Affinity for phosphatidylinositol 4,5-bisphosphate determines muscarinic agonist sensitivity of Kv7 K+ channels

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Kv7 K+-channel subunits differ in their apparent affinity for PIP$_2$ and are differentially expressed in nerve, muscle, and epithelia in accord with their physiological roles in those tissues. To investigate how PIP$_2$ affinity affects the response to physiological stimuli such as receptor stimulation, we exposed homomeric and heteromeric Kv7.2, 7.3, and 7.4 channels to a range of concentrations of the muscarinic receptor agonist oxotremorine-M (oxo-M) in a heterologous expression system. Activation of M$_4$ receptors by oxo-M leads to PIP$_2$ depletion through G$_q$ and phospholipase C (PLC). Chinese hamster ovary cells were transiently transfected with Kv7 subunits and M$_4$ receptors and studied under perforated-patch voltage clamp. For Kv7.2/7.3 heteromers, the EC$_{50}$ for current suppression was 0.44 ± 0.08 µM, and the maximal inhibition (Inhib$_{\text{max}}$) was 74 ± 3% (n = 5–7). When tonic PIP$_2$ abundance was increased by overexpression of PIP 5-kinase, the EC$_{50}$ was shifted threefold to the right (1.2 ± 0.1 µM), but without a significant change in Inhib$_{\text{max}}$ (73 ± 4%, n = 5). To investigate the muscarinic sensitivity of Kv7.3 homomers, we used the A315T pore mutant (Kv7.3T) that increases whole-cell currents by 30-fold without any change in apparent PIP$_2$ affinity. Kv7.3T currents had a slightly right-shifted EC$_{50}$ as compared with Kv7.2/7.3 heteromers (1.0 ± 0.8 µM) and a strongly reduced Inhib$_{\text{max}}$ (39 ± 3%). In contrast, the dose–response curve of homomeric Kv7.4 channels was shifted considerably to the left (66 ± 8 nM), and Inhib$_{\text{max}}$ was slightly increased (81 ± 6%, n = 3–4). We then studied several Kv7.2 mutants with altered apparent affinities for PIP$_2$ by coexpressing them with Kv7.3T subunits to boost current amplitudes. For the lower affinity (Kv7.2 (R452E, R459E, and R461E) triple mutant was also coexpressed with Kv7.3T) channels, the EC$_{50}$ and Inhib$_{\text{max}}$ were similar to Kv7.4 or Kv7.3T homomers (0.12 ± 0.08 µM and 79 ± 6% [n = 3–4] and 0.58 ± 0.07 µM and 27 ± 3% [n = 3–4], respectively). The very low-affinity Kv7.2 (R452E, R459E, and R461E) triple mutant was also coexpressed with Kv7.3T. The resulting heteromer displayed a very low EC$_{50}$ for inhibition (32 ± 8 nM) and a slightly increased Inhib$_{\text{max}}$ (83 ± 3%, n = 3–4). We then constructed a cellular model that incorporates PLC activation by oxo-M, PIP$_2$ hydrolysis, PIP$_2$ binding to Kv7-channel subunits, and K$^+$ current through Kv7 tetramers. We were able to fully reproduce our data and extract a consistent set of PIP$_2$ affinities.

INTRODUCTION

A wide spectrum of ion channels and transporters are regulated by the plasma membrane abundance of the lipid phosphatidylinositol (PI) 4,5-bisphosphate (PIP$_2$). Upon stimulation of G$_{q/11}$-coupled receptors, activation of phospholipase C hydrolyzes PIP$_2$, producing cytosolic inositol trisphosphate (IP$_3$) and membrane-bound diacylglycerol, leading to three possible modes of action on ion channels: IP$_3$-mediated Ca$^{2+}$ signals, diacylglycerol-mediated activation of protein kinase C, and depletion of PIP$_2$ via consumption by PLC activity (Gamer and Shapiro, 2007). M-type K$^+$ currents are produced by voltage-gated Kv7 (KCNO) subunits in a variety of neuronal, muscle, and epithelial tissues, where they control excitability, action potentials, and K$^+$ transport (Jentsch, Brown and Adams, 1980; Constanti and Brown, 1981).

M channels are composed of Kv7.2/7.3 heteromers, but some channels also contain Kv7.5, and Kv7.2 or Kv7.3 homomers are also neurally expressed (Wang et al., 1998; Cooper et al., 2001; Roche et al., 2002; Pan et al., 2006). In the inner ear and auditory cortex, Kv7.4 homomers dominate, and in the cardiovascular system, Kv7.1, Kv7.4, and Kv7.5 are expressed in various combinations (Kubisch et al., 1999; Kharkovets et al., 2000; Loussouarn et al., 2006; Mackie and Byron, 2008).

The term M current comes from its depression by muscarinic receptor stimulation in sympathetic neurons (Brown and Adams, 1980; Constanti and Brown, 1981). After intense study, a wide spectrum of evidence indicates that this muscarinic action arises from the need of M channels for membrane PIP$_2$ to be functional (Zhang et al., 2003; Li et al., 2005; Suh et al., 2006) and the depletion of PIP$_2$ abundance in neurons by muscarinic agonist (Suh and Hille, 2002; Ford et al., 2003; Winks et al., 2005).
Furthermore, cellular modeling has been used to quantify this system, yielding a biophysically satisfying framework that produces sufficient changes in PIP2 abundance upon receptor stimulation to account for the observed depression of M current, given known rates of lipid enzymes, reasonable estimates of channel/PIP2 affinities and plausible densities of PIP2 molecules, and the signaling molecules relevant to this Gq/11-mediated system (Suh et al., 2004; Horowitz et al., 2005).

