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European Journal of Pharmacology 462 (2003) 25–32
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Activation of muscarinic m5 receptors inhibits recombinant KCNQ2/KCNQ3 K^+ channels expressed in HEK293T cells

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Received 10 January 2003; accepted 14 January 2003

Abstract

A variety of G-protein-coupled receptors regulate membrane excitability via M-type K^+ current (M-current) modulation. Muscarinic m1 and m3 acetylcholine receptors have both been implicated in the modulation of M-current. The muscarinic m5 receptor, like muscarinic m1 and m3 receptors, couples to phospholipase C via a pertussis toxin-insensitive G protein. Since a number of other receptors which activate phospholipase C also modulate M-current, we investigated if muscarinic m5 receptors could modulate recombinant M-type (KCNQ2/KCNQ3) K^+ channels after heterologous expression in human embryonic kidney (HEK) 293T cells. Application of Oxo-tremorine M to HEK293T cells expressing muscarinic m1, m3, or m5 receptors produced a similar robust inhibition of M-current, whereas muscarinic m2 and m4 receptor stimulation was without effect. Muscarinic m1, m3, or m5 receptor stimulation decreased the deactivation time constants of M-current at -50 mV. The inhibition of M-current by stimulation of muscarinic m1, m3, or m5 receptors was insensitive to overnight treatment with pertussis toxin or cholera toxin, which interfere with $G_{i/o}$ and G_s G-protein signaling. These data suggest that muscarinic m1, m3, and m5 receptors inhibit M-channels via the activation of a common G protein.

Keywords: K^+ current; M-current; Oxo-tremorine M; Muscarinic receptor; Channel expression

1. Introduction

The M-type K^+ current (M-current) is a slowly activating and non-inactivating K^+ current that plays a dominant role in regulating membrane excitability, not only because it is one of few sustained time- and voltage-sensitive currents active in the range of action potential initiation, but also because of its modulation by many neurotransmitters via receptors coupled to the $G_{q/11}$ protein (Marrion, 1997, review; Brown and Yu, 2000, review). The current was termed M since it was first described to be inhibited by the activation of muscarinic acetylcholine receptors. Among the five subtypes of muscarinic receptor (m1–m5), m1, m3, and m5 are coupled to pertussis toxin-insensitive G proteins (e.g. $G_{q/11}$) and have similar biochemical characteristics, one of which is the receptor-mediated hydrolysis of phosphoinositol bisphosphate and the generation of inositol trisphosphate and diacylglycerol (Eglen and Nahorski, 2000, review). However, thus far, only the muscarinic m1 receptor has been implicated in the modulation of M-current since pirenzepine, a selective muscarinic m1 receptor inhibitor, prevented the muscarinic inhibition of M-current in rat sympathetic neurons (Marrion et al., 1989; Bernheim et al., 1992). Although the finding that muscarinic inhibition of M-current and seizure activity were abolished in m1 gene knockout mice further supports the exclusive involvement of muscarinic m1 receptor in M-current modulation (Hamilton et al., 1997), several studies imply that the muscarinic m1 receptor may not be the only muscarinic receptor subtype responsible for M-current inhibition in the central and/or peripheral nervous system. For example, the degree of muscarinic inhibition of M-current recorded from CA1 pyramidal cells in m1 knockout mice was not significantly different compared to that of wildtype controls (Rouse et al., 2000). These authors also demonstrated that m1-toxin was not able to block M-current inhibition. In addition, although not proven in native neuronal cells, muscarinic m3 receptors have been implicated in M-like channel modulation in NG 108-15 neuroblastoma × glioma cells transfected with m3 DNA (Robbins et al., 1991). On the other hand, muscarinic m2 and m4 receptors are preferentially coupled to pertussis toxin-sensitive G proteins and activation of either muscarinic m2 or m4 receptors failed to

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doi:10.1016/S0014-2999(03)01323-2

produce muscarinic inhibition in NG 108-15 cells transfected with m2 or m4 DNA (Robbins et al., 1991). However, muscarinic m2 receptors have been suggested to mediate M-current inhibition in guinea-pig olfactory cortex neurons since M-current suppression was insensitive to the selective muscarinic m1 receptor antagonist, pirenzepine, but was inhibited by gallamine or 4-diphenyl-acetoxy-*N*-methylpiperidine (Constanti and Sim, 1987).

Compared to the other four muscarinic receptor subtypes, little is known about the physiological functions of the last-identified subtype, the muscarinic m5 receptor, because of the lack of strongly selective muscarinic m5 receptor agonists, antagonists or antibodies as well as the lack of endogenous systems that predominantly express this receptor subtype (Reever et al., 1997; review). Although a few studies have shown that activation of muscarinic m5 receptors modulates Ca^{2+} channels (Pemberton and Jones, 1997; Pemberton et al., 2000), the potential role of muscarinic m5 receptors in M-current modulation has not be reported. Given the fact that the muscarinic m5 receptor, like the muscarinic m1 and m3 receptors, is associated with the $G_{q/11}$ protein and both native and cloned M-channels (e.g. heteromeric KCNQ2/KCNQ3 channel) have been shown to be modulated by activation of many $G_{q/11}$-protein-coupled receptors (Cruzblanca et al., 1998; Filippov et al., 1998; Shapiro et al., 2000; Guo and Schofield, 2002a), we sought to determine if activation of muscarinic m5 receptors could also modulate M-current. In the present studies, we tested if KCNQ2/KCNQ3 channel currents (M-current) could be inhibited by activation of muscarinic m5 receptors transiently transfected into human embryonic kidney (HEK) 293T cells.

