G-protein coupled receptors are not considered to exhibit voltage sensitivity. Here, using Xenopus oocytes, we show that the M2 muscarinic receptor (m2R) is voltage-sensitive. The m2R-mediated potassium channel (GIRK) currents were used to assay the activity of m2R. We found that the apparent affinity of m2R toward acetylecholine (ACh) was reduced upon depolarization. Binding experiments of \(^{3}\text{H}\)ACh to individual oocytes expressing m2R confirmed the electrophysiological findings. When the GIRK channels were activated either by overexpression of G\(\beta\gamma\) subunits or by injection of GTP\(\gamma\)S, the ratio between the currents measured at \(-60\) mV and \(+40\) mV was the same as for the basal activity of the GIRK channel. Thus, the steps downstream to agonist activation of m2R are not voltage-sensitive. We further found that, in contrast to m2R, the apparent affinity of m1R was increased upon depolarization. We also found that the voltage sensitivity of binding of \(^{3}\text{H}\)ACh to oocytes expressing m2R was greatly diminished following pretreatment with pertussis toxin. The cumulative results suggest that m2R is, by itself, voltage-sensitive. Furthermore, the voltage sensitivity does not reside in the ACh binding site, rather, it most likely resides in the receptor region that couples to the G-protein.

It is of common knowledge that voltage-gated ionic channels are voltage sensors; changes in membrane potential, usually depolarization, cause conformational changes in the channel protein, and, as a result, the channel opens. Ligand-gated channels also show voltage sensitivity, although much weaker, implying that these proteins also sense changes in membrane potential. Notable among them is the N-methyl-d-aspartic acid receptor (1, 2) but also non-N-methyl-d-aspartic acid glutamate channels (3, 4) and nicotinic channels (5).

In contrast, G-protein-coupled receptors (GPCRs), involved in the majority of signal transduction processes (6), are not considered to be voltage-sensitive. Surprisingly, voltage dependence was demonstrated in processes linked to one group of GPCRs, the muscarinic receptors. It was shown that m2R inhibits ACh release in a voltage-dependent manner; the inhibition is strong at resting potential and weakens upon depolarization (7–9). In other studies it was shown, in mouse pancreatic acinar cells, that ACh-induced Ca\(^{2+}\) response, mediated by muscarinic receptors, is sensitive to the membrane potential (10, 11). Also, binding of \(^{3}\text{H}\)ACh to muscarinic receptors in rat brain synaptosomes (12) or synaptoneurosomes (13) was strong at resting potential and was reduced under depolarization achieved by increasing KCl concentration. Finally, electrical pulses were shown to reduce binding of muscarinic receptor antagonist to synaptosomes (14).

Important questions are whether the muscarinic receptors, representatives of the GPCRs, are by themselves voltage-sensitive, and if so, whether it is this sensitivity that underlies the above-mentioned voltage-dependent processes. However, so far this possibility was not directly investigated and was even not thoroughly considered.

To be able to directly test this possibility we used an expression system, Xenopus laevis oocytes, which enables studying each receptor in isolation regarding its ability to sense changes in membrane potential. The results imply that m2R and, most likely, m1R exhibit, by themselves, voltage sensitivity.

EXPERIMENTAL PROCEDURES

Preparation of cRNA and Oocytes—cDNA plasmids of the two subunits of the GIRK (GIRK1 and GIRK2), m2R, m1R, and the \(\alpha\), \(\beta\), and \(\gamma\) subunits of the G-protein (Go\(\alpha\), G\(\beta\)1, and G\(\gamma\)) were linearized with the appropriate restriction enzymes (15). The linearized plasmids were transcribed in vitro using a standard procedure (16).

Preparation of cRNA and Oocytes—cDNA plasmids of the two subunits of the GIRK (GIRK1 and GIRK2), m2R, m1R, and the \(\alpha\), \(\beta\), and \(\gamma\) subunits of the G-protein (Go\(\alpha\), G\(\beta\)1, and G\(\gamma\)) were linearized with the appropriate restriction enzymes (15). The linearized plasmids were transcribed in vitro using a standard procedure (16).

X. laevis oocytes were isolated and incubated in ND96 solution composed of ND96 (in mM: 96 NaCl, 2 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 5 Hepes, 10 pyruvate, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin (16). A day after their isolation, the oocytes were injected with the relevant cRNAs. For the experiments with m1R, 200 pg of cRNA of m1R were injected per oocyte. For the experiments with m2R, cRNAs of m2R (70 pg) and GIRK1 and GIRK2 (200 pg each) were injected. In addition, cRNA of Go\(\alpha\) (1000 pg) was injected to decrease the basal GIRK current (I\(K\)) and to improve the relative activation by the agonist (17). Injection of Go\(\alpha\) proved to decrease I\(K\) by about 3-fold. G\(\beta\)1 and G\(\gamma\) subunits were injected in the amounts indicated under “Results.” Chemicals were purchased from Sigma Israel (Rehovot, Israel), unless otherwise stated.

Current Measurements—The currents were measured 4–7 days after cRNA injection and were recorded using the standard two-electrode voltage clamp technique (Axoclamp 2B amplifier, Axon Instruments, Foster City, CA). Each oocyte was placed in the recording bath containing ND96 solution and was impaled with two electrodes pulled from 1.5-mm Clark capillaries (CEI, Pangboure, England). Both electrodes were filled with 100 mM KCl solution to prevent elevation of \([\text{K}^+]_o\) (the recording and the injecting electrode resistances were 15 and 5 M\(\Omega\), respectively). The m2R-mediated GIRK currents were measured in a 24 mM \(K^+\) solution (in mM: 72 NaCl, 24 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 5 Heps, with pH adjusted to 7.5 with KOH) (18). The m1R-mediated chloride currents were measured in ND96 solution. Current = voltage (I-V)
curves were obtained by applying two 6-s voltage ramps from −100 mV to +40 mV and back to −100 mV. pCLAMPs software (Axon Instruments) was used for data acquisition and analysis.

