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Rapid desensitization of the TRH receptor and persistent desensitization of its constitutively active mutant

[1]Ilona Zaltsman, [1]Hagit Grimberg, [1]Monica Lupu-Meiri, [1]Ilana Lifschitz & *,[1]Yoram Oron

[1]Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv 69978, Israel

1 We studied rapid desensitization of the thyrotropin-releasing hormone receptor (TRH-R) or the m1-muscarinic receptor (m1-R) to a short challenge of threshold TRH concentration and persistent desensitization due to constitutive activity of a mutant TRH-R.

2 Xenopus oocytes expressing TRH-Rs and/or m1-Rs were challenged for 15 s with threshold concentrations of TRH ([TRH]) and then immediately with supraoptimal [TRH] or acetylcholine ([ACh]). The threshold challenge caused desensitization of 50–57% of responses to subsequent supraoptimal stimulation with TRH or ACh.

3 The homologous desensitization was reversible within 60 s after removal of the agonist.

4 The protein kinase C (PKC) inhibitor, chelerythrine, inhibited the control responses by 30–40%, without affecting the desensitized responses. Chelerythrine or the phosphatase inhibitor, okadaic acid, had little effect on the kinetics of resensitization, indicating limited involvement of PKC.

5 In oocytes coexpressing wild type TRH-Rs or m1-Rs with a constitutively active TRH-R mutant (C335Stop TRH-R), a persistent desensitization (33–57%) of the responses to TRH or ACh was observed. Additionally, there was a complete loss of the rapid desensitization induced by threshold [TRH].

6 Chlorodiazepoxide (CDE), a competitive binding antagonist of TRH-Rs and an inverse agonist of C335Stop TRH-Rs, abolished the persistent desensitization induced by C335Stop TRH-Rs and enabled the rapid desensitization, conferring the wild type phenotype on C335Stop TRH-Rs. Chelerythrine had qualitatively the same effect as CDE.

7 In conclusion, unlike the rapid desensitization, the persistent desensitization caused by the constitutively active C335Stop TRH-Rs is largely mediated by PKC. It abrogates, however, the rapid desensitization, suggesting a common mechanistic step(s).
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Keywords: TRH receptors; muscarinic receptors; constitutive activity; rapid homologous/heterologous desensitization; persistent homologous/heterologous desensitization; protein kinase C; inverse agonism

Abbreviations: ACh, acetylcholine; CDE, chlorodiazepoxide; C335Stop TRH-R, a consititutively active mutant of the thyrotropin-releasing hormone receptor truncated at cys335; $InsP_3$, inositol 1,3,5-trisphosphate; m1-R, muscarinic m1-receptor; PKC, protein kinase C; TRH-R, thyrotropin-releasing hormone receptor; WT, wild type

Introduction

Different types of desensitization provide regulatory feedback in normal physiological responses to natural agonists. Of the many types of desensitization described in the literature, we concentrated on two: rapid desensitization, induced by a short challenge with threshold concentrations of agonists, and persistent desensitization exhibited by constitutively active receptors.

Constitutively active receptors were initially identified following introduction of specific mutations in receptor sequences (Kjelsberg *et al*., 1992), and later were identified as causes of human disease (Shenker *et al*., 1993; Parma *et al*., 1993; Arvanitakis *et al*., 1998). Moreover, there is evidence that wild type TRH-Rs receptors exhibit a limited degree of constitutive activity (Jinsi-Parimoo & Gershengorn, 1997). We have previously shown that TRH-R mutant truncated at cys335 residue (C335Stop TRH-R) is constitutively active (Matus-Leibovitch *et al*., 1995). We demonstrated that C335Stop TRH-R caused persistent homologous and heterologous desensitization (Matus-Leibovitch *et al*., 1995), which was either fully or partially abolished by the inverse agonist, CDE (Heinflink *et al*., 1995; Grimberg *et al*., 1999).

In *Xenopus* oocytes expressing TRH-Rs, a 10–30 s challenge with threshold concentrations of an agonist caused extensive desensitization to a subsequent challenge with a supraoptimal homologous or heterologous agonists (Lipinsky *et al*., 1995).