2. Materials and methods

2.1. Materials

HEK293T cells were kindly provided by Dr. Jamboor K. Vishwanatha (University of Nebraska Medical Center, Omaha). The drugs and chemicals used in these experiments were obtained as follows: K^+ methylsulfate ($KMeSO_4$) was from ICN (Aurora, OH). HEPES, EGTA, GTP lithium salt, ATP sodium salt, Oxo-tremorine M, pirenzepine, and atropine were from Sigma/Aldrich (St. Louis, MO). Penicillin–streptomycin, fetal calf serum, minimal essential medium (MEM), and Opti-MEM were from GIBCO/BRL Life Technologies (Gaithersberg, MD). Mirus TransIT-LT1 reagent was from Pan Vera (Madison, WI). Pertussis toxin (PTX) and cholera toxin (CTX) were from Calbiochem (La Jolla, CA).

2.2. Cell culture and transfection

The method for cell culture and transfection was as previously described (Guo and Schofield, 2002a). Briefly, HEK293T cells were cultured in MEM supplemented with 10% fetal calf serum under an atmosphere containing 5% CO_2. The cells were transfected with human KCNQ2, rat KCNQ3 cDNA, and pEGFP-N1 encoding the green fluorescent protein as follows. A mixture of 0.5 μg each of KCNQ2/pcDNA3.1, KCNQ3/pcDNA 3.1, and pEGFP-N1, plus 4.5 μl of TransIT-LT1 reagent (Mirus, Madison WI) was made in 75 μl of opti-MEM and preincubated for 20 min. The mixture was then applied to cell culture wells containing HEK293T cells at ~ 90% confluence. For the cotransfection of different muscarinic receptors and KCNQ2/KCNQ3 cDNAs, 0.5 μg of m1–m5/pcDNA3.1 cDNA was added to the above mixture in 6 μl TransIT-LT1 reagent. After 24-h incubation, the cells were plated on coverslips in 35-mm culture dishes, which subsequently served as the recording chamber. Cells transfected only with the pEGFP-N1 vector served as controls. All currents were recorded from the transfected cells within 48 h of plating.

2.3. Electrophysiological recordings

Unless otherwise stated, whole cell M-current was recorded in the conventional open-tip whole cell configuration where the membrane under the pipette tip was ruptured with a pulse of negative pressure. The internal (pipette) solution contained (mM): $KMeSO_4$ 110, KCl 15, EGTA 0.3, $MgCl_2$ 4, HEPES 10, Na_2ATP 4, Li_2GTP 0.5, and was adjusted to pH 7.4 with KOH. In a few experiments, whole cell M-current was recorded by the nystatin perforated-patch technique. For this method, patch pipettes were first tip-filled with an internal solution containing (mM) KCl 55, K_2SO_4 75, HEPES 10, $MgCl_2$ 8, adjusted to pH 7.4 with KOH, and then back-filled with the same internal solution containing 240 μg/ml nystatin made from a freshly prepared stock solution (60 mg/ml) of nystatin in dimethyl sulfoxide. The bath solution contained (mM) NaCl 150, KCl 2.0, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, and glucose 11, adjusted to pH 7.4 with NaOH. The osmolalities of the bath and pipette solutions were adjusted with sucrose to 305 and 285 mosMol/kg, respectively. Drugs were applied to cells via a custom made multi-barreled gravity-fed perfusion system with polyethylene tubes connected to different solution reservoirs. To avoid possible problems due to desensitization, the muscarinic receptor agonist, Oxo-tremorine M, was applied only once for any given cell.

Transfected HEK293T cells were voltage-clamped using an Axopatch 1-C or 200A amplifier (Axon Instruments, Foster City, CA). Patch electrodes were fabricated from N51A borosilicate capillary tubing (Garner Glass, Claremont, CA) using a Model P80-PC micropipette puller (Sutter, Novato, CA) and fire-polished to final resistances of < 2 MΩ when filled with the internal solutions. Under the open-tip configuration, series resistance was 3.7 ± 0.4 MΩ ($n = 15$), whereas with nystatin perforated-patch conditions, series resistance was normally less than 20 MΩ. In both cases, ~ 80% series resistance compensation was applied. Membrane currents were filtered at 1.0 kHz (− 3 dB) using a four-pole low-pass Bessel filter, digitized at 5 kHz with a 12-bit

analog-to-digital converter (GW instruments, Somerville, MA) and stored for analysis using a Macintosh Quadra 800 computer. Voltage protocols were generated from a 12-bit digital-to-analog converter (GW Instruments) using the S3 data acquisition package (Dr. S.R. Ikeda, NIAAA, NIH, Bethesda, MD).