**[3H]ACh Binding Experiments—**Binding experiments were performed 3–4 days following cRNAs injection, employing a device that was designed to enable brief incubation periods (Fig. 1A). Standard pipetor tips (volumes up to 200 μl) were trimmed 4 mm from the edge, and a 3-cm-long glass pipette (2-mm outer diameter) was inserted to the cut tip end. The distal end of the glass pipette was fire-polished to prevent damage of the oocytes. The process of handling the oocyte is shown from left to right in Fig. 1A.

The oocyte was first dropped (using the modified tips, which enable the lifting of the oocyte with a very small and accurate volume of solution) into a small chamber with 200 μl of either ND96 or high K+ (in mM: 2 NaCl, 96 KCl, 1 CaCl2, 1 MgCl2, 5 Hepes, with pH adjusted to 7.5 with KOH) solution containing a given concentration of labeled ACh ([3H]acetylcholine iodide, specific activity, 80 Ci/mmol, American Radiolabeled Chemicals, Inc., St. Louis, MO). The oocyte was incubated in this solution for 30 s, a duration that was found to provide the maximal ratio between total and nonspecific binding (Fig. 1B, see below). For m1R-expressing oocytes, the optimal incubation time was found to be 60 s. Then the oocyte was removed from this chamber using the same modified tip, and rapidly dropped into a washing chamber filled with 4000 μl of ligand-free ND96 or high K+ solution. In less than a second the oocyte reached the bottom of the cone-shaped washing chamber. Using a second pipetor also with a modified tip, the oocyte, with 5 μl of the physiological solution, was rapidly removed through the side arm (an additional second), and was dropped into a vial containing 3 ml of scintillation liquid.

To determine the optimal incubation time, total and nonspecific bindings were measured following various durations of incubation with [3H]ACh. As illustrated in Fig. 1B, both the total binding and the nonspecific binding increased as the duration of incubation was prolonged. With 10 s of incubation no specific binding was obtained. The maximal ratio between the total binding and the nonspecific binding was obtained when the oocytes were incubated for 30 s. Longer incubation durations yielded high nonspecific binding and, hence, decreased the signal-to-noise ratio.

Specific binding was obtained by subtracting the nonspecific binding (unjected oocytes) from the total binding. The specific binding was further ascertained, because it was abolished following the addition of 100 μM unlabeled (cold) ACh or 100 μM unlabeled atropine (antagonist of the various types of muscarinic receptor) to a solution containing 50 nM [3H]ACh (Fig. 1C). The number of binding sites expressed in the oocyte was determined by subtracting the nonspecific binding from the binding of the muscarinic receptors antagonist quinuclidinyl benzilate (QNB) to m2R-expressed oocytes. Fig. 1D shows that the specific binding of QNB is the same in ND96 and high K+ solution (−20 fmol/oocyte). (A similar experiment was done with m1R-expressed oocytes.)

**Statistical Evaluation—**Significance was checked by Student’s two-tailed or one-tailed t test. Results are given as mean ± S.E.

**RESULTS**

**Membrane Potential Affects the Dependence of m2R-mediated GIRK Response on ACh Concentration—**Xenopus oocytes were injected with cRNAs of proteins involved in the pathway leading to activation of K+ currents by m2R via βγ subunits of the G-proteins: m2R, the subunits of the GIRK channel (GIRK1 and GIRK2), and the Gα3 subunit (17).

GIRK channels are inward rectifiers: they conduct inward K+ currents better than outward. Fig. 2A shows a typical current-voltage (I-V) relation of the basal K+ current (Iag-V) via the expressed GIRK channels in a high K+ solution (24 mM K+). IK is believed to arise due to the presence of free endogenous Gβγ (19). The Iag-V relation is linear at potentials negative than −50 mV, and inward rectification occurs at more positive potentials.

We then wished to investigate whether there is additional voltage sensitivity in m2R-induced response. To this end, the dependence of the ACh-induced K+ current (INaCh) on ACh concentration (dose-response, DR) was measured at two holding potentials: −60 mV and +40 mV. Fig. 2 (B and C) depicts the basic experimental protocol for one ACh concentration. The oocyte was voltage-clamped to either −60 mV (Fig. 2B) or +40 mV (Fig. 2C), in a low K+ (2 mM K+) solution. ND96. IK was developed upon replacement of the ND96 by the 24 mM K+ solution. Then, ACh (100 nM) was added, and INaCh appeared.

![Fig. 1. The binding experiments technique. A, protocol of the experiment. The oocyte was dropped into 200 μl of [3H]ACh in the desired concentration. After a period of incubation, the oocyte was taken to the washing chamber that contained 4000 μl of ligand-free solution and rapidly (after 1–2 s) removed to the scintillation liquid. B, effect of incubation duration on [3H]ACh binding. Eight injected oocytes (with RNA of m2R) (total binding; black bars) and eight unjected oocytes (nonspecific binding; white bars) were incubated for different periods (10 s, 30 s, 2 min, and 5 min), and the average [3H]ACh binding for each case was measured. C, the specific [3H]ACh binding was determined by subtraction of the nonspecific binding measured in unjected oocytes (CTRL) or in the presence of unlabeled ACh (cold AC) or unlabeled atropine (Atr) from the total binding measured in m2R-injected oocytes (TOT, black bar) (n = 8 in each bar). D, the number of muscarinic binding sites (S.B.) was determined by subtracting the nonspecific binding measured in unjected oocytes (CTRL) from the binding of QNB to m2R-expressed oocytes (QNB) in ND96 and in high K+ solution.](http://www.jbc.org/22483)
I_{ACh} was terminated upon washout of ACh. Finally, returning to ND96 solution abolished I_K.

