Kinetics of the functional loss of different muscarinic receptor isoforms in *Xenopus* oocytes

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Native *Xenopus* oocytes express two isoforms of muscarinic receptors that mediate qualitatively different physiological responses. Oocytes of the majority of donors (common) express M_3 -like receptors (M_3 Rs) at comparable densities at both the animal and vegetal hemispheres of the cell. Rare (variant) donors possess oocytes that express mainly M_1 -like receptors (M_1 Rs), localized predominantly at the animal hemisphere. We have investigated the apparent degradation of these two isoforms and its relationship to their hemispheric distribution. Cycloheximide (CHX) caused a time-dependent decrease in receptor-mediated responses and [3 H]quinuclidinyl benzylate (QNB) binding in oocytes from both types of donors. The $t_{1/2}$ values ranged between 3 and 7 h. Removal of CHX resulted in rapid recovery of the response. This implied rapid degradation and turnover of both types of receptors. The loss of M_1 Rs was more rapid than that of M_3 Rs. Moreover, the decrease was more rapid and more extensive on the animal hemisphere in both types of donors. Injection of oocytes expressing either receptor isoform with specific antisense oligonucleotides complementary to either m_1 or m_3 muscarinic receptors (from mouse) showed receptor loss at approximately the same rate as that calculated from experiments with CHX. Furthermore, oocytes of variant donors express M_1 Rs exclusively on the animal hemisphere, while the residual activity found on the vegetal hemisphere of the cell was mediated by M_3 Rs. Inhibition of putative receptor glycosylation with tunicamycin caused a rapid decrease in receptor-mediated responses and radioligand binding on M_1 Rs, but had virtually no effect on M_3 Rs. The expression of cloned m_1 muscarinic receptors, however, was not affected by tunicamycin, suggesting that glycosylation is not a general prerequisite for the functional expression of muscarinic receptors.

INTRODUCTION

Xenopus oocytes have been extensively used as a convenient model system to investigate the molecular events of biological signal transduction mediated by both intrinsic and acquired cell membrane receptors [1–3]. This giant cell allows facile biochemical and electrophysiological manipulations at a single-cell level. Moreover, oocytes express foreign receptors that appear to efficiently couple to the intrinsic signal transduction pathway, i.e. phosphatidylinositol 4,5-bisphosphate hydrolysis and generation of the two intracellular messengers inositol 1,4,5-trisphosphate and diacylglycerol, resulting in the mobilization of cellular and external Ca^{2+} . The physiological response to these events is the increased gating of chloride channels, measurable as complex depolarizing current [3].

We have reported that native oocytes express two types of muscarinic responses [4–6], and that the two phenotypes of oocytes differ in their hemispheric distribution of muscarinic receptors [7]. Recently we have shown that the oocytes of a majority of donors express M_3 -like muscarinic receptors (M_3 Rs), while oocytes of a small fraction of donors express predominantly M_1 -like muscarinic receptors (M_1 Rs) [8].

In the present work, we have studied the loss of function of the two types of native muscarinic receptors following inhibition of protein synthesis with cycloheximide (CHX), and its relationship to their hemispheric distribution. We have also investigated the role of receptor glycosylation in their functional expression. Our results suggest that, despite the similarity in their structure and mechanism of action, the two receptor subtypes are processed differently by the oocyte.

MATERIALS AND METHODS

Experimental animals

Adult *Xenopus* females, purchased from the South African Xenopus Facility (Noordhock, South Africa 7985), were maintained and fed as described [9]. The animals were cold-anaesthetized and ovary fragments were dissected into ND96 medium (composition given below). Stage 5 or 6 oocytes [10] were separated and maintained at 20 °C in NDE96 medium. When the same donors were used repeatedly, dissections were spaced 2 to 3 weeks apart to allow full recovery of the animals from surgery. Where indicated, oocytes were defolliculated with collagenase [6].