2.4. Data analysis and statistics

Currents were analyzed and fitted using Igor Pro software (Wave Metrics, Lake Oswego, OR) on a Macintosh Quadra 800 computer. All data are expressed as mean ± S.E.M. Analysis of variance coupled with the Student–Newman–Keuls post hoc test was used to determine statistical significance. $P < 0.05$ was considered significant.

3. Results

3.1. Activation of muscarinic m5 receptors inhibit KCNQ2/KCNQ3 channel currents

Nontransfected HEK293T cells or HEK293T cells transfected only with pEGFP-N1 vector expressed small endogenous voltage-dependent outward currents which were activated at positive potentials (Guo and Schofield, 2002a). These currents started to activate positive to − 20 mV and displayed no appreciable tail currents at − 60 mV. When expressed in HEK293T cells, heteromeric KCNQ2/KCNQ3 channels produced a typical M-current with slow deactivation kinetics (Fig. 1). To avoid contamination with the endogenous current, M-currents were generated by holding the cells at − 20 mV and applying 0.5–1.5 s hyperpolarizing pulses to − 50 mV to elicit characteristic slow current deactivation. M-current amplitude was measured as the difference between the first 10 ms and the last 10 ms of the deactivation tail at − 50 mV.

Since some studies suggest that HEK293 cells express endogenous muscarinic m1 receptors (Conklin et al., 1992; Mundell and Benovic, 2000), we investigated if Oxo-tremorine M could inhibit recombinant M-channels via the endogenous muscarinic receptors. In all cells tested, Oxo-tremorine M (10 μM) had no effect on M-current elicited from HEK293T cells expressing only KCNQ2/KCNQ3 cDNAs ($n = 9$) (Fig. 1A). To test whether Oxo-tremorine M can modulate M-current via muscarinic m5 receptors, we cotransfected HEK293T cells with KCNQ2/KCNQ3 and m5 cDNAs. In all HEK293T cells tested after

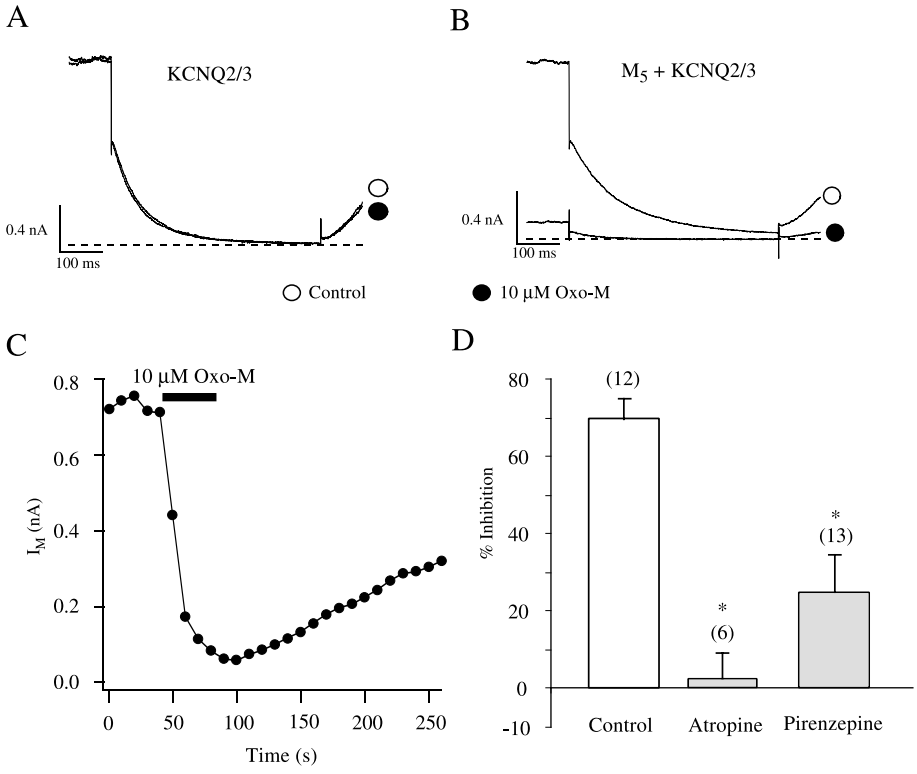

Fig. 1. Inhibition of KCNQ2/KCNQ3 channel currents (M-current) by Oxo-tremorine M via muscarinic m5 receptors. (A) Superimposed M-current traces elicited from a HEK293T cell transfected only with KCNQ2/KCNQ3 cDNAs. Current relaxation was elicited by a hyperpolarizing step to − 50 mV from a holding potential of − 20 mV in the absence and presence of 10 μM Oxo-tremorine M applied by superfusion. The dashed line represents the zero current level. (B) Superimposed M-current traces elicited from a HEK293T cell cotransfected with KCNQ2/KCNQ3 and m5 cDNAs in the absence and presence of 10 μM Oxo-tremorine M applied as in A. (C) Time course of the Oxo-tremorine M effect from currents recorded from the cell shown in B. (D) The mean percentage inhibition of M-current by 10 μM Oxo-tremorine M in control, and in the presence of 10 μM atropine or 10 μM pirenzepine. Data are the mean ± S.E.M. The numbers in parentheses represent the number of cells tested, * indicates $P < 0.05$ compared to the control group.