Employing this basic experimental protocol, full DR curves at the two holding potentials were constructed. To compensate for the intrinsically different I_K obtained at the two holding potentials of −60 mV and +40 mV in a single oocyte, and to be able to compare between oocytes varying in their I_K-V relation, we measured fractional I_{ACh}. That is, for each holding potential, I_{ACh} at any particular ACh concentration was normalized to I_{ACh} obtained at a saturating concentration of ACh at the same holding potential.

One way to construct DR curves is to apply various concentrations of ACh in a random order and wash out the ACh between applications. However, such protocols require periods of recording that may exceed the time when the oocytes are still viable. We have, therefore, applied the various ACh concentrations in an increasing order without washing out between applications. Such a continuous exposure to ACh may distort the true DR curve, because I_{ACh} is known to desensitize (20–22). To check whether this is the case under our experimental conditions, the following experiment was conducted (Fig. 3A). Three concentrations (15, 100, and 10,000 nM) of ACh were applied, and the ratio between I_{ACh} induced by 15 nM and I_{ACh} induced by 10,000 nM was measured. Then, ACh was washed out for 4 min and only the lowest and the highest ACh concentrations were reapplied without the intermediate concentration, and again the ratio between I_{ACh} (15 nM) and I_{ACh} (10,000 nM) was measured (at −60 mV) twice: before and after 10-min wash with ACh-free 24 mM K⁺ solution. The responses to 5, 15, and 30 nM at the first application were 8, 15, and 45% from the maximal response, respectively, and 6, 15, and 43% from the maximal response after the 10-min wash.

Another problem that may arise concerns the duration of the experiments. Even without wash between ACh applications, an experiment during which four concentrations of ACh are applied at two holding potentials lasts about 20 min. As seen in Fig. 3B (see also Ref. 18), during such a period of time, I_K itself undergoes changes, its amplitude declines. Hence, a DR curve measured at the beginning of the experiment may differ from that measured at a later time during the experiment. To check for this possibility, we established a DR curve, then washed with ACh-free 24 mM K⁺ solution for 10 min, and the same DR was established again. It can be seen (see Fig. 3B) that I_K indeed declined with time and, in correlation with this reduction, I_{ACh} increased with time. However, when I_{ACh} at any ACh concentration was normalized to the maximal I_{ACh} obtained with 10,000 nM ACh, the DR curves were remarkably similar before and after the 10-min wash. The responses to 5, 15, and 30 nM were 8, 15, and 45% of the maximal response, respectively, at the first application, and 6, 15, and 43% of the maximal response after the 10-min wash (similar results were obtained in two additional experiments).

Next, full DR curves at two holding potentials were established. In the example of Fig. 4A, the oocyte was first voltage-clamped to −60 mV. Following the appearance of I_K, ACh concentrations of 15, 30, 60, and 10,000 nM were applied in an ascending order (Fig. 4A, ACh 1, 2, 3, and 4, respectively). As mentioned before, for each holding potential, I_{ACh} at any ACh concentration was normalized to I_{ACh} obtained at 10,000 nM.
M₂ Muscarinic Receptor Is Voltage-sensitive

Following wash with ACh-free 24 mM K⁺ solution, the holding potential was switched from −60 mV to +40 mV, and the same ACh concentrations as for the holding potential of −60 mV were reapplied (framed by the dashed-line rectangle in Fig. 4A and shown at higher resolution in Fig. 4B). At +40 mV we could not detect any I_{ACh} at 15 nM ACh, even though currents as small as 5 nA are clearly detectable with our experimental system. Had the response evoked by 15 nM ACh been 20% of I_{ACh} at 10,000 nM, as it was at −60 mV, the current would have been 80 nA and thus easily detectable. Furthermore, the response to 30 nM was only 11% (in comparison to 45% at −60 mV) and the response to 60 nM was 32% (in comparison to 59% at −60 mV) of the response to 10,000 nM ACh.

Similar experiments were done on 45 oocytes taken from 16 X. laevis females. The order of the holding potentials was randomly selected, i.e., first +40 mV and then −60 mV or vice versa. The results of all these experiments are depicted in Fig. 4C where full DR curves obtained at −60 mV (squares) and +40 mV (triangles) are shown. The shift to the right of the DR curve at +40 mV is clear (The two graphs are significantly different (p < 0.05)).

The shift in apparent affinity at +40 mV may be caused by cellular changes due to the long depolarization, rather than by a specific effect of depolarization on the m2R system. To check for this possibility, in some of the experiments, after measuring a DR curve at −60 mV, the holding potential was switched to +40 mV for a period of 10 min. Then, the holding potential was switched again to −60 mV and a DR curve was measured again. Results from three such experiments are depicted in Fig. 4D. It is evident from this figure that the DR curves established at −60 mV before (squares) and after (circles) the depolarization period were practically identical, indicating that depolarization as such did not cause any damage or any other long lasting effect on the DR relation.

