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Recovery from Muscarinic Modulation of M Current Channels Requires Phosphatidylinositol 4,5-Bisphosphate Synthesis

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Summary

Suppression of M current channels by muscarinic receptors enhances neuronal excitability. Little is known about the molecular mechanism of this inhibition except the requirement for a specific G protein and the involvement of an unidentified diffusible second messenger. We demonstrate here that intracellular ATP is required for recovery of KCNQ2/KCNQ3 current from muscarinic suppression, with an EC_{50} of ~0.5 mM. Substitution of nonhydrolyzable ATP analogs for ATP slowed or prevented recovery. ADPβS but not ADP also prevented the recovery. Receptor-mediated inhibition was irreversible when recycling of agonist-sensitive pools of phosphatidylinositol-4,5-bisphosphate (PIP_2) was blocked by lipid kinase inhibitors. Lipid phosphorylation by PI 4-kinase is required for recovery from muscarinic modulation of M current.

Introduction

The M current (I_M) is a voltage- and time-dependent potassium current first described in sympathetic neurons (Brown and Adams, 1980) and subsequently observed in several other neurons, including hippocampal and cortical pyramidal cells (for review, see Marrion, 1997). The I_M is a subthreshold voltage-gated K^+ current that stabilizes the membrane potential and controls neuronal excitability; its receptor-mediated suppression increases responses to excitatory synaptic inputs (Jones et al., 1995; Wang and McKinnon, 1995). Agonists for gonadotropin-releasing hormone receptors, angiotensin II receptors, purinergic receptors, substance P receptors, bradykinin receptors, and muscarinic M_1 receptors all inhibit I_M (Shapiro et al., 1994a; Simmons et al., 1994; Choi and Lovinger, 1996; Cruzblanca et al., 1998), increasing neuronal excitability.

The precise signal transduction mechanism for the decrease of I_M is not known. The data clearly indicate that inhibition of I_M by these receptors follows activation of an NEM- and pertussis toxin-insensitive G protein of the $G_{q/11}$ class (Pfaffinger et al., 1988; Caulfield et al., 1994; Shapiro et al., 1994b; Haley et al., 1998); it involves a diffusible cytoplasmic signal (Selyanko et al., 1992; Marrion, 1993) and is interrupted when cytoplasmic calcium is held below normal levels (Beech et al., 1991). The same signal may modulate L- and N-type Ca^{2+} channels in sympathetic neurons (Hille, 1994; Shapiro et al., 1994a). Its identity is a major question in this field. Knowing that the G protein $G_{q/11}$ normally activates phospholipase C (PLC), a natural suggestion would be that the diffusible message is a product generated by PLC, either inositol-1,4,5-trisphosphate (IP_3) or diacylglycerol or both. However, repeated tests of IP_3, Ca^{2+}, and protein kinase C as candidate messengers have proven negative (Bosma and Hille, 1989; Beech et al., 1991; Cruzblanca et al., 1998; del Río et al., 1999; Shapiro et al., 2000). An alternative and more recent hypothesis is that a *decrease* in the concentration of the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) is the signal that depresses I_M. Several ion channels open in the presence of PIP_2 and close when PIP_2 is depleted from the membrane (Huang et al., 1998; Shyng and Nichols, 1998; Sui et al., 1998; Hilgemann et al., 2001). After PIP_2 is hydrolyzed by PLC, the action of several lipid kinases using ATP is required to resynthesize it. This paper tests the need for ATP and lipid kinases in the *recovery* from the muscarinic inhibition of I_M.

The role of intracellular ATP in the regulation of I_M remains confusing. Pfaffinger et al. (1988) found that I_M fell to below 20% of its initial value after 8 min of dialysis with ATP-free whole-cell pipette solutions, and Tokimasa and Akasu (1990) reported a similar fall after 15 min of dialysis with adenylyl-imidodiphosphate (AMP-PNP) replacing all ATP. In contrast, Simmons et al. (1990) found that I_M remained above 80% of its initial value with a similar AMP-PNP pipette solution, and others suggested that intracellular ATP levels did not affect rundown of I_M (Chen and Smith, 1992). More recently, Simmons and Schneider (1998) found that metabolic substrates such as glucose and pyruvate in the extracellular solution allow I_M to be maintained in the absence of added ATP. These studies emphasized rundown of current. Is there also a role of ATP in receptor-mediated modulation?

Here we coexpress channel subunits KCNQ2 and KCNQ3 with M_1 muscarinic receptors to reconstitute modulation of I_M in a heterologous expression system (Shapiro et al., 2000). To verify that our conclusions apply to native neurons, we also study modulation of endogenous I_M in sympathetic neurons. Our emphasis is on recovery from muscarinic inhibition. We demonstrate that the *recovery* of the I_M from muscarinic inhibition is tightly dependent upon the cytosolic level of ATP—even though ATP levels do not affect the *induction* of muscarinic inhibition. We also provide evidence that a membrane lipid kinase, PI 4-kinase, but not PI 3-kinase or protein kinases, is required in this ATP-mediated recovery of I_M.

Results

Muscarinic Suppression and Recovery of I_M via PLC

Cotransfection of the plasmids for the two channel subunits KCNQ2 and KCNQ3 in tsA cells yields slowly activating and deactivating currents with I_M-like voltage dependence, kinetics, and pharmacology that can be suppressed by the application of the muscarinic agonist oxotremorine-M (oxo-M) (Shapiro et al., 2000). Figure 1A shows a family of currents over a range of test potentials before and during oxo-M application, and after washout

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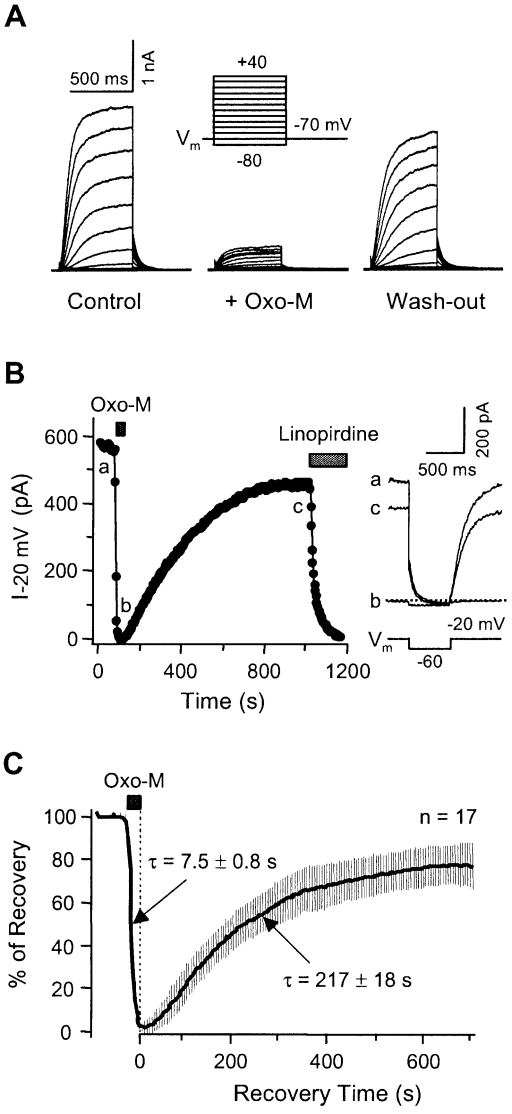

Figure 1. Muscarinic Modulation of KCNQ2/KCNQ3 Channels in tsA Cells

(A) Families of current elicited by voltage steps from −80 to 40 mV, in 10 mV intervals, before and during the application of 10 μM oxo-M, and after washout of oxo-M. Holding potential −70 mV. *Inset* shows the pulse protocol. (B) Reversible regulation of current at −20 mV by short muscarinic stimulation. Oxo-M and linopirdine (30 μM) were bath-applied as marked. Pulses to −60 mV were given every 4 s, and points are plotted at the same intervals. *Inset* shows the current waveforms. *Dashed line* in the current traces is the zero-current level. (C) Recovery from the muscarinic suppression. Oxo-M was applied for 20 s. Recovery of the current was calculated as a percentage of the preapplication level. n = 17, mean ± SEM.

of the oxo-M. Here cells were held at −70 mV, and voltage steps given from −80 to 40 mV evoked the K^+ current. Oxo-M suppresses the outward KCNQ2/KCNQ3 current at all voltages. The 10 μM concentration of oxo-M used throughout this paper is supramaximal.

We first review the basic parameters of inhibition and recovery. Figure 1B shows the time course of oxo-M effects, now measuring KCNQ2/KCNQ3 current as we do in the rest of paper as the steady holding current at −20 mV. Induction of receptor-mediated inhibition occurred with a mean exponential time constant τ of 7.5 ± 0.8 s—slower than the measured solution exchange time (2.4 s). The suppression is mediated by the M_1 muscarinic receptors since it was almost completely abolished when oxo-M was applied in the presence of 10 μM pirenzepine and it was absent when cells were not cotransfected with the receptor (n = 3) (data not shown). Linopirdine (30 μM), a selective blocker of I_M (Lamas et al., 1997), completely inhibited the expressed current (Figure 1B). The slow recovery after muscarinic suppression of the current followed an exponential time course with a time constant τ of 217 ± 18 s (Figure 1C). The mean recovery of the current from muscarinic suppression in these experiments was to 77% ± 9% (n = 17) of the preceding level, measured 10 min after washout of oxo-M. Figure 2A shows the muscarinic modulation of I_M in superior cervical ganglion (SCG) neurons after overnight culture. I_M was strongly suppressed by 10 μM oxo-M in the same time as for tsA cells (induction τ of 7.8 ± 1.3 s), but the recovery was twice as fast (τ of 108 ± 23 s [n = 7]).

