositide responses following sustained activation of m^3-muscarinic receptors. Manuscript in preparation.

that is susceptible to desensitization in CHO-m3 cells. If this phenomenon is generally applicable to PIC-linked muscarinic receptors in other cell types then it might explain data from previous studies in SK-N-SH cells (3), 1321N1 astrocytoma cells (4), and transfected CHO cells (5), which report that diminished muscarinic receptor responsiveness requires extended (tens of minutes to hours) periods of agonist pre-exposure. These studies have monitored the accumulation of inositol phosphates in the presence of lithium as a measure of muscarinic receptor activation of PIC. Because labeled inositol phosphates are generally allowed to accumulate for 20 min or more, a desensitization event that affects only the response that occurs at an early time point (i.e., 10 sec) is likely to be overlooked. Experiments by Masters *et al.* (4), for example, demonstrate that muscarinic receptor-mediated phosphoinositide hydrolysis in 1321N1 cells desensitizes only after pre-exposure to agonist for >2.5 hr. However, in the same cell line a 2-min agonist pre-exposure desensitizes the calcium mobilization response (32). It, therefore, appears as though calcium mobilization is desensitized despite there being a full phosphoinositide response in these cells. We would argue that total accumulated inositol phosphate levels reflect both early and late $Ins(1,4,5)P_3$ production (and possibly alternative lipid, phosphoinositide and phosphoinositide-4-phosphate, hydrolysis). Hence, Masters *et al.* (4) would not necessarily have been able to detect the desensitization of an early peak $Ins(1,4,5)P_3$ response that was responsible for mobilization of intracellular calcium stores.

There are, however, a number of studies that report relatively rapid muscarinic receptor desensitization despite monitoring of the accumulation of inositol phosphates as a measure of receptor activation. Studies in astrocytes (11), cerebellar granule cells (9), and cultured corticostriatal neurons (12) show a significant decrease in inositol phosphate accumulation after a 15-min pre-exposure to muscarinic agonist. This is difficult to rationalize in light of the fact that, in each of these preparations, accumulation of inositol phosphates in the presence of lithium is linear for at least 30 min of agonist stimulation, suggesting that under these conditions there is no detectable desensitization of the inositol phosphate response. In these studies, pre-exposure to agonist should affect the linearity of inositol phosphate accumulation. It would be interesting to know whether, after desensitization, the rate of inositol phosphate accumulation is lower during the total period of subsequent agonist stimulation or only at early time points. If the latter is the case then a mechanism of desensitization similar to that reported here may be operating in these cell types.

Menniti *et al.* (13), working on AR4-2J cells, have recently demonstrated that bombesin mediates a rapid rise in $[^3H]Ins(1,4,5)P_3$ that falls to a sustained steady state level within 20 sec. In a similar fashion to the m3 muscarinic receptor response described here, the early peak phase of $Ins(1,4,5)P_3$ production in AR4-2J cells is desensitized after a short period of pretreatment with bombesin. An interesting observation made by this group (13) is that the rate of total inositol phosphate accumulation, in the presence of lithium, decreases after 20 sec of bombesin stimulation. This correlates with the fall in peak $Ins(1,4,5)P_3$ and suggests that receptor desensitization and not an increase in $[^3H]Ins(1,4,5)P_3$ metabolism is responsible for the reduced levels of $[^3H]Ins(1,4,5)P_3$ reported after 20 sec of agonist stimulation. In a similar study,[3] we have demonstrated that the rate of total inositol phosphate accumulation (in the presence of lithium) in CHO-m3 cells decreases after 15 sec of agonist stimulation. This change in rate correlates with a decrease in mass $Ins(1,4,5)P_3$ observed after 10 sec of agonist stimulation (see Fig. 1), indicating that the fall in $Ins(1,4,5)P_3$ after 10 sec is mediated by a change in PIP_2 hydrolysis rather than a change in $Ins(1,4,5)P_3$ metabolism. The diminution of $Ins(1,4,5)P_3$ synthesis could, therefore, be mediated by either depletion of PIP_2 or an uncoupling of the receptor from PIC. This being the case, a 5-min pre-exposure of CHO-m3 cells to carbachol is likely to initiate a similar desensitization event. For this reason we feel that an increase in $Ins(1,4,5)P_3$ metabolism is not likely to play a major role in the desensitization response described here, although we accept that our data do not completely rule out this possibility.