This study was undertaken to compare the mechanisms of the two types of desensitization: rapid physiological and persistent related to constitutive activity. Rapid desensitization may greatly affect the modulation of cellular responses to homologous or heterologous stimulation under normal physiological conditions, whereas persistent desensitization may prove important in different phathological states resulting from mutated receptors that exhibit constitutive activity. In this report we demonstrate that the persistent desensitization caused by the constitutively active C335Stop TRH-Rs abrogates the rapid desensitization evoked by short challenge of the WT TRH-R to threshold concentrations of agonists and may, therefore, alter the normal modulation of physiological responses.

Methods

Xenopus laevis *oocytes*

Defolliculated oocytes were obtained from mature *Xenopus* females, essentially as previously described (Shapira *et al*., 1990). *In vitro*-transcribed cRNAs for WT TRH-Rs (1–5 ng/

*Author for correspondence; E-mail: pharm14@post.tau.ac.il

oocyte), C335Stop TRH-Rs (5–10 ng/oocyte) or m1-Rs (1 ng/oocyte) were injected 24–48 h before the assay as described previously (Oron *et al*., 1987; Lupu-Meiri *et al*., 1993; Matus-Leibovitch *et al*., 1995). Greater amounts of the mutant receptor mRNA was required in order to obtain measurable responses (Matus-Leibovitch *et al*., 1995). Coexpression of different receptors (m1-Rs, gastrin-releasing peptide-Rs, neuromedin B-Rs and TRH-Rs), at the amounts of cRNAs used here, does not inhibit either of the responses, suggesting that neither the expression mechanism, nor the post-receptor signal transduction components are limiting (Matus-Leibovitch *et al*., 1995; Shapira & Oron, unpublished).

Electrophysiology

Two-electrode voltage clamp measurements were performed at $V_H = -80$ to -100 mV, as previously described (Lupu-Meiri *et al*., 1990; 1993). Chloride currents were continuously recorded. Responses to threshold [TRH] (0.1–10 nM) exhibited latencies of approximately 15 s and their amplitudes were less than 5% of the control responses to 10 μM of the hormone. Rapid desensitization to threshold agonist concentration was assayed as previously described (Lipinsky *et al*., 1995), except when C335Stop TRH-Rs were coexpressed with m1-Rs. Because of the very low sensitivity of the mutant receptor to TRH (Matus-Leibovitch *et al*., 1995), 10 μM TRH were used as a pre-challenge in order to produce rapid desensitization. Intracellular injections of $InsP_3$ were performed using a Drummond microinjector (volume 4.6–9.2 nl/oocyte) as previously described (Shapira *et al*., 1996).

Statistics

All experiments were performed on a large number of oocytes (n) from a number of donors (N). Results were presented as mean ± s.e.mean. All results were analysed by either paired or unpaired Students t-test with $P < 0.05$ considered as significant difference between two populations.

Materials

Collagenase type IA and $InsP_3$ (K^+ salt) were purchased from Sigma (Rehovot, Israel). *In vitro* translation was done with a Riboprobe® kit (Promega, Madison, WI, U.S.A.), modified to contain higher concentration of T7 polymerase and m7G(5′)ppp(5′)G (Boehringer-Mannheim, Mannheim, Germany). All other chemicals were of analytical grade.

Drugs

Chelerythrine hydrochloride and okadaic acid were purchased from Alomone Labs (Jerusalem, Israel). TRH, CDE, ACh were purchased from Sigma (Rehovot, Israel).

Results

Rapid homologous desensitization of the TRH response

When oocytes expressing receptors coupled to the phosphoinositides-phospholipase C pathway are challenged with very low concentrations of agonists, a significant, though variable desensitization (25–60%) of the subsequent response to supraoptimal agonist concentration is observed (Lipinsky *et al*., 1995). In the present series of experiments, oocytes expressing WT TRH-Rs were challenged with threshold [TRH] (0.1–1.0 nM), which resulted in a small response (116 ± 25 nA, $n = 22$, N = 5) with prolonged latency (14.6 ± 1.5 s, $n = 22$, N = 5). Ten to fifteen seconds after the challenge with threshold [TRH], within the period of the latency of the response, oocytes were challenged with supraoptimal [TRH] (10 μM). The resulting response was 57 ± 7% desensitized (2500 ± 203 nA, $n = 30$, N = 4), when compared to oocytes of the same batch challenged directly with 10 μM TRH (6240 ± 360 nA, $n = 27$, N = 4). These results (Figure 1) confirmed our previous report of rapid homologous desensitization of the TRH response in oocytes (Lipinsky *et al*., 1995).