cotransfection with KCNQ2/KCNQ3 and m5 cDNAs, 10 μM Oxo-tremorine M inhibited M-current by 70 ± 5% (n = 12) (Fig. 1B). The percentage M-current inhibition was calculated as:

$$(I_{pre} - I_{post})/I_{pre} \times 100$$

where I_{pre} and I_{post} are the M-current before and after Oxo-tremorine M application, respectively. Fig. 1C shows that Oxo-tremorine M (10 μM) produced a rapid inhibition of the time-dependent M-current which was at least partially reversible. To confirm that the muscarinic modulation of recombinant M-channels is mediated by muscarinic m5 receptors and is not due to some nonspecific action (Shapiro et al., 2000), atropine, a nonselective muscarinic receptor antagonist, was applied prior to Oxo-tremorine M application. In all cells tested (n = 6), pretreatment with atropine (10 μM) completely prevented 10 μM Oxo-tremorine M-induced M-current inhibition (Fig. 1D). Atropine (10 μM) had no measurable affect on M-current in the absence of Oxo-tremorine M. Since the muscarinic m5 receptor has been shown to have intermediate affinity for pirenzepine (Bonner et al., 1988), we tested if muscarinic m5 receptor-mediated M-current inhibition was blocked by 10 μM pirenzepine. Among the 13 cells transfected with m5 cDNA, the effects of 10 μM pirenzepine were somewhat variable, producing complete inhibition in 5 cells but only partial inhibition in the remainder. The average inhibition of M-current by 10 μM Oxo-tremorine M after pirenzepine pretreatment was 25 ± 10% (n = 13) (Fig. 1D), a value significantly different from the control group (P < 0.05).

3.2. Similar M-current inhibition by activation of muscarinic m1, m3, and m5 receptors

With the exception of muscarinic m1 receptors, the modulation of KCNQ2/KCNQ3 channels by other muscarinic receptors has not been tested (Shapiro et al., 2000; Selyanko et al., 2000). Therefore, we investigated if activation of other muscarinic receptors could also modulate KCNQ2/KCNQ3 channels. Fig. 2A and B shows that, in addition to muscarinic m5 receptor activation, muscarinic m1 and m3 receptor activation also significantly inhibited the M-current. On the other hand, 10 μM Oxo-tremorine M did not produce M-current inhibition in cells