Another possibility to explain the shift in the DR curves would be that voltage-gated channels existing in the oocyte relay changes in membrane potential to m2R (23). Oocytes often possess endogenous atypical voltage-gated Na⁺ channels of an unknown molecular origin. They are dormant at rest but can be induced into a functional state by long lasting depolarization (24). To avoid a possible effect of these channels on the obtained results, each batch of oocytes was checked for the existence of such Na⁺ channels, employing standard protocols (24), prior to conduction of the DR experiments. Batches that were found to possess those channels were discarded.

Membrane Potential Affects the Dependence of the m2R-mediated GIRK Response on Oxotremorine Concentration—Binding experiments, employing [³H]ACh, conducted in synaptosomes (12) or synaptoneurosomes (13), revealed that the m2Rs comprise two affinity states, and depolarization shifts the high affinity population into a low affinity state. Yet the DR curves, obtained using ACh (Fig. 4C), did not reveal the existence of the two affinity states. Because this aspect is important for the analysis of the DR curves, we repeated the DR experiments with another muscarinic receptor agonist, oxotremorine (OXO), hoping that DR curves with OXO as an agonist will reveal the two affinity states populations. Fig. 5 reveals that, with OXO as an agonist, the high and low affinity populations that comprise m2R (12, 13, 23) became apparent. It is further seen that at −60 mV the two populations coexist, whereas at +40 mV the low affinity population predominates. This observation indicates that as in synaptosomes and synaptoneurosomes (12, 13), ACh at the same holding potential. 10,000 nM was selected as a reference concentration, because at this concentration saturation is reached even at the depolarizing (+40 mV) holding potential (20,000 nM ACh did not produce an additional response at either holding potential). The corresponding responses to 15, 30, and 60 nM were 20, 45, and 59% of the response to 10,000 nM.

**Fig. 4. Full DR curves for m2R. A**, experimental protocol. The oocyte was voltage-clamped to −60 mV in ND96. After switching to 24 mM K⁺ solution, four ACh concentrations were applied sequentially (1, 2, 3, and 4 stand for 15, 30, 60, and 10,000 nM, respectively). After 10-min wash with ACh-free 24 mM K⁺ solution, the holding potential was switched to +40 mV and the above-described protocol was repeated (dashed rectangle). B, enlargement of the dashed rectangle shown in A. C, full DR curves assembled from the various experiments conducted at −60 mV (squares) and at +40 mV (triangles). The number of repetitions for each ACh concentration varied between 6 and 25. The two graphs are significantly different (p < 0.05). The lines here and below (Figs. 5 and 8C) were generated by first fitting Equation 1 (dashed lines) or Equation 2 (solid lines) to the data points obtained at the resting potential (−60 mV). From this fit both K₁ and K₂ were established. These Kₐ values were then preserved when fitting the curves under depolarization, and only the fraction of the population at each affinity state was adjusted to best fit the experimental data. D, DR curves at −60 mV before (squares) and after (circles) 10 min of depolarization. Results from three oocytes.
also in oocytes, where m2R is expressed in isolation, depolarization shifts the high affinity population into a low affinity state (see “Discussion” for more details).

The observed dependence of the DR curves of either ACh or OXO on membrane potential may reside in any one of the following steps and/or components participating in the GIRK channel activation: 1) binding of ACh to m2R and consequent activation of the coupled G-protein; 2) GDP/GTP exchange and consequent dissociation of the G-protein into activated Ga subunit and Gβγ dimer; and 3) activation of the GIRK channel by the Gβγ subunits. To examine whether it is the receptor that is voltage-sensitive, the steps that are downstream to step 1), i.e. steps 2) and 3), were isolated, and the voltage dependence of the GIRK channel activation by factors downstream to m2R activation was tested.

**Activation of GIRK Channels by Overexpression of Gβγ Is Not Voltage-sensitive**—It is possible that Gβγ not only activates the GIRK channels, but, as suggested recently (25), high levels of Gβγ also directly affect the rectification properties of the channel (possibility 3). Such a behavior could cause an increase in outward currents at high doses of ACh, producing the results seen in Figs. 4C and 5 (however, this still cannot explain the absence of any response at +40 mV at 15 nM ACh). To test for this possibility, we expressed several concentrations of Gβγ subunits in the oocytes together with the GIRK channel, but without m2R. In this case, essentially the same I-V relation was obtained as in control oocytes (Fig. 5). The shift in apparent affinity seen at +40 mV (Fig. 4C) is similar in control and in Gβγ-overexpressing oocytes (Fig. 6C). To be able to compare results from various oocytes showing natural scatter in the I_{K(CF)}/I_{K(40)} ratio, the average ratio obtained from control oocytes of the same batch was taken as 1 and the rest of the data points (of the individual control oocytes as well as the oocytes expressed with Gβγ) were normalized to this value.

It is possible that by overexpression we did not reach sufficiently high concentrations of Gβγ subunits and therefore could not observe the changes in inward rectification of I_K seen in a previous study (25). This seems unlikely, because I_K obtained with the highest concentration of Gβγ (5000 pg/oocyte) reached a level of 10,000 nA in −60 mV, which is in the same range of I_K plus I_{ACH} obtained with the saturated concentration of ACh. Nevertheless, to check for this possibility directly, we compared the I_{K(V)} relation to the I_{ACH(V)} relation induced by saturating concentration of ACh, 10 μM. I_{ACH(V)} was normalized to I_{K(V)} by taking the corresponding currents at −90 mV to be equal. Fig. 2A shows that the two I-V curves are virtually the same, indicating that activation of the GIRK channel by the highest concentration of Gβγ employed in our experiments did not alter the I-V relation. We may therefore conclude that under our experimental conditions the m2R-mediated voltage sensitivity seen in Figs. 4C and 5 is not caused by direct effects of the Gβγ subunits on the properties of the GIRK channel.