The rapid homologous desensitization of the response to TRH was also rapidly reversible. When oocytes expressing WT TRH-Rs were challenged with 0.1–1.0 nM TRH for 15 s and then the agonist was washed off, the response to supraoptimal [TRH] rapidly resensitized (Figure 2). Whereas challenge with threshold concentration of the agonist resulted in 44 ± 2% desensitization of the response to 10 μM TRH ($n = 36$, N = 5), 30 s wash decreased the degree of desensitization to 22 ± 2% ($n = 13$, N = 2) and by 5 min it was completely abolished (107 ± 1% of the control response, $n = 12$, N = 2).

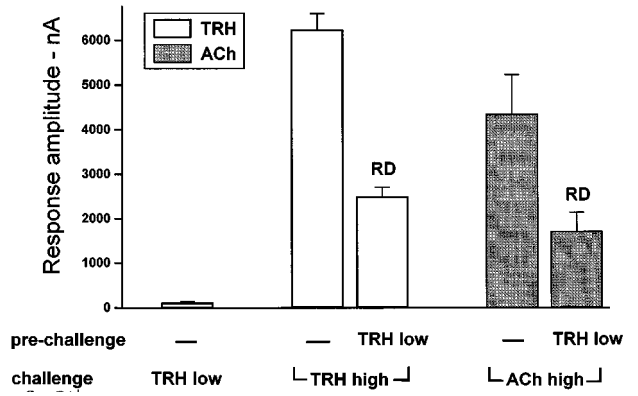

Figure 1 Rapid desensitization of the TRH or ACh responses by threshold stimulation of TRH-Rs. Oocytes expressing TRH-Rs alone, or together with m1-Rs, were either challenged with a supramaximal concentration of TRH or ACh alone, or 15 s after a pre-challenge with low [TRH] (0.1–1.0 nM, rapid desensitization – RD). The response to threshold stimulation with low [TRH] alone is shown in the left-most column. The results are the mean ± s.e.mean of 22–197 individual measurements in oocytes of 4–13 frogs.

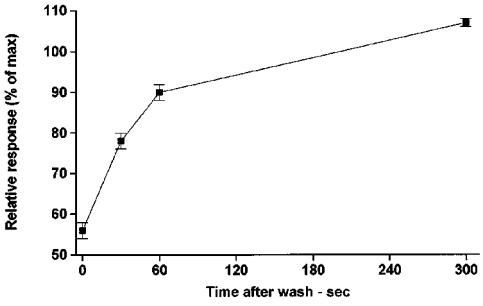

Figure 2 Recovery from rapid desensitization. Oocytes expressing TRH-Rs were pre-challenged with low [TRH] (0.1–1.0 nM). Fifteen seconds later the agonist was washed off and cells were challenged with 10 μM TRH at various times. Desensitization was calculated by comparing the responses to 10 μM TRH after pre-challenge with the response to 10 μM TRH in naive cells. Each point represents results from 12–36 oocytes from 2–5 frogs.

Rapid heterologous desensitization of the muscarinic response

We previously reported that in oocytes coexpressing TRH-Rs and m1-Rs, a rapid desensitization of the response to TRH was observed following a threshold challenge with ACh (Lipinsky *et al*., 1995). Here we examined the effect of threshold challenge with TRH on the muscarinic response in oocytes expressing both receptors. A threshold challenge with 0.1–1.0 nM TRH caused rapid and extensive desensitization of the subsequent (10–15 s later) response to ACh (to 50±7% of naive controls challenged with 10 μM ACh alone, $n=197$, N=13, Figure 1). These results demonstrated that threshold stimulation of the TRH-Rs results in pronounced rapid heterologous desensitization of the muscarinic response.