Single-channel analysis of Kv7.2–7.5 channels has revealed widely divergent activities of the channels in intact cells that can be ascribed to widely differential apparent affinities for PIP2. Whereas Kv7.3 homomers have a very high saturating open probability (P_o) and high PIP2 apparent affinity, Kv7.2 and Kv7.4 homomers display dramatically lower values, and Kv7.2/7.3 heteromers have intermediate values for saturating P_o and PIP2 apparent affinity, as one might expect for heteromeric channels containing subunits with divergent affinities (Li et al., 2005). We have recently localized the region of the channel that accounts for divergent P_o and PIP2 apparent affinity to a highly basic interhelical linker domain in the C terminus that is critical for PIP2 interactions (Hernandez et al., 2008). Kv7.3 homomers in particular possess several unique properties. Although they display much greater apparent affinity for PIP2 and saturating P_o than the others (Li et al., 2004, 2005; Hernandez et al., 2008) and their unitary conductance is also the highest, the whole-cell currents from heterologously expressed Kv7.3 homomers are at least 20-fold lower. We have suggested this aspect to be the result of a high fraction of wild-type (WT) Kv7.3 channels being dormant, a condition wholly reversed by a single point mutation in the inner pore at the 315 position from an alanine to a hydrophilic threonine or serine (Zaika et al., 2008), and for many of the experiments shown here, we exploit the high expression of Kv7.3 (A315T) channels, which we will refer to as Kv7.3{T} in this paper.

The widely divergent apparent affinity for PIP2 of Kv7 channels predicts a corresponding divergence in sensitivity to stimulation of PLC-linked M1 receptors because the degree of PIP2 depletion should correlate with receptor stimulation, Gq/11 activation, and PLC activity. Thus, Kv7.3 is predicted to be relatively insensitive to muscarinic agonists, whereas Kv7.2 or Kv7.4 is predicted to be more sensitive. In addition, Kv7.2 mutants with decreased PIP2 apparent affinity (Hernandez et al., 2008) should be more sensitive than WT channels, and increased tonic PIP2 abundance should render channels less sensitive. In this study, we test these ideas on mammalian cells heterologously expressing various Kv7 channels and M1 receptors and quantified agonist sensitivity as the dose–response relationship between muscarinic agonist concentration and Kv7-current inhibition. Furthermore, we modified a cellular model that simulates the Gq/11-mediated PLC signaling system, including PIP2 metabolism and channel–PIP2 interactions (Suh et al., 2004; Horowitz et al., 2005; Jensen et al., 2009), to model the relationship between receptor occupancy, PIP2 affinity, and channel activity. The data and model are highly congruent, providing a satisfying biophysical basis for our observations.

MATERIALS AND METHODS

cDNA constructs

Human Kv7.2, Kv7.3, and Kv7.4 (GenBank/EMBL/DDBJ accession nos. AF110020, AF071478, and AF105202, respectively) were provided by D. McKinnon (Kv7.2; State University of New York at Stony Brook, Stony Brook, NY) and T. Jentsch (Kv7.3 and Kv7.4; Zentrum für Molekulare Neurobiologie, Hamburg, Germany). Plasmids were subcloned into pcDNA3.1 using standard techniques. Mouse type 1b PI(4)P5-kinase (PIP5-kinase) was provided by L. Pott (Ruhr-University, Bochum, Germany; Bender et al., 2002). Mutations were made by PCR using the QuikChange method (Agilent Technologies) and verified by sequencing. The Kv7.3 (A315T) mutant (Kv7.3{T}) and all Kv7.2 mutants were made by site-directed mutagenesis using standard techniques and verified by dye-termination sequencing.

Cell culture and transfections

Chinese hamster ovary (CHO) cells were used for electrophysiological analysis as described previously (Gamper et al., 2005). Cells were grown in 100-mm tissue culture dishes (Falcon; BD) in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum and 0.1% penicillin and streptomycin in a humidified incubator at 37°C (5% CO2) and passed every 3–4 d. Cells were discarded after ∼30 passages. For transfection, cells were plated onto poly-L-lysine–coated coverslip chips and transfected 24 h later with Polyfect reagent (QIAGEN) according to the instructions of the manufacturer. For electrophysiological experiments, cells were used 48–96 h after transfection. As a marker for successfully transfected cells, cDNA encoding green fluorescent protein was cotransfected together with the cDNAs of the genes of interest. We found that >95% of green fluorescent cells expressed Kv7 currents in control experiments.