Muscarinic M_1 receptors usually couple via the G proteins $G_{q/11}$ to PLC. To test whether activation of PLC is required for suppression of I_M, we compared the actions of a PLC inhibitor U73122 with those of an inactive analog. For both tsA cells and SCG neurons, we found that 1 μM U73122 is relatively ineffective at blocking muscarinic suppression (although it did slow the induction), but 3 μM had a strong effect. We did not study higher concentrations since they reduced the size of I_M considerably. The inactive control compound U73343 had only a weak effect. Figure 2B shows experiments with SCG neurons incubated with U73122 or U73343 for 5 min after breakthrough, and Figure 2C summarizes the experiments on both cell types. We conclude that the pathway for muscarinic receptor-mediated inhibition of I_M and KCNQ2/KCNQ3 channels requires the activation of PLC. The small increase of I_M with 1 μM U73122 may indicate that there is some tonic activity of PLC that depresses current even without receptor stimulation.

Intracellular ATP Is Required for Recovery from Muscarinic Inhibition

To explore a role for ATP in recovery from muscarinic inhibition, we stimulated transfected tsA cells several times with oxo-M (10 μM) in the presence of various concentrations of ATP in the pipette solution. When the pipette contained 3 mM ATP, successive 20 s exposures to oxo-M repeatedly suppressed the current with a similar induction time course (τ ≈ 7.2 s) (Figure 3A). The recovery was strong, although never quite complete (cf. Figure 3D). Closer inspection shows that the channel recovery from muscarinic inhibition slowed with repeated stimulation of the cell (Figure 3B). The recovery rate differed among cells, but it was always slightly slower in the second and the third stimulations (n = 5). When the ATP in the pipette was decreased to 0.3 mM, the extent of channel recovery was dramatically diminished even in the first agonist exposure (Figure 3C). As summarized in Figure 3D, the recovery with 0.3 mM ATP was significantly less than with 3 mM ATP and diminished further in the subsequent stimulations. Although it affected *recovery* from inhibition, the reduction

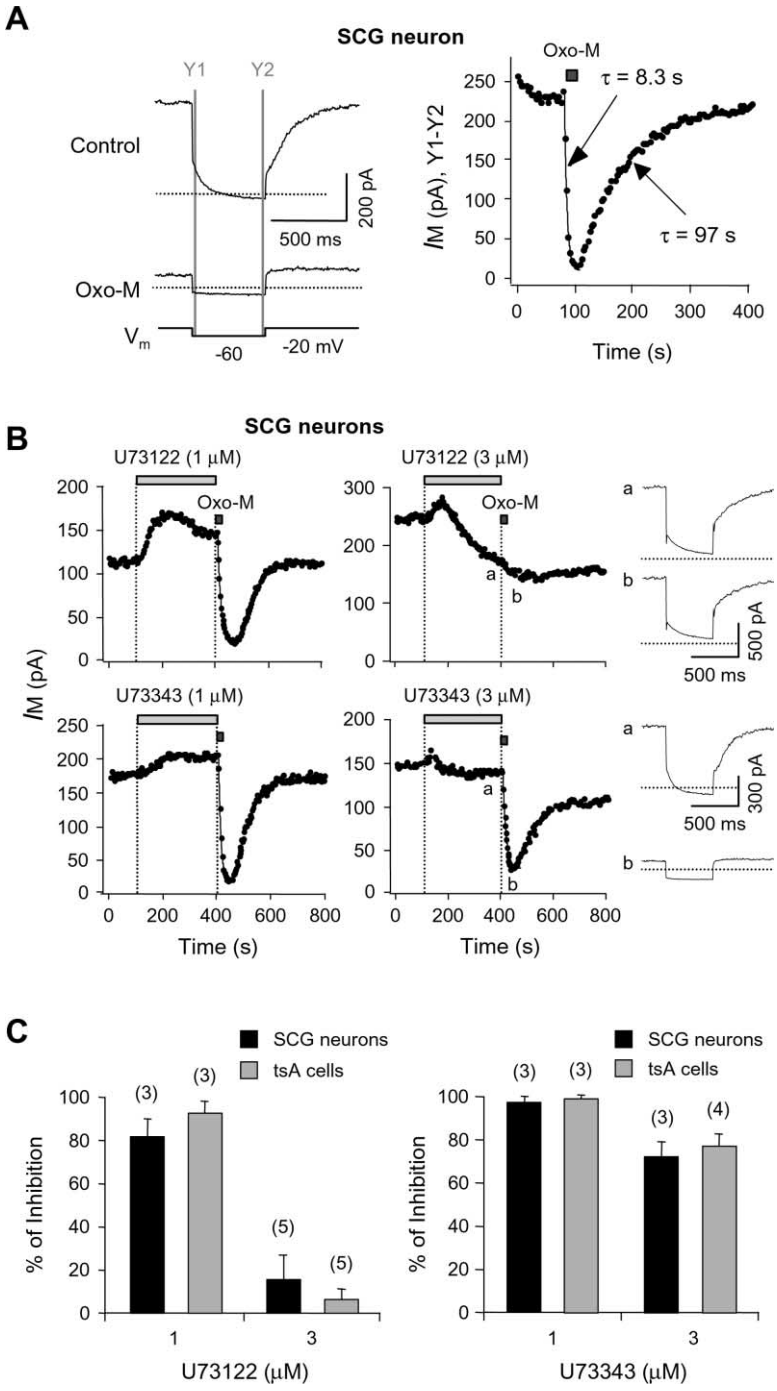

Figure 2. Muscarinic Modulation of I_M via PLC

(A) Currents in SCG neurons elicited by the pulse protocol shown below, before (control) and during application of 10 μM oxo-M (Oxo-M). Left: typical time-dependent modulation of the I_M by bath application of oxo-M for 20 s. The amplitude was measured as the difference between the current 10–20 ms after the beginning of the voltage step (Y1) and the current at the end of the step (Y2). Membrane capacitance, 38 pF. (B) Effects of aminosteroids on muscarinic modulation of I_M in SCG neurons. Five minutes after aminosteroid treatment began, oxo-M was added for 20 s. (C) Summary of effects of aminosteroids on muscarinic inhibition in SCG neurons and tsA cells.

of intracellular ATP concentration did not slow or diminish the *induction* of inhibition by oxo-M (Figure 3E).

In order to check whether the depression of recovery from muscarinic suppression might represent a rapid rundown of the channel in low intracellular ATP concentrations, we investigated the effect of cytosolic ATP on the *maintenance* of KCNQ2/KCNQ3 currents. As is summarized in Figure 3F, there was no significant difference in the spontaneous slow loss of channel activity (rundown) between control and ATP-deficient cells; the mean channel activity fell to 42% ± 7% (n = 8) and 48% ± 5% (n = 7) of the initial level after 30 min with and without 3 mM ATP in the pipette solution, respectively. Thus, the decrease of channel recovery with 0.3 mM ATP pipette solution is not due to a more rapid rundown of the channels but to ATP dependence of recovery from the muscarinic receptor-mediated suppression.

Nonhydrolyzable ATP Analogs Exert Dominant-Negative Effects on Recovery

We next tested whether the hydrolysis of phosphate groups from ATP is needed for recovery. We replaced ATP with nonhydrolyzable ATP analogs in the pipette solution. Both AMP-PNP and adenylylmethylene diphos-

