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Muscarinic Potentiation of I_K in Hippocampal Neurons: Electrophysiological Characterization of the Signal Transduction Pathway

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Central cholinergic (muscarinic) systems play an important role in learning and memory. In mammalian central neurons, muscarinic stimulation depresses several voltage-activated K^+ currents and modulates synaptic transmission. Using whole-cell voltage-clamp recordings in hippocampal CA1 neurons of rat brain slices, we report that activation of muscarinic receptors potentiates a voltage-activated, sustained K^+ current (I_K-type). This potentiation of I_K is mediated by activation of protein kinase C and involves a G-protein-linked, intracellular Ca^{2+}-dependent process. This underlying second messenger pathway may prove to be important in the mediation of other previously reported muscarinic neuronal actions.

Central cholinergic (muscarinic) systems play an important role in learning and memory. Despite many studies, the actions of muscarinic receptor (mAChR) activation on central neurons are still not fully understood (see review by Nicoll et al., 1990). In mammalian hippocampal neurons, muscarinic stimulation depresses several voltage-activated K^+ currents (Benardo and Prince, 1982; Halliwell and Adams, 1982; Cole and Nicoll, 1983; Gähwiler and Brown, 1985; Nakajima et al., 1986; Storm, 1989) and modulates other ionic events (MacVicar and Tse, 1989; Blitzer et al., 1990; Burgard and Sarvey, 1990; Markram and Segal, 1990). Stimulation of phosphoinositide turnover has been implicated in some of these muscarinic actions (Baraban et al., 1985; Malenka et al., 1986; Dutar and Nicoll, 1988).

In the present experiments, we studied a voltage-activated, delayed rectifier-like potassium current (I_K) in CA1 neurons of rat hippocampus, using the technique of whole-cell recordings in brain slices. We demonstrate here that stimulation of postsynaptic mAChRs potentiates this I_K-type current. The potentiation of I_K is mediated by activation of protein kinase C (PKC), and the intracellular pathway coupling the stimulation of mAChRs to the potentiation of I_K involves a G-protein-linked, intracellular Ca^{2+}-dependent process.

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Materials and Methods

Experimental methods have been described previously (Zhang et al., 1991). Briefly, 30-50-d-old Wistar rats were anesthetized with halothane (Fluothane, Ayerst Laboratories, Montreal) and decapitated. Transverse brain slices (400 μm) were obtained and maintained in artificial cerebrospinal fluid (ACSF) that contained (in mM) NaCl, 125; KCl, 2.5; NaH_2PO_4, 1.25; $MgCl_2$, 2; $CaCl_2$, 2; $NaHCO_3$, 25; and glucose, 10; with pH 7.4, aerated with 5% CO_2, 95% O_2. The osmolarity of the ACSF was 300 ± 5 mOsm. If required, Ca^{2+} and NaH_2PO_4 were omitted and TTX (0.5 μM), 2 mM $CoCl_2$, or 5 mM EGTA was added to the ACSF. The standard patch pipette (internal) solution contained (in mM) K-gluconate, 150; EGTA, 1.1; $CaCl_2$, 0.1; Mg-ATP, 2; Tris-GTP, 0.2; and HEPES, 10; with pH 7.25 adjusted with KOH and osmolarity of 280 ± 5 mOsm. No significant rundown of I_K was seen using this internal solution. When necessary, the composition of the internal solution was modified (see Table 1) and the pH and osmolarity appropriately adjusted. The following substances were used for the modification of internal solutions: GTP-γS or GDP-βS (lithium salts), K-ATP, 5'-adenylimidodiphosphate (AMP-PNP; tetralithium salt), adenosine 5'-O-(3-thiotriphosphate) (ATP-γS; tetralithium salt), 1,2-bis(2-aminophenxy) ethane-N',N',N',-N'-tetra-acetic acid (BAPTA; tetrapotassium salt, Molecular Probes, OR), and heparin (sodium salt).

Recordings were made from submerged slices in a chamber with a perfusion rate of 3-5 ml/min at room temperature (23-24°C) except where indicated. To diminish junction potentials, an Ag-AgCl reference electrode was placed into a plastic tube containing an agar-saline bridge, with the internal solution on the reference electrode side and ACSF on the bath side. The voltage offset from 0 mV after withdrawing the patch pipette from the recorded neuron was usually less than 5 mV, which may represent a residual Donnan-type potential (cf. Barry and Lynch, 1991), owing to the relatively large soma and dendritic tree of CA1 neurons. Corrections for this voltage offset were applied to the data.