The involvement of PKC in rapid desensitization

We previously reported that chelerythrine, a specific inhibitor of PKC, appeared to partially prevent rapid homologous desensitization (Lipinsky *et al*., 1995). In the present study we performed this experiment for both homologous and heterologous desensitization. Indeed, treatment with chelerythrine decreased the degree of homologous desensitization (from 57±7% in the untreated oocytes to 29±2% with chelerythrine). The analysis of the results (Figure 3), however, indicated that the major action of chelerythrine was to inhibit the control response (from 6240±360 to 4060±302 nA upon 40 min treatment with 20 μM of the inhibitor, $n=27-30$, N=3). The response after desensitization increased only modestly and not significantly following incubation with the inhibitor (from 2676±264 in untreated controls, to 2901±268 nA). Hence, the apparent partial prevention of desensitization by chelerythrine could be accounted for by the inhibition of the control response, rather than by an increase of the desensitized response.

We observed a similar pattern for the heterologous rapid desensitization (Figure 4). In this series of experiments, chelerythrine treatment decreased the desensitization of the muscarinic response from 30 to 26%. Chelerythrine inhibited the control and the desensitized responses to a similar degree (by 42–44%). These results suggested that PKC had no role in either homologous or heterologous rapid desensitization.

This conclusion was supported by kinetic experiments. We hypothesized that if the mechanism of the rapid desensitization involves PKC, treatment with chelerythrine will accelerate the recovery form desensitization following washing off the agonist (see Figure 2). Conversely, treatment with okadaic acid, a phosphoserine/phosphothreonine phosphatase inhibitor, would slow down the kinetics of response recovery upon the removal of agonist. Our results suggest that neither okadaic acid nor chelerythrine affected the kinetics of response recovery (not shown).

Persistent desensitization of the TRH or the ACh response by C335Stop TRH-Rs

In order to test the extent of persistent desensitization exhibited by the constitutively active TRH-R mutant, C335Stop TRH-R, this mutant was coexpressed with either WT TRH-Rs or m1-Rs. C335Stop TRH-R desensitized the WT TRH-R response from 1579±283 to 1057±193 nA ($n=32-38$, N=4), i.e. by 33%. Similar results were observed for m1-R-mediated responses. When C335Stop TRH-Rs were coexpressed with m1-Rs, stimulation with 10 μM ACh yielded responses of 1878±311 nA ($n=229$, N=14), i.e. 57% desensitization when compared to the control response in oocytes coexpressing m1-Rs with WT TRH-Rs (4355±879 nA, $n=173$, N=13). These results confirmed our previous reports of persistent desensitization (Matus-Leibovitch *et al*., 1995, Heinflink *et al*., 1995; Grimberg *et al*., 1999).

To test whether persistent desensitization due to the constitutive activity of C335Stop TRH-R was caused by depletion of cellular Ca^{2+} stores, we measured responses to microinjected $InsP_3$. Injection of 0.92 pmol $InsP_3$ resulted in a mean response of 149±21 nA in control oocytes and of 145±24 nA in oocytes expressing C335Stop TRH-Rs ($n=21-22$, N=2), suggesting that depletion of Ca^{2+} stores was not the cause of persistent desensitization.

The relationship between rapid and persistent desensitization

Persistent desensitization resulting from constitutive activity abrogated rapid desensitization. In this series of experiments, the response to 10 μM TRH in oocytes expressing WT TRH-Rs alone was 1579±263 nA ($n=38$, N=4). In cells coexpressing WT and C335Stop TRH-Rs the response was 1057±193 nA ($n=32$, N=4), exhibiting 33% persistent desensitization. After a 15 s challenge with 1 nM TRH, 10 μM of the agonist elicited a response of 1348±324 nA ($n=29$,

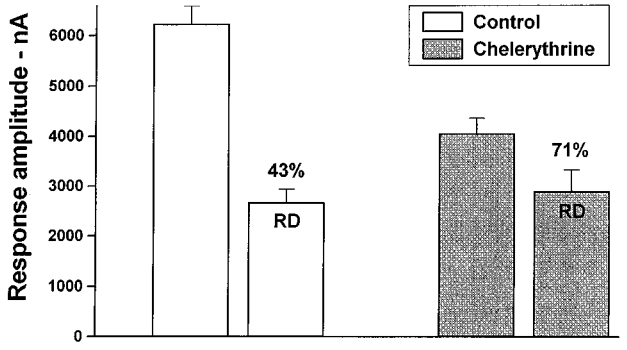

Figure 3 The effect of chelerythrine on rapid homologous desensitization. Oocytes expressing TRH-Rs were challenged either with 10 μM of TRH alone (control) or 15 s following pre-challenge with 0.1–1.0 nM TRH (rapid desensitization, RD). The numbers over the columns represent rapid desensitization as per cent of control responses. Where indicated, oocytes were preincubated for 40 min with chelerythrine (20 μM). Results are presented as mean± s.e.mean of 27–30 oocytes from three frogs.