**Activation of GIRK Channels by Injection of GTPγS Is Not Voltage-sensitive**—The shift in apparent affinity seen at +40 mV (Fig. 4C) could be achieved if GDP/GTP exchange and the consequent dissociation of the G-protein into α and βγ subunits would be voltage-dependent. To isolate this step, the non-hydrolysable GTP analogue, GTPγS, was injected into oocytes expressing GIRK channels prior to the recording. Five min later, I_K at −60 mV and +40 mV was measured, and the ratio I_{K(−60)/I_{K(40)}} was determined as described above. It can be
seen that injection of the oocytes with 200 pmol of GTPγS increased \( I_{K} \) at both holding potentials (Fig. 6B). However, the ratio \( I_{K} - I_{K, +40} \), after the injection of GTPγS is similar to the ratio obtained in control oocytes that were not injected with GTPγS (Fig. 6C). The magnitude of \( I_{K} \) in oocytes injected with GTPγS was in the same range of \( I_{K} \) plus \( I_{ACh} \) obtained with moderate concentrations of ACh. We could not inject higher concentration of GTPγS, because the oocytes did not survive such injections. The cumulative results support the conclusion that the steps downstream to the agonist activation of m2R are not voltage-sensitive.

**Effect of Depolarization on the Binding of [3H]ACh to Oocytes Expressing m2R**—To directly examine whether it is the first step in the signal transduction, the binding of agonist to m2R (step 1 above), which is voltage-sensitive, we measured binding of [3H]ACh to oocytes expressing m2R.

Although earlier studies, conducted in complex systems (synaptoneurosomes (13) and synaptosomes (12)) revealed that binding of [3H]ACh to muscarinic receptors was strong at resting potential and was reduced under depolarization, the cause of this phenomenon was either attributed to voltage-gated Na+ channels (13) or was not at all investigated (12).

To maximize specific binding of [3H]ACh, large quantities of m2R cRNA (1000 pg) were injected, yielding ~20 fmol of binding sites/oocyte (as measured by the muscarinic receptor antagonist QNB, Fig. 1D). Changes in membrane potential were induced by modifying the extracellular KCl concentration and were measured with standard intracellular microelectrodes. Five oocytes taken from the same batch that had been used for the binding experiments served to determine the average membrane potential of the oocytes of that batch. The resting potential (measured in ND96 solution) of the oocytes expressing m2R alone was ~38 ± 4 mV (n = 29, from six batches). Upon shift to a high K+ solution (96 mM KCl) the oocytes were depolarized to +5 ± 2 mV. To increase the difference between the two levels of membrane potential so as to maximize the chances of detecting possible effect of membrane potential on the binding of [3H]ACh, the resting potential of the oocytes was made more negative by injecting the oocytes with cRNAs of GIRK1 and GIRK2 subunits. Indeed, a resting potential of ~87 ± 2 mV was obtained (n = 37, from eight batches).

To measure binding of [3H]ACh, the injected oocytes were placed in either ND96 solution (resting potential, ~87 ± 2 mV) or in high K+ solution (depolarization, +5 ± 2 mV) and were exposed to various concentrations of [3H]ACh. After 30 s of exposure to [3H]ACh the oocytes were quickly removed, washed, and placed into scintillation fluid (see Fig. 1A). For each membrane potential, we used as controls (nonspecific binding) oocytes injected with cRNAs of GIRK1 and GIRK2 but without m2R. Oocytes that were injected with cRNAs of GIRK1, GIRK2, and m2R, provided the total binding. Specific binding (see “Experimental Procedures” for details) was obtained by subtracting the control from the total binding. Results of one such experiment with one concentration of [3H]ACh (50 nM) at the two membrane potentials are depicted in Fig. 7A. It can be seen that the specific binding of [3H]ACh to m2R is voltage-dependent: it is higher at resting potential than under depolarization.

Fig. 7B depicts results obtained from many such experiments where ACh concentrations ranged between 50 and 2000 nM (n = 12–45 oocytes for each concentration). With higher concentration of [3H]ACh, the nonspecific binding was too high and masked the specific binding. Fig. 7B shows that, at each ACh concentration, the average specific binding was reduced upon membrane depolarization (the two graphs are significantly different; p < 0.05).

The differences in binding seen in Fig. 7 (A and B) may not stem from changes in membrane potential induced by high K+. Rather, K+ itself may affect, by some unknown mechanism, the binding parameters. To check for this possibility, the same binding experiments as in Fig. 7 (A and B) (binding in ND96 and in high K+ solutions) were repeated, but now with oocytes injected only with m2R cRNA. Recall that, without coexpressed GIRK, the membrane potential was ~38 mV in ND96 and +5 mV in 96 mM KCI, a much smaller difference than when GIRK was also expressed where the resting potential was ~87 mV. It is seen (Fig. 7C) that, without the injection of GIRK cRNA, the difference between specific binding at resting potential (~38 mV) and depolarization was much smaller than that observed when GIRK cRNA was also injected (Fig. 7A; the two bars are not significantly different, p = 0.79). In particular, the specific
binding of 50 nM [3H]ACh in the 96 mM K+ solution was 80 ± 7% (n = 13) of that in the ND96 solution (in comparison to 20% in Fig. 7B when GIRK cRNA was injected), indicating that the reduction in binding seen at high K+ solution is not due to changes in K+ concentration per se but, rather, results from K+ -induced changes in membrane potential.