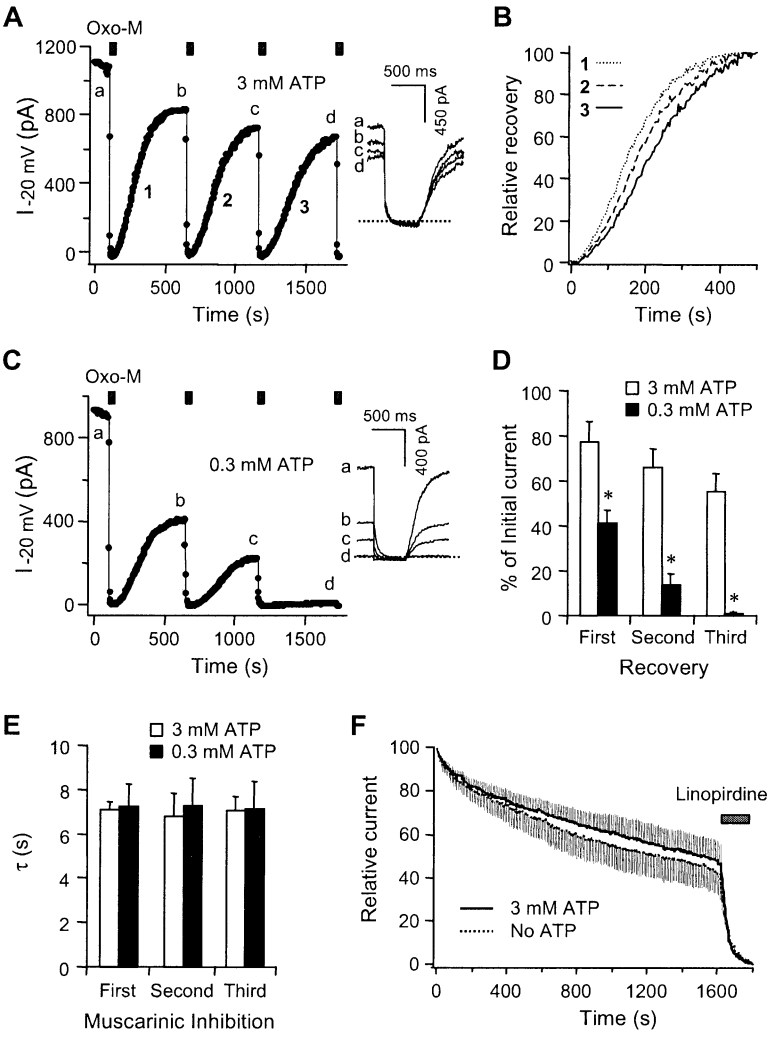

Figure 3. Importance of Intracellular ATP for Recovery of KCNQ2/KCNQ3 Currents from Muscarinic Suppression

(A) Time course of current at −20 mV with 3 mM ATP in the pipette solution. Oxo-M was bath-applied four times for 20 s. *Inset* shows current traces from before each oxo-M exposure. Typical traces from more than five experiments. (B) Time course of recovery from the repetitive stimulations with oxo-M shown in (A) The time to half recovery was 133 s after the first oxo-M exposure (1), 160 s after the second (2), and 197 s after the third (3). (C) The same kind of experiment as in (A) except with 0.3 mM ATP. The plot is typical of five experiments. (D) Summary of recovery from repeated oxo-M stimulations in five experiments. *$p < 0.01$, compared with 3 mM ATP. (E) Time constant (τ) for current inhibition in repeated muscarinic stimulations with pipette solutions containing 3 mM or 0.3 mM ATP. (F) Time course of rundown of KCNQ2/KCNQ3 current with and without intracellular ATP. Measurements started 3 min after breaking through to whole-cell recording. *Solid line*, with 3 mM ATP ($n = 7$); *dotted line*, without ATP ($n = 8$). Linopirdine (30 μM) was added at the end of experiments.

phonate (AMP-PCP) are expected to be competitive inhibitors of reactions requiring hydrolyzable ATP. In the experiments shown in Figures 4A–4C, the pipette solution contained different mole fractions of AMP-PNP and ATP. With a lowered mole fraction of hydrolyzable ATP in the pipette, the rate and extent of muscarinic suppression of KCNQ2/KCNQ3 current were not affected but the recovery was greatly reduced (see also Figure 4G). By 7 min after washout of oxo-M, the current had recovered to only 32% ± 7% of the preceding level with 2 mM ATP plus 1 mM AMP-PNP and to 12% ± 5% with 1 mM ATP plus 2 mM AMP-PNP, significantly lower than the control 72% ± 9% ($p < 0.01$) (Figure 4F). Moreover, in the presence of 4 mM AMP-PNP or AMP-PCP without added ATP, there was almost no recovery from oxo-M (Figures 4D–4F). Evidently, hydrolyzable ATP is prerequisite for the recovery of KCNQ2/KCNQ3 channels from muscarinic suppression. Similarly, in SCG neurons, complete substitution of AMP-PNP for ATP blocked the recovery from muscarinic suppression without affecting the induction or rate of inhibition (Figures 4H and 4I).

Figure 4J summarizes the effects of AMP-PNP and AMP-PCP on channel recovery in tsA cells in the presence of a constant concentration of 1 mM ATP. The recovery observed with 1 mM ATP was inhibited by addition of AMP-PNP or AMP-PCP in a concentration-dependent manner. In all cases, the recovery was to less than 10% of the preceding level when 3 mM AMP-PNP or AMP-PCP was added along with 1 mM ATP in the pipette. These results indicate that nonhydrolyzable forms of adenine nucleotides act as inhibitors of the ATP-mediated channel recovery and that hydrolysis of ATP and probably a phosphorylation reaction with a relatively low affinity for ATP are required for the recovery of KCNQ2/KCNQ3 and M current channels.

AMP-PNP is commercially available only as the lithium salt. Since millimolar Li^+ disrupts phosphoinositide turnover during the stimulation of PLC-coupled receptors (Jenkinson et al., 1994), we needed to control for effects of Li^+. However, the recovery of the channel was not much affected by Li^+ alone. When the pipette solution contained 3 mM ATP and 16 mM LiCl, recovery was to 65% ± 8% ($n = 4$, $p > 0.1$ versus control) 7 min after the washout of oxo-M (data not shown). Furthermore, AMP-PCP, a sodium salt, blocked the recovery as much as AMP-PNP did. Therefore, we presumed that the inhibition from muscarinic suppression is due to the nonhydrolyzable ATP analog itself.