Perforated-patch electrophysiology

Pipettes were pulled from borosilicate glass capillaries (1B150F-4; World Precision Instruments) using a Flaming/Brown micropipette puller (P-97; Sutter Instrument Co.) and had resistances of 1–2 MΩ when filled with internal solution and measured in standard bath solution. Membrane current was measured with a pipette and membrane capacitance cancellation and was sampled at 5 ms and filtered at 1 kHz by an amplifier (EPC-9) and PULSE software (HEKA/Instrutech). In all experiments, the perforated-patch method of recording was used with 200–600 pg/ml amphotericin B in the pipette (Rae et al., 1991). Amphotericin was prepared as a stock solution as 60 mg/ml in DMSO. In these experiments, the access resistance was typically 10 MΩ 5–10 min after seal formation. Cells were placed in a 500-µl perfusion chamber through which solution flowed at 1–2 ml/min. Inflow to the chamber was by gravity from several reservoirs, selectable by activation of solenoid valves (Warner Scientific). Bath solution exchange was complete by <30 s. Experiments were performed at room temperature. The amplitude of the Kv7 current was usually defined as the holding current at 0 mV. In some cells, a more precise measurement was the NEX91-sensitive current at the holding potential of 0 mV. CHO cells had negligible endogenous

Published October 26, 2009
macroscopic K+ currents under our experimental conditions, and 10 μM XE991 completely blocked the K+ current in Kv7-transfected CHO cells but had no effect on currents in nontransfected cells (Gamper et al., 2005). Dose–response data of channel current inhibition versus [oxotremorine-M] ([oxo-M]) were fit using Prism software (version 5.01; GraphPad Software, Inc.) for Windows by nonlinear regression using a Hill equation of the form

\[ \text{Inhib} = \frac{\text{Inhib}_{\text{max}}}{1 + \left( \frac{[\text{oxo-M}]}{x} \right)^n} \]

where Inhib is the fractional inhibition of the Kv7 current, \( \text{Inhib}_{\text{max}} \) is the maximal inhibition, \( x \) is [oxo-M], \( x_{\text{null}} \) is the [oxo-M] at which inhibition = 50% of maximal inhibition, and \( n \) is the Hill coefficient, which we constrained to the value of one. All results are reported as mean ± SEM.

Solutions and materials
The external Ringer’s solution used to record Kv7 currents in CHO cells contained 160 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES, pH 7.4, with NaOH. The pipette solution contained 160 mM KCl, 5 mM MgCl2, 5 mM HEPES, and 10 mM EGTA, pH 7.4, with KOH and added amphotericin B (200–600 μg/ml). Reagents were obtained as follows: Dulbecco’s modified Eagle’s medium, fetal bovine serum, penicillin, and streptomycin were purchased from Invitrogen; amphotericin B was obtained from Sigma-Aldrich. XE991 was purchased from Tocris.

Modeling
A kinetic model was implemented in the Virtual Cell modeling and simulation framework (www.xcell.org). From within Virtual Cell, the model can be accessed by any user under Shared Models/b Falken/Hernandez Falkenburger Shapiro 2009. The rate constants and equations that constitute the model and initial values for the parameters are listed in Tables S1–S3. The model is a simplified adaptation of a model describing PIP2 metabolism and Kv7.2/7.3 current by Hille and co-workers (Suh et al., 2004; Horowitz et al., 2005). Basal levels of PIP2 were assumed to be 5,000 molecules/μm² based on the results of earlier modeling that well accounted for the onset and recovery rates of muscarinic suppression of Kv7.2/7.5 current (Suh et al., 2004) and from the calculated membrane-bound fraction of the PIP2/IP3-binding PH-PLCδ1 probe (Horowitz et al., 2005). Xu et al. (2003) used a value of 4,000/μm² based on biochemical assays, but the exact number does not affect the conclusions drawn from the modeling (Fig. S1). Our model has only one invariant reaction rate for PIP2 synthesis (syn) that lumps together activity of phosphatidylinositol (PI) 4-kinase and PIP 5-kinase. Syn prevents PIP2 levels from falling to zero during application of muscarinic agonist (oxo-M) and mediates PIP2 recovery after washout of agonist. The rates of receptor-independent steady-state PIP2 degradation via PIP2 phosphatase and basal PLC activities were lumped together (deg), whose value was chosen to keep PIP2 levels stable at rest. The model simulates extracellular concentrations of oxo-M with a 10 s time constant for solution exchange, as determined by calibration experiments. For dose–response curves, oxo-M was modeled as applied for 100 s to ensure that steady state was reached as in the experiments. PLC is activated by muscarinic receptor agonist with an EC50 of 1.6 μM, a value chosen to allow significant PIP2 changes over the entire oxo-M concentration range, which is the prerequisite to observe changes in Kv7 currents. The time constant for PLC activation was taken from fluorescence resonance energy transfer (FRET) measurements of PLC binding to G protein (Jensen et al., 2009).

The amplitudes of Kv7 currents were implemented in two steps: PIP2 binding to Kv7-channel subunits and generation of current by four Kv7 subunits. The Ka values for PIP2 binding were 500 μM⁻² for Kv7.2 (WT), 75 μM⁻² for Kv7.3, 2,500 μM⁻² for Kv7.4, 10 μM⁻² for Kv7.2 (EEE), 75 μM⁻² for Kv7.2 (R463E), and 5,000 μM⁻² for Kv7.2 (R463Q). By analogy to the Hodgkin and Huxley term n, which combines the sensing state of four voltage sensors to determine the voltage-dependent open probability of the entire channel, we calculated the PIP2-dependent open probability of the entire channel as the product of the PIP2 saturation of the four channel subunits (Table S2). For the heteromeric channels consisting of WT or mutant Kv7.2 and 7.3 subunits, we assumed the channel tetramers to consist of two subunits of each type, which is sufficient for our purposes here. Because the PIP2 saturation of Kv7.3 subunits does not change much in response to oxo-M, the changes in Kv7.2/7.3 current are predominantly governed by the PIP2 occupancy of the Kv7.2 subunits with an exponent of 2 and a Ka of 500. This implementation is thus similar to the earlier models (Suh et al., 2004; Horowitz et al., 2005), which used a Ka of 1,000 μM⁻² for the entire Kv7.2/7.3 channel and an exponent of 1.8.