Electrophysiology

Electrophysiological methods were described in detail elsewhere [6]. Briefly, all experiments were performed under a two-electrode voltage clamp using a DAGAN 8500 or Axoclamp intracellular clamp. Oocytes were routinely voltage-clamped at –100 mV to minimize potassium current. Acetylcholine (ACh) was added rapidly and directly to the bath in > 1 ml of ND96 in order to avoid the dead time of the perfusion system and the gradual build up of the agonist concentration.

Assay of hemispheric responsiveness to ACh

The method was described in detail in [6]. Briefly, a special lucite perfusion bath was constructed with depressions (0.8–1.2 mm diameter; 3 mm depth) drilled in its bottom. An oocyte was gently pressed into a depression that was slightly smaller than its diameter, in a way that allowed the exposure of only one

Abbreviations used: ACh, acetylcholine; CHX, cycloheximide; M_1 R, amphibian M_1 -muscarinic receptor isoform; M_3 R, amphibian M_3 -muscarinic receptor isoform; m_1 R, rat m_1 -muscarinic receptor; QNB, quinuclidinyl benzylate; TM, tunicamycin.

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hemisphere of the cell to bath solution. The oocyte was maintained under two-electrode voltage clamp and ACh was applied directly to the bath in a large volume of the medium.

Radioligand binding

The protocol was similar to that described by us recently [6,7,11]. Briefly, single oocytes were incubated for > 3 h in ND96 solution (20 μ l/oocyte) with 1.0 nM-[³H]quinuclidinyl benzylate ([³H]QNB) at 4 °C. Oocytes were rapidly washed 5–6 times in 4 ml of ice-cold ND96 solution and counted for radioactivity. Non-specific binding, determined in the presence of 1 μ M-atropine, was 50–75% of total binding. Receptor numbers were calculated from the specific radioactivity of the QNB and the efficiency of counting (30%).

Assay of hemispheric receptor distribution

Oocytes were incubated with the ligand and the excess label was washed off as described above. Each oocyte was inserted into an orifice (approx. 1 mm in diameter) made in an agar disc (5 mm in diameter; 3 mm thickness; 2.5% agar dissolved in hot ND96) with the equator perpendicular to the surface of the disc. The oocyte in the disc was quickly frozen on a block of solid CO₂. The two hemispheres were cut apart under a binocular microscope with a precooled razor blade and the halves were counted for radioactivity in 4.5 ml of Hydroluma (LUMAC) scintillation cocktail. Oocytes incubated in the presence of 1 μ M-atropine (to determine non-specific binding) were processed in the same way, and the mean non-specific binding of each hemisphere was subtracted from the corresponding mean total binding.

Intracellular injections of antisense oligonucleotides

The oligonucleotides were synthesized on a 380B Applied Biosystems DNA synthesizer and purified on a 20% acrylamide/8 M-urea preparative sequencing gel. For each muscarinic receptor subtype, two different oligomers were synthesized, based on the published rat muscarinic ACh receptor mRNA sequences [12]. They were complementary to nucleotide sequences corresponding either to the *N*-terminus or the third cytoplasmic loop. Oligomers were complementary to bases 189–225 (anti-m₁/N) and 1169–1210 (anti-m₁/CL) of the m₁ receptor (m₁R), and to bases 47–97 (anti-m₃/N) and 1184–1231 (anti-m₃/CL) of the m₃ receptor. The specificity of the different oligonucleotides has been previously shown by Northern analysis in rat brain, where a hybridizing band could be detected with both the appropriate size and correct brain region distribution, and by *in situ* hybridization histochemistry [13,14].

The oligonucleotides were dissolved in distilled water, and 9.6 ng (30–50 nl) was injected into single oocytes using a VWR digital microdispenser (10 μ l maximal delivery) with the capillary pulled to a 5–20 μ m tip. Control oocytes were sham-injected with the same volume of distilled water.

Expression of cloned m₁Rs

In vitro transcribed mRNA from cloned rat brain m₁R was injected into defolliculated oocytes in a volume of 50 nl. Responses were assayed 16–24 h later.

Analysis of results

All experiments were repeated several times in oocytes from different frogs. The number of oocytes assayed for each condition is denoted by *n* and the number of donors by *N*. Experiments were performed by assaying four or more oocytes within each experiment, and means $\pm$ S.E.M. values were determined. Statistical significance was determined by Student's *t* test.