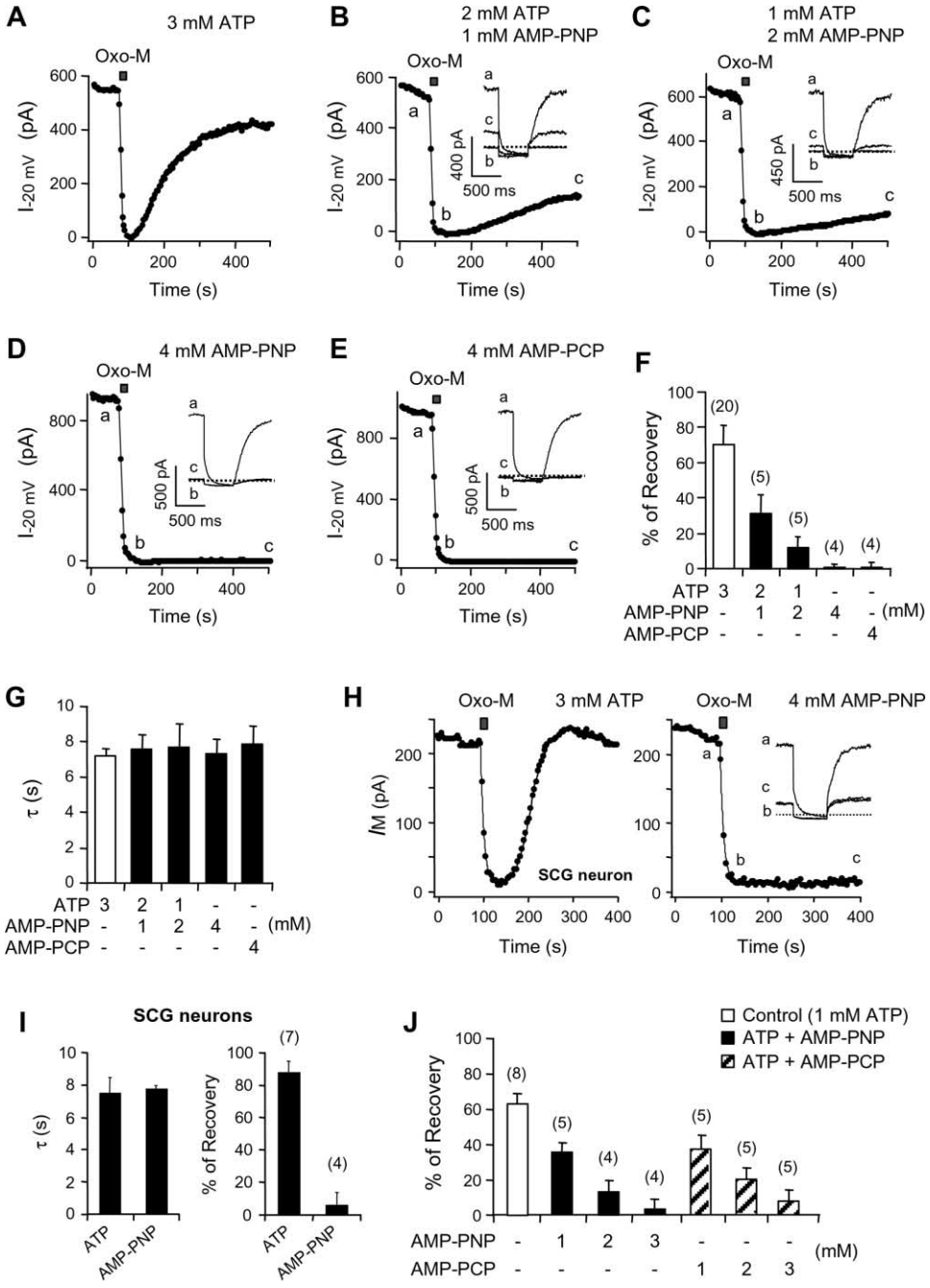

Figure 4. Nonhydrolyzable ATP Analogs Slow Recovery

Different combinations of ATP, AMP-PNP, and AMP-PCP were included in the pipette solution to test the ATP dependence of recovery from muscarinic suppression. All experiments except (H) and (I) are with tsA cells. The pipette solutions contained: (A) 3 mM ATP without AMP-PNP, (B) 2 mM ATP and 1 mM AMP-PNP, (C) 1 mM ATP and 2 mM AMP-PNP, or (D and E) 4 mM AMP-PNP or 4 mM AMP-PCP and no ATP. The *insets* show selected current traces. (F) Summary of recovery from inhibition under the various conditions of (A)–(E), measured 7 min after washout of oxo-M and normalized to the value before the stimulation. (G) Time constant (τ) of muscarinic inhibition with different combinations of ATP and ATP analogs in pipette solution. (H) Modulation of I_M in SCG neurons. The pipette solution contained 3 mM ATP or 4 mM AMP-PNP. Oxo-M was applied for 20 s as indicated. *Inset* shows selected current traces. I_M was taken 8–10 min after pipette breakthrough. (I) Summary of time constant (τ) and recovery from inhibition with ATP and AMP-PNP in pipette solution. Recovery was measured 5 min after washout of oxo-M and normalized to the value before the stimulation. (J) AMP-PNP and AMP-PCP reduce ATP-mediated channel recovery. The pipette contained 1 mM ATP and the listed analogs. The control had only 1 mM ATP.

ADP with Glucose Supports Recovery of KCNQ2/KCNQ3 Currents

In bullfrog sympathetic ganglion cells, extracellular glucose can help to maintain I_M in the absence of pipette ATP (Simmons and Schneider, 1998). Thus it is possible that ATP can be resynthesized from precursors by energy metabolism during our recordings (our bathing solution contains 8 mM glucose). We tested this possibility

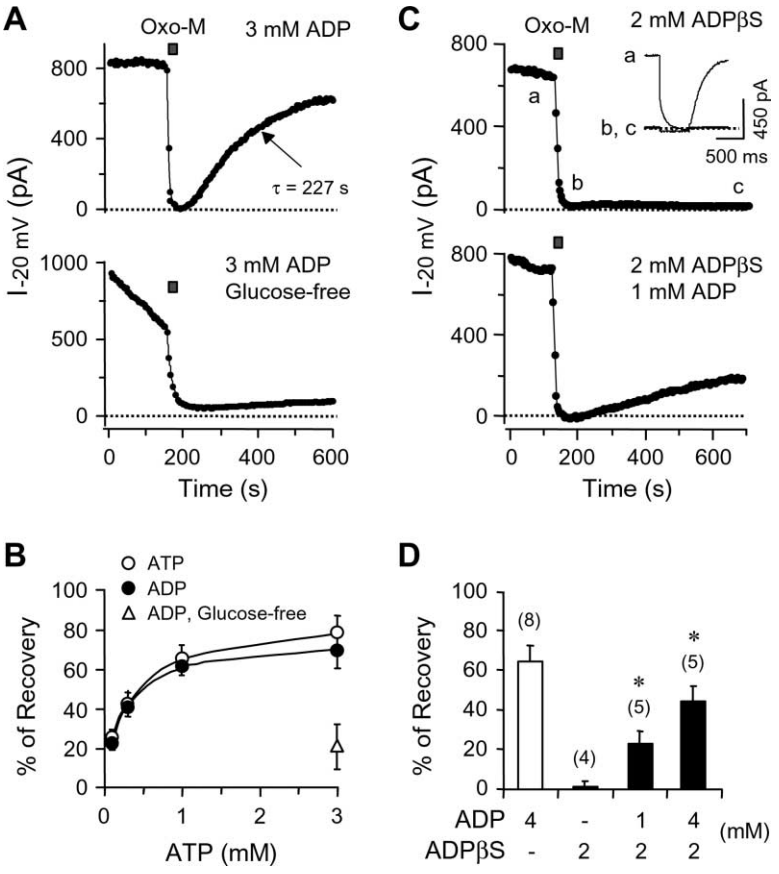

Figure 5. ADP Substitutes for ATP

Currents were recorded after waiting at least 5 min from breakthrough to allow for dialysis of nucleotides. (A) Current at −20 mV recorded with 3 mM ADP instead of ATP in a bath with or without 8 mM glucose. Oxo-M was applied as indicated. (B) Dose-response relation for recovery with ATP or ADP in the pipette. Mean of four to seven experiments. (C) Currents at −20 mV with 2 mM ADPβS or 2 mM ADPβS and 1 mM ADP. Oxo-M was applied as indicated. (D) Summary of channel recovery with combinations of ADPβS and ADP, measured 7 min after washout of oxo-M and normalized to the value at the beginning of the stimulation. *$p < 0.01$, compared with 2 mM ADPβS.

by totally replacing ATP with ADP in the pipette solution. Figure 5A shows that KCNQ2/KCNQ3 current still recovers well from muscarinic inhibition with 3 mM ADP in the pipette. The concentration-dependent effects of ADP and ATP on the recovery of the current are virtually identical (Figure 5B). About 0.5 mM of either nucleotide permits half-maximal recovery. Substitution of ADP changed neither the oxo-M-induced suppression nor the rundown of the KCNQ2/KCNQ3 channels. Extracellular glucose is essential for this action of ADP. The recovery with 3 mM ADP is no better than with ADP- and ATP-free pipette solutions if the extracellular glucose is removed (Figures 5A, lower panel and 5B), and spontaneous reduction is accelerated. Another pathway for synthesis of ATP from ATP uses adenylate kinase (2ADP → ATP + AMP). However, addition of the adenylate kinase inhibitor P^1,P^5-di(adenosine-5′) pentaphosphate Ap5A (200 μM) to the pipette solution did not alter the ADP-mediated recovery of the KCNQ2/KCNQ3 current (67% ± 8%, n = 7).

To further examine whether the efficacy of ADP is due to ATP synthesis from ADP, we used adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS), which, being a poor substrate for phosphorylation by kinases, is a competitive inhibitor of ATP synthesis. With an ATP-free pipette solution containing 2 mM ADPβS, there was no recovery at all (Figure 5C, *upper*). Partial recovery could be restored by including ADP along with ADPβS (Figures 5C, *lower* and 5D). However, ADP was significantly less effective with ADPβS than without. Apparently, ATP synthesis from ADP suffices to maintain channel activity and also can support recovery from muscarinic inhibition.

G Protein Activity Is Not Needed for Recovery from Muscarinic Suppression

We then tested if GTP has a role in recovery, using tsA cells dialyzed with various GTP or GDP analogs. Since some of the analogs slowed inhibition, we used longer agonist exposures, 5 min in oxo-M. With the standard pipette solution, the KCNQ2/KCNQ3 current was suppressed by oxo-M throughout the 5 min exposure to oxo-M (Figure 6A, top). Thus, muscarinic receptors remain active for the full time without desensitizing (Suh and Kim, 1995; Willars et al., 1998). Within 7 min after washout of oxo-M, the current recovered to 52% ± 7% of the preagonist value. The GDP analog guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS) generally acts as a competitive antagonist of GTP binding to G proteins. Substitution of the intracellular GTP by GDPβS slowed the reduction of muscarinic inhibition 6-fold ($\tau = 45.8 \pm 3.6$ s, n = 6) and decreased the maximum suppression of current (Figure 6A, bottom). However, including GDPβS did not hinder the recovery of the channel; mean recovery was to 60% ± 4% with 0.03 mM GTP plus 0.7 mM GDPβS and to 44% ± 12% with 1 mM GDPβS (Figure 6B). In contrast, in cells dialyzed with the nonhydrolyzable GTP analogs guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) or guanylyl-imidodiphosphate (GMP-PNP), the current did not recover after the 5 min stimulation with oxo-M (Figure 6B). Presumably, with these analogs, activity of the