It could be claimed that the results seen in Fig. 7B do not imply that membrane potential affects m2R itself. Rather, the GIRK channel coexpressed with m2R could relay changes in membrane potential to m2R. This possibility seems unlikely, because the binding of [3H]ACh to the M1-muscarinic receptor (m1R) measured in Xenopus oocytes (see below) shows voltage dependence that is in opposite direction to that found in m2R, although in both cases the oocytes expressed GIRK channels. Furthermore, although a minor, intrinsic voltage sensitivity was previously suspected in GIRK channels (26), recent studies suggest that there is no intrinsic voltage-dependent gating or rectification in GIRK channels and in homologous IRK channels (27, 28). Also, GIRK channels certainly cannot underlie a similar voltage dependence of agonist binding to m2R found in synaptosomes (12), because GIRK channels are almost never found in presynaptic nerve terminals (19). These considerations render unlikely the possibility that the voltage dependence of ACh binding depends on GIRK channels.

Membrane Potential Affects the Dependence of the m1R-mediated Chloride Response on ACh Concentration—The results so far showed that depolarization reduces the affinity of m2R toward its agonists, i.e. ACh and OXO. Such a reduction in affinity could be achieved if depolarization causes repulsion of the positively charged ACh and OXO from the m2R binding site and thereby would decrease the apparent affinity of the receptor toward its agonist. Alternatively, depolarization may cause a conformational change in m2R and thereby affect its affinity to its agonist. The conformational change in m2R could be caused by either membrane potential affecting directly the binding site or by affecting other site(s) in the receptor.

To distinguish between these possibilities we repeated the experiments described above employing another member of the muscarinic receptor family. We selected the m1R, because it possesses a similar binding site to that of m2R (see Fig. 10A) and is activated by the same positively charged ACh but is coupled to a different G-protein. This receptor mediates opening of calcium-dependent chloride channels via activation of phospholipase C and a consequent release of calcium from internal stores (29, 30).

Xenopus oocytes were injected with cRNAs of m1R, and the dependence of ACh-induced Cl− current (ICl) on ACh concentration was measured at the same two holding potentials as before: −60 mV and +40 mV. The basic experimental protocol is depicted in Fig. 8A. An oocyte expressing the m1R was placed in ND96 solution and voltage-clamped to the desired holding potential (−60 mV or +40 mV). Then, ACh (0.5 μM, Fig. 8A) was applied for 10 s, causing evolvement of ICl. The amplitude of this response was used as a measure of the level of activation of m1R.

The I-V relation of the Cl− channel (Fig. 8B) was measured as follows (31): The oocytes were treated with the Ca2+-free ND96 solution to make the oocyte permeable to Ca2+. Then, CaCl2 (3 mM) was added to the ND96 solution to induce the Ca2+-dependent Cl− current, and the I-V curve was established (see “Experimental Procedures”).

To check for possible voltage sensitivity of m1R, full DR curves at the two holding potentials were constructed. Because ICl is known to desensitize and long periods of wash (more than 20 min) are required for recovery from desensitization, we measured, in each oocyte, responses to only one submaximal concentration of ACh and to a saturated concentration of ACh (200 μM) at each of the two holding potentials. The holding potentials were administered in a random order, i.e. first +40 mV and then −60 mV or vice versa. As before, the DR curves were assembled by normalizing the responses to each ACh concentration to the maximal response obtained by the saturated ACh concentration in the same oocyte, at each holding potential.

Such experiments were done in 56 oocytes taken from seven X. laevis females. The results of all these experiments are depicted in Fig. 8C, where full DR curves obtained at −60 mV (circles) and +40 mV (squares) are shown. Fig. 8C suggests that also m1R is voltage-sensitive, but in contrast to m2R,
depolarization increases its apparent affinity toward ACh.

**Effect of Depolarization on the Binding of [3H]ACh to Oocytes Expressing m1R**—Measurements of binding of [3H]ACh to oocytes injected with 500 pg of m1R cRNA (yielding ~14 fmol of binding sites/oocyte as measured with QNB) were done as for the m2R-injected oocytes. Here as well, to increase the difference between the holding potentials, cRNAs of the GIRK channel subunits (GIRK1 and GIRK2) were injected. Fig. 8D shows that binding to oocytes expressing m1R is significantly higher under depolarization (ND96; n = 41) than at resting potential (high K+; n = 57; The two bars are significantly different, p < 0.05).

The electrophysiological and binding experiments show that also the m1R is voltage-sensitive. Furthermore, as in the case of m2R, it is the binding of ACh that is voltage-sensitive. Note that depolarization increased binding of [3H]ACh in m1R but decreased binding in m2R, whereas in both cases the oocyte were expressed the GIRK channel. These results rule out the possibility that it is the GIRK channel that relays changes in membrane potential to the receptor.

The observation that m1R and m2R exhibit voltage sensitivity in opposite directions suggests that the voltage sensitivity in either m1R or m2R does not reside in the receptor binding site. These results further diminish the likelihood that the voltage sensitivity of m2R is due to repulsion of the positively charged agonists from the receptor.

The opposite effects of depolarization on the DR curves of m1R and m2R strengthen the conclusion reached earlier (based on the experiments described in Fig. 6C) according to which the herein observed voltage sensitivity does not reside in the GDP/GTP exchange and consequent dissociation of the G-protein into activated Go and Gβγ dimer.

**Effect of PTX on the Binding of [3H]ACh to Oocytes Expressing m2R**—The observation that m2R and m2R exhibit different voltage sensitivity and acknowledging that they share a common binding site, but differ in their associated G-protein, raise the possibility that the voltage sensitivity resides in the receptor regions that are coupled to their corresponding G-proteins. Previous studies demonstrated a substantial increase in the affinity of binding of agonist to GPCRs when the latter are actually attached to their coupled G-proteins (32, 33). Hence, it is possible that the receptor-G-protein complex comprises the high affinity state, whereas the receptor alone comprises the low affinity state, and membrane potential determines the distribution between these two affinity states by changing the conformation of the receptor, thereby affecting its association with its G-protein.

