

Low Molecular Weight mRNA Encodes a Protein That Controls Serotonin 5-HT_{1c} and Acetylcholine M₁ Receptor Sensitivity in *Xenopus* Oocytes

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ABSTRACT Serotonin 5-HT_{1c} and acetylcholine M₁ receptors activate phosphoinositidase, resulting in an increased formation of IP₃ and 1,2 diacylglycerol. In *Xenopus* oocytes injected with mRNA encoding either of these receptors, Ca²⁺ released from intracellular stores in response to IP₃ then opens Ca²⁺-gated Cl⁻ channels. In the present experiments, oocytes expressing a transcript from a cloned mouse serotonin 5-HT_{1c} receptor were exposed to identical 15-s pulses of agonist, administered 2 min apart; the second current response was two to three times that of the first. However, in those oocytes coinjected with the 5-HT_{1c} receptor transcript and a low molecular weight fraction (0.3–1.5 kb) of rat brain mRNA, the second current response was ~50% of the first. Thus, the low molecular weight RNA encodes a protein (or proteins) that causes desensitization. Experiments using fura-2 or a Ca²⁺-free superfusate indicated that desensitization of the 5-HT_{1c} receptor response does not result from a sustained elevation of intracellular Ca²⁺ level or require the entry of extracellular Ca²⁺. Photolysis of caged IP₃ demonstrated that an increase in IP₃ and a subsequent rise in Ca²⁺ do not produce desensitization of either the IP₃ or 5-HT_{1c} peak current responses. Furthermore, in oocytes coinjected with the low molecular weight RNA and a transcript from the rat M₁ acetylcholine receptor, the M₁ current response was greatly attenuated. Our data suggest that the proteins involved in attenuation of the M₁ current response and desensitization of the 5-HT_{1c} current response may be the same.

INTRODUCTION

Desensitization is an adaptive phenomenon that results in the attenuation of responses to an agonist after prolonged or repeated application of agonist. By controlling the sensitivity of the transductive process, desensitization may act to preserve the fidelity of frequency encoded inputs (Klein et al., 1989). In those cases

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where receptor activation leads to the increase of an intracellular messenger such as Ca^{2+} , desensitization acts as a safeguard of cell function by preventing intracellular messengers from reaching toxic levels (Klein et al., 1989). While the advantages afforded the cell by desensitization are clear, the underlying mechanisms are not fully understood for those receptors that evoke a cellular response by increasing intracellular Ca^{2+} .

Heterologous expression of such receptors in *Xenopus* oocytes is one method of studying desensitization in a system that is amenable to a wide variety of experimental manipulations. Recent studies (Moriarty et al., 1988; Boton et al., 1990; Singer et al., 1990) have focused on the 5-HT_{1c} receptor pathway in oocytes injected with mRNA from rat brain. It is presumed that after agonist binding a G protein is activated, then phosphoinositidase C, which hydrolyzes phosphatidylinositol 4,5 bisphosphate, giving rise to 1,2 diacylglycerol (DAG) and inositol trisphosphate (IP_3) (Dascal et al., 1986). DAG stimulates the activity of protein kinase C (PKC) (Moran and Dascal, 1989) and IP_3 leads to an increase in intracellular Ca^{2+} by releasing Ca^{2+} from intracellular stores and by directly or indirectly increasing the permeability of the plasma membrane to Ca^{2+} (Snyder et al., 1988). Elevated intracellular Ca^{2+} is manifested as an increase in conductance due to Ca^{2+} -activated Cl^- channels (Takahashi et al., 1987). The peak of the Cl^- conductance exhibits marked desensitization upon a second exposure to agonist. This series of events presents many sites for modulation, and products resulting from the pathway activation may provide the negative feedback controlling the sensitivity of the system (Boton et al., 1990). To sort through the myriad of interactions and gain understanding of the mechanism of desensitization, investigators have taken advantage of the fact that the oocytes can be readily injected with drugs, purified regulatory proteins, and intracellular messengers (Dascal et al., 1986). Such experiments have led Dascal and his co-workers (Boton et al., 1990; Singer et al., 1990) to suggest that there are two different types of desensitization involving two or more distinct sites. Despite the fact that these studies have improved our understanding of desensitization, its mechanistic basis remains to be determined.

Identification of the component(s) involved in desensitization may be aided by experiments with fractionated mRNA. Working with oocytes injected with size-fractionated mRNA, Lübbert et al. (1987b) found that the oocytes injected with those fractions encoding the receptor did not exhibit the typical prolonged desensitization observed with unfractionated mRNA. Thus, it was concluded that some messages contained in fractions other than the one coding for the receptor were likely to be responsible for the desensitization. As a step toward identifying the protein(s) involved in this desensitization, we coinjected mRNA transcribed from the mouse 5-HT_{1c} receptor clone with fractionated rat brain mRNA. For comparison we also injected mRNA transcribed from a rat M_1 receptor clone along with that from the 5-HT_{1c} receptor clone.

METHODS

RNA Preparation

Total RNA was extracted from the brains and hearts of 14–15-d-old rats using a modified LiCl/urea method (Dierks et al., 1981). Poly(A) RNA was prepared by column chromatography

using oligo-dT cellulose type III (Collaborative Research, Inc., Bedford, MA) and stored in sterile water at -80°C . Poly(A) RNA from brain was fractionated on a 6–20% sucrose gradient (Goldin et al., 1986). Fractions of equal volume were collected, ethanol-precipitated, and resuspended in a volume of water equal to that of the RNA solution applied to the gradient. Consecutive fractions were pooled in groups of three, ethanol-precipitated, and resuspended in volumes of sterile water equivalent to each of the three fractions. Our definition of the dose of fractionated RNA as microgram equivalents per microliter used for injection is illustrated by the following example. Suppose 100 μg of poly(A) RNA is applied to the gradient and each fraction after several ethanol precipitations is resuspended in 50 μl of H₂O. The actual mass of RNA in each fraction will depend on its size range, but we say that each fraction contains 100 $\mu\text{g}/50 \mu\text{l}$ or 2 μg eq/ μl . To determine the size range of the RNAs in the pooled fractions, RNAs were separated on formaldehyde gels (Sambrook et al., 1989) and assayed for poly(A) by hybridization to gel blots with ³²P-labeled poly(dT) (Goldin et al., 1986).