Solutions and chemicals

The composition of ND96 was (in mM): NaCl 96, KCl 2, MgCl₂ 1, CaCl₂ 1.8, Na-Hepes 5, pH 7.5. NDE96 included additionally sodium pyruvate (2.5 mM) and cefotaxime (50 μ g/ml). Ach, atropine, collagenase (type IA), tunicamycin (TM) (a mixture of A, B, C and D types [15]) and CHX were purchased from Sigma. [³H]QNB was from Amersham. *In vitro* transcribed mRNA of cloned m₁R was a gift from Dr. J. Battey (NINDS, NIH, Bethesda, MD, U.S.A.). All other chemicals were of analytical grade.

RESULTS

Effect of CHX on hemispheric responses and receptor distribution

In the following series of experiments we have investigated the effect of inhibition of protein synthesis on the functional expression of M₃Rs and M₁Rs in oocytes of common and variant donors respectively.

Incubation of oocytes expressing the M₃Rs with CHX (10 μ g/ml, a concentration shown to fully inhibit total protein synthesis in oocytes [16,17]) resulted in a rapid decrease of the response measured on the animal hemisphere of the cell. The activity followed roughly first-order kinetics, with a *t*_{1/2} of approx. 5 h (Fig. 1a). The activity measured on the vegetal hemisphere decreased at a slightly lower rate (*t*_{1/2} 7 h). These results were confirmed by muscarinic ligand binding experiments. In oocytes incubated with CHX for 5–7 h, specific [³H]QNB binding decreased from 1.1 $\pm$ 0.2 to 0.5 $\pm$ 0.2 fmol/oocyte on the animal

Fig. 1. Effect of CHX on muscarinic responses

Oocytes from common (a) or variant (b) donors were incubated with 10 μ g of CHX/ml for 2–24 h. At the indicated times, oocytes were assayed for responses to 0.1 mM-ACh on the animal (◆) and vegetal (■) hemispheres. □, Control (no CHX). The data are presented as percentages of control amplitudes obtained in oocytes of the same batch incubated in the absence of CHX, and are means. Each time point for each hemisphere is the mean of 6–10 determinations from three common and four variant donors. The same number of oocytes was assayed in control responses.

hemisphere ($P < 0.025$) and from 0.9 ± 0.2 to 0.4 ± 0.1 fmol/oocyte on the vegetal hemisphere ($P < 0.025$) ($n = 7-11$ oocytes for each condition; a similar number of cells was used for non-specific binding, $N = 3$).

In oocytes of variant donors that express mainly M_1 Rs, CHX inhibited the response measured on the animal hemisphere with an apparent $t_{1/2}$ of 3 h. The decrease of activity on the vegetal hemisphere was slower ($t_{1/2}$ 5 h). Despite prolonged incubation with CHX, the response did not continue to decrease, but exhibited residual activity of 20 and 50 % of control on the animal and vegetal hemispheres respectively (see Fig. 1*b*). Binding experiments confirmed that the loss of responses corresponded to the loss of receptor binding activity. CHX treatment reduced [3 H]QNB specific binding from 1.4 ± 0.4 to 0.6 ± 0.3 fmol/oocyte on the animal hemisphere ($P < 0.05$, $n = 14$; $N = 2$). The binding on the vegetal hemisphere was too low to determine with sufficient accuracy (usually below 0.3 fmol/oocyte).

We have tested the reversibility of the effect of CHX in oocytes of variant donors. Oocytes were exposed to CHX for 8 h, and displayed 15 % of the control response in this series of experiments. At 1 h after the removal of CHX, the response recovered to 30 % of control values, and by 2.5 h the response had completely recovered.

Inhibition of receptor synthesis by antisense oligonucleotides

The results described in the previous section do not necessarily pertain to the turnover of the receptor molecule. Inhibition of the synthesis of another protein, putatively required for the receptors' functional expression, would have brought about the same results.