To get a sense of how the model works, we varied several parameters over a wide range and modeled the results (Fig. S1). We first modeled the effects of varying the Ka of a subunit for PIP2 on the dose–response relationship between muscarinic agonist and current inhibition for a homomeric channel (Fig. S1 A). When the model used values of Ka that varied from 75 to 2400, two trends were evident. At the high end of the range (i.e., low-affinity subunits), a change in the Ka mostly results in a shift of the dose–response curve, with little impact on the maximum inhibition. However, at the low end of the range (i.e., high-affinity channels), a change in the Ka results in little displacement of the dose–response curve (quantified by the EC50) but a big effect on the maximum inhibition. This can be understood in that maximum PLC activity does not reduce the PIP2 abundance to zero but rather to a steady-state value that reflects the equilibrium between PLC-mediated consumption and PIP2 synthesis. Thus, for the high-affinity channels whose overall n² affinity is near those steady-state values, the predominant effect of altering Ka in the low range is a change in maximal inhibition. However, for the low-affinity channels, the subunit occupancy at maximal [oxo-M], which is very low, will change little when Ka is altered in this high range. Thus, the predominant effect is a shift in the dose–response curve, as the amount of PLC activity needed for the same degree of PIP2 unbinding changes accordingly. Fig. S1 B shows a similar analysis for a heteromeric channel in which two subunits are Kv7.2 and the other two have Ka values over the same wide range. One can see that the same principles apply but to a lesser extent, as expected from the invariant affinity of the Kv7.2 subunits that buffer the overall response.

PIP2 abundance has been suggested to vary considerably among cell types. For a given cell, this value reflects the equilibrium between tonic PIP2 synthesis by PI kinases (syn in our model) and degradation by tonic PIP2 phosphatases and basal PLC activity (deg in our model). In Fig. S1 C, we model the effect on the dose–response curve for Kv7.2/3 heteromers of doubling the tonic PIP2 abundance to 10,000 μM⁻² by doubling the value of syn. In Fig. S1 D, we model the effect of the same maneuver on the relationship between [oxo-M] and PIP2 abundance. It can be seen that there are some modest changes: the curve in Fig. S1 C is a little shifted to the left and the maximal inhibition is a little less, and the curve in Fig. S1 D has a steeper fall toward a steady-state value that is a little larger.

Online supplemental material
Tables S1, S2, and S3 present information on model parameters, the formulations describing the Kv7 currents, and initial values of the parameters, respectively. Fig. S1 characterizes the basic behavior of the model, as described in the Materials and methods section. Online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.200910313/DC1.
We studied CHO cells transfected with various Kv7 channels and M₁ muscarinic receptors under perforated-patch whole-cell voltage clamp. The inhibition of the current was quantified over a range of concentrations of the muscarinic agonist oxo-M. Our first experiments evaluated the muscarinic agonist sensitivity of WT Kv7.2/7.3 heteromers under control conditions and in cells in which tonic PIP₂ abundance was artificially increased. In control, the Kv7.2/7.3 current was inhibited by oxo-M in a concentration-dependent manner (Fig. 1, A and C) with an EC₅₀ of 0.44 ± 0.08 µM (n = 5–7) and a maximal current inhibition of 74 ± 3% (Fig. 1 B, open circles). The EC₅₀ value is comparable with that obtained previously in sympathetic neurons (0.30–0.68 µM; Bernheim et al., 1992; Haley et al., 2000; Winks et al., 2005; Robbins et al., 2006; Zaika et al., 2007) but is on the low end of that reported in mammalian expression systems (79–95%; Selyanko et al., 2000; Shapiro et al., 2000; Gamper et al., 2003; Suh et al., 2004, 2006; Horowitz et al., 2005). Likewise, the maximal inhibition is well within the range of that reported in sympathetic neurons (61–93%; Beech et al., 1991; Bernheim et al., 1992; Cruzblanca et al., 1998; Haley et al., 2000; Winks et al., 2005; Robbins et al., 2006; Zaika et al., 2007) but is on the low end of that reported in mammalian expression systems (79–95%; Selyanko et al., 2000; Shapiro et al., 2000; Gamper et al., 2003; Suh et al., 2004, 2006; Horowitz et al., 2005). We suspect that the precise values of both parameters will depend on the specific density of receptors or PLC molecules for each type of native, or heterologously transfected, cell.