A DNA fragment for the mouse 5-HT_{1c} receptor (Lübbert et al., 1987a) was used as a hybridization probe to isolate several clones from cDNA libraries of normal mouse brain (Lonberg et al., 1988) and choroid plexus tumor (Lübbert et al., 1987a). A composite clone containing the entire protein coding region for the mouse 5-HT_{1c} receptor was constructed from overlapping cDNA clones. This 2.3-kb cDNA was subcloned into transcription vector pGEM3 (Promega Corp., Madison, WI) and the resultant plasmid was designated M1c2.3-pGEM3. Analysis of the translated amino acid sequences of the mouse 5-HT_{1c} receptor and that of the rat (Julius et al., 1988) indicated 97% homology. DNA from the mouse 5-HT_{1c} and rat M₁ receptor clones contained in pGEM 3 and pGEM 1, respectively, and DNA from the G protein γ subunit clones contained in pGEM 3Z were linearized; the mRNAs were then prepared by *in vitro* transcription.

Oocyte Preparation

Portions of an ovary were removed from *Xenopus laevis* anesthetized with MS-222 (Sigma Chemical Co., St. Louis, MO). To free the oocytes from the ovary and the investing follicle cells, small portions of the ovary were placed in OR-2 (82.5 mM NaCl, 2.0 mM KCl, 1.0 mM MgCl₂, and 5.0 mM HEPES, pH 7.5) containing 0.2% collagenase (Boehringer Mannheim Biochemicals, Indianapolis, IN) and gently agitated with an orbital shaker for 2 h. Stage V and VI oocytes were selected and placed in incubation medium ND-96 (96.0 mM NaCl, 2.0 mM KCl, 1.0 mM MgCl₂, 1.8 mM CaCl₂, and 5.0 mM HEPES, pH 7.5) supplemented with 50 $\mu\text{g}/\text{ml}$ of gentamicin (Sigma Chemical Co.) and 2.5 mM Na-pyruvate (Sigma Chemical Co.). After a second selection 12–24 h later, the oocytes were injected with 50 nl of RNA with a 510 injector (Drummond Scientific Co., Broomall, PA) using pipettes pulled on a 720 puller (David Kopf Instruments, Tujunga, CA) and broken manually to an outside diameter of 18 μm . The oocytes were allowed to translate for 3 d at 18–20°C in incubation medium which was changed daily.

Electrophysiology

Using a two-electrode technique, oocytes were clamped at -80 mV with a Dagan Corp. (Minneapolis, MN) 8500 instrument. Electrodes were filled with 3 M KCl and had a resistance of 1–2 M Ω . Permanent records were made on a chart recorder. Data treated statistically are given as the mean \pm SEM. Oocytes positioned with the animal hemisphere directed upward were placed atop a grooved Sylgard well (Dow Corning Corp., Midland, MI) in a chamber with a glass coverslip bottom. In this manner fluid could be exchanged beneath as well as above the oocyte and it was possible to focus on the oocyte with an IMT-2 inverted microscope (Olympus Corp., Lake Success, NY) equipped with a 40 \times objective (Carl Zeiss Inc., Thornwood, NY). The chamber volume was 200 μl . With fura-2 (Molecular Probes, Inc., Eugene, OR) the dead time of the system was determined to be ~ 6 s with equilibration of concentration achieved in 14 s. Control solution was ND-96 and experimental solution was made with the addition of agonist.

Two types of Ca^{2+} -free solutions were used. One was made by adding 0.5 mM EGTA to an ND-96 solution in which CaCl_2 was omitted. A second Ca^{2+} -free solution, OR-Mg (Boton et al., 1989), pH 7.5, contained 82.0 mM NaCl, 2.0 mM KCl, 20.0 mM MgCl_2 , 5.0 mM HEPES, and 0.5 mM EGTA. Flow rate was increased from 2–3 ml/min to 20 ml/min 6 s before superfusion with solution containing agonist. The rate of flow remained elevated during exposure to agonist and 45 s after returning to control solution. Solution temperature was maintained between 22 and 24°C.

Optical Methods

A Delta Scan system (Photon Technology International, South Brunswick, NJ) was coupled to the inverted microscope allowing fura-2 measurements of intracellular Ca^{2+} . The center of the field presented to the photomultiplier was $\sim 100 \mu\text{m}$ from the equatorial region of the oocyte and the size of the field was $52 \times 52 \mu\text{m}$. Fura-2 containing 100 mM KCl and 5 mM HEPES, pH 7.0, was injected via a Drummond pipette to give a final concentration of 50 μM in the ooplasm. A 540-nm short pass interference filter was placed in front of the photomultiplier, allowing the room to be illuminated by a safelamp fitted with a Kodak (Rochester, NY) ML-2 filter. The fura-2 data were digitally filtered with an exponential time constant of 2 s.

A 0.1-mM stock concentration of caged IP_3 (myoinositol 1,4,5-trisphosphate, $\text{P}^{4(5)}$ -1-(2-nitrophenyl)ethyl ester) (Calbiochem Corp., La Jolla, CA) was prepared in 5 mM HEPES, pH 7.0. Oocytes were injected with 5 pmol of caged IP_3 (Parker and Ivorra, 1990) and a xenon short arc flashlamp was used to release IP_3 . Light passed through a 308-nm cutoff filter, entered through the epifluorescence port of the inverted microscope, and was focused onto the vegetal hemisphere of the oocyte with the 40 \times objective. The field of illumination was $\sim 500 \mu\text{m}$. The flash energy was adjusted to give near-threshold responses that would facilitate upon the second flash and thus mimic the responses to agonist.

RESULTS

Desensitization in Coinjected Oocytes

The top traces of Fig. 1 depict the typical current responses to 10^{-9} M 5-HT obtained from oocytes injected with transcript of the 5-HT_{1c} clone. Previous investigations have shown that this current response is the result of a Ca^{2+} -activated Cl^- conductance (Dascal et al., 1986). Examination of the top trace of Fig. 1 *B* reveals that the second response to agonist also contains a smaller, less well-defined current that follows the peak current. As shown previously (Lübbert et al., 1987*b*), this secondary current becomes more pronounced if the agonist concentration is increased to 10^{-7} M. As shown in the upper trace, when there was a 2-min interval between exposures to agonist, the peak current response induced by the second exposure to agonist was larger than that elicited by the first exposure. This facilitation has been reported before in oocytes injected with 10 ng of brain poly(A) RNA and exposed to 5-HT concentrations less than 5×10^{-8} M (Lübbert et al., 1987*b*). Fig. 1, *A* and *B*, also illustrate the variability of facilitation among oocytes. The second response was 1.2 ± 0.2 and 3.4 ± 1.1 ($n = 3$) times the first for the oocytes in *A* and *B*, respectively.