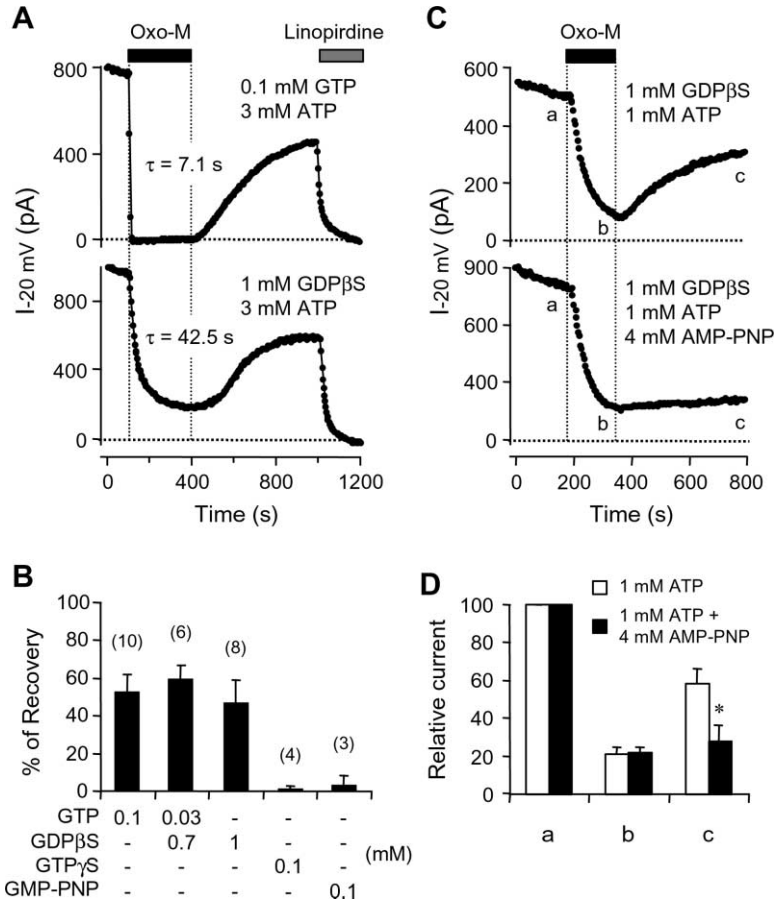

Figure 6. GTP Analogs Slow Induction of Muscarinic Modulation

(A) Current with the standard 0.1 mM GTP (top) or 1 mM GDPβS in the pipette (bottom). Oxo-M applied for 5 min as indicated. (B) Summary of recovery with different GTP analogs. Number of experiments given in parentheses. (C) Currents with 1 mM of ATP and GDPβS without (top) or with (bottom) 4 mM AMP-PNP. Oxo-M was applied for 3 min. (D) Summary of seven experiments like that in (C), showing current before (a), during (b), and 7 min after (c) oxo-M. Pipette solutions contain 1 mM GDPβS instead of GTP. *$p < 0.01$, compared with 1 mM ATP alone.

G proteins cannot be shut off by the normal GTPase activity. Thus, although intracellular GTP is required for receptor-mediated channel *inhibition*, the *recovery* is not dependent on GTP and G proteins.

The recovery of current from muscarinic suppression was analyzed using ATP analogs in the presence of 1 mM GDPβS instead of GTP to further exclude the involvement of GTP and G proteins in recovery. As shown in Figure 6C, when we decreased the concentration of hydrolyzable ATP in the pipette to 1 mM, the extent of recovery was not significantly affected. However, the addition of 4 mM of the nonhydrolyzable ATP analog AMP-PNP along with 1 mM ATP dramatically blocked the recovery. Relative current 7 min after oxo-M washout was only 23% ± 7% ($n = 7$) with 1 mM ATP plus 4 mM AMP-PNP, significantly lower than the control group 58% ± 6% ($n = 7$, $p < 0.01$) (Figure 6D). Similarly, 4 mM AMP-PCP with 1 mM ATP suppressed the recovery in the presence of 1 mM GDPβS (data not shown). Thus, as before, hydrolyzable ATP is important for the recovery from muscarinic suppression but not for the induction of suppression, whereas GTP is exclusively involved in the induction of receptor-mediated suppression and is not involved in recovery.

Inhibition of the Resynthesis of PIP_2 Prevents Recovery of I_M

The need for hydrolyzable ATP suggests that a kinase mediates recovery. To test this hypothesis, we examined the effects of various kinase inhibitors. In a variety of cells, the receptor-mediated activation of PLC depletes PIP_2 in the plasma membrane, and the replenishment of PIP_2 requires ATP and lipid kinases, such as PI 4-kinase. Therefore, using the inhibitors wortmannin and phenylarsine oxide (PAO) (Nakanishi et al., 1995; Meyers and Cantley, 1997; Varnai and Balla, 1998), we asked if inhibition of PI 4-kinase might affect recovery of the KCNQ2/KCNQ3 current from muscarinic inhibition. Many recent studies report that wortmannin inhibits the replenishment of PIP_2 after muscarinic receptor-mediated depletion (Willars et al., 1998; Sorensen et al., 1998; Xie et al., 1999). As shown in Figure 7A, when we applied wortmannin in the bath, the recovery of current was clearly slowed and reduced in a concentration-dependent manner; the current recovered to only 18% ± 7% at 10 μM and to 2% ± 3% at 50 μM wortmannin (Figure 7B). In tsA cells treated with 50 μM wortmannin, the current no longer recovered, even 10 min after the *washout* of wortmannin (data not shown), consistent with previous reports that the inhibitory effect of wortmannin is persistent (Nakanishi et al., 1995). In SCG neurons, the actions of wortmannin were similar and recovery was almost completely blocked by 50 μM wortmannin (Figures 7C and 7D). Despite its profound effect on *recovery*, wortmannin did not affect the *inhibition* of I_M or of KCNQ2/KCNQ3 current during oxo-M application.

PAO, an arsenical that blocks the formation of PIP_2 from PI by inhibiting PI 4-kinase (Wiedemann et al., 1996;

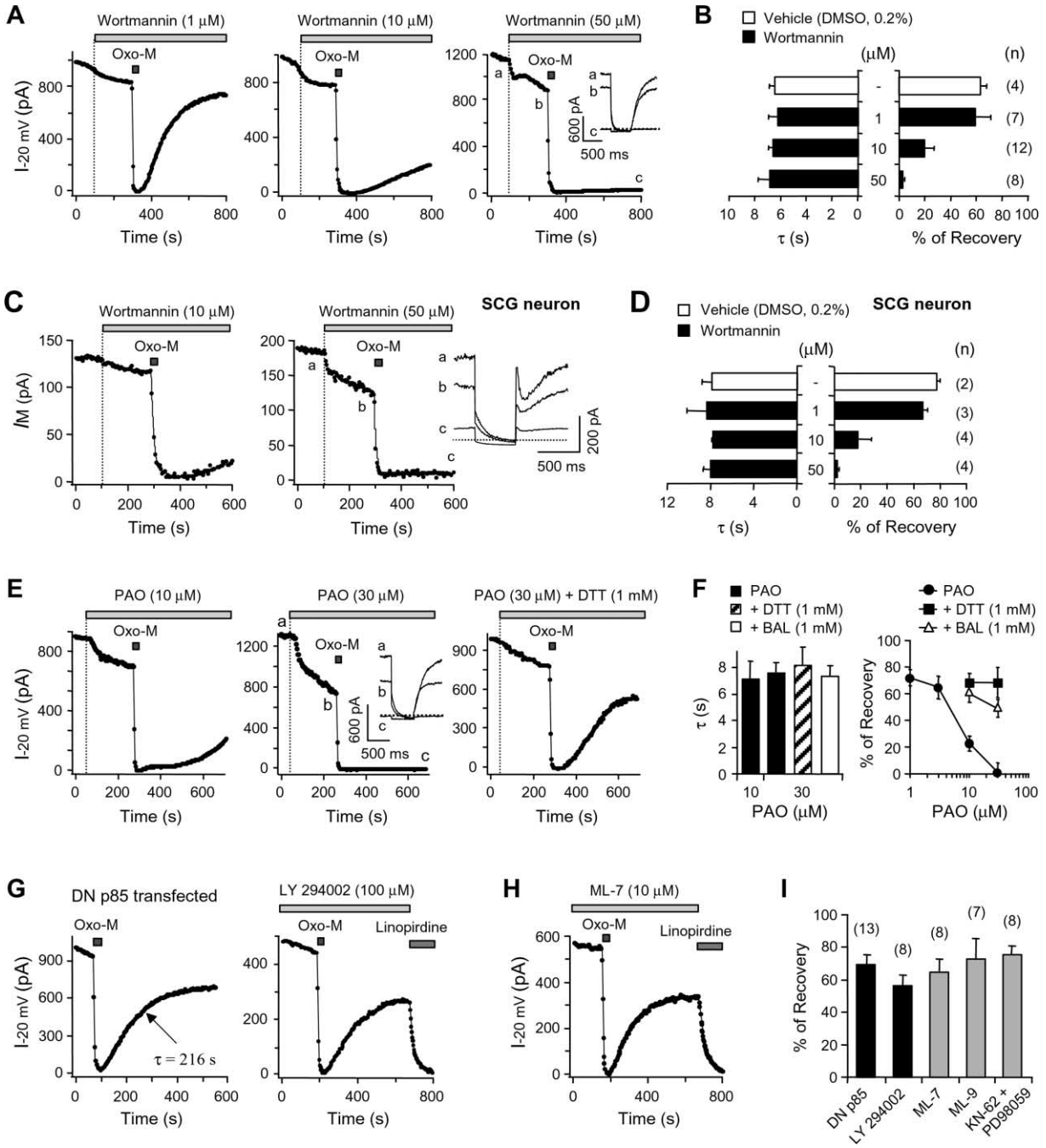

Figure 7. Recovery from Muscarinic Suppression with Wortmannin and Other Kinase Inhibitors

(A) Inhibition and recovery of currents in tsA cells treated with 1, 10, or 50 μM wortmannin. Three minutes after wortmannin treatment began, oxo-M was added for 20 s. (B) Summary of experiments like those in (A). (C) Modulation of I_M with wortmannin in SCG neurons. Experiments done as in (A). (D) Summary of experiments like those in (C). (E) Inhibition and recovery in tsA cells treated with 10 or 30 μM PAO in the absence or presence of 1 mM DTT. (F) Summary of effects of PAO on inhibition and recovery of current. Mean of five to ten experiments. (G) Lack of effect of PI 3-kinase on channel recovery. *Left*, Muscarinic modulation and recovery in cells transfected with a dominant-negative PI 3-kinase regulatory subunit plasmid DN p85 (1 μg). *Right*, Recovery in cells preincubated with 100 μM LY 294002 for 1 hr and studied in the presence of LY 294002. (H) Lack of effect of MLCK inhibition on channel recovery. Recovery in cells preincubated with 10 μM of the MLCK inhibitor ML-7 for 30 min and studied in the presence of ML-7. Applications of 30 μM linopirdine are marked. Preincubation with LY 294002, ML-7, ML-9, or the protein kinase inhibitors did not change the initial KCNQ2/KCNQ3 current recorded at −20 mV. (I) Summary of recovery in the presence of the kinase inhibitors. Includes cells preincubated with 40 μM ML-9 or 2 μM KN-62 and 50 μM of PD 98059, inhibitors for CaMK and MEK, for 30 min and then stimulated with oxo-M for 20 s in the presence of the inhibitors. Number of cells in parentheses.