We have previously shown that specific inhibition of receptor synthesis by short oligonucleotides complementary to either m_1 Rs or m_3 Rs caused a significant decrease in muscarinic responses measured 2.5–5 h after the injection of the antisense DNA [8]. These experiments were instrumental in the identification of the muscarinic receptors in oocytes of common or variant frogs as M_3 and M_1 isoforms respectively.

We have now repeated these experiments and analysed the responses to the activation of the muscarinic receptors in each hemisphere. Moreover, in order to test if the results obtained with CHX represented the true degradation rate of the receptors, we assayed the muscarinic responses in oocytes from common or variant donors at times that corresponded to their respective half-lives, as calculated from experiments with CHX.

Oocytes from common donors were injected with 9.6 ng of the anti- m_3 oligonucleotide/cell and were assayed 3.5–4.5 h later at the animal hemisphere, and 4.5–6.0 h later at the vegetal hemisphere. As was predicted from the results of the experiments with CHX, the muscarinic responses decreased to 53 and 50 % of control values at the animal and the vegetal hemispheres respectively. Oocytes injected with anti- m_1 oligonucleotides showed a negligible effect (88–94 % of values obtained in sham-injected controls; Table 1).

Similarly, oocytes from variant donors were injected with the same amount of anti- m_1 oligonucleotide and assayed between 2.5 and 5 h after injection. While the responses at the animal hemisphere decreased to 51 % of the control values, there was no effect on the activity assayed at the vegetal hemisphere. This unexpected result was further confirmed by control experiments. The injection of anti- m_3 oligonucleotides had no effect on the response measured at the animal hemisphere. At the vegetal hemisphere, however, the injection of anti- m_3 oligonucleotides resulted in a significant decrease in the response (to 60 % of control values; Table 1). These results suggested that oocytes from variant donors may express two different receptor subtypes in the same cell.

Table 1. Effect of antisense oligonucleotides on muscarinic responses

Oocytes from common or variant donors were injected with 9.6 ng of either anti- m_1 or anti- m_3 antisense oligonucleotides. Hemispheric responses to 0.1 mM-ACh were assayed 3.5–4.5 h (for common/animal), 4.5–6.0 h (common/vegetal) or 2.5–3.5 h (for variant/animal) and 4.0–5.0 h (variant/vegetal) after injection. The results are presented as mean amplitudes $\pm$ S.E.M. and as percentages of control responses assayed at the same time points. NS, not significant.

	Response		<i>n</i> , <i>N</i>	<i>P</i>
	(nA)	(% of control)		
Variant donors				
Control animal	3310 ± 421	100	55, 3	–
+ anti- <i>m</i> ₁	1689 ± 313	51	38, 3	< 0.005
+ anti- <i>m</i> ₃	3356 ± 473	101	34, 3	NS
Control vegetal	1013 ± 175	100	28, 3	–
+ anti- <i>m</i> ₁	1102 ± 288	109	23, 3	NS
+ anti- <i>m</i> ₃	609 ± 136	60	25, 3	< 0.05
Common donors				
Control animal	104 ± 28	100	16, 3	–
+ anti- <i>m</i> ₁	92 ± 27	88	18, 3	NS
+ anti- <i>m</i> ₃	55 ± 8	53	25, 3	< 0.03
Control vegetal	149 ± 41	100	14, 3	–
+ anti- <i>m</i> ₁	140 ± 32	94	21, 3	NS
+ anti- <i>m</i> ₃	75 ± 14	50	23, 3	< 0.025

Effect of TM on hemispheric responses and receptor distribution

Our data suggest that the two muscarinic receptor isoforms are degraded at different rates. Since all of the cloned muscarinic receptors possess at least one glycosylation consensus sequence close to the *N*-terminus of the molecule [12,18], we have investigated the role of glycosylation in the functional loss of hemispheric distribution of the receptors.