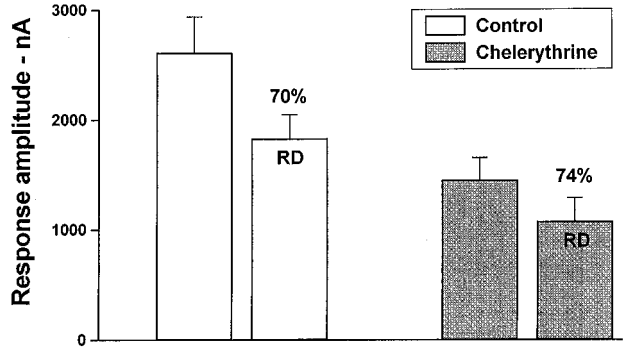

Figure 4 The effect of chelerythrine on rapid heterologous desensitization. Oocytes expressing TRH-Rs and m1-Rs were challenged either with 10 μM of ACh alone (control) or 15 s following pre-challenge with 0.1–1.0 nM TRH (RD). Where indicated, oocytes were preincubated for 40 min with chelerythrine (20 μM). The numbers over the columns represent desensitization as per cent of control responses. Results are presented as mean± s.e.mean of 29–173 oocytes from 3–13 frogs.

N = 4), i.e. a 28% enhancement instead of the rapid desensitization exhibited by the WT TRH-Rs (see Figure 1).

We have previously reported that CDE, acting as an inverse agonist, largely abolished the persistent desensitization and caused a significant increase in the amplitude of responses mediated by C335Stop TRH-R (Heinflink *et al.*, 1995; Grimberg *et al.*, 1999). To assess the role of constitutive activity in the abrogation of rapid desensitization, we used CDE on oocytes coexpressing WT and C335Stop TRH-Rs (Figure 5). As expected, a 2 h treatment with 20 μM of the inverse agonist abolished the persistent desensitization of the TRH response, causing its increase from 1057 ± 193 to 2249 ± 356 nA ($n = 32-34$, N = 4). More interestingly, following treatment with CDE the rapid desensitization protocol yielded responses of 1710 ± 234 nA ($n = 29$, N = 4). Hence, suppression of constitutive activity abolished persistent desensitization and caused recovery of rapid desensitization. Similar results were obtained when chelerythrine was used to partially abolish persistent desensitization. The response to TRH increased from 1057 ± 193 to 1489 ± 242 nA ($n = 32$, N = 4), while a 15 s challenge with 1 nM TRH and then with 10 μM of the agonist yielded responses of 813 ± 212 nA ($n = 32$, N = 4), i.e. rapid desensitization of 45%.

A similar qualitative pattern was seen in heterologous desensitization. In oocytes expressing m1-Rs alone, 10 μM ACh elicited a response of 4355 ± 879 nA ($n = 173$, N = 13). Coexpression of C335Stop TRH-Rs and m1-Rs, resulted in a response of 1914 ± 484 nA ($n = 73$, N = 6), i.e. 56% persistent desensitization. The rapid desensitization protocol using TRH for the first challenge, however, failed to decrease the response to ACh (1970 ± 515 nA $n = 70$; N = 6). Thus, the constitutively active C335Stop TRH-Rs, when coexpressed with m1-Rs completely abolished the rapid desensitization.

Suppression of the constitutive activity of C335Stop TRH-R by CDE abrogated the heterologous persistent desensitization. The response to 10 μM ACh increased from 1914 ± 484 to 4302 ± 514 nA ($n = 79$, N = 6, Figure 6). On the other hand, rapid desensitization protocol with 10 μM TRH in cells treated with CDE yielded a response to 10 μM ACh of 2841 ± 490 nA ($n = 77$, N = 6), i.e. partial recovery of rapid desensitization. Incubation with chelerythrine produced similar results. The response to 10 μM ACh increased to 2680 ± 598 nA ($n = 29$, N = 3) and a complete recovery of rapid desensitization was observed (1067 ± 276 nA response to 10 μM ACh, i.e. 60% desensitization after a 15 s challenge with 10 μM TRH; $n = 28-32$, N = 3–4, Figure 6). Hence, abrogation of persistent homologous or heterologous desensitization (be it *via* inhibition of the constitutively active receptor with CDE or inhibition of PKC with chelerythrine) resulted in a recovery of rapid desensitization.