Whole-cell patch recordings were performed as described by Hamill et al. (1981). The resistance to ground of the whole-cell seal was 2-5 GΩ before breaking through the membrane, and the series resistance was then usually less than 20 MΩ. Signals were recorded with an Axoclamp 2A amplifier, and the discontinuous single electrode voltage-clamp mode was used. The sampling rate was 4-6 kHz, and the single-pole low-pass filter was set at 1 kHz. Data were digitized via a 12-bit A/D interface (TL-A, Axon Instruments, Foster City) and stored and analyzed using the PCLAMP software package (Axon Instruments, version 5.5) on an IBM AT.

In slices perfused with a medium containing 0.5 μM TTX, 2 mM Co^{2+} or 5 mM EGTA and zero added Ca^{2+}, several types of voltage-activated K^+ currents were recorded in CA1 neurons, namely, I_A, I_M, I_Q, and a sustained outward current (Spigelman et al., 1992; see also review by Storm, 1990). The slowly inactivating I_D (Storm, 1990) was not observed under the present recording conditions. The sustained outward current observed in this study is designated as a delayed rectifier-like potassium current (I_K), because of its slow activation kinetics and sensitivity to external tetraethylammonium (TEA) (Storm, 1990). Neurons were usually voltage clamped near −30 mV to inactivate I_A and to monitor the activation time constant of this I_K-type current. Recorded under these conditions, outward currents evoked by depolarizing voltage steps may represent a partially inactivated I_K, but its pharmacological properties

and dynamics were similar to those previously described (Segal and Barker, 1984; Numann et al., 1987; Storm, 1990; Oyama et al., 1992). Bath application of TEA (3–5 min) depressed I_K by 57 ± 8% with 5 mM and by 87 ± 6% with 30 mM (n = 11 and 5, respectively). I_K was not significantly inhibited by 1–2 mM 4-aminopyridine (n = 4). The time constant, τ_n, for activation was obtained by fitting the Hodgkin–Huxley expression developed for the fast sodium current described in the squid giant axon (Hodgkin and Huxley, 1952),

$$I = I_{max}(n_\infty - (n_\infty - n_0))\exp(-t/\tau_n)^x(h_\infty - (h_\infty - h_0)\exp(-t/\tau_h)),$$

to only well-clamped currents. In this equation, I_{max} is the maximal current in the absence of inactivation, n and h are unitless variables between 0 and 1, specifying the degree of activation and inactivation respectively at a given potential, and the subscripts "0" and "∞" indicate the steady state values of these variables at the potential prior to and at the end of a long voltage step, respectively. τ_n and τ_h are the time constants for activation and inactivation, respectively, and X is an integer greater than or equal to 1 and represents the order of activation. τ_n was 80–100 msec at a step voltage to −15 mV and 30–50 msec at a step voltage to 0 mV at room temperature.

In acutely dissociated hippocampal neurons, others have reported a sustained K^+ current that has an activation time constant of a few milliseconds and is depressed by external Co^{2+} and Cd^{2+} (Sah et al., 1988). The I_K described here was not blocked by Co^{2+} or by zero added Ca^{2+}.

Due to space-clamp limitations and the large amplitude of I_K, we were not able to examine the complete I–V relation of I_K with confidence, particularly after application of carbachol. This proved true even when the transmembrane K^+ gradient was decreased by 50% either by decreasing the internal [K^+] and/or increasing the external [K^+].

Blockade of I_M by cholinergic analogs was observed in some neurons, but further characterization of this blockade was limited because of rapid rundown of the I_M current under the present recording conditions (Pfaffinger, 1988; Brown et al., 1989). The voltage-activated Cl^- current previously reported (Madison et al., 1987) was not detected in the present experiments (n = 3).

Results were collected from CA1 neurons that had resting potentials more negative than −50 mV and voltage-activated potassium currents (Spigelman et al., 1992). Dialyzed with the standard internal solution and in normal ACSF, these neurons had a mean input resistance of 96 ± 8 MΩ at room temperature and 59 ± 3 MΩ at 33°C (n = 7). Mean values ± SEM are presented throughout the text. Drugs were purchased from Sigma (St. Louis) except where indicated.