TM (2 μ g/ml) has been shown to fully inhibit glycosylation in *Xenopus* oocytes without affecting protein synthesis [19]. TM affected the hemispheric responses to ACh differently in oocytes from the two populations of donors. In oocytes from common donors (expressing mainly M_3 Rs), even prolonged treatment with TM had no effect on the response to ACh measured at either hemisphere (Fig. 2*a*). These results were confirmed by binding experiments with [3 H]QNB. The number of binding sites on the animal hemisphere was 1.3 ± 0.3 (control) and 1.1 ± 0.3 (TM) fmol/oocyte, while on the vegetal hemisphere the corresponding values were 0.8 ± 0.3 and 1.0 ± 0.2 fmol/oocyte ($n = 16$; $N = 2$).

In oocytes from variant donors, however, the response to ACh measured at the animal hemisphere of the cell decreased rapidly (to 43 and 14 % of untreated controls after 3 and 7 h respectively of exposure to TM; Fig. 2*b*). Treatment with TM for 15 h did not result in greater inhibition. The response measured at the vegetal hemisphere was largely unaffected by TM, and in some experiments was even increased (Fig. 2*b*). Here also, radioligand binding corresponded to the electrophysiological results. A major decrease in QNB binding was observed at the animal hemisphere (from 2.0 ± 0.6 to 0.6 ± 0.4 fmol/oocyte; $P < 0.025$, $n = 16$; $N = 1$). The binding values at the vegetal hemisphere were too low to determine accurately in oocytes from these donors [7]. Washing the oocytes free of TM resulted in a complete recovery of the response at the animal hemisphere 16 h later, indicating that the effect of TM was reversible (results not shown).

Fig. 2. Effect of TM on muscarinic responses

Oocytes from common (a) or variant (b) donors were incubated with 2 μ g of TM/ml for 2–24 h. At the indicated times, oocytes were assayed for responses to 0.1 mM-ACh on the animal ($\blacklozenge$) and vegetal ($\blacksquare$) hemispheres. $\square$, Control (no TM). The data are presented as mean percentage of control amplitudes obtained in oocytes of the same batch incubated in the absence of TM. Each time point for each hemisphere is the mean of 6–10 determinations from three common and four variant donors. The same number of oocytes was assayed in control responses.

Effects of CHX and TM on the expression of m_1 Rs

The M_1 and M_3 receptor isoforms have been tentatively identified by classical pharmacological techniques and by inhibition of biosynthesis using specific antisense oligonucleotides [8] synthesized according to the sequences published for the rat receptor species [12]. Although all of the muscarinic receptor subtypes possess at least one glycosylation consensus sequence, the amino acid sequence of the frog muscarinic receptors is unknown. Hence it was feasible that the lack of TM sensitivity in oocytes from common frogs may have resulted from the lack of such a sequence in the amphibian receptor. We have, therefore, expressed cloned m_1 Rs in oocytes and tested the effect of TM on their hemispheric expression.

Oocytes were assayed approx. 24 h after injection of m_1 R mRNA. The response recorded at the animal hemisphere was 2130 ± 315 nA and that at the vegetal hemisphere was 309 ± 94 nA ($n = 50$; $N = 6$). The mean animal/vegetal ratio of hemispheric responses was 7.8 ± 1.5 ($N = 6$). Incubation with CHX resulted in a rapid decrease in the responses measured on both hemispheres with a $t_{1/2}$ of approx. 3–4 h (Fig. 3). It appears, therefore, that the characteristics of the acquired m_1 Rs are similar to those of the native M_1 R isoform.

Incubation of oocytes (injected with the cloned m_1 R mRNA) with TM (2 μ g/ml) had virtually no effect at short incubation times. Treatment with TM for 24 h resulted in a minor ($15 \pm 6\%$; $n = 25$; $N = 4$) decrease in the amplitude measured at the animal hemisphere, and a slightly larger decrease at the vegetal hemisphere. Hence muscarinic receptors that have been shown to possess a potential glycosylation consensus sequence can be effectively expressed without glycosylation.

Effect of TM and CHX on animal/vegetal amplitude ratios

When the ratios of the response amplitudes measured at the two hemispheres were calculated, there was little change in oocytes of common donors treated either with CHX or TM (Fig.