To check for this possibility, we compared binding of [3H]ACh to oocytes (expressing m2R and GIRK channels) treated with pertussis toxin (PTX) to binding of [3H]ACh to PTX-un-treated oocytes. Binding was measured (as described above) 12-20 h following injection of PTX. We first ensured that m2R was indeed uncoupled from its G-protein. This was done by electrophysiological measurements that showed that IACh was abolished. Only then the binding experiments were conducted. It can be seen (Fig. 9) that at both membrane potentials PTX decreased the specific binding. However, it affected more significantly the binding to oocytes at resting potential (~87 mV) than to oocytes depolarized to +5 mV, causing the difference in binding at the two membrane potentials to almost disappear. (The two curves that depict the PTX treated binding do not significantly differ (p = 0.31).)

These results are compatible with the mechanism suggested above. Accordingly, depolarization decreases the likelihood of formation of the m2R-G-protein complex and hence reduces the affinity of m2R toward its agonist. At resting potential, where most of the receptors are in a high affinity state (Table I), that is in the m2R-G-protein complex state, the effect of PTX was strong, whereas under depolarization, where most of the receptors reside in their low affinity state (that is the receptor alone), the effect of PTX was greatly reduced.

**DISCUSSION**

We showed here, for the first time, that GPCRs are by themselves voltage-sensitive. This conclusion is based on the following observations. 1) Electrophysiologically measured DR curves showed that depolarization decreased the apparent affinity of m2R and increased the apparent affinity of m1R toward ACh. 2) Binding measurements of [3H]ACh to individual oocytes expressed with either m2R or m1R confirmed the electrophysiological findings. 3) Overexpression of Gβγ and injection of GTPγS into oocytes ruled out the possibility that the voltage sensitivity resides in steps and/or components that are downstream of m2R activation. 4) Pretreatment of m2R-expressing oocytes with PTX greatly diminished the voltage dependence of [3H]ACh binding seen under control conditions (point 2).

Based on the above conclusions, we suggest the following mechanism for the action of membrane potential on m2R and most likely also on m1R. Depolarization causes conformational changes in a site different from the agonist binding site. The conformational change occurs in a site that affects the association of m2R with its corresponding G-protein and thereby alters the affinity of m2R (or m1R) toward the agonist. Below, we discuss in some detail the mechanism suggested above. We did not completely rule out the possibility that depolarization causes a conformational change in the receptor coupling site of the G-protein. However, we view this possibility as unlikely, because no intrinsic voltage sensitivity of any G-protein has ever been reported and these peripheral membrane proteins are not expected to be exposed to the membrane’s electrical field.

**Where in the Receptor Could the Voltage Sensitivity Reside?**—Intuitively, the most obvious domain in the receptor that could serve as a voltage sensor would be the binding site itself. The binding site is conserved among m1R and m2R and is buried within a cavity, formed by the transmembrane segments (6, 10), and thus can sense the changes in the membrane electrical field. However, because of our finding showing that depolarization affects in an opposite way the binding of ACh to the two receptor subtypes, it seems unlikely that the binding site itself is the voltage sensor.

A clue as to what this domain may be comes from a preliminary theoretical search for possible voltage-dependent motifs in m2R. Conducting a computerized analysis of proteins sequences, we found that the cytosolic region of m2R touching the
fifth transmembrane domain contains a rich excess of positive amino acids residues. Although this region is believed to be mainly cytosolic, it could potentially confer voltage-dependent conformational changes in the receptor via movements of the fifth transmembrane domain region. These movements may affect the three-dimensional structure of the agonist binding site.

The possibility that the voltage sensitivity resides in the above-mentioned cytosolic region is particularly interesting, because this region is involved in the association with the G-protein (6). Our finding, that pretreatment of m2R-expressing oocytes with PTX almost completely abolished the voltage dependence of [3H]ACh binding, provides strong support for the idea that the region associated with the G-protein is the voltage sensor. If this is the case, we would expect differences in the amino acid composition between m1R and m2R in this region, such as to favor the herein observed opposite effect of depolarization on the apparent affinity of these two GPCRs. Indeed, comparing the amino acid composition in the G-protein-coupling region of m1R and m2R reveals a cluster of positively charged amino acids in m2R (amino acids 215–255) that does not exist in m1R (Fig. 10B).

A Possible Mechanism That May Underlie the Depolarization-mediated Shift in Affinity—Muscarinic receptors were shown to exhibit two affinity states (Refs. 12 and 13 and see Fig. 5). Thus, following earlier studies (12, 13) we analyzed our data employing Michaelis-Menten equations, assuming two affinity states, \( K_a^H \) and \( K_a^L \) in Equations 1 and 2.

\[
Y = \frac{B_{max}^H X}{K_a^H + X} + \frac{(1 - B_{max}^H) X}{K_a^L + X} \tag{Eq. 1}
\]

Here, \( Y \) is the amplitude of the corresponding current at any agonist concentration, \( B_{max}^H \) is the saturation level of the response of the high affinity population (expressed as a fraction of 1), \( X \) is the concentration of the agonist, \( K_a^H \) denotes the \( K_a \) of the high affinity state, and \( K_a^L \) denotes the \( K_a \) of the low affinity state.