A number of studies have demonstrated that long term desensitization of muscarinic receptor responsiveness is associated with a loss of cell surface receptor number and probably involves proteolytic degradation of internalized receptors (3, 5, 33). In addition, Thompson and Fisher (3, 10) have recently reported that muscarinic receptors in SK-N-SH cells are rapidly sequestered from the plasma membrane after agonist stimulation. The data presented here indicate that neither of these processes is involved in desensitization of the peak $Ins(1,4,5)P_3$ response. Despite the receptor binding analysis being carried out at 4°, to prevent return of sequestered receptors to the plasma membrane (3, 10), no change in [^{3}H]NMS binding was observed after agonist exposure for periods of up to 10 min. Because the $Ins(1,4,5)P_3$ peak is desensitized after 5 min of agonist pre-exposure the mechanism of desensitization is neither receptor sequestration nor receptor internalization.

The mechanism of m3 receptor desensitization has yet to be determined but may be mediated by changes in $Ins(1,4,5)P_3$ metabolism, PIP_2 availability, or receptor uncoupling. Based on ongoing research the authors' bias is towards receptor uncoupling, where changes at the level of the receptor, G protein (G_q), or PIC may be important. The insensitivity of the m3 receptor-mediated inositol polyphosphate response in CHO-m3 cells to pertussis toxin treatment suggests the involvement of G_q in this response.[2] Because the $G_{q\alpha}$ gene family comprises five members (34) there is the possibility of heterogeneity in coupling efficiencies between m3 receptors and different $G_{q\alpha}$ subunits and in the susceptibility of a given receptor/G_q combination to desensitization. One proposal is that the early and late phases of $Ins(1,4,5)P_3$ production are mediated by m3 receptor coupling to different G_q proteins that possess different sensitivities to desensitization. The differential mechanisms of phosphoinositide hydrolysis, mediating early and late $Ins(1,4,5)P_3$ responses, may form the basis for differential susceptibilities to desensitization.

Alternatively, the desensitization of m3 receptors reported here may be mediated via agonist-induced receptor phosphorylation in a way analogous to the mechanism of rapid desensitization of β-adrenoceptors. A precedence for the involvement of receptor phosphorylation in muscarinic desensitization has been set by the work of Kwatra *et al.* (35, 36). This group have demonstrated that chick heart muscarinic receptor desensitization (not PIC linked) correlates with agonist-mediated recep-

[3] A. B. Tobin, S. T. Safrany, and S. R. Nahorski, unpublished observations.

tor phosphorylation via a kinase distinct from PKC, protein kinase A, and calcium/calmodulin-dependent protein kinase (35, 36). A strong candidate for mediating m3 receptor phosphorylation, however, is PKC, because this kinase is activated upon muscarinic receptor occupancy (33). Pretreatment with tumor-promoting phorbol esters is known to decrease muscarinic receptor-mediated phosphoinositide hydrolysis and cell surface receptor number (24, 28, 33). However, a recent study in corticostriatal neurons reported that PKC inhibitors were unable to prevent agonist-mediated muscarinic receptor desensitization (12). Clearly further studies are required to determine the molecular mechanism of m3 receptor desensitization and the role, if any, that receptor phosphorylation may play.