**RESULTS**

Figure 1. Agonist-induced Kv7.2/7.3-current suppression is reduced by overexpression of PIP 5-kinase. (A) Averaged time course of normalized current amplitude during sequential application of a range of concentrations of oxo-M to CHO cells coexpressing Kv7.2/7.3 channels and M₁ receptors, without (open circles) or together with PIP 5-kinase (closed circles). Times of applications of oxo-M at the given concentrations are indicated by the arrows. (B) Concentration dependence of current inhibition by oxo-M from cells expressing Kv7.2/7.3 alone (open circles) or together with PIP 5-kinase (closed circles). The lines represent the fits of experimental data by a Hill equation, with the values given in the text. Each point represents the mean ± SEM from n = 5–7 experiments. (C) Current waveforms before and after the application of a range of concentrations of oxo-M to CHO cells not coexpressing (left) or coexpressing PIP 5-kinase (right). The dashed line in the current traces is the zero current level, and the pulse protocol used is shown in the bottom in the left panel.
greater activity of molecules in the C_{q/11} signaling pathway being required in the cells transfected with PIP_5-kinase relative to the activity required to reduce PIP_2 abundance in control cells to the level needed for a given amount of current inhibition. In our model, an increase in syn by 1.7-fold is required to produce our observed effect on the EC_{50} value, which is a little less than the twofold increase whose effect we modeled (Fig. S1, C and D). Our modeling also predicts a significant decrease in Inhib_{max}, which we did not observe. The relatively mild effects observed of PIP_5-kinase transfection, compared with previous studies, are probably caused either by less expression of PIP_5-kinase molecules than before or by a higher level of tonic PIP_2 abundance in the membrane in the present studies.

The muscarinic sensitivity of Kv7 channels correlates with their apparent affinity for PIP_2

Our previous work indicates the apparent affinity of PIP_2 for Kv7.3 homomers to be high and that of Kv7.2 or Kv7.4 homomers to be much lower, with Kv7.2/7.3 heteromers being intermediate (Li et al., 2005; Hernandez et al., 2008). To ask whether the sensitivity to muscarinic suppression for Kv7 and Kv7.4 homomers is a good indicator of their apparent PIP_2 affinity, we quantified the dose–response relationship of muscarinic agonist and current suppression for Kv7.3 and Kv7.4 homomers. Many laboratories report only tiny Kv7.3 homomeric currents in heterologous systems (Etxeberria et al., 2004; Schenzer et al., 2005; Zaika et al., 2008), which would preclude the dose–response analysis desired here. Thus, for the Kv7.3 experiments, we used the A315T pore mutant, which displays large whole-cell currents that enable such analysis, with no changes in the unitary P_o of the channel at saturating voltages, indicating that the A315T mutation does not alter the high PIP_2 apparent affinity of Kv7.3 subunits (Zaika et al., 2008). We will call this mutant Kv7.3T in this paper. Compared with Kv7.2/7.3 heteromers, the most dramatic difference for Kv7.3T homomers was the strongly reduced maximal inhibition of the current at high oxo-M concentrations. Thus, at 10 µM oxo-M, the Kv7.3T current was suppressed by only 39 ± 3% (n = 5–7; Fig. 2, A and C), whereas there was only a slight shift of the oxo-M dose–response curve to higher concentrations (EC_{50} = 1.0 ± 0.76 µM, n = 5–7; Fig. 2 B). As discussed below, these data suggest that at supramaximal concentrations of muscarinic agonist, the equilibrium between PLC-mediated consumption and synthesis of PIP_2 results in a PIP_2 abundance at which most high-affinity Kv7.3T channels are still bound by PIP_2 molecules. This hypothesis is further explored in the modeling to follow.
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...barrel motif common to other PIP2-binding domains (Hernandez et al., 2008). Interestingly, that study found mutation of two conserved basic residues in Kv7.2 (R463 and R467) within the cationic cluster to display opposite effects on PIP2 apparent affinity that depended on the nature (charge reversal vs. charge neutralization) of the substituted residues, observations explained by molecular modeling and docking simulations (Hernandez et al., 2008). To ask whether muscarinic agonist sensitivity correlates with the observed changes in PIP2 apparent affinity of one such Kv7.2 mutant pair, we studied R463E and R463Q, which were previously shown to have a much higher and lower apparent affinity for PIP2, respectively (Hernandez et al., 2008). However, because cells transfected with those mutants display only small whole-cell currents (Hernandez et al., 2008), we studied the oxo-M concentration dependence for inhibition of R463E and R463Q mutant subunits coexpressed with Kv7.3 (Fig. 4). For Kv7.2 (R463E)/7.3, the muscarinic sensitivity was sharply reduced, manifested by a reduction in the maximal inhibition of the current to 27 ± 3% and a slight shift of the EC50 to higher concentrations (0.58 ± 0.07 µM, n = 3–4), compared with Kv7.2/7.3 (Fig. 4, A and B). In contrast, mutation of R463 to the lower affinity Kv7.4 homomers displayed an inhibition of ~75% at 1 µM oxo-M (Fig. 3, A and C), a concentration that produced only ~19% inhibition of the Kv7.3 current (Fig. 2, B and C), and ~48% inhibition of the Kv7.2/7.3 current (Fig. 3 B, dashed line). Analyzed over a wide range of agonist concentrations, the dose dependence of Kv7.4-current suppression by oxo-M was shifted to lower concentrations by approximately sevenfold (EC50 = 66 ± 8 nM, n = 3–4) compared with Kv7.2/7.3 channels and by 15-fold compared with Kv7.3 channels. Interestingly, there was no significant difference from Kv7.2/7.3 channels with regard to maximal suppression (81 ± 6%; Fig. 3 B). Thus, the activity of molecules in the Gq/11 signaling pathway required to reduce PIP2 levels enough to cause unbinding of PIP2 from low apparent affinity Kv7.4 channels must be significantly less than the activity required to cause PIP2 unbinding from higher affinity Kv7.2/7.3 channels or highest affinity Kv7.3 channels. These ideas are also tested in our modeling below.