The DR curves, and in particular that of Fig. 4C, could not be well fitted with Equation 1 (dotted line). We, therefore, attempted to fit the DR curves assuming two binding sites (in addition to the two affinity states, \( K_a^H \) and \( K_a^L \)) where the affinity of each binding site \( (K_a^i) \) is taken to be equal as in Equation 2.

\[
Y = \frac{B_{max}^H X^2}{(K_a^H + X)^2} + \frac{(1 - B_{max}^H) X^2}{(K_a^L + X)^2} \tag{Eq. 2}
\]

Indeed, the DR curves were better fitted by Equation 2 and hence we extracted the values of the various parameters at the two holding potentials using Equation 2 (see Table I). Table I shows that the high affinity population comprised 86% at −60 mV, and it was reduced to 43% at +40 mV. It is further seen that for m2R the difference between \( K_a^H \) and \( K_a^L \) for OXO is much higher (200-fold) than for ACh (13-fold). This difference may explain the observation that the high and low affinity populations that comprise m2R are clearly apparent in the DR curve for OXO but almost unnoticeable in the DR curve for ACh (see later for discussion comparing the \( K_a \) values obtained here and those obtained in other studies).

What Is the Nature of the Two Affinity States?—As mentioned under “Results,” it was suggested (32, 33) that the affinity, toward the agonist, of the complex (receptor-G-protein) is higher than the affinity of the receptor alone. The observation that pretreatment with PTX reduced the fraction of the high affinity population (a fraction that is high at resting potential but also exists under depolarization, see Table I) and as a result almost completely abolished the voltage dependence of [3H]ACh binding to m2R is compatible with the following mechanism. Depolarization reduces the likelihood of m2R to associate with its G-protein, presumably by causing a conformational change in the receptor region that couples with its G-protein, and thereby increases the fraction of the low affinity population.

What May Be the Physiological Significance of Our Findings?—GPCRs play a key role in most of the signal transduction processes. Based on our findings, we believe that voltage sensitivity will be found to be a general characteristic of many other members of GPCRs. If this will indeed be the case, then changes in membrane potential may provide a new mechanism for modulation of signal transduction processes in excitable cells.

We will illustrate here the physiological significance of GPCRs being voltage-sensitive by discussing one fundamental process in which GPCRs play a major role; release of neurotransmitter and, in particular, release of ACh. Presynaptic autoreceptors (often belonging to the GPCR family) are known to modulate release (34). In particular, m2R was found to inhibit and m1R to enhance release of ACh. Furthermore, the m2R-mediated inhibition of ACh release was found to be voltage-dependent (8). But, inhibitory autoreceptors such as m2R were suggested to do more than modulating release; it was suggested...
that they lie at the very heart of control of the action potential-evoked transmitter release from fast synapses (35). In the case of control of ACh release, it was suggested that m2R does so by a direct association of m2R with proteins of the exocytotic machinery, which indeed was found to take place (36). It was further shown that at resting potential ACh release is under tonic block imposed by m2R (8, 37). Upon action potential-mediated depolarization two independent processes occur. One, opening of voltage-gated Ca\(^{2+}\) channels and a consequent influx of Ca\(^{2+}\). The other, depolarization-mediated relief of the tonic block imposed by m2R. The free release machinery, together with the Ca\(^{2+}\) that had entered, enable initiation of ACh release. Upon membrane repolarization, termination of release occurs because of reinstatement of the block (35, 37, 38).

Linking the herein described findings with the earlier mentioned physiological observations implies that it is m2R, a GPCR, that is the key player and in fact induces the voltage-dependent processes associated with control of ACh release. Thus, at resting potential m2R is in a high affinity state, and, hence, even the low concentration of ACh present in the synaptic cleft suffices to keep it occupied and, consequently, to physically interact with the release machinery and keep release blocked. Under depolarization, m2R shifts to a low affinity state, ACh dissociates, and the ACh-unoccupied m2R detaches from the release machinery. The free release machinery, together with the Ca\(^{2+}\) that had entered, promotes release. Upon membrane repolarization, m2R shifts back to its high affinity state and the block is reinstated. The findings here provide a strong and direct support for the mechanism detailed above for m2R-mediated control of ACh release (35).

Comparing the herein estimated K\(_d\) values with values obtained in previous studies shows a reasonable similarity where K\(_d\)^\(H\) is concerned but a large discrepancy where K\(_d\)^\(L\) is concerned. For example, in \({}^3\)H[ACh binding experiments done in rat brain synaptosomes (12), K\(_d\)^\(H\) was found to be 20 nM (in comparison to 23 nM here), and K\(_d\)^\(L\) was found to be 20 \(\mu\)M (in comparison to 301 nM here). Similarly, K\(_d\)^\(H\) for OXO, measured in cells containing muscarinic receptor subtypes 1–4 (39), was found to be 2.5 nM (similar to our results) and K\(_d\)^\(L\) was found to be 9 \(\mu\)M (in comparison to 463 nM here). One explanation to this discrepancy may be that, in recombinant receptor systems (such as the system used here), the amount of the injected m2R cRNA may affect the apparent affinity (33). Indeed, in experiments designed to specifically examine this possibility, we found that, when a lower concentration of the m2R cRNA was injected, the difference between the high and the low K\(_d\) values was (for ACh) about 500-fold, approaching the difference found in synaptosomes.

However, even if the above explanation accounts for some of the differences between oocytes and synaptosomes, additional possibilities must be considered. An attractive explanation may be that the voltage sensitivity of a weak voltage sensor, such as the M\(_2\)-receptor, is amplified by other proteins with which it forms complexes in its natural environment.

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Yair Ben-Chaim, Oded Tour, Nathan Dascal, Itzchak Parnas and Hanna Parnas

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