Contrasting with this facilitation are the lower traces of *A* and *B*, which represent the current responses obtained for those oocytes coinjected with 5-HT_{1c} transcript and poly(A) RNA from rat brain (*A*) or rat heart (*B*). The second response was $65 \pm 20\%$ ($n = 5$) of the first for those oocytes injected with 50 ng of poly(A) RNA from rat

brain and $42 \pm 6\%$ ($n = 9$) of the first in those oocytes injected with 85.7 ng of poly(A) RNA from heart. Thus, for both types of poly(A) RNA, the peak of the second 5-HT-induced current was less than that of the first. The fact that mRNA from both brain and heart code for the proteins that desensitize argues for some lack of tissue specificity. Encouraged by these findings, we next sought to determine which fraction(s) of brain mRNA code for the proteins responsible for the desensitization. (We wish to note, for clarity, that the signal due to the poly(A)-encoded 5-HT_{1c} receptor itself is negligible for 10^{-9} M 5-HT [Lübbert et al., 1987b]).

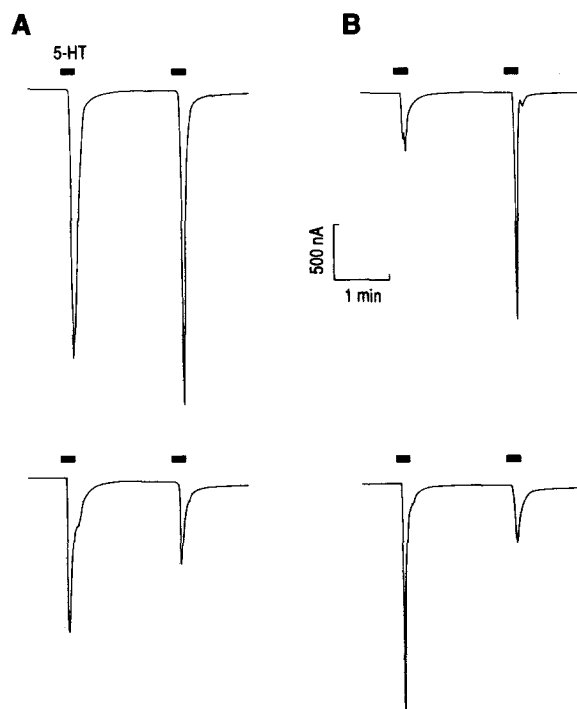


FIGURE 1. Voltage-clamp currents in *Xenopus* oocytes. Oocytes injected with 0.7 ng of transcript from the cDNA of the mouse 5-HT_{1c} receptor (*upper traces*) are compared with oocytes coinjected with the same amount of transcript and 50 ng of poly(A) RNA (*lower traces*) from rat brain (A) or 85.7 ng of poly(A) RNA from rat heart (B). Membrane potential was held at -80 mV. Change-over to superfusate containing 10^{-9} M 5-HT is indicated by the bar above the trace. Data are from typical experiments conducted with separate oocytes. A and B are from different batches of oocytes.

Fractionation of the Messages That Encode the Desensitizing Protein(s)

The effect of different low molecular weight mRNA fractions on the ratio of peak current amplitudes evoked by the first and second 5-HT exposures is displayed in Fig. 2. Substantial desensitization, $>60\%$, occurred in those oocytes injected with fractions J and K. The ³²P poly(dT)-probed RNA blot (Fig. 2B) reveals that the desensitizing protein is encoded by RNA in the size range of 0.3–1.5 kb. In oocytes coinjected with tRNA and the 5-HT_{1c} transcript, recovery was $148 \pm 29\%$ ($n = 9$) vs. $254 \pm 60\%$ ($n = 3$) for oocytes injected with the 5-HT_{1c} transcript alone. Although the injection of tRNA from *E. coli* did result in reduction in the extent of facilitation, this injection did not lead to desensitization, indicating that a nonspecific effect of low molecular weight RNA was not responsible for bringing about desensitization.

The effect of varying the amount of low molecular weight RNA component (0.3–1.5 kb) was explored (Fig. 3). The data indicate that marked desensitization is

present even with fivefold less RNA than our standard injection. This would suggest a lower limit of ~ 17.1 ng-eq/oocyte for the amount of message necessary to code for desensitization equivalent to the undiluted 0.3–1.5-kb RNA (see Methods for a definition of weight equivalents of RNA). Thus, the amount of 0.3–1.5-kb RNA that

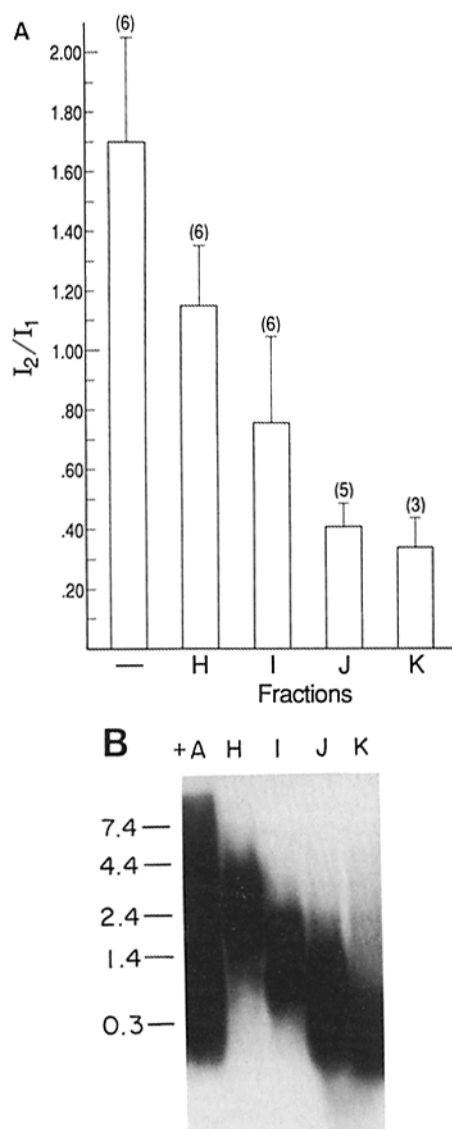


FIGURE 2. (A) Distribution of desensitization obtained with fractions of low molecular weight mRNA. Individual fractions recovered from the sucrose gradient were combined with the 5-HT_{1c} receptor transcript. Each oocyte was injected with 0.7 ng of transcript for the 5-HT_{1c} receptor. The amount of fractionated mRNA was 85.7 ng-eq/oocyte. The interval between exposures to 5-HT was always 2 min. The peak current responses evoked by the first and second exposures to 5-HT are denoted by I_1 and I_2 , respectively. Bars represent mean \pm SEM. (B) Northern blot of unfractionated and fractionated rat brain mRNA. RNA was electrophoresed and transferred to Hybond H. The poly(A) RNA was visualized by hybridization with ³²P-oligo-(dT) probe. Unfractionated mRNA is denoted by +A. H–K designate the consecutive pooled fractions. RNA sizes are indicated to the left.

was injected in subsequent experiments was never less than 80.0 ng-eq/oocyte to ensure strong desensitization.