Fig. 3. Effect of CHX on the muscarinic responses in oocytes expressing m_1 Rs

Defolliculated oocytes (to suppress endogenous muscarinic responses [6]) were injected with 0.25–0.50 ng of cloned rat brain m_1 R mRNA. Oocytes were assayed 16–24 h after injection, 2 and 4 h after the addition of CHX (10 μ g/ml). $\blacksquare$, Control (no CHX); $\square$, animal hemisphere; $\blacklozenge$, vegetal hemisphere. Each point represents 20–30 determinations from four experiments performed on oocytes from different donors.

Fig. 4. Ratios of animal/vegetal responses in control ($\square$), TM-treated ($\blacksquare$) or CHX-treated ($\blacklozenge$) oocytes

The data taken from Figs 1 and 2 were calculated as mean ratios of amplitudes of responses to ACh assayed at the animal and vegetal hemispheres of common (a) and variant (b) oocytes. For variant oocytes, the mean control ratio (9.5 at all times of incubation) was compared to mean ratios at different times of incubation.

4a). Similarly, in oocytes injected with cloned m_1 R mRNA, the ratio remained the same after either treatment (results not shown). However, ratio of the amplitudes at the two hemispheres decreased dramatically in oocytes from variant donors when treated with either CHX or TM (from 9.5 ± 1.0 in untreated controls to 3.3 ± 0.5 or 1.5 ± 0.1 in oocytes treated for 6–8 h with CHX or TM respectively; see Fig. 4b). This pronounced change reflected the different rates of the apparent degradation of the

two receptor isoforms, rather than the slight differences in the kinetic rates between the two hemispheres (see Fig. 1a).

DISCUSSION

The work presented in this paper attempts to establish a model system in which questions related to receptor turnover, asymmetric distribution and, eventually, sorting, can be meaningfully addressed with relatively simple tools. The facile expression of different receptors or their mutants in a cell that is inherently asymmetrical and does not require special treatment to maintain its polarity are two additional desirable properties of the oocyte.

Using hemispheric assay of receptor-mediated responses and hemispheric ligand binding in *Xenopus* oocytes, we were able to show that two native muscarinic receptors exhibit different hemispheric distributions and apparent degradation kinetics. The most striking observation, however, was the relatively rapid course of the loss of receptor function and, when assayed, the even more rapid course of its recovery. These data suggest that receptors of the seven-transmembrane-segment family undergo very rapid turnover in the non-stimulated oocyte. Similar results have been obtained for the native angiotensin II and the acquired thyrotropin-releasing hormone receptors (N. Matus-Leibovitch, M. C. Gershengorn & Y. Oron, unpublished work). This assumption implies that steady-state receptor expression in oocytes should have been achieved within 3–4 half-lives of degradation, i.e. 10–20 h after the injection of mRNA. According to our experience (as well as that of other investigators), optimal responses are achieved 16–24 h after the injection of mRNA. This 4–6 h difference may represent the absolute period required for the diffusion of the injected mRNA to the endoplasmic reticulum. This may also explain the very rapid kinetics of recovery from inhibition by CHX.

The apparent degradation of receptors appeared to proceed at a slightly faster rate at the animal hemisphere of the cell. Moreover, different receptors were degraded at different rates. Thus the M_3 Rs had a half-life of 5 h, while that of the native M_1 Rs and of the acquired m_1 Rs was 3 h. The results obtained with the specific antisense oligonucleotides confirmed that the CHX data pertain to the degradation of the receptors, rather than to another protein required for their activity. Another outcome of these data is the implicit conclusion that receptors are either the most rapidly turning over component or, alternatively, that their amount is limiting when the protein components of the signal transduction pathway are considered.

The use of antisense oligonucleotides also allowed us to identify M_3 Rs as the predominant species on the vegetal hemisphere of oocytes from variant frogs. Working on the assumption of a homogeneous population of receptors, we would have had to assume that glycosylation affects receptors differently at the two hemispheres. These results suggest that antisense oligonucleotides may remain important tools for specific identification of distinct molecular species within cells that allow their injection.