Table 1. Effects of Kinase Inhibitors on Recovery of KCNQ2/KCNQ3 Channles from Muscarinic Suppression

Kinase Inhibitor (Dose)	Affected Kinase	Recovery (% of Initial)	n
None	None	72 ± 6	20
Wortmannin (10 μM)	PI 3- and 4-kinase, MLCK	18 ± 7*	12
K-252a (10 μM)	MLCK, PKC, PKA	75 ± 7	5
Bisindolylmaleimide I (3 μM)	PKC	68 ± 6	4
H-89 (10 μM)	PKA	64 ± 5	4
KN-62 (2 μM)	CaMK	66 ± 8	5
PD 98059 (50 μM)	MEK	74 ± 6	5
SB 203580 (10 μM)	ERK	71 ± 5	4
AG213 (100 μM)	Tyrosine kinase	67 ± 4	5
Genistein (50 μM)	Tyrosine kinase	70 ± 5	4

Cells were preincubated with SB 203580, AG213, and genistein for 1 hr, KN-62 and K-252a for 30 min, or H-89 and bisindolylmaleimide I for 5 min. The channel recovery was measured 7 min after washout of oxo-M and was normalized to the value at the beginning of the stimulation. None, Untreated control. *$p < 0.01$, compared with the control.

Varnai and Balla, 1998; Sorensen et al., 1998), also prevented the recovery of the KCNQ2/KCNQ3 current after muscarinic inhibition. As shown in Figure 7E, PAO significantly blocked the recovery of current at 10 μM (23% ± 4%, n = 5), and almost completely blocked it at 30 μM (2% ± 4%, n = 10). This inhibitory effect of PAO was prevented by addition of vicinal dithiol sulfhydryl reagents to the PAO solution: dithiothreitol (DTT) gave full prevention and 2,3-dimercaptopropanol (BAL) gave partial prevention (Figures 7E and 7F). POA forms stable inactive complexes with DTT and BAL (Schaefer et al., 1994). The *induction* of muscarinic inhibition was not affected by PAO (Figure 7F).

Wortmannin also inhibits PI 3-kinase and myosin light chain kinase (MLCK) (Okada et al., 1994; Nakanishi et al., 1995). Therefore, we needed to compare the effects of wortmannin with those of other kinase inhibitors. In experiments illustrated in Figure 7G and summarized in Figure 7I, the recovery of KCNQ2/KCNQ3 current was not significantly different from the control in cells cotransfected with a dominant-negative PI 3-kinase regulatory subunit (DN p85) (Dhand et al., 1994; Poser et al., 2000) or treated with the PI 3-kinase inhibitor LY 294002 (Vlahos et al., 1994). In addition, treatment of cells with the MLCK inhibitors ML-7 (Figures 7H and 7I) (Watanabe et al., 1998; Aromolaran et al., 2000) and ML-9 (Watanabe et al., 1998; Samizo et al., 1999) had little effect on the recovery. In these experiments, the cells were preincubated with the MLCK inhibitors for 30 min. The presence of large currents upon subsequent breakthrough showed that maintenance of KCNQ2/KCNQ3 currents does not require this kinase. MLCK can be activated by two different pathways: a Ca^{2+}/calmodulin-dependent kinase-mediated pathway and a mitogen-activated protein kinase kinase (MEK)-mediated pathway (Pfitzer, 2001). However, when we treated the cells with a mixture of the selective inhibitors for these pathways, KN-62 and PD 98059, there was no significant difference in the recovery compared to the control (72% ± 4%, n = 8) (Figure 7I) and no change in the muscarinic inhibition or the maintenance of KCNQ2/KCNQ3 current.

Table 1 compares the magnitudes of recovery of current from muscarinic inhibition in the presence of various kinase inhibitors. The application of 10 μM wortmannin clearly blocked recovery. In contrast, no significant differences were found between the control group and the groups treated with the serine-threonine protein kinase inhibitors bisindolylmaleimide I and H-89. The same was the case for KN-62, an inhibitor of Ca^{2+}/calmodulin-dependent kinase; AG213 and genistein, inhibitors of tyrosine kinases; and SB 203580 and PD 98059, inhibitors of extracellular signal-regulated kinase (ERK) and MEK, respectively. This pharmacological profile points to PI 4-kinase as the relevant kinase for recovery of KCNQ2/KCNQ3 current.

Because of ongoing endogenous hydrolysis of PIP_2, irreversible inhibition of PI 4-kinase by wortmannin or PAO will deplete PIP_2 from unstimulated cells (Wiedemann et al., 1996; Willars et al., 1998). Therefore, we asked if long treatments with the kinase inhibitors decrease channel activity without application of agonist. As shown in Figure 8, during 13 min exposures to wortmannin or PAO, the KCNQ2/KCNQ3 current ran down more quickly than in control. The faster rundown was evident in 10 μM wortmannin (Figure 8B) and dramatic in 50 μM wortmannin (Figure 8C) and 30 μM PAO (Figure 8D). Addition of DTT blocked the effect of PAO on rundown (Figure 8E). As before with wortmannin- and PAO-treated cells, application of oxo-M inhibited current rapidly, and there was little recovery. These results are consistent with the hypothesis that the amplitude of the KCNQ2/KCNQ3 current mirrors the PIP_2 level in the membrane.

Discussion

This paper distinguishes the *induction* of muscarinic inhibition of I_M from the *recovery*. In broad strokes, we find that GTP is needed only for the induction of inhibition, and ATP hydrolysis is needed only for the recovery. Some lipid kinase inhibitors do not change the induction, but block the recovery. Many other kinase inhibitors do not affect recovery.

Observations with Nucleotides and Recovery

In our work, reducing the ATP in the pipette from 3 mM to 0.3 mM reduces recovery from brief muscarinic inhibition in a cumulative manner so that after three exposures to oxo-M there is no recovery. Replacing some of the

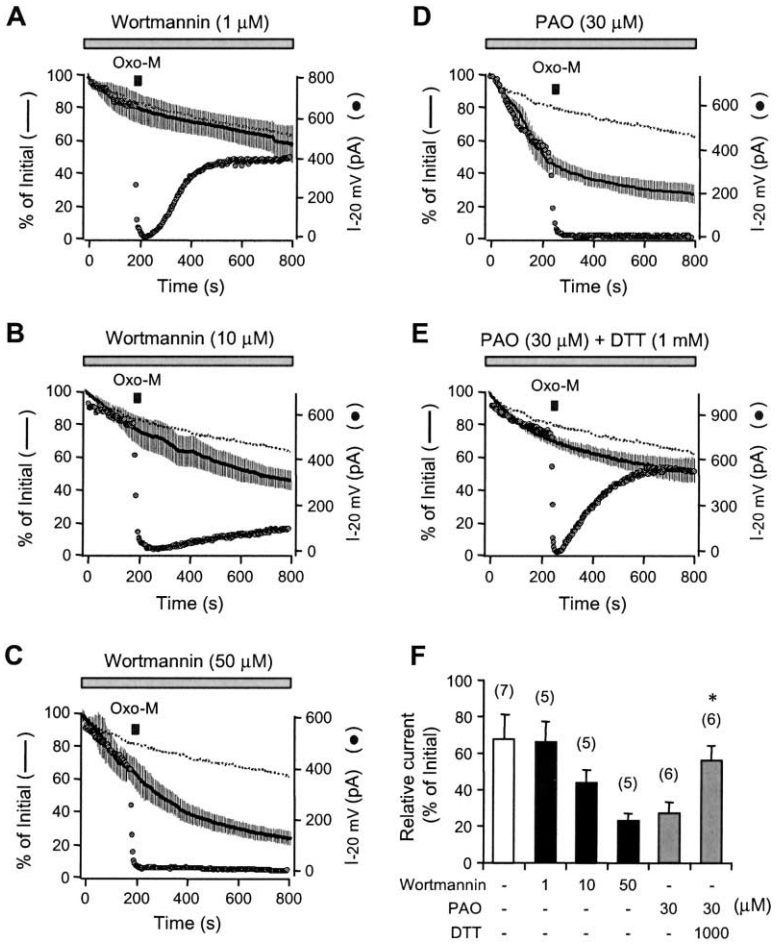

Figure 8. Wortmannin and PAO Accelerate Rundown of KCNQ2/KCNQ3 Current

Rundown of currents with 1 μM (A), 10 μM (B), 50 μM (C) of wortmannin, or 30 μM of PAO without (D) and with (E) 1 mM DTT in the bath (left; solid line). To show the effect of wortmannin or PAO on the muscarinic modulation, oxo-M was applied for 20 s to a single cell 3 or 4 min after starting treatment with wortmannin or PAO treatment (right; circles). Dotted line shows the spontaneous rundown of the current taken from Figure 3F. (F) Summary of the relative current 13 min after application of inhibitors. *$p < 0.01$, compared with 30 μM PAO alone.