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References

1. Dohlman, H. G., J. Thorner, M. G. Caron, and R. J. Lefkowitz. Model systems for the study of seven-transmembrane-segment receptors. *Annu. Rev. Biochem.* **60**:653–688 (1991).
2. Hausdorff, W. P., M. G. Caron, and R. J. Lefkowitz. Turning off the signal: desensitization of β-adrenergic receptor function. *FASEB J.* **4**:2881–2889 (1990).
3. Thompson, A. K., and S. K. Fisher. Relationship between agonist-induced muscarinic receptor loss and desensitization of stimulated phosphoinositide turnover in two neuroblastomas: methodological considerations. *J. Pharmacol. Exp. Ther.* **252**:744–752 (1990).
4. Masters, S. B., M. T. Quinn, and J. H. Brown. Agonist-induced desensitization of muscarinic receptor-mediated calcium efflux without concomitant desensitization of phosphoinositide hydrolysis. *Mol. Pharmacol.* **27**:325–332 (1985).
5. Hu, J., S.-Z. Wang, and E. E. El-Fakahany. Effects of agonist efficacy on desensitization of phosphoinositide hydrolysis mediated by m1 and m3 muscarinic receptors expressed in Chinese hamster ovary cells. *J. Pharmacol. Exp. Ther.* **257**:938–945 (1991).
6. Sugiya, H., K. A. Tennes, and J. W. Putney. Homologous desensitisation of substance P-induced inositol polyphosphate formation in rat parotid acinar cells. *Biochem. J.* **244**:647–653 (1987).
7. Nakahata, N., and T. K. Harden. Regulation of inositol trisphosphate accumulation by muscarinic cholinergic and H_1-histamine receptors on human astrocytoma cells. *Biochem. J.* **241**:337–344 (1987).
8. Morrison, W. J., and S. D. Shukla. Desensitization of receptor-coupled activation of phosphoinositide-specific phospholipase C in platelets: evidence of distinct mechanisms for platelet-activating factor and thrombin. *Mol. Pharmacol.* **33**:58–63 (1987).
9. Xu, J., and D.-M. Chuang. Muscarinic acetylcholine receptor-mediated phosphoinositide turnover in cultured cerebellar granular cells: desensitization by receptor agonists. *J. Pharmacol. Exp. Ther.* **242**:238–244 (1987).
10. Thompson, A. K., and S. K. Fisher. Preferential coupling of cell surface muscarinic receptors to phosphoinositide hydrolysis in human neuroblastoma cells. *J. Biol. Chem.* **266**:5004–5010 (1991).
11. Pearce, B., C. Morrow, and S. Murphy. Characteristics of phorbol ester and agonist induced down-regulation of astrocyte receptors coupled to inositol phospholipid metabolism. *J. Neurochem.* **50**:936–944 (1987).
12. Eva, C. S., R. Gamalero, E. Genazzani, and E. Costa. Molecular mechanisms of homologous desensitization and internalization of muscarinic receptors in primary cultures of neonatal corticostriatal neurons. *J. Pharmacol. Exp. Ther.* **253**:257–265 (1990).
13. Menniti, F. S., H. Takemura, K. G. Oliver, and J. W. Putney. Different modes of regulation for receptors activating phospholipase C in the rat pancreatoma cell line AR4-2J. *Mol. Pharmacol.* **40**:727–733 (1991).
14. Peralta, E. G., A. Ashkenazi, J. W. Winslow, J. Ramachandran, and D. J. Capon. Differential regulation of PI hydrolysis and adenylate cyclase by muscarinic receptor subtypes. *Nature (Lond.)* **334**:434–437 (1988).
15. Berridge, M. J. Inositol trisphosphate and diacylglycerol: two interacting second messengers. *Annu. Rev. Biochem.* **56**:159–193 (1987).
16. Berridge, M. J., and R. F. Irvine. Inositol phosphates and cell signalling. *Nature (Lond.)* **341**:197–204 (1989).
17. Nahorski, S. R. Inositol polyphosphates and neuronal calcium homeostasis. *Trends Neurosci.* **10**:444–448 (1988).
18. Ashkenazi, A., E. G. Peralta, J. W. Winslow, J. Ramachandran, and D. J. Capon. Functionally distinct G-proteins selectively couple different receptors to PI hydrolysis in the same cell. *Cell* **56**:487–493 (1989).