Putative PIP2-interacting residues within an interhelical domain in the C terminus control muscarinic sensitivity

We have localized the primary binding site of PIP2 to M channels to a C-terminal domain that consists of a cluster of conserved basic amino acids within a seven–β-sheet barrel motif common to other PIP2-binding domains (Hernandez et al., 2008). Interestingly, that study found mutation of two conserved basic residues in Kv7.2 (R463 and R467) within the cationic cluster to display opposite effects on PIP2 apparent affinity that depended on the nature (charge reversal vs. charge neutralization) of the substituted residues, observations explained by molecular modeling and docking simulations (Hernandez et al., 2008). To ask whether muscarinic agonist sensitivity correlates with the observed changes in PIP2 apparent affinity of one such Kv7.2 mutant pair, we studied R463E and R463Q, which were previously shown to have a much higher and lower apparent affinity for PIP2, respectively (Hernandez et al., 2008). However, because cells transfected with those mutants display only small whole-cell currents (Hernandez et al., 2008), we studied the oxo-M concentration dependence for inhibition of R463E and R463Q mutant subunits coexpressed with Kv7.3 (Fig. 4). For Kv7.2 (R463E)/7.3, the muscarinic sensitivity was sharply reduced, manifested by a reduction in the maximal inhibition of the current to 27 ± 3% and a slight shift of the EC50 to higher concentrations (0.58 ± 0.07 µM, n = 3–4), compared with Kv7.2/7.3 (Fig. 4, A and B). In contrast, mutation of R463 to the lower affinity Kv7.4 homomers displayed an inhibition of ~75% at 1 µM oxo-M (Fig. 3, A and C), a concentration that produced only ~19% inhibition of the Kv7.3 current (Fig. 2, B and C), and ~48% inhibition of the Kv7.2/7.3 current (Fig. 3 B, dashed line). Analyzed over a wide range of agonist concentrations, the dose dependence of Kv7.4-current suppression by oxo-M was shifted to lower concentrations by approximately sevenfold (EC50 = 66 ± 8 nM, n = 3–4) compared with Kv7.2/7.3 channels and by 15-fold compared with Kv7.3 channels. Interestingly, there was no significant difference from Kv7.2/7.3 channels with regard to maximal suppression (81 ± 6%; Fig. 3 B). Thus, the activity of molecules in the Gq/11 signaling pathway required to reduce PIP2 levels enough to cause unbinding of PIP2 from low apparent affinity Kv7.4 channels must be significantly less than the activity required to cause PIP2 unbinding from higher affinity Kv7.2/7.3 channels or highest affinity Kv7.3 channels. These ideas are also tested in our modeling below.

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Thus, the currents from Kv7.2 (EEE)/7.3T channels were inhibited by 60% at 0.1 µM oxo-M, a concentration that produced an inhibition of Kv7.2 (R463Q)/7.3T channels of only 30% (Fig. 4 C). In summary, our results suggest that sensitivity to muscarinic agonist via Gq/11-coupled M1 receptors, PLC activity, and consumption of PIP2 correlates well with apparent PIP2 affinity across the family of homomeric and heteromeric M-type channels both for WT Kv7 variants and for the Kv7.2 mutants. They also suggest that the PIP2 affinity of one type of subunit in a heteromer has strong effects on the overall PIP2 affinity and subsequent muscarinic sensitivity of the overall tetrameric channel. Thus, PIP2 binding to all subunits in the tetramer is likely to be required for the channel to be activatable by voltage.

A cellular model of Gq/11 signaling provides a biophysical model of our observations

We used a simplified version (Fig. 5 A) of a more comprehensive kinetic model developed previously (Suh et al., 2004) to show that our experimental findings can be explained by the basic principles of Gq/11 signaling.
and channels regulated by PIP2 abundance, which we assume to tonically be 5,000 molecules/µm² of plasma membrane. In our model here, Kv7.2, 7.3, and 7.4 subunits or mutant Kv7.2 subunits possess distinct affinities for PIP2, quantified as the Kₘ for PIP2 binding, which was empirically determined to best fit the data. For Kv7.2–7.4, the Kₘ values were 500, 75, and 2,500, and for the Kv7.2 mutants R463E, R463Q, and the EEE mutant, they were 75, 5,000, and 10⁶, respectively. The model supposes binding of one PIP2 to one Kv7 subunit. Because each channel is composed of four subunits, the current of a Kv7 channel is proportional to the product of the fractional PIP2 saturation of four subunits (see Materials and methods and Table S2). We assumed heteromeric Kv7.2/7.3 channels to consist of two Kv7.2 (WT or mutant) and two Kv7.3 subunits, which is reasonable for our purposes here. For homomeric channels, current was modeled as being proportional to the fourth power of PIP2-bound subunits. This implementation differs from the model by Suh et al. (2004) in which PIP2 bound to a channel particle instead of to a subunit particle, which did not allow “mixing” of Kv7.2 and 7.3 subunits to form a heteromeric channel. Thus, we first determined the affinity of homomeric channels to determine the affinity of each type of WT subunit and then adjusted the affinity of WT or mutant Kv7.2 subunits to reproduce the dose–response relationships observed experimentally with Kv7.2/7.3 heteromers. Our model reflects the affinity of PIP2 for Kv7.2, 7.3, and 7.4 subunits to be intermediate, high, and low, respectively, and the R463E and R463Q mutants of Kv7.2 to give that subunit high (similar to Kv7.3) and low (similar to Kv7.4) affinities for PIP2, respectively. Finally, in our model, the EEE mutant possesses a profoundly low PIP2 affinity, which is in accord with the very low affinity predicted in our earlier docking simulations and seen in single-channel patches (Hernandez et al., 2008).