Fig. 4 compares the peak current responses evoked by the first exposure to 5-HT in oocytes injected with the transcript from the 5-HT_{1c} and those coinjected with

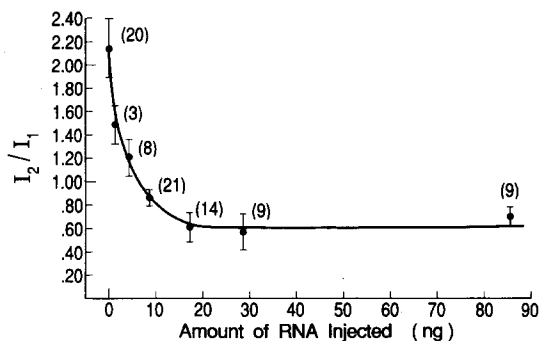


FIGURE 3. Effect of dilution of fraction J upon desensitization of the 5-HT_{1c} current response. The final concentration of the receptor transcript was always 0.7 ng/oocyte. The amount of fraction J RNA injected is indicated on the ordinate. The peak current responses evoked by the first and second exposure to 5-HT are denoted by I_1 and I_2 , respectively. Five batches of oocytes were used. Bars represent mean \pm SEM.

receptor clone transcript and 0.3–1.5-kb RNA. The coinjected oocytes showed a 20% reduction of peak current response. Thus, some proteins encoded by 0.3–1.5-kb RNA may act to reduce the peak amplitude without the requirement of a prior exposure to agonist. Alternatively, some component of this fraction acts to decrease the expression of the receptor, perhaps simply because of competition between injected RNAs during translation.

Fig. 5 depicts the effect of increasing the time between exposures of agonist on the extent of the recovery of the peak current response in oocytes injected with 5-HT_{1c} transcript and 0.3–1.5-kb RNA. With a 2-min interval between exposures to 5-HT, the peak current response recovered <10% even when the test concentration of 5-HT was increased to 10^{-8} M. In fact, the extent of recovery actually declined. If,

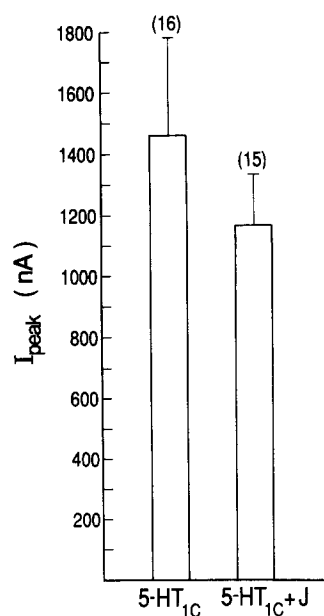


FIGURE 4. Effect of fraction J on the peak of the first 5-HT_{1c} current response. Amplitudes of the first current response with and without the coinjection of fraction J RNA are compared. The amounts of 5-HT_{1c} receptor transcript and fraction J mRNA injected per oocyte were 0.7 ng and 85.7 ng-eq, respectively. Three different batches of oocytes were used. Bars represent mean \pm SEM.

however, the interval was increased to 8 min, the recovery was nearly 20% when the test concentration of 5-HT was 10^{-9} M. As with oocytes injected with rat brain poly(A) RNA (Boton et al., 1990), recovery was more complete for longer intervals. Therefore, the desensitization was not permanent and the lack of responsiveness cannot be attributed to a general loss of cell viability.

Role of Calcium in Desensitization

Fura-2 experiments. To ascertain whether the rise of intracellular Ca^{2+} correlates with the increase in Cl^- conductance evoked by 5-HT, the level of Ca^{2+} was monitored with fura-2 while the oocyte was voltage clamped. The results of a typical experiment in which the oocyte was injected with message coding for the 5-HT_{1c} receptor are displayed in Fig. 6. When the Cl^- conductance peaked the Ca^{2+} level had risen to

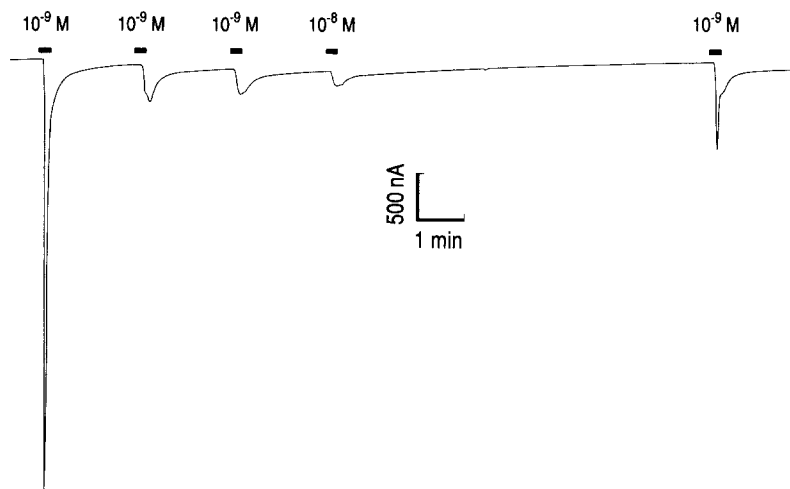


FIGURE 5. Effect of increasing the interval between exposures to 5-HT upon recovery from desensitization in oocytes coinjected with 5-HT_{1c} receptor transcript and fraction J. The amounts of 5-HT_{1c} receptor transcript and fraction J mRNA injected per oocyte were 0.7 ng and 85.7 ng-eq, respectively. Bars above the current traces indicate exposures to 5-HT.

only 17% of its maximum; Ca^{2+} concentration peaked when the Cl^- conductance had declined to 20% of its maximum. Due to complicating factors such as compartmentalization (Lupu-Meiri et al., 1988; Dreyfus et al., 1989) and the fact that the fura-2 ratio may include contributions from Ca^{2+} at points distant from the plasma membrane, it is not possible to conclusively determine the concentration of Ca^{2+} at the plasma membrane with fura-2. The complexity of Ca^{2+} release is further illustrated by recent imaging data (Brooker et al., 1990) indicating that it takes several minutes for Ca^{2+} to spread uniformly across the oocyte after agonist activation of the PIC pathway. Therefore, it would seem possible that the Ca^{2+} level may remain elevated even though the Cl^- conductance has returned to baseline, in which case Cl^- conductance would fail to serve as a reliable indicator of intracellular Ca^{2+} . There is evidence that the Cl^- conductance does desensitize to elevated Ca^{2+} (Boton et al.,

1989; Singer et al., 1990), suggesting that desensitization brought about by the protein(s) coded for by 0.3–1.5-kb RNA may be due, at least in part, to a maintained elevation of intracellular Ca²⁺.