19. Buckley, N. J., T. I. Bonner, C. M. Buckley, and M. R. Brann. Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol. Pharmacol.* **35**:469–476 (1989).
20. Harden, T. K., L. A. Petch, S. F. Traynelis, and G. L. Waldo. Agonist-induced alteration in the membrane form of muscarinic cholinergic receptors. *J. Biol. Chem.* **260**:13060–13066 (1985).
21. Challiss, R. A. J., I. H. Batty, and S. R. Nahorski. Mass measurements of inositol (1,4,5)trisphosphate in rat cerebral cortex slices using a radioreceptor assay: effects of neurotransmitters and depolarisation. *Biochem. Biophys. Res. Commun.* **157**:648–691 (1988).
22. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
23. Merrit, J. E., and T. J. Rink. Regulation of cytosolic free calcium in fura-2-loaded rat parotid acinar cells. *J. Biol. Chem.* **262**:17362–17369 (1987).
24. Lai, W. S., T. B. Rogers, and E. E. El-Fakahany. Protein kinase C is involved in desensitisation of muscarinic receptor induced by phorbol esters but not by receptor agonists. *Biochem. J.* **267**:23–29 (1990).
25. Lambert, D. G., and S. R. Nahorski. Muscarinic-receptor-mediated changes in intracellular Ca^{2+} and inositol 1,4,5-trisphosphate mass in a human neuroblastoma cell line, SH-SY5Y. *Biochem. J.* **265**:555–562 (1990).
26. Lambert, D. G., R. A. J. Challiss, and S. R. Nahorski. Accumulation and metabolism of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in muscarinic receptor-stimulated SH-SY5Y neuroblastoma cells. *Biochem. J.* **273**:791–794 (1991).
27. Lambert, D. G., and S. R. Nahorski. Second messenger responses associated with stimulation of neuronal muscarinic receptors expressed by human neuroblastoma SH-SY5Y. *Prog. Brain Res.* **84**:31–42 (1990).
28. Felder, C. C., M. O. Poulter, and J. Wess. Muscarinic receptor operated Ca^{2+} influx in transfected fibroblast cells is independent of inositol phosphates and release of intracellular calcium. *Proc. Natl. Acad. Sci. USA* **89**:509–513 (1992).
29. Waldo, G. L., J. K. Northup, J. P. Perkins, and T. K. Harden. Characteristics of an altered membrane form of the β-adrenergic receptor produced during agonist-induced desensitization. *J. Pharmacol. Exp. Ther.* **258**:13900–13908 (1983).
30. Ferris, C. D., A. M. Cameron, R. L. Huganir, and S. H. Snyder. Quantal calcium release by purified reconstituted inositol 1,4,5-trisphosphate receptors. *Nature (Lond.)* **356**:350–352 (1992).
31. Irvine, R. F. "Quantal" Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates: a possible mechanism. *FEBS Lett.* **263**:5–9 (1990).
32. McDonough, P. M., J. H. Eubanks, and J. H. Brown. Desensitisation and recovery of muscarinic and histaminergic calcium mobilisation in 1321N1 astrocytoma cells. *Biochem. J.* **249**:135–141 (1988).
33. Kopp, R., P. Mayer, and A. Pfeiffer. Agonist-induced desensitization of cholinergically stimulated phosphoinositide breakdown is independent of endogenously activated protein kinase C in HT-29 human colon carcinoma cells. *Biochem. J.* **269**:73–78 (1990).
34. Simon, M. I., M. P. Strathmann, and N. Gautam. Diversity of G proteins in signal transduction. *Science (Washington D. C.)* **252**:802–808 (1991).
35. Kwatra, M. M., E. Leung, A. C. Maan, K. K. Machn, J. Ptasienski, R. D. Green, and M. M. Hosey. Correlation of agonist-induced phosphorylation of chick heart muscarinic receptors with receptor desensitization. *J. Biol. Chem.* **262**:16314–16321 (1987).
36. Kwatra, M. M., and M. M. Hosey. Phosphorylation of the cardiac muscarinic receptor in intact chick heart and its regulation by a muscarinic agonist. *J. Biol. Chem.* **261**:12429–12432 (1986).

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