Discussion

This report describes the relationship between two types of desensitization: rapid, brought about by previous challenge with threshold concentration of an agonist, and persistent, resulting from constitutive activity of the receptor. Although the definition of rapid varies considerably in different reports (e.g. from seconds to hours, Saucier *et al.*, 1998; Waugh *et al.*, 1999), this protocol demonstrates desensitization within the latency period of the response, i.e. 10–15 s. The present findings suggest that, upon removal of the desensitizing signal, the duration of this desensitization is very short (<1 min). These properties make rapid desensitization a likely candidate for transient suppression of the homologous and heterologous signals under physiological conditions. Despite our previous suggestion (Lipinsky *et al.*, 1995), the present results imply that rapid desensitization is not mediated by PKC. The lack of effect of okadaic acid on the extent and kinetics of rapid desensitization or on its recovery strengthen this conclusion and imply further that serine or threonine phosphorylation(s) are not involved.

Pei *et al.* (1994) have previously reported persistent desensitization due to constitutive activity of the β2-adrenergic receptor. We have recently described persistent homologous and heterologous desensitization attributable to the constitutive activity of C335Stop TRH-R. We show that persistent desensitization precludes rapid desensitization. The persistent desensitization of C335Stop TRH-R is abrogated by the inverse agonist, CDE and by the PKC inhibitor, chelerythrine. The re-sensitized response exhibits rapid desensitization. This suggests that both types of desensitization share a common mechanistic step.

The mechanism of the persistent desensitization of C335Stop TRH-R is unclear. On the one hand, Pei *et al.* (1994) have shown that the persistent desensitization of the response mediated by the constitutively active β 2-AR mutant

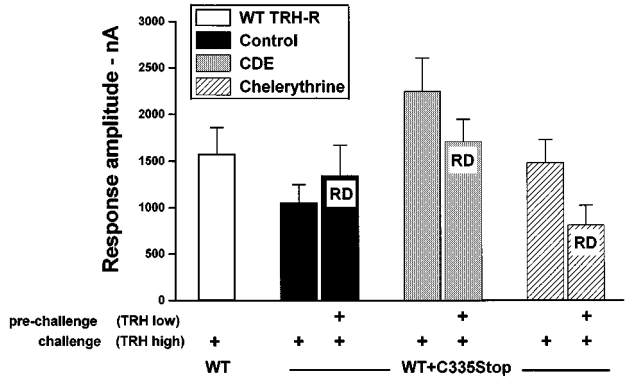

Figure 5 The effect of persistent desensitization on rapid homologous desensitization. Oocytes coexpressing WT and C335Stop TRH-Rs were tested for rapid desensitization (RD) without any treatment (control), following 2 h incubation with 20 μM CDE, or following 40 min incubation with 20 μM chelerythrine. Responses to 10 μM TRH in oocytes expressing WT TRH-Rs alone are shown in the left-most column. Each column represents results obtained in 32–38 oocytes from four frogs.

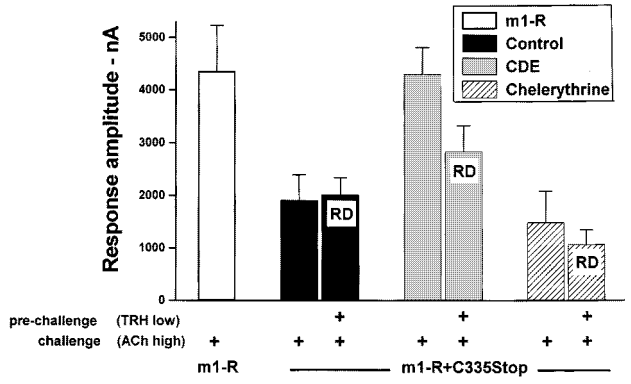

Figure 6 The effect of persistent desensitization on rapid heterologous desensitization. Oocytes coexpressing m1-Rs and C335Stop TRH-Rs were tested for rapid desensitization (RD) without any treatment (control), following 2 h incubation with 20 μM CDE, or following 40 min incubation with 20 μM chelerythrine. Responses to 10 μM ACh in oocytes expressing m1-Rs alone are shown in the left-most column. Each column represents results obtained in 28–173 oocytes from 3–13 frogs.