To address this possibility, Ca²⁺ levels were monitored with fura-2 in oocytes injected with unfractionated rat brain poly(A) RNA and exposed twice to 10⁻⁷ M 5-HT with a 9–10-min interval between exposures, an interval sufficient for the Ca²⁺ level to return to baseline. In the four oocytes tested, the peak current upon a second exposure to 5-HT was 43 ± 5% of that evoked by the first exposure to the agonist. The fura-2 waveforms were quite variable between paired exposures to 5-HT (data not shown), vitiating any quantitative comparison of the Ca²⁺ release. Despite this limitation, fura-2 could be used to determine whether Ca²⁺ levels returned to baseline. These data indicate that the oocytes exhibited profound desensitization, even though both the Cl⁻ conductance and Ca²⁺ level returned to baseline before the

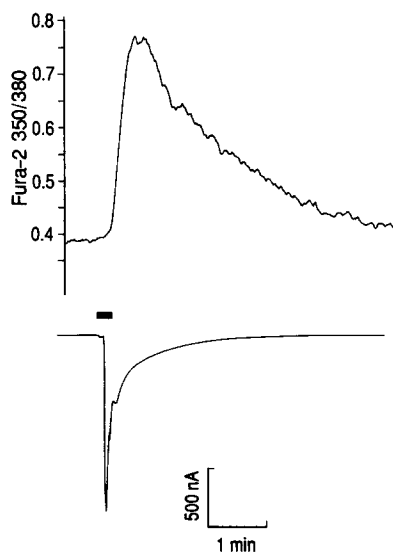


FIGURE 6. Comparison of the time course of the change in intracellular Ca²⁺ and Cl⁻ conductance. Ca²⁺ (upper trace) and Cl⁻ conductance (lower trace) were simultaneously measured. The bar above the current trace indicates exposure to 5 × 10⁻⁹ M 5-HT. The increase of the 350/380 nm ratio indicates an elevation of intracellular Ca²⁺.

second exposure to 5-HT. Thus, a sustained, elevated Ca²⁺ level is not responsible for delaying the recovery of the mechanism generating the peak current response.

Ca²⁺-free solutions. Receptor-linked inositide responses in a number of cells are influenced by the level of extracellular Ca²⁺ (Baird et al., 1989). In oocytes, Snyder et al. (1988) found that the second phase of the increase in Cl⁻ conductance evoked by IP₃ injections requires the entry of extracellular Ca²⁺. In addition, our preliminary fura-2 work with oocytes that were not voltage clamped revealed that injections of IP₃ result in a two-phase Ca²⁺ increase, with the second phase requiring the entry of extracellular Ca²⁺ (data not shown). To determine whether the recovery of 5-HT current responses is governed by external Ca²⁺, oocytes were superfused with Ca²⁺-free solution. Results from experiments with Ca²⁺-free solution (Fig. 7) indicate that the return to baseline of the Cl⁻ conductance is rapid with no slow phase as expected. Fig. 7 and data summarized in Table I reveal that removal of extracellular

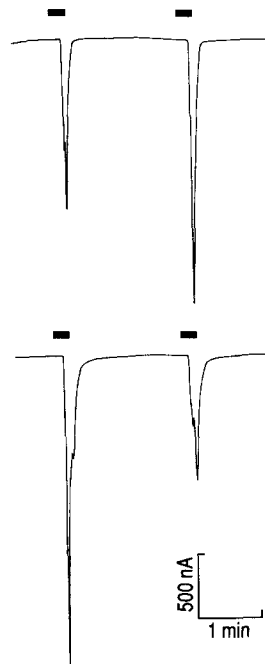


FIGURE 7. Role of extracellular Ca^{2+} in facilitation and desensitization of the 5-HT_{1c} current response. Bars above the current traces indicate exposures to 10^{-9} M 5-HT. The amounts of 5-HT_{1c} receptor transcript and fraction J mRNA injected per oocyte were 0.7 ng and 85.7 ng-eq, respectively. The effects of Ca^{2+} -free superfusate (0.5 mM EGTA added to ND-96 without CaCl_2) on facilitation in an oocyte not injected with fraction J (*upper trace*) and desensitization in an oocyte injected with fraction J (*lower trace*) are depicted.

Ca^{2+} did not prevent facilitation in oocytes injected with transcript from the 5-HT_{1c} or desensitization in oocytes coinjected with receptor transcript and 0.3–1.5-kb RNA. Therefore, neither phenomenon requires extracellular Ca^{2+} .

Photolysis of Caged IP_3 in Oocytes Exhibiting Desensitization

To test whether the oocytes injected with 0.3–1.5-kb RNA become desensitized by an increase in IP_3 , electrophysiological assays were conducted during the photolysis of injected caged IP_3 . Typical responses obtained are displayed in Fig. 8. The two light flashes (arrows) that preceded the exposures to 5-HT resulted in peak current responses exhibiting a rapid return to baseline. The second current response was 2.0 ± 0.5 ($n = 2$) times the first for those oocytes injected with the 5-HT_{1c} receptor

TABLE I

Calcium "Free" Superfusate Does Not Prevent Facilitation or Desensitization

Superfusate	Facilitation	Desensitization
ND-96*	$249 \pm 21\%$ (5)	$35 \pm 17\%$ (3)
OR-Mg*	$234 \pm 60\%$ (6)	$64 \pm 18\%$ (3)
ND-96 w/o CaCl_2	$270 \pm 58\%$ (3)	41% (1)

Composition of solutions is given in the Methods. Each oocyte was injected with 0.7 ng of 5-HT_{1c} receptor transcript. The effect of Ca^{2+} "free" superfusate on desensitization was determined in oocytes coinjected with 85.7 ng-eq of 0.3–1.5-kb RNA. Facilitation and desensitization computed as $I_2/I_1 \times 100$, where I_1 and I_2 are the peak current responses to the first and second exposures to 5×10^{-9} M 5-HT. Data are given as mean \pm SEM. *Data are from the same batch of oocytes.

transcript alone (upper trace) and 2.5 ± 0.5 ($n = 3$) times for those oocytes coinjected with the receptor transcript and 0.3–1.5-kb RNA (lower trace). Thus, the second current response evoked by photolysis of caged IP₃ underwent facilitation as reported by Parker and Miledi (1989). In oocytes coinjected with 0.3–1.5-kb RNA, the second peak current response evoked by 5-HT was $65 \pm 11\%$ ($n = 3$) of the first, demonstrating that 0.3–1.5-kb RNA encodes the desensitizing protein(s). We conclude that the desensitizing proteins do not influence the current response triggered by the photolysis of caged IP₃. However, it remains possible that the photochemically produced IP₃ desensitized the first 5-HT response slightly.