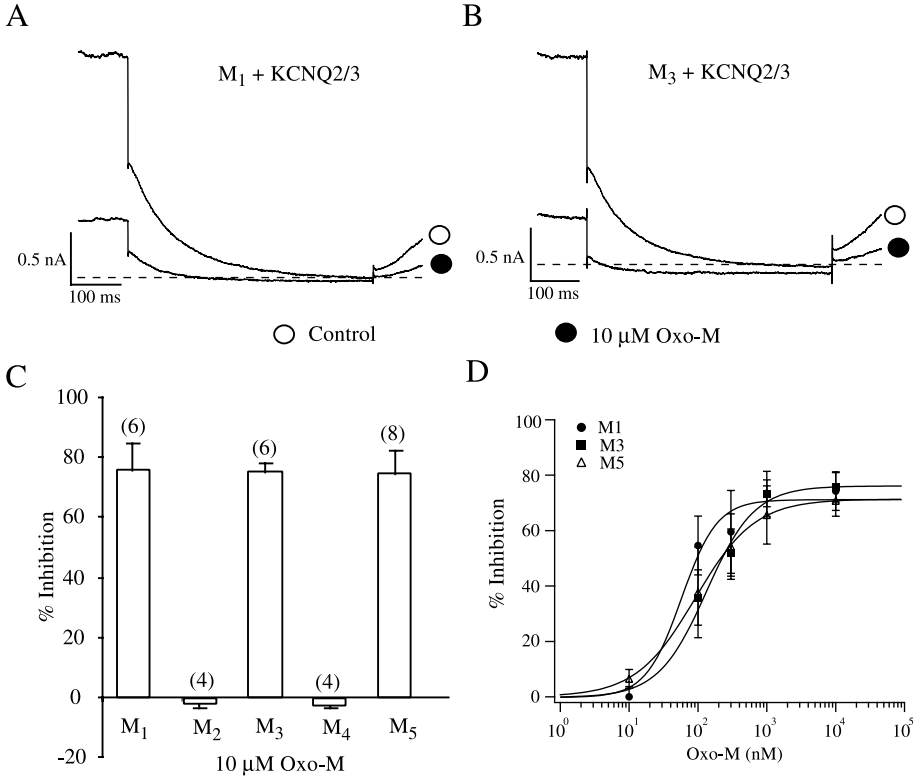

Fig. 2. Effect of Oxo-tremorine M on M-current elicited from HEK293T cells cotransfected with different muscarinic receptors and KCNQ2/KCNQ3 cDNAs. (A, B) Superimposed M-current traces elicited from a cell cotransfected with m1 (A) or m3 (B) and KCNQ2/KCNQ3 cDNAs in the absence and presence of 10 μM Oxo-tremorine M applied as in Fig. 1A. The dashed line represents the zero current level. (C) Mean inhibition, elicited by 10 μM Oxo-tremorine M, of KCNQ2/KCNQ3 channels expressed in HEK293T cells cotransfected with different muscarinic receptor cDNAs. (D) Concentration–response relationship of M-current inhibition by Oxo-tremorine M recorded from HEK293T cells transfected with muscarinic m1, m3, and m5 receptors. The solid lines represent the best fit to a Hill equation. Data are the mean ± S.E.M. The numbers in parentheses represent the number of cells tested. Each point on the dose–response curves represents the mean inhibition from 3 to 14 cells.

expressing muscarinic m2 or m4 receptors. Fig. 2C shows the effect of 10 μM Oxo-tremorine M on M-current elicited from HEK293T cells cotransfected with KCNQ2/KCNQ3 channels and one of the five different muscarinic receptors under perforated-patch conditions. In addition, there was no significant difference between muscarinic m1, m3, and m5 receptor-mediated M-current inhibition by 10 μM Oxo-tremorine M between perforated-patch recording and open-tip recording configurations (data not shown).

Of the muscarinic receptor subtypes, it has been suggested that muscarinic m3 receptors couple most effectively to M-channels since both maximal concentrations of acetylcholine and muscarine completely inhibited M-like current in NG 108-15 cells transfected with m3 DNA, but only partially inhibited M-like current in cells transfected with m1 DNA (Robbins et al., 1991). In our studies, the mean percentage inhibition of M-current by 10 μM Oxo-tremorine M was not significantly different between m1-, m3-, or m5-transfected cells ($74 \pm 6\%$ ($n = 13$), $75 \pm 4\%$ ($n = 14$), and $70 \pm 5\%$ ($n = 12$) for m1, m3, and m5, respectively; $P > 0.05$). To determine if there was a difference in efficacy and potency of Oxo-tremorine M at muscarinic m1, m3, or m5 receptors in producing M-current inhibition, we studied the dose–response relationships of activation of different muscarinic receptors on M-current inhibition. Fig. 2D shows superimposed concentration–response curves for M-current inhibition generated from HEK293T cells cotransfected with muscarinic m1, m3, or m5 receptors, and KCNQ2/KCNQ3 channels. At each concentration, the points represent the mean inhibition from 3 to 14 cells. At all concentrations tested, there was no significant difference between M-current inhibition resulting from stimulation of the three receptor subtypes ($P > 0.05$). The solid lines are fits of the data to a Hill equation. The fitted maximum inhibition was 71%, 76%, and 71% for m1, m3, and m5, respectively. Half-maximal inhibition was attained at 56, 130, and 92 nM for m1, m3, and m5, respectively. The Hill coefficients were 1.7, 1.2, and 1.0 for m1, m3, and m5, respectively. These data suggest that stimulation of muscarinic m1, m3, and m5 receptors by Oxo-tremorine M display similar efficacy and potency on M-current inhibition.

3.3. Effect of activation of muscarinic m1, m3, and m5 receptors on M-current gating

It has been suggested that in rat sympathetic neurons, inhibition of M-current by activation of muscarinic or purinergic receptors is not associated with a change in the deactivation kinetics of M-current (Marrion et al., 1989; Boehm, 1998; Filippov et al., 1998). However, in previous studies, we found that activation of histamine H_1 receptors significantly decreased both the fast and slow time constants of KCNQ2/KCNQ3 channel deactivation (Guo and Schofield, 2002b). Therefore, we investigated the effect of the activation of the different muscarinic receptors on the kinetic components of KCNQ2/KCNQ3 channel current deactivation. The deactivation process of M-current was well fitted with a double-exponential equation both in the absence and presence of Oxo-tremorine M as follows:

$$I_M = I_{M(f)} \exp(-t/\tau_f) + I_{M(s)} \exp(-t/\tau_s) + I_{ss}$$

where $I_{M(f)}$ and $I_{M(s)}$ are the amplitudes of the fast and slow components, respectively, and τ_f and τ_s are the fast and slow time constants of deactivation, respectively. I_{ss} is the steady state component. Fig. 3A and B shows examples of the current deactivation phase of M-current elicited from an m5-transfected cell in the absence and presence of 1 μM Oxo-tremorine M. In the current records shown, only 1 of every 50 data points is plotted in order to clearly reveal the superimposed fit. The solid lines are a fit of the data to a double-exponential equation. Oxo-tremorine M (1 μM) decreased both the fast and slow deactivation time constants of M-current. A similar effect of Oxo-tremorine M on M-current gating was observed in m1- and m3-transfected cells. The bar graphs of Fig. 3C and D show the fast and slow deactivation time constants of M-current elicited from m1-, m3-, and m5-transfected cells, in the absence and presence of 1 μM Oxo-tremorine M, respectively. The fast time constant decreased from 67 ± 5 ms ($n = 16$) in the absence of Oxo-tremorine M to 34 ± 5, 50 ± 4, and 42 ± 4 ms in the presence of 1 μM Oxo-tremorine M (for m1 ($n = 6$), m3 ($n = 6$), and m5 ($n = 4$), respectively; $P < 0.05$ cf. control). There were no significant differences in the fast time constants of deactivation in the presence of 1 μM Oxo-tremorine M between m1- ($n = 6$), m3- ($n = 6$), and m5- ($n = 4$) transfected cells, respectively. The slow time constant decreased from 227 ± 28 ms in the absence of Oxo-tremorine M to 93 ± 13, 120 ± 29, and 116 ± 14 ms in the presence of 1 μM Oxo-tremorine M (for m1 ($n = 6$), m3 ($n = 6$), and m5 ($n = 4$), respectively; $P < 0.05$ cf. control). There were no significant differences in the slow time constants of deactivation in the presence of 1 μM Oxo-tremorine M between m1- ($n = 6$), m3- ($n = 6$), and m5- ($n = 4$) transfected cells, respectively. Although Oxo-tremorine M decreased both the fast and slow components of M-current deactivation, Oxo-tremorine M had no significant effect on the relative amplitudes of the two current components (data not shown).

3.4. A pertussis toxin- and cholera toxin-insensitive G protein is involved in muscarinic m1, m3, and m5 receptor-mediated M-current inhibition

In sympathetic neurons, inhibition of M-current by muscarinic and bradykinin receptors has been shown to be mediated by the α subunit of the $G_{q/11}$ heterotrimer

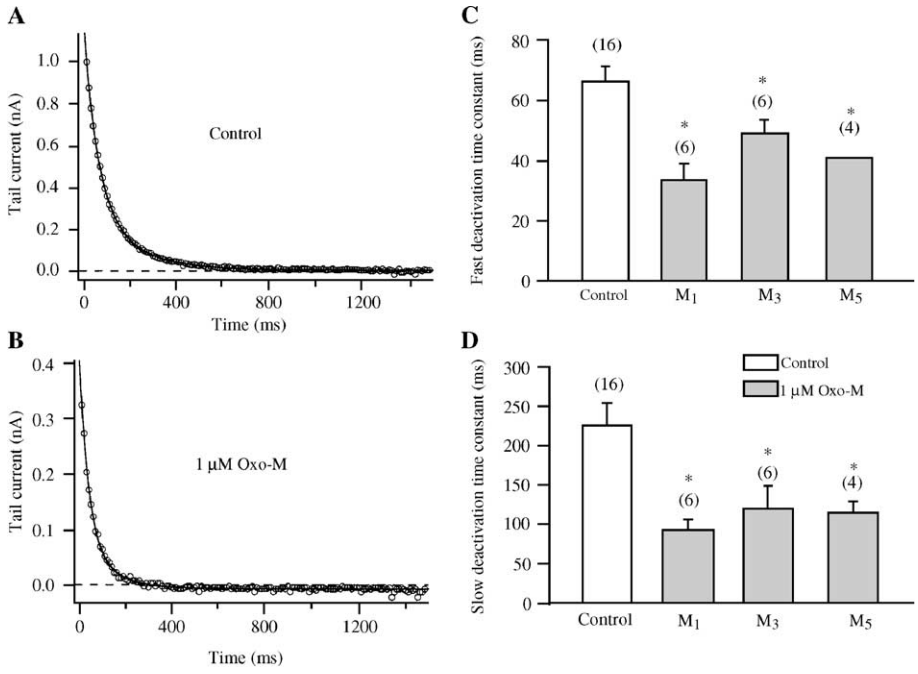

Fig. 3. Effects of Oxo-tremorine M on M-current deactivation. (A, B) Deactivation tail currents of M-current elicited from a HEK293T cell cotransfected with m5 and KCNQ2/KCNQ3 cDNAs in the absence and presence of 1 μM Oxo-tremorine M, respectively. In the current records shown, only 1 of every 50 points is plotted. The solid lines are fits to a double-exponential function (see text). (C, D) The effects of 1 μM Oxo-tremorine M on the fast and slow time constants of M-current elicited from HEK293T cells cotransfected with m1, m3, or m5, and KCNQ2/KCNQ3 cDNAs, respectively. Data are the mean ± S.E.M. The numbers in parentheses represent the number of cells tested.