was accompanied by phosphorylation by β-AR kinase (βARK). They postulated that the conformation of the constitutively active receptor mutant mimics that of the agonist-occupied species and, thus, is a good substrate for βARK. On the other hand, the response to TRH is rapidly inhibited by activation of PKC, the persisitent desensitization of C335Stop TRH-R is antagonized by the PKC inhibitor, chelerythrine, and is precluded in cells in which PKC was down-regulated (Grimberg *et al*., 1999).All this evidence points to PKC as the mediator of persistent desensitization, rather than a receptor kinase (GRK) species. The constitutively active and persistently desensitized C335Stop TRH-R mutant lacks all concensus phosphorylation sites in the C-terminus. Hence, the target of PKC activity must be either within the intracellular loops of the receptor or further down-stream in the signal transduction cascade. We have previously shown that the TRH-evoked mobilization of Ca^{2+} in oocytes (Matus-Leibovitch *et al*., 1995) or AtT20 cells (Matus-Leibovitch *et al*., 1995; Grimberg *et al*., 1999) expressing C335Stop TRH-Rs exhibited prolonged latency and diminished amplitude. Since constitutive activity did not promote depletion of cellular Ca^{2+} pools, it must affect steps proximal to $InsP_3$-induced Ca^{2+} mobilization.

We have previously shown (Lipinsky *et al*., 1995) that rapid desensitization resulted in a diminished and delayed Ca^{2+} mobilization in oocytes. Since rapid desensitization precedes Ca^{2+} mobilization (Lipinsky *et al*., 1995), desensitization could not be attributed to Ca^{2+} pool depletion. Our rapid desensitization protocol (threshold concentration of agonist and short times of exposure) implies very low receptor occupancy and precludes significant involvement of GRKs.

The information relating to the mechanism of rapid desensitization in other model systems is meager. This is mainly due to the small number of reports investigating very rapid events. Yu & Hinkle (1997) have described rapid desensitization (10 s exposure) of TRH-Rs transfected in HEK293 cells. They have used, however, either rapid exposure to high [TRH] (1 μM) or prolonged exposure to threshold concentrations (1 nM). Although they demonstrated that both $InsP_3$ generation and Ca^{2+} mobilization were impaired, the depletion of Ca^{2+} pools appeared to be the major cause of TRH-induced homologous and heterologous desensitization. Severi *et al*. (1991) studied rapid (30 s) desensitization of cholecystokinin-induced smooth muscle cells contraction. Homologous desensitization was accompanied by desensitization of $InsP_3$ and Ca^{2+} responses and was not inhibited by PKC antagonists. Prolonged challenge (5 min) resulted in heterologous desensitization and appeared to be mediated by PKC. Waugh *et al*. (1999) described rapid (10 s) desensitization of the response to the stimulation of m1-Rs. The authors suggested that casein kinase1α was involved in receptor phosphorylation and desensitization. It should be emphasized, however, that very high agonist concentrations were used in that study (EC_{50} 8.2 μM). Barak *et al*. (1999) investigated rapid (30 s) desensitization of the substance P-mediated response in HEK293 cells. Their report suggests very rapid participation of GRKs in the desensitization process, which probably reflects the high concentration of substance P (0.1 μM) that was used.

Despite the evidence presented here that PKC does not mediate rapid desensitization, the central role of this enzyme(s) in the modulation of the response is still obvious. The study of these phenomena is complicated by the large number of PKC isozymes and by the many potential targets within the signal transduction pathway. Further investigations in defined systems, expressing single PKC subtypes and using the rapid desensitization protocol may help clarify the mechanism(s) involved.

Rapid desensitization caused by low agonist concentration appears to be an excellent candidate for transient, rapidly reversible modulation of homologous and heterologous responses. It brings about the attenuation of responses to high concentrations of agonists, provided they arrive within the short interval of the desensitization. Constitutive activity causes persistent desensitization and eliminates this regulatory mechanism, thus interfering with normal physiological processes. This hypothesis makes the study of these two phenomena interesting in the context of diseases associated with constitutively active mutations of serpentine receptors.

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