Low Molecular Weight RNA Encodes a Protein That Attenuates the M₁ Response

We also conducted experiments to determine whether proteins encoded by 0.3–1.5-kb RNA desensitize the acetylcholine M₁ current response (Fig. 9). To provide a

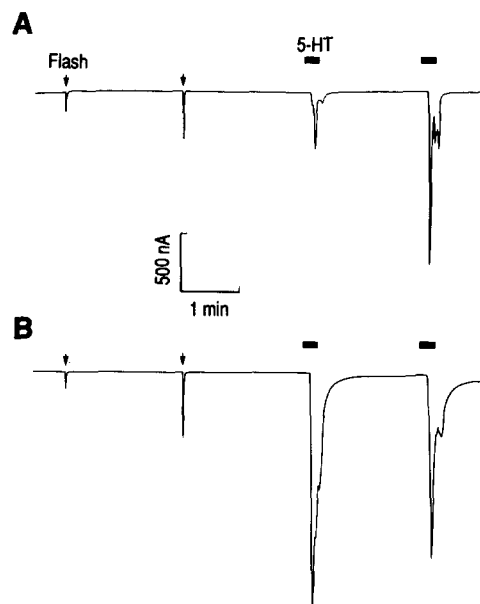


FIGURE 8. Comparison of current responses evoked by 5-HT and increases in IP₃. The photolysis of caged IP₃ is indicated by the arrows and exposure to 10⁻⁹ M 5-HT by the bars. The amounts of 5-HT_{1c} receptor transcript and fraction J mRNA injected per oocyte were 0.7 ng and 85.7 ng-eq, respectively. The current responses of an oocyte not injected with fraction J (*upper trace*) are compared with those of an oocyte injected with fraction J (*lower trace*). Data are from typical experiments conducted with separate oocytes.

comparison of the M₁ and 5-HT_{1c} current responses, transcripts of the two receptors were coinjected. In those oocytes injected with only the receptor transcripts (upper traces), the amplitude of the initial M₁ current response was 508 ± 199 nA ($n = 4$). The responses displayed facilitation; the second response was 2.9 ± 0.5 times the first. Interestingly, when these same oocytes were next exposed to 5-HT, the second 5-HT_{1c} current response was only 1.1 ± 0.2 times the first. Thus, it appears that the facilitatory effect involves a common component and can be saturated. Those oocytes in which 0.3–1.5-kb RNA was injected along with the two receptor transcripts (lower traces) responded differently. As shown in the lower traces, there is almost complete attenuation of the M₁ current response even for the first application of agonist. In three oocytes tested, both the first and second M₁ current responses were attenuated

to 13 ± 9 and 23 ± 15 nA, respectively. For three reasons it is unlikely that this attenuation is the result of poor translation of the injected messages by these oocytes. First, the initial 5-HT_{1c} response was robust ($3,775 \pm 978$ nA). Second, desensitization occurred; the second 5-HT_{1c} current response was $41 \pm 20\%$ of the first. Third, although the maximal current response to 5×10^{-8} M ACh was <30 nA, M₁ responses of 865 ± 176 nA ($n = 2$) were evoked when the concentration of ACh was

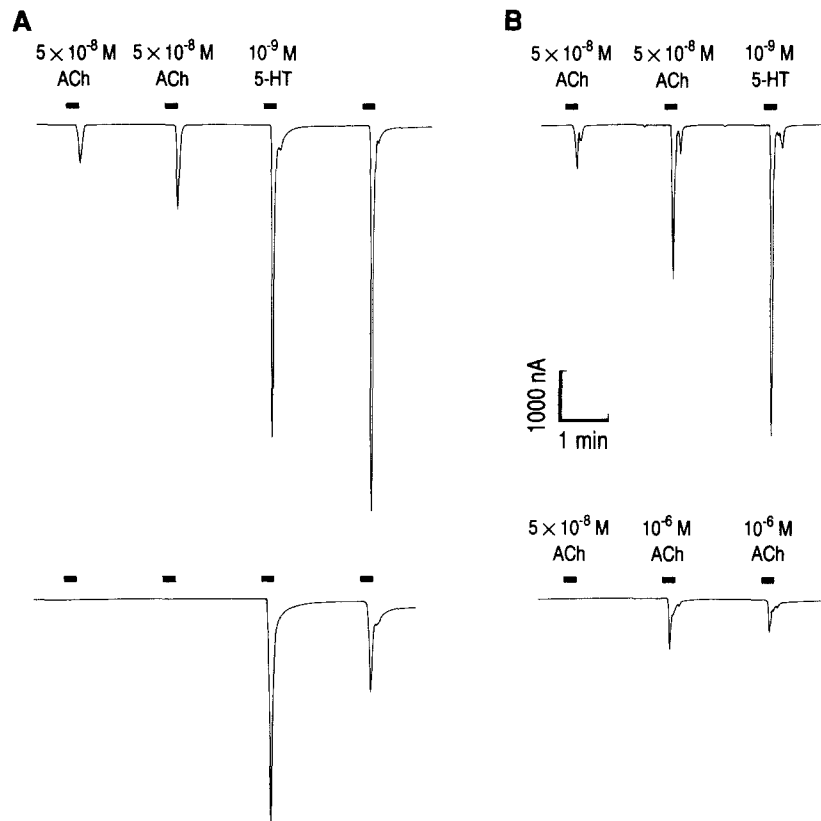


FIGURE 9. Comparison of current responses evoked by activating the muscarinic M₁ and serotonergic 5-HT_{1c} receptors. Each oocyte was coinjected with 0.5–0.7 ng of each transcript made from the cDNAs of the rat M₁ and mouse 5-HT_{1c} receptors. The upper traces are from oocytes injected with only the transcripts from the cDNAs of the receptors. The lower traces are oocytes coinjected with 80.0–85.7 ng-eq of fraction J. Data are from typical experiments conducted with separate oocytes (oocytes A and B from different batches of oocytes).

increased to 10^{-6} M. These currents were not endogenous M₁ responses, since noninjected oocytes from the same batch did not exhibit increases in Cl⁻ conductance when exposed to 10^{-6} M ACh. Taken together, these results suggest that some protein(s) coded by the low molecular weight fraction attenuate the response to ACh.