ATP with nonhydrolyzable analogs or just adding the nonhydrolyzable analogs to a constant ATP concentration slows recovery or even stops it. Pfaffinger et al. (1988) reported a similar observation for I_M in frog sympathetic neurons. In our study, ADP seems to substitute fully for ATP, apparently because despite the steady dialyzing action of the whole-cell pipette, glucose-dependent energy metabolism readily phosphorylates much of the added ADP to ATP within the cell. Inclusion of ADPβS, an inhibitor of ATP regeneration (Eckstein and Goody, 1976), together with the ADP, depressed the ability of ADP to support recovery. An analog of GDP had a different effect. We found that putting GDPβS in the pipette slows the induction of inhibition dramatically—presumably by reducing the probability for GTP to activate G proteins during receptor activation—without affecting the recovery after removal of agonist. Pfaffinger et al. (1988) also reported strong slowing of inhibition of I_M in neurons with GDPβS. All of these experiments suggest that recovery requires transfer of a phosphate from ATP to regenerate a phosphorylated product that had been depleted by the preceding muscarinic inhibition. GTP is not required during this recovery.

We also looked at rundown in several of the same conditions. This is relevant from two points of view. First, we needed to be able to show that lack of recovery after muscarinic suppression of current is not merely due to "normal" spontaneous rundown of the channels. Second, it is possible that rundown and slowed recovery reflect the same process. If so, rundown might be a tonic receptor-mediated inhibition that accumulates because recovery is compromised. On the first point, we found that normal rundown alone does not account for the poor recovery from inhibition seen with low-ATP solutions.

On the second point, we favor the concept that rundown and agonist-induced inhibition are the same process. Using substitutions of adenine nucleotide analogs and manipulations of bath glucose, Simmons and Schneider (1998) concluded that *maintenance* of I_M in neurons requires low amounts of hydrolyzable ATP. They point out that in all reports where channels are well maintained in the absence of pipette ATP (Simmons et al., 1990; Chen and Smith, 1992; this paper), metabolic substrates such as glucose and/or pyruvate are included in the bath. In those studies reporting more rapid rundown without pipette ATP (Pfaffinger et al., 1988; Tokimasa and Akasu, 1990), substrates were not supplied. In the Simmons and Schneider proposal, when no ATP is supplied in the pipette but metabolic substrates are available, residual ADP in the cell would be phosphorylated to yield a low but nonzero ATP level. From our work, this proposed low ATP level is insufficient to permit rapid recovery after muscarinic inhibition,

but if significant ADP is added to the pipette solution, it may be phosphorylated to provide enough ATP for recovery. We suggest that the low ATP without added adenine nucleotide suffices for a low activity of lipid kinases needed to compensate slow rundown but is insufficient for the major phosphorylation needed after agonist-induced inhibition.

The action of the PLC inhibitor U73122 is subtle and requires further discussion. A previous study from this laboratory showed that the mechanism of muscarinic suppression of I_M differs from that of bradykinin-induced suppression (Cruzblanca et al., 1998; see also Delmas et al., 2002). One striking difference was that muscarinic suppression was only slightly blocked by 1 μM U73122 whereas bradykinin suppression was strongly blocked. This led us to propose that PLC is not used in muscarinic suppression of I_M. Studying the muscarinic pathway in tsA cells and SCG neurons, we now confirm the weak effect of 1 μM U73122, but find that 3 μM U73122 does block I_M modulation. The effect is specific since the inactive analog U73343 spares most of the muscarinic suppression. Therefore, we now conclude that PLC, a Ca^{2+}-requiring enzyme, is involved in the bradykinin and muscarinic pathways alike, although the muscarinic pathway is less sensitive to U73122. The Ca^{2+} requirement would explain why the modulation of M current fails when cytoplasmic-free Ca^{2+} is lowered (Beech et al., 1991).

The IC_{50} for U73122 is in the low micromolar range in many assays involving PI turnover, and its effects are concentration and time dependent. Both the 1 and the 3 μM concentrations that we used should inhibit a significant fraction of the PLC, slowing but not abolishing its activity. This is a range of *partial* block of PLC. Indeed our measurements here involve block of responses to 20 s exposures to oxo-M, but if we lengthen the oxo-M exposure to 3 min, even 3 μM U73122 is insufficient to prevent a slow muscarinic suppression of I_M (data not shown). Higher concentrations of U73122 were not easy to explore because they strongly decreased I_M without receptor stimulation. The difference between the concentrations required to block muscarinic and bradykinin actions probably reflect quantitative differences in the efficiency of coupling of bradykinin and muscarinic receptors to their effectors (Haley et al., 2000; Delmas et al., 2002).

The Lipid Kinase and PI-Polyphosphate Hypothesis

Receptors that activate the G proteins $G\alpha_{q/11}$, including the M_1 muscarinic receptor, turn on the activity of phosphoinositide-specific PLC, which cleaves PIP_2 into two labile second messengers IP_3 and diacylglycerol. If PLC is active long enough, it will deplete the membrane of various phosphoinositides, including PIP_2 and PI-4-P, faster than they are regenerated (Willars et al., 1998). Depletion takes several seconds. Generation of more PIP_2 requires a series of steps that include phosphorylating phosphatidylinositol on the inositol 4 and 5 positions by lipid kinases. Regeneration of the phosphorylated forms may take several minutes. These lipid kinases require higher concentrations of ATP for maximal activity (~1 mM) than do many protein kinases and ATP-driven reactions (a few micromolar) (Balla, 1998).

Several laboratories working on a variety of ion channels and transporters have provided evidence that functions of these membrane proteins are either enhanced or turned off by PIP_2 (Hilgemann et al., 2001), and they have been seeking a physiological regulation that is mediated by PIP_2 breakdown or synthesis. In many cases, the effect of PIP_2 on these membrane proteins is documented but its physiological role and the endogenous regulatory agonists are not yet known.

For M current modulation, the hypothesis would be as follows: activity of I_M (and presumably all other KCNQ) channels depends on the presence of certain phosphatidylinositol phosphates in the membrane, including PIP_2, and activation of M_1 and other $G\alpha_{q/11}$-coupled receptors suppresses current by depleting the membrane of these essential lipid phosphates. The second messenger for suppression of current is therefore the depletion of an essential cofactor rather than the production of an inhibitor. Formally, this is analogous to vertebrate photoreceptors where the *breakdown* of cGMP is the light signal. Recovery then would require a sequence of reactions that regenerate the essential lipid phosphates. These steps would underlie the classical slow muscarinic regulation of I_M electrical activity (Brown and Adams, 1980). Rundown of I_M may also reflect gradual loss of PIP_2 from the membrane.

As a test, we sought to block ATP-dependent recovery with a panel of kinase inhibitors. Among those we tested, only wortmannin and PAO worked. They totally blocked recovery at 50 and 30 μM, respectively. Wortmannin is an inhibitor of PI 3-kinase at nanomolar concentrations, and of PI 4-kinase at micromolar concentrations (Okada et al., 1994; Nakanishi et al., 1995). For blocking recovery of current from muscarinic suppression, wortmannin was effective only at concentrations of $\geq$10 μM. Our tests of other ways to inhibit PI 3-kinase do not support a role for them in recovery. Thus, LY 294002, a selective inhibitor of PI 3-kinase, and expression of a dominant-negative PI 3-kinase regulatory subunit (DN p85) failed to block the recovery of KCNQ2/KCNQ3 channel. Furthermore, PAO, an inhibitor of PI 4-kinase in vitro (Yue et al., 2001) and in vivo (Wiedemann et al., 1996; Linseman et al., 1999), also inhibited recovery in a concentration-dependent manner. Formation of complexes with BAL or DTT rendered PAO inactive. These results imply that the effects of lipid kinase inhibitors on recovery are mediated by inhibiting the activity of PI 4-kinase, an essential enzyme for the regeneration of PIP_2.

We found that wortmannin and PAO also accelerated rundown of channels without prior muscarinic inhibition. When PI 4-kinase is inhibited, the cellular content of PIP_2 falls slowly. For example, it is reported that membrane PIP_2 levels decrease to $<$20% of the control level within 15 min in unstimulated SH-SY5Y neuroblastoma cells incubated with wortmannin (Willars et al., 1998). This would explain the gradual channel rundown in wortmannin and PAO.