which is not sensitive to pertussis toxin (Jones et al., 1995; Haley et al., 1998). Although it is generally accepted that muscarinic m1, m3, and m5 receptors are coupled to a $G_{q/11}$ protein, it has been suggested that activation of these three muscarinic receptors can stimulate other signal transduction pathways, such as activation of adenylate cyclase (Eglen and Nahorski, 2000, review). Therefore, we tested if a common G protein was involved in all three subtypes mediating M-current inhibition by using the G-protein toxins, pertussis toxin, and cholera toxin. In our experiments, overnight pretreatment with either 500 ng/ml pertussis toxin or cholera toxin did not prevent muscarinic m1, m3, or m5 receptor-mediated M-current inhibition (Fig. 4). The M-current inhibition by 10 μM Oxo-tremorine M after pertussis toxin treatment was 80 ± 7% (n = 8), 61 ± 8% (n = 12), and 63 ± 9% (n = 9) for m1, m3, and m5, respectively, values not significantly different from the respective control groups (P>0.05). The M-current inhibition by 10 μM Oxo-tremorine M after cholera toxin treatment (66 ± 6% (n = 6), 65 ± 8% (n = 6), and 61 ± 11 (n = 6) for m1, m3, and m5, respectively) was also not significantly different from the respective control groups (P>0.05). In rat SCG neurons, norepinephrine inhibition of N-type Ca^{2+} channels is partially pertussis toxin-sensitive (Schofield, 1991) whereas vasoactive intestinal polypeptide inhibition of Ca^{2+} channel is cholera toxin-sensitive (Zhu and Ikeda, 1994). To ensure that pertussis toxin and cholera toxin were effective, we used the norepinephrine and vasoactive intestinal polypeptide-induced N-type Ca^{2+} channel modulation as positive controls. Overnight treatment of rat SCG neurons with 500 ng/ml pertussis toxin or cholera toxin significantly reduced 10 μM norepinephrine or vasoactive intestinal polypeptide-induced inhibition of N-type Ca^{2+} current, respectively (data not shown).

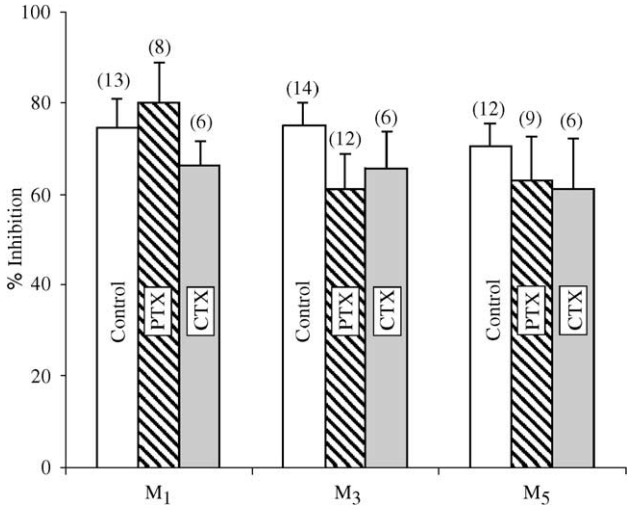

Fig. 4. Mean inhibition of M-current by 10 μM Oxo-tremorine M with and without pertussis toxin (PTX) or cholera toxin (CTX) treatment measured from HEK293T cells cotransfected with m1, m3, or m5, and KCNQ2/KCNQ3 cDNAs. Neither pertussis toxin nor cholera toxin prevents KCNQ2/KCNQ3 channel inhibition by Oxo-tremorine M. Data are the mean ± S.E.M. The numbers in parentheses represent the number of cells tested.

4. Discussion

These studies reveal that, in addition to muscarinic m1 and m3 receptors, activation of muscarinic m5 receptors can also modulate recombinant M-channels in a heterologous expression system. The observation that there were no significant differences between the efficacy and potency of M-current inhibition mediated by muscarinic m5 or m1, and m3 receptors coupled with similar changes in M-current gating, and a lack of response to G-protein toxins, suggest that these muscarinic receptors share a common signal transduction pathway to elicit M-channel modulation.

Although muscarinic receptors have been cloned for more than a decade, difficulties in pharmacologically distinguishing between the different subtypes has severely hampered investigation of the physiological functions of these receptors in native mammalian tissues (Eglen and Nahorski, 2000, review). This has particularly hindered studies of the muscarinic m5 receptor. Thus far, most functional studies of the muscarinic m5 receptor have been limited to cell lines with heterologously expressed muscarinic m5 receptors since few tissues or cell lines have been identified which predominantly express native muscarinic m5 receptors (Reever et al., 1997, review; Eglen and Nahorski, 2000, review). However, despite these obstacles, it has been found that muscarinic m5 receptor proteins are enriched in the central nervous system, including the cortex, hippocampus, and olfactory tubercle, and have a unique distribution profile as shown by selective muscarinic m5 receptor labeling (Reever et al., 1997, review). Moreover, one of the physiological functions of muscarinic m5 receptors has been suggested to include ion channel modulation (e.g. Ca^{2+} channel modulation). Our studies provide another example of the involvement of muscarinic m5 receptors in channel modulation, suggesting that a potential physiological function of the muscarinic m5 receptor subtype may be the modulation of central nervous system excitability via the inhibitory action of acetylcholine on M-channels, provided that muscarinic m5 receptors and M-channels are colocalized in the same cells.