Fig. 10 illustrates the size distribution of the message(s) that code for attenuation of the M₁ current response. The inset in the figure shows examples of oocytes that were exposed to ACh once, then to 5-HT twice. Desensitization was present in oocytes

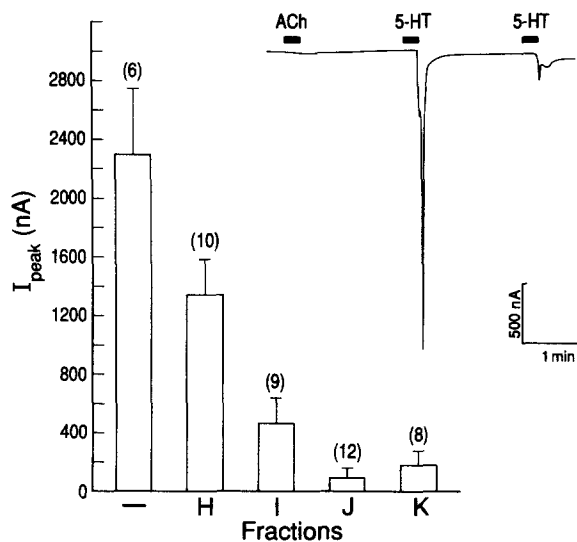


FIGURE 10. Distribution of attenuation of M₁ current response with fractions of low molecular weight RNA. Individual fractions recovered from a sucrose gradient were combined with the 5-HT_{1c} and M₁ receptor transcripts. Each oocyte was injected with 0.5 ng of receptor transcript and 80.0 ng-eq of fractionated RNA. The interval between agonist exposures was 2 min. Two different batches of oocytes were used. Bars represent mean \pm SEM. (Inset) Example of current responses obtained from an oocyte injected with fraction J. Bars above the current traces indicate exposures to 10^{-6} M ACh and 10^{-9} M 5-HT.

coinjected with fraction J. These data indicate that within the resolution of the sucrose gradients the messages coding for attenuation of the M₁ response and desensitization of the 5-HT_{1c} response are located in the same molecular weight range and could be identical.

Tests with G Protein γ Subunits

Noting that attenuation of the M₁ response by proteins encoded by 0.3–1.5-kb RNA is remarkably similar to that caused by the injection of $\beta\gamma$ subunits of G proteins from brain and erythrocytes (Moriarty et al., 1988), we next sought to determine if two recently cloned γ subunits of G proteins from brain (Gautam et al., 1989, 1990) were responsible for attenuation. This hypothesis is attractive because of the relatively low molecular weights of the G γ subunits and their mRNAs. A comparison of the amplitudes of the M₁ response in those oocytes injected with either G γ (₂) (4.4 kb) or G γ (₃) (1.0 kb) with those oocytes injected with only the receptor transcripts can be found in Table II. The data indicate that the transcripts from the two G γ subunits do not code for the proteins involved in attenuation.

TABLE II
G Protein γ Subunits Do Not Attenuate the ACh Response

ACh response amplitude		
Control	G γ (2)	G γ (3)
1,763 \pm 391 (11)	1,666 \pm 197 (9)	1,935 \pm 470 (10)

ACh concentration was 10^{-6} M. Control oocytes were injected with 0.67 ng of the M₁ receptor transcript. To determine if either γ subunit reduced the amplitude of the ACh (M₁) response, each oocyte was coinjected with 4.0 ng of G γ (2) or G γ (3). The maximal response of 10 oocytes not injected with RNA was 10 nA. Data are given as mean \pm SEM.

DISCUSSION

Apparent Size of Proteins Involved in Sensitivity Control

Our results indicate that a low molecular weight fraction of rat brain mRNA (0.3–1.5 kb) codes for the protein(s) that brings about desensitization of the 5-HT_{1c} and attenuation of the M₁ responses in oocytes. Although we have no direct evidence, it is tempting to suggest that the same protein is responsible for both effects, because the size ranges of the messages encoding these proteins are the same and the phenomena are similar. While it is impossible to assign to any protein or group of proteins a definitive role in desensitization or attenuation, certain potential regulatory proteins may be evaluated on the basis of size. Because the mRNAs possess noncoding regions which typically represent 50% of the mRNA (Sutcliffe, 1988), the maximum size of the proteins translated is probably 30 kD or less. On the less likely assumption that the mRNAs involved possess no noncoding regions, the maximum size of the

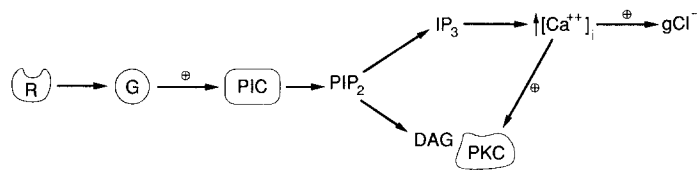


FIGURE 11. Simplified diagram of the major events involved in the transduction pathway triggered by serotonin or acetylcholine. On binding of agonist the receptors activate a GTP-binding protein which increases the activity of PIC, resulting in the metabolism of PIP₂ (phosphatidylinositol 4,5 biphosphate) to DAG and IP₃ (inositol 1,4,5 trisphosphate). Ca²⁺ released from the IP₃ stores evokes a number of cellular responses, including an increase of conductance in the Cl⁻ channels of the plasma membrane. Metabolites of IP₃ may also serve to facilitate the increase of intracellular calcium. DAG, in conjunction with Ca²⁺, activates PKC, which regulates diverse cellular events including elements of the transduction pathway. Following several steps products from the metabolism of IP₃ and DAG are used to resynthesize PIP₂ (not shown).

proteins translated could be 60 kD or less; allowing for uncertainties associated with SDS-PAGE estimates of molecular weights, it would be prudent to consider proteins with apparent molecular weights even as large as 65 kD.

Site of Sensitivity Control

Considering the complexity of the pathway that links receptor activation with the increase in chloride conductance (Fig. 11), there are several potential sites at which modulatory proteins may act. Singer et al. (1990) found that IP₃ injections decreased the response to application of 5-HT 30–300 min later and therefore suggested that IP₃ plays a role in desensitization. There are many technical differences between the experiments of Singer et al. (1990) and the present study. However, our experiments with caged IP₃ indicate that the injection of the desensitizing RNA fraction, 0.3–1.5 kb, does not prevent the facilitation of the IP₃-induced current response initiated by the photolysis of caged IP₃ (Fig. 8 B), rendering it unlikely that desensitization

involves a step after the generation of IP₃. Nonetheless, Ca²⁺ or metabolites of IP₃ could be responsible for triggering desensitization, if they work in concert with some other products of the cascade such as 1,2 diacylglycerol, which with Ca²⁺ would be expected to increase the activity of PKC (Nishizuka, 1988).