Similarly, 30 min preincubations with wortmannin were previously reported to reduce I_M of bullfrog sympathetic neurons (Tokimasa et al., 1995), and at that time the effect was attributed to inhibition of MLCK. Wortmannin is indeed an inhibitor of MLCK at submicromolar concentrations, and previous studies provide evidence that I_M in bullfrog sympathetic ganglia can be regulated

by MLCK. They found that application of a peptide inhibitor for MLCK decreases I_M, whereas intracellular perfusion of catalytic subunits of MLCK enhances I_M (Akasu et al., 1993; Tokimasa et al., 1995). Our data show that applications of two specific inhibitors for MLCK, ML-7 and ML-9, or of a nonselective MLCK inhibitor K-252a (Nakanishi et al., 1988) neither blocked the ATP-dependent recovery from muscarinic inhibition nor accelerated rundown. In addition, blockage of the known pathways for MLCK activation using inhibitors for Ca^{2+}/calmodulin-dependent kinase and MEK had little effect on either the maintenance of KCNQ2/KCNQ3 channels or the recovery from muscarinic suppression. Thus, MLCK is not likely to be involved in the ATP-mediated recovery of KCNQ2/KCNQ3 current in tsA cells.

Here we have developed two lines of evidence consistent with the lipid-kinase hypothesis. First, recovery requires high concentrations of hydrolyzable ATP and second, it is blocked by inhibitors of PI 4-kinase. Additional direct biochemical experiments and work with excised patches of the type that has been successful with some other channels and transporters would test these conclusions. In other examples, domains of basic residues in combination with hydrophobic ones have been identified as binding sites for PIP_2 on the membrane protein (Fan and Makielski, 1997; Hilgemann et al., 2001). Hilgemann et al. (2001) point out that in the known examples of regulation by PIP_2, additional signaling molecules also participate. Perhaps for I_M PIP_2 is just one of several regulatory signals mediating muscarinic inhibition (see, for example, Cruzblanca et al., 1998). It is generally accepted that the signal(s) for modulation of I_M can diffuse in the cytoplasm or, if in the membrane, past the seal of an on-cell pipette (Selyanko et al., 1992; Marrion, 1993). If lipids are the signal, we will have to understand what component is diffusible in this manner. The case of I_M stands out as one of the first recognized examples of ion channel modulation by known physiological inputs (Brown and Adams, 1980) and with a known physiological effect on neuronal excitability. Our laboratory has previously given evidence that N- and L-type Ca^{2+} channels can also be modulated by M_1 muscarinic receptors and by angiotensin II receptors using a second messenger pathway that may be the same as that for modulation of I_M by these receptors (Hille, 1994; Shapiro et al., 1994a).

Experimental Procedures

Cells

Plasmids encoding KCNQ2 (GenBank AF110020) and KCNQ3 (GenBank AF091247) were kindly provided by David McKinnon (State University of New York, Stony Brook, NY), and the plasmid containing mouse M_1 receptor was provided by Neil Nathanson (University of Washington, Seattle, WA). The KCNQ2 and KCNQ3 genes were subcloned into the pcDNA3 expression plasmid (Invitrogen, San Diego, CA) as previously described (Shapiro et al., 2000) and transiently transfected with the muscarinic M_1 receptor in human tsA-201 (tsA) cells. Cells were grown in tissue culture dishes (Falcon, Oxnard, CA) in tsA growth medium (DMEM plus 10% fetal bovine serum with 0.2% penicillin/streptomycin) at 37°C (5% CO_2) and passaged approximately every 7 days. Plasmids were transfected with lipofectamine 2000 transfection reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's directions. The next day, the cells were plated onto poly-L-lysine-coated coverslip chips and used within 2 days for electrophysiological experiments. As a marker for successfully transfected cells, 0.1 μg of cDNA encoding green fluorescent protein was cotransfected with channel and receptor cDNA.

SCG neurons were taken from 4- to 5-week-old male rats (Sprague-Dawley) and cultured for 1 day (Beech et al., 1991). Rats were quickly anesthetized with CO_2 and decapitated. Ganglia were dissociated and the neurons suspended twice in DMEM supplemented with 10% heat-inactivated horse serum. Cells were plated on glass coverslips coated with poly-L-lysine, incubated at 37°C (5% CO_2) overnight, and used within 24 hr.

Current Recording and Analysis

The whole-cell configuration of the patch-clamp technique was used to voltage-clamp and dialyze cells at room temperature (22°C–25°C). Electrodes were pulled from glass hematocrit tubes (VWR Scientific, Seattle, WA). They had resistances of 1.3–2.5 MΩ. The whole-cell access resistance was 2–4 MΩ and series-resistance errors were compensated >60%. Fast and slow capacitances were compensated prior to the applied test-pulse sequences. When measuring the rates of induction and recovery from muscarinic inhibition of the current, we applied test and control solutions rapidly to the 100 μl chamber (flow rate of 1.5 ml/min) in the vicinity of the recorded cell. Tests using junction potential measurements on an open pipette showed that solutions are changed with a mean exponential time constant of 2.4 s.

The KCNQ2/KCNQ3 currents from tsA cells were studied by holding the cell at −20 mV and applying a 500 ms hyperpolarizing step to −60 mV every 4 s. The amplitude of the current usually was defined as the outward current at the holding potential sensitive to block by 30 μM linopirdine. Like HEK293 cells, tsA cells do have small endogenous voltage-gated K^+ currents (Yu and Kerchner, 1998). These endogenous currents have an activation rate >10-fold faster than KCNQ2/KCNQ3 channels, show little observable tail current (deactivation) at −60 mV, and are just starting to activate at −20 mV (data not shown). Thus, the KCNQ2/KCNQ3 current was distinguished easily from the endogenous current. In all experiments with pipette solutions containing nucleotide analogs, we waited longer than 5 min before applying oxo-M to allow for the dialysis into the cytoplasm. The M current amplitude in SCG neurons was measured from deactivation current records at −60 mV as the difference between the average of a 10 ms segment, taken 10–20 ms into the hyperpolarizing step, and the average during the last 10 ms of that step.

Solutions and Materials

The external Ringer's solution used to record KCNQ currents contained (in mM): 160 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 8 glucose, adjusted to pH 7.4 with NaOH. SCG neurons were superfused with an external Ringer's solution having 0.5 μM tetrodotoxin. The standard pipette solution contained (in mM): 175 KCl, 5 $MgCl_2$, 5 HEPES, 0.1 1,2-bis(2-aminophenoxy)ethane *N,N,N',N'*-tetraacetic acid (BAPTA), 3 Na_2ATP, and 0.1 Na_3GTP, titrated to pH 7.4 with KOH. Bisindolylmaleimide I, wortmannin, LY 294002, ML-7, ML-9, K-252a, H-89, (S)-5-isoquinolinesulfonic acid (KN-62), AG213, genistein, and PD 98059 were dissolved in DMSO. The final concentration of DMSO never exceeded 0.3%. All other substances were dissolved in water. Final dilutions were made with external solution.

Reagents were obtained as follows: oxotremorine methiodide (oxo-M) (Research Biochemicals, Natick, MA); BAPTA (Molecular Probes); DMEM, fetal bovine serum, lipofectamine, and penicillin/streptomycin (Life Technologies, Gaithersburg, MD); ATP, GTP, GDPβS, GMP-PNP, GTPγS, linopirdine, AMP-PNP, AMP-PCP, ADPβS, ADP, wortmannin, LY 294002, KN-62, genistein, PAO, BAL, DTT, Ap5A, and pirenzepine (Sigma, St. Louis, MO); ML-7, ML-9, SB 203580, K-252a, H-89, and AG 213 (CN Biosciences, La Jolla, CA). U73122 and U73343 were obtained from Research Biochemicals. The drugs were dissolved in chloroform to a final concentration of 5 mM and aliquotted into Effendorf tubes. After evaporation of the solvent under nitrogen, tubes were stored in 4°C. On the day of experiment, one aliquot was dissolved in dimethyl sulfoxide to a concentration of 2.5 mM. The dominant-negative PI 3-kinase regulatory subunit p85 was kindly provided by Daniel R. Storm (University of Washington, Seattle, WA).

Data Analysis

Data acquisition and analysis used the Pulse/Pulse Fit 8.11 software in combination with an EPC-9 patch clamp amplifier (HEKA, Lambrecht, Germany). Further data processing was performed with Excel (Microsoft) and Igor (WaveMetrics, Oregon). Time constants were measured by exponential fits. All quantitative data are expressed as the mean ± SEM. Comparison between two groups was analyzed using Student's unpaired t test, and differences were considered significant at a level $p < 0.05$.

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Note Added in Proof

While this paper was under review, a related paper on muscarinic (M_1) receptor signaling to TRPM7 channels appeared: Runnels, L.W., Yue, L., and Clapham, D.E. (2002). The TRPM7 channel is inactivated by PIP_2 hydrolysis. Nat. Cell Biol. *4*, 329–336. It shows that muscarinic suppression of that current is due to hydrolysis of PIP_2 and that its reversal is slowed by wortmannin and speeded by added PIP_2. In cell-attached patches, the TRP7M channels can be modulated by bath-applied agonist.