The first important observation is the relationship between PIP2 density and concentration of receptor agonist (Fig. 5 B). The activation of PLC by o xo-M was informed by measurements of muscarinic agonist-induced changes in FRET between Goq and PLC (Jensen et al., 2009) and adjusted to produce significant changes in PIP2 density over the entire range of o xo-M concentrations in which current inhibition was observed. Steady-state PIP2 density reflects the equilibrium between consumption by PLC and synthesis by PI kinases, with half-maximal PIP2 reduction at ~2.0 µM o xo-M (Fig. 5 B). PIP2 synthesis during o xo-M application is greater than zero (and in fact stimulated as compared with baseline values; Gamper et al. 2004). PLC activity saturates at a finite value, as there are a finite number of PLC molecules that can be activated. As a consequence, there is a defined, nonzero minimum level for PIP2 in the presence of maximal o xo-M concentrations (~500 µM in Fig. 5 B). As a consequence, the inhibition of all Kv7 channels will saturate at high [oxo-M] (Fig. 5 C). Inhib_max is predicted to diverge between Kv7 channels, depending on their affinity for PIP2. Low-affinity channels will have lost virtually all their bound PIP2 molecules at minimal PIP2 densities, whereas a substantial proportion of high-affinity channels will retain their bound PIP2. The small maximum inhibition of Kv7.3 current at supramaximal o xo-M is thus explained by the fact that, because of their high PIP2 affinity, they retain much of their bound PIP2 even at maximal PLC activity (Fig. 5 C, dashed line). Another consequence of the high affinity of Kv7.3 for PIP2 is that the channel is relatively PIP2 saturated at rest, and the predicted relationship between PIP2 abundance and Kv7.3 current is relatively flat at resting PIP2 densities (Fig. 5 D, dashed line). Low concentrations of o xo-M are therefore predicted to have virtually no effect on Kv7.3 current, in accord with the data. Conversely, Kv7.4 has a low affinity for PIP2, and the PIP2 concentration curve for Kv7.4 is relatively steep in the area of resting PIP2 (Fig. 5 D, dotted line). This results in strong current reduction even by small changes of PIP2 abundance and augmented inhibition at saturating [oxo-M]. Current inhibition thus requires only little o xo-M, low activation of receptors, Go_q/11, and PLC, explaining the left-shifted concentration curve as compared with Kv7.2/7.3 channels (Fig. 5 C, dotted line). The comparison between values for EC_50 and maximal inhibition for the data and model values for Kv7.3, 7.4, and 7.2/7.3 channels are nicely in accord (Table I), providing satisfying support for our thinking.

The next set of modeling has to do with the behavior of the Kv7.2 mutants. Again, we assumed a 1:1 stoichiometry in the channel tetramers made from coexpressed Kv7.2 mutant subunits and Kv7.3 subunits, with current proportional to the sequential product of four PIP2-bound subunits. We assume that the changes in muscarinic sensitivity for the higher or lower PIP2 affinity Kv7.2 mutants studied here can be ascribed to the same biophysical principles as for the WT channels. In the model, the best fit for the data from Kv7.2 (R463E) and (R463Q) subunits were with PIP2 affinities similar to that of Kv7.3 or Kv7.4 subunits, respectively, and the PIP2 affinity needed for the Kv7.2 (EEE) mutant had to be made extremely low. Thus, for Kv7.2 (R463E)/Kv7.3 channels, the relationship between current inhibition and [oxo-M] is predicted to be flat at low [oxo-M] with relatively low inhibition at saturating [oxo-M], which is in accord with the data, like that of Kv7.3 homomers. Conversely, for Kv7.2 (R463Q)/7.3 channels, the relationship between current inhibition and [oxo-M] is predicted to be relatively steep at low [oxo-M], resulting in strong current reduction by small changes in PIP2 abundance and augmented inhibition at saturating [oxo-M], all similar to the response of Kv7.4 homomers. In the model, the o xo-M sensitivity of Kv7.2 (EEE)/Kv7.3 channels is only slightly greater than that for Kv7.2.
Two related types of single-channel experiments have suggested that the family of Kv7 channels possesses divergent apparent affinities for PIP2. The first are cell-attached patch recordings in which channel Po at saturating voltages varied widely, consistent with the tonic PIP2 abundance supporting a divergent level of channel opening. The second are inside-out patch data in which Po measured over a wide range of concentrations of a water-soluble PIP2 analogue revealed a parallel differential PIP2 apparent affinity (Hernandez et al., 2008). These results predicted a corresponding divergent dose–response relationship between PLC-linked receptor agonist and Kv7-channel inhibition. That prediction was verified here. A main result of this work is that the effect of alterations in PIP2 affinity on the sensitivity of the channels to muscarinic agonist depends on whether the channels have a relatively low or high apparent affinity for PIP2. For the former, reductions in PIP2 affinity do not much affect the Inhibmax because it is already quite high but rather shift the dose–response relation of oxo-M versus inhibition to lower concentrations. Conversely, for the latter, reductions in affinity do not much change the EC50 of the dose–response relation, but rather the