A dominant role for muscarinic m1 receptors in inhibiting the M-current of rat sympathetic neurons was first identified pharmacologically by Marrion et al. (1989) using pirenzepine and 11-2[2-[(diethylamino)methyl]-1-piperidinyl]-acetyl-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepine-6-one (AF-DX116). Bernheim et al. (1992), using a careful pharmacological approach employing Schild analysis, later showed that pirenzepine blockade was competitive. In our studies, muscarinic m5 receptor-mediated M-current inhibition was also sensitive to pirenzepine, suggesting that caution should be used when classifying muscarinic receptor subtypes involved in M-current inhibition using only a single concentration of a selective antagonist. Further studies to determine the potency of pirenzepine on M-current inhibition in cells expressing both muscarinic m1 and m5 receptors would compliment the pharmacological classification of muscarinic receptor subtypes involved in M-current inhibition.

The observation that muscarinic m1, m3, and m5 receptor activation produced a similar degree of M-current inhibition conflicts with the expression studies from transfected NG 108-15 neuroblastoma × glioma cells, where M-current inhibition elicited by activation of muscarinic m3 receptors was much stronger than that by activation of muscarinic m1 receptors (Robbins et al., 1991). Although the different expression systems may account for this discrepancy, it is also possible that heteromeric KCNQ2/KCNQ3 K^+ channels differ from the M-like channels expressed in NG 108-15 cell since it has been shown that both *ether-a-go-go-related* (mErg1) and KCNQ2/KCNQ3 channels contribute to M-like current in NG 108-15 (Selyanko et al., 1999). Moreover, both currents from heterologously expressed mouse Erg1a channels and the presumed-mouse Erg1a component of the M-like current, after blockade of KCNQ2/KCNQ3 channels, were inhibited by activating muscarinic m1 receptors (Selyanko et al., 1999).

Although muscarinic inhibition of M-current in rat sympathetic neurons was not associated with changes in the kinetics of the current (Marrion et al., 1989), our data from HEK293T cells clearly indicate that Oxo-tremorine M decreases both the fast and slow time constants of M-current deactivation. This observation is not an artifact of voltage error produced by series resistance since in most experiments, the tail currents were <2 nA, and the series resistance was normally <5 MΩ under open-tip configuration. Since $\sim$ 80% compensation was applied, less than 2 mV voltage errors were expected. Moreover, there was no correlation between the deactivation time constants and tail current amplitude ($r=0.26$ for both slow and fast deactivation time constants, $n=70$; data not shown). Single-channel studies will be required to determine if the mechanism underlying the effects of Oxo-tremorine M on channel gating involves a shift of the channels into a short open-time mode as has been shown for muscarinic inhibition of M-current in bullfrog sympathetic neurons (Marrion, 1993).

In our studies, KCNQ2/KCNQ3 channels were inhibited by activation of muscarinic m1, m3, and m5 receptors, but not m2 or m4 receptors. These data are consistent with results obtained from NG 108-15 cells (Robbins et al., 1991). In addition, the data support the notion that a common property of M-channel modulation is the activation of $G_{q/11}$-protein-coupled receptors (Marrion, 1997, review). Although muscarinic m1, m3, and m5 receptors are preferentially coupled to $G_{q/11}$ proteins, several studies have suggested that muscarinic receptor subtypes are promiscuous in their coupling to G proteins, and could therefore couple to G_s and G_i in addition to $G_{q/11}$ (Eglen and Nahorski, 2000). For example, in CHO cells, muscarinic m1 and m3 receptors can interact with both $G_{q/11}$ and G_i proteins (Offermanns et al., 1994). However, our studies suggest that in HEK293T cells, a common G protein is involved in muscarinic m1, m3, and

m5 receptor-mediated M-current inhibition since neither pertussis toxin nor cholera toxin prevents muscarinic M-current inhibition in cells transfected with either muscarinic m1, m3, or m5 receptors.

Although our findings suggest that a common G protein (probably $G_{q/11}$) is involved in mediating M-current inhibition by muscarinic m1, m3, and m5 receptors, further studies are required to determine if a common signal transduction pathway impinges on the KCNQ2/KCNQ3 channels to produce M-current inhibition.

Acknowledgements

KCNQ2 and KCNQ3 cDNAs were obtained from Dr. David McKinnon, SUNY at Stonybrook, NY, and were subcloned into pcDNA3.1 by Dr. Stephen Ikeda (NIAAA, NIH). The cDNA clones for human muscarinic m1–m5 receptors were provided by the Guthrie cDNA Resource Center (http://www.guthrie.org/AboutGuthrie/Research/cDNA).

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