It is very important to note that when the M₁ response is attenuated, the 5-HT_{1c} response can still be evoked (Fig. 9 B). If one assumes that IP₃ generated by the activation of either receptor activates the same pool of IP₃ receptors, then attenuation of the M₁ response must be confined to the components of the pathway that precede the formation of IP₃. Of course, unfractionated poly(A) rat brain RNA contains messages for both the M₁ receptor and the low molecular weight factor responsible for attenuation described in this paper; yet oocytes injected with poly(A) produce robust responses to ACh (Dascal et al., 1986). This difference may result from the stoichiometry of the attenuating protein(s) and M₁ receptors encoded by poly(A). It is also possible that the protein(s) responsible for attenuation is but one of several diverse factors that modulate the chain of events leading to activation of PIC. Because both receptors couple to PIC via G proteins, and only the M₁ current response is nearly completely attenuated before application of agonist, it seems logical that this attenuation occurs before PIC. However, more than one type of PIC exists and it is possible that each receptor couples via distinct G proteins to different PICs. Thus, attenuation may occur at the receptors, G proteins, and/or PICs.

Type of Sensitivity Control

The fact that desensitization of the peak 5-HT_{1c} current response is not permanent suggests a role for reversible covalent modification of some component(s) of the transduction pathway. An attractive possibility is that phosphorylation/dephosphorylation reactions may modulate the activity of one or more of the proteins of the cascade. Perhaps 0.3–1.5-kb RNA encodes proteins with modulatory functions analogous to those of the rhodopsin (Hurley, 1987; Uhl et al., 1990) and β -adrenergic (Hausdorff et al., 1990; Lohse et al., 1990) systems. For instance, the α_1 -adrenergic receptor, which couples to the PIC pathway, undergoes an agonist-independent phosphorylation that impairs the coupling of the receptor with its G protein (Lefkowitz and Caron, 1988).

Attenuation of the M₁ response occurs without requiring a prior exposure to agonist; unlike desensitization of the 5-HT_{1c} response, attenuation is agonist independent. Attenuation could nonetheless still involve phosphorylation. Perhaps 0.3–1.5-kb RNA encodes the proteins necessary to maintain phosphorylation of the M₁ receptor. If the scheme outlined above is correct, then the 5-HT_{1c} and M₁ receptors differ in terms of their degree of phosphorylation and/or G protein coupling after phosphorylation because the 5-HT_{1c} current response is not significantly attenuated, while the M₁ current response is attenuated by > 95%.

Single Protein Hypothesis

Both 5-HT_{1c} desensitization and M₁ attenuation may share a single type of protein. One possibility is GAP-43 (1.5 kb) (Karns et al., 1987), which disrupts phospholipid metabolism (Van Dongen et al., 1985) and possibly receptor–G protein interaction (Strittmatter et al., 1990). It is also plausible that 0.3–1.5-kb RNA codes for one or

more proteins that modulate the activity of kinases and phosphatases. One possibility that we find intriguing is that an expressed phosphatase inhibitor promotes a phosphorylated, inactive form of the 5-HT_{1c} and M₁ receptors. The M₁ receptor would be inactivated by an agonist-independent phosphorylation; the 5-HT_{1c} receptor, on the other hand, would undergo inactivation by an agonist-dependent phosphorylation and dephosphorylation would be slowed by the phosphatase inhibitor. Interestingly, two phosphatase inhibitors, I-1 (18.7 kD) and I-2 (22.8 kD) (Cohen, 1989), appear to inhibit the activity of at least some oocyte phosphatases (Huchon et al., 1981; Foulkes and Maller, 1982).

Other Possibilities

5-HT_{1c} desensitization. The proteins encoded by 0.3–1.5-kb RNA may modulate the activity of those elements of the phosphoinositide phospholipase C (PIC) pathway in such a manner as to enhance the metabolism of IP₃, thereby reducing the half-life of IP₃. As a consequence, less Ca²⁺ would be mobilized from internal stores and the Cl⁻ current would be smaller. Phospholipase A₂ (PLA₂), which ranges in weight from 12 to 18 kD (Rhee et al., 1989), might be encoded by 0.3–1.5-kb RNA and should therefore be considered as well.

Ca²⁺ binding proteins could be coded for by 0.3–1.5-kb RNA. These proteins affect the activity of a broad range of target enzymes including several kinases (Kennedy, 1989). The results we obtained with Ca²⁺-free superfusate indicate that, if Ca²⁺ binding proteins are involved in desensitization, then entry of external Ca²⁺ is not necessary. Our fura-2 data obtained from oocytes injected with poly(A) RNA indicate that a maintained elevation of the level of intracellular Ca²⁺ is not required for desensitization. Even so, elevating the intracellular Ca²⁺ concentration may provide an important component of the trigger mechanism of desensitization.

As noted above, we cannot definitely eliminate the possibility that the IP₃ receptor and points beyond are also targets of modulation. Whether the IP₃-gated stores recover their ability to release an equivalent amount of Ca²⁺ after the initial 5-HT response has yet to be answered. If, as in other cells, there is communication between the IP₃-sensitive and -insensitive stores (Gill et al., 1989; Schulz et al., 1989), then a reduction in the amount of Ca²⁺ released could conceivably result from the reduced refilling of either store. Our finding that desensitization is present even when intracellular Ca²⁺ levels return to baseline renders it unlikely that desensitization is caused by attenuation of the Ca²⁺ pumps.

M₁ attenuation. We do not yet know whether attenuation of the M₁ response arises from a shift in the dose–response relation or from a frank suppression. In any case, this effect may involve G proteins. For instance, the attenuation resembles the results of Moriarty et al. (1988, 1989) who found that the β (35–36 kD) and γ (5–8 kD) subunits of G proteins injected as a complex attenuated the responses of endogenous muscarinic as well as that of exogenous receptors that couple to PIC. Our data indicate that neither Gγ₍₂₎ nor Gγ₍₃₎ attenuate the M₁ response, but do not permit us to reject the notions that some type of Gγ is involved in this attenuation or that the attenuation requires coinjection of both β and γ subunit RNAs. Another possibility to consider is that attenuation of the M₁ response may involve one of the low molecular weight GTP-binding proteins (20–25 kD) (Hoshijima et al., 1990).

Assuming that the M₁ receptor was to bind more efficiently to one of these G proteins, and further, that this protein was to interact preferentially with membrane-bound effectors other than PIC or no effector at all, then there would be less receptor to interact with the PIC pathway. This speculation implies that the 5-HT_{1c} and M₁ receptors differ in affinities for different G proteins, since the 5-HT_{1c} current response is not significantly attenuated by 0.3–1.5-kb RNA.

Possibilities for Future Research

The present data allow us to suggest several mechanisms that could underlie either desensitization of the 5-HT_{1c} response or attenuation of the M₁ response. We now have a clear indication of the size messages that code for the protein(s) responsible for these phenomena and our assay may provide an appropriate method for the expression cloning of the proteins involved. Knowing the primary amino acid sequence would help in deciding how and where these protein(s) act.

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