The large differences in the amplitudes of the responses mediated by M_1 Rs and M_3 Rs do not allow the analysis of the receptor population on the animal hemisphere of variant oocytes. Hence it is possible that M_3 Rs are indeed expressed on both hemispheres, but their contribution to the electrophysiological response would amount to 1–10 % of the amplitude. One could investigate this question using receptor binding of specific ligands (e.g. pirenzepine). The high non-specific binding and the low number of receptors, however, preclude this type of analysis in native oocytes.

The co-expression of two subtypes of muscarinic receptors in the same cell is interesting, although its physiological significance is unknown. It is possible that different donors express both

receptors in different proportions, and this may explain the well-known variability of responses among different donors and in the same donor during different seasons.

The experiments aimed at elucidating the role of glycosylation in receptor expression yielded a number of potentially important conclusions. We demonstrated that glycosylation is not an absolute prerequisite for functional expression of muscarinic receptors. Both native M_3 Rs and acquired m_1 Rs were fully expressed despite inhibition of glycosylation by TM. Glycosylation may be necessary, however, for the functional expression of some receptors. Native M_1 Rs appear to be as sensitive to glycosylation inhibition as to the inhibition of their synthesis with either CHX or anti- m_1 oligonucleotides. The differential sensitivity of various membrane glycoproteins has been also described by Sumikawa *et al.* [19]. While kainate receptors and K^+ and Ca^{2+} voltage-activated channels were only slightly affected, γ -aminobutyric acid receptors and voltage-activated Na^+ channels were almost completely inhibited by TM.

Liles & Nathanson [20] studied the role of glycosylation in the expression of muscarinic receptors in N1E-115 neuroblastoma cells. They reported that TM caused a rapid decrease in the number of receptors in that model system. More recently, van Koppen & Nathanson [21] have shown that cardiac m_2 Rs mutated in the consensus glycosylation sequence were functionally expressed to the same extent as were the wild type receptors. They concluded that glycosylation is not required for muscarinic m_2 receptor expression and function, and proposed that the previously observed inhibition by TM [20] may have been caused by perturbation of another glycoprotein, possibly required for receptor expression.

We cannot exclude the possibility that the effect of TM on M_1 R expression in oocytes may be indirectly mediated by inhibition of the glycosylation of another protein which is necessary for this receptor's expression and activity. This possibility, however, seems unlikely. This putative protein would have to be specific for M_1 R, since both M_3 R and m_1 R were unaffected. Moreover, the kinetics of the inhibition of M_1 R expression were similar when CHX, specific anti- m_1 oligonucleotides and TM were used. Hence the half-life of the putative protein required for the expression of the receptor would have to be equal to or shorter than that of the receptor.

Based on studies reported on the nicotinic receptor and on the Na^+ channel, several mechanisms of receptor expression inhibition by TM are possible. The unglycosylated receptor may fail to insert into the membrane. This hypothesis is strengthened by the report of Mishina *et al.* [22], who observed a complete failure of insertion of a nicotinic receptor clone that was point-mutated in the single glycosylation site on the α subunit. Furthermore, Sumikawa & Miledi [23] reported impaired insertion and accumulation of the uninserted receptor in *Xenopus* oocytes treated with TM. A similar finding was reported by Prives & Olden [24] in cultured muscle cells.

Alternatively, similar results would be observed in the case of increased degradation of the newly synthesized unglycosylated receptor [24,25]. Pitta *et al.* [26] provide evidence that glycosylation of vesicular stomatitis virus glycoprotein is involved in protein folding and oligomerization, and that the effects on transport is only secondary.

The evidence described above suggests that inhibition of glycosylation of some receptors may result in a lack of function due to a variety of reasons. These may include faulty insertion and/or sorting of the receptor protein. Indeed, the hemispheric activity ratios of M_1 R changed dramatically upon treatment with either CHX or TM. However, since different receptor subtypes are expressed on each hemisphere, this effect is due to different degradation rates of the two subtypes rather than to a change in

the sorting mechanism. The definitive resolution of such questions will therefore have to be deferred until specific antibodies against receptor subtypes are available.

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