<table>
<thead>
<tr>
<th>Channel</th>
<th>Data</th>
<th>Model</th>
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<tr>
<td></td>
<td>EC50</td>
<td>Inhibmax</td>
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<tr>
<td></td>
<td>μM</td>
<td>%</td>
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<tr>
<td>Kv7.2wt/7.3wt</td>
<td>0.44 ± 0.08</td>
<td>74 ± 3</td>
</tr>
<tr>
<td>Kv7.3T</td>
<td>1.0 ± 0.76</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>Kv7.4wt</td>
<td>0.066 ± 0.008</td>
<td>81 ± 6</td>
</tr>
<tr>
<td>Kv7.2 (R463E)/7.3T</td>
<td>0.58 ± 0.07</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>Kv7.2 (R463Q)/7.3T</td>
<td>0.12 ± 0.08</td>
<td>79 ± 6</td>
</tr>
<tr>
<td>Kv7.2 (EEE)/7.3T</td>
<td>0.032 ± 0.004</td>
<td>83 ± 3</td>
</tr>
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The values for both data and model were taken from Hill equation fits in both cases.
large effect is an increase in Inhibmax. Thus, the current from Kv7 channels does not generally go to zero upon maximal stimulation of M1 receptors, but rather Inhibmax depends on the equilibrium between PLC-mediated hydrolysis and PIP2 synthesis and the affinity of the particular channel. All of these phenomena are well simulated by our model incorporating PIP2 metabolism, receptor-mediated PLC activation, and channels regulated by PIP2 binding with quantifiable affinities. What adds impact to our results is that agonist-dependent PIP2 depletion or creation is highly receptor and cell dependent. Thus, PIP2 binding/unbinding is hypothesized to often occur not via PIP2 depletion but rather via alteration of the PIP2 affinity of PIP2-regulated channels by myriad signaling molecules (Gβγ, Ca2+/calmodulin, pH, Na+, PKC, PKA, etc.; Gamper and Shapiro, 2007). Therefore, the changes of PIP2 affinity by mutagenesis or change of subunit composition in these experiments somewhat mimic this physiological mechanism of lipid signaling. For example, whereas Kv7.3 homomers are only slightly inhib- ited at maximal depletion of PIP2 by PLC, a reduction in the channel’s affinity for PIP2 will put its Kp in the range where such physiological depletions will have a much stronger effect on the currents. In addition, the strong subunit dependence of muscarinic agonist sensitivity predicts that transcriptional control of subunit expression in distinct regions of the nervous system will have profound consequences for muscarinic receptor control over neuronal excitability.

The cellular modeling makes predictions for the values of EC50 and Inhibmax for muscarinic suppression of the Kv7 subunits that we studied. Those predicted and experimental values are compared in Table I. For clarity, we will use Kv7.2/7.3 heteromers as the baseline to compare with the others in this discussion. Several trends are clearly in accord between the two sets of values. First, they both show high-affinity channels (Kv7.3T and Kv7.2 (R463E)/Kv7.3T) to have sharply reduced Inhibmax but only slightly increased EC50 values. Second, they both show low-affinity channels (Kv7.4, Kv7.2 (R463Q)/Kv7.3T, and Kv7.2 (EEE)/Kv7.3T) to manifest strongly decreased EC50 values. The model also predicts the sig- nificant current remaining after maximal receptor stimulation for intermediate affinity channels, which is in accord with the experimental observations. However, a clear discrepancy is the near complete elimination of the current by low-affinity channels predicted by the model, which we did not observe in the data. An explanation may be the existence of “protected” pools of PIP2 (perhaps sequestered by MARCKS [myristoylated ala- nine-rich C-kinase substrate] proteins or other PIP2-sequestering moieties; Gambhir et al., 2004; McLaughlin et al., 2005; Milosevic et al., 2005) that cannot be consumed by PLC, preventing Kv7-channel activity from ever going to zero. Alternatively, the CHO cells that we use for our heterologous expression system may be en-
were found, relative to Kv7.2, to be 2.2 (Kv7.3), 3.8 (Kv7.2 R463E), –5.7 (Kv7.2 R463Q), and –11.2 (Kv7.2 EEE) kJ/M (docking with Kv7.4 was not modeled; Hernandez et al., 2008). Although there are quantitative differences between the present modeling results and the previous docking simulations, there is qualitative agreement that lends credence to the docking simulations and adds to an internally consistent body of data and modeling across single-channel patches, receptor-mediated modulation in intact cells, homology/docking modeling, and the model simulations. Clearly, the next step will be to perform biochemical affinity measurements for the PIP2-binding domains of the various M-type channels to test whether our conclusions from signaling cascades translate into quantitative measurements of physical molecular interactions.

We thank P. Reed for expert technical assistance and B. Hille for discussions.

This work was supported by National Institutes of Health grant R01 NS043394 (to M.S. Shapiro), American Heart Association grant-in-aid 075071Y (to M.S. Shapiro), and the Human Frontier Science Program (to B. Falkenburger).

Lawrence G. Palmer served as editor.

Submitted: 7 August 2009
Accepted: 8 October 2009

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