Results

Muscarinic potentiation of I_K

When neurons were voltage clamped at a holding potential (Vh) of −60 mV and perfused with a medium containing 0.5 μM TTX, 5 mM EGTA, or 2 mM $CoCl_2$ and zero added Ca^{2+}, depolarizing voltage steps evoked a transient outward current followed by a sustained current (Fig. 1*A*, a1). When clamped at −40 mV or more positive potentials, the transient current was mainly inactivated leaving the sustained component (Fig. 1*A*, a2). Bath application of 30 mM TEA greatly depressed the sustained current evoked at both holding potentials with only a minor effect on the transient current (Fig. 1*A*), suggesting that the transient and sustained currents represent I_A and I_K, respectively (Spigelman et al., 1992; cf. Storm, 1990). Bath application of 50 μM carbachol (3–4 min), a stable cholinergic agonist, reversibly increased the amplitude of I_K without significantly changing I_A (n = 8; Fig. 1*B*) or I_Q (not shown). In neurons pretreated with 1 μM atropine, an mAChR antagonist, no carbachol-mediated potentiation of I_K was observed (−11.2 ± 9%; n = 6), suggesting a muscarinic action of carbachol on I_K.

Since pyramidal neurons receive defined cholinergic innervation originating from the septum, endogenously released ACh may also potentiate I_K. In neurons clamped at holding potentials near −30 mV to inactivate the action potentials and I_A, depolarizing voltage steps evoked a sustained outward current at 33°C, which represented mainly the Ca^{2+}-independent I_K. In six of seven neurons tested, these sustained currents were reversibly enhanced (by 74 ± 12%) following high-frequency stimulation of synaptic afferents in the presence of 1 μM eserine, an AChE inhibitor (Cole and Nicoll, 1983; Gähwiler and Brown, 1985) (Fig. 1*C*). Similar high-frequency stimulation did not cause significant change (by 18 ± 21%; n = 8) in I_K in neurons pretreated with 1 μM atropine. Application of 1 μM eserine alone did not cause consistent changes in the sustained outward current. These results suggest that potentiation of I_K by either synaptic stimulation or exogenous application of a cholinergic agonist is mediated by activation of mAChRs.

Generally, the enhancement of I_K started 1–2 min after the onset of carbachol application and persisted for another 5–25 min during washout (Figs. 2, 3*A*). This enhancement of I_K could be observed repeatedly in the same cell, and moreover could be elicited even 70 min after starting the whole-cell recording (Fig. 2). Some desensitization of the carbachol-induced potentiation of I_K was observed following multiple carbachol treatments; however, this desensitization could be reduced by briefly exposing slices (2–3 min) to the normal Ca^{2+}-containing medium during wash intervals (Fig. 2). Since I_K was stable prior to carbachol treatment and the carbachol-induced potentiation of I_K was reproducible during prolonged recordings, these observations suggest a muscarinic modulation of I_K rather than a restoration of a washed-out component of I_K.

To monitor the time course of I_K activation and to inactivate I_A, neurons were clamped near −30 mV to inactivate I_A, and I_K was then evoked by depolarizing voltage steps. The I–V relation of I_K (peak current vs. step voltage) showed a shift toward more negative potentials after application of 50 μM carbachol (Fig. 3*E*). The net potentiated current (Fig. 3*D*) obtained by subtracting the control current from the current during carbachol application showed a similar voltage dependence as the control current (Fig. 3*F*). The activation time constant, τ_n, of I_K was increased during the maximal carbachol-induced potentiation (control value, 41 ± 4 msec; 57 ± 4 msec after 50 μM carbachol; n = 5).

Table 1. Composition of internal solution

Solutions	EGTA/Ca^{2+} (mM)	Tris-GTP (μM)	Mg-ATP (mM)	Others
Standard	1.1/0.1	200	2	
A	1.1/0.1		2	200 μM GTP-γS
B	1.1/0.1		2	200 μM GDP-βS
C	1.1/0.1	200	2	400 μM IP_3
D	1.1/0.1	200	2	3 mg/ml heparin
E		200	2	20 mM BAPTA
F	1.1/0.1	200	2	200 μM H-7 or Ro-31-7549
G	1.1/0.1	200		2 mM K-ATP
H	1.1/0.1	200		2 mM AMP-PNP with or without 2 mM $MgCl_2$
I	1.1/0.1	200		2 mM ATP-γS with 2 mM $MgCl_2$

All internal solutions contained 150 mM K-gluconate and 10 mM HEPES, except for solution E, which contained 100 mM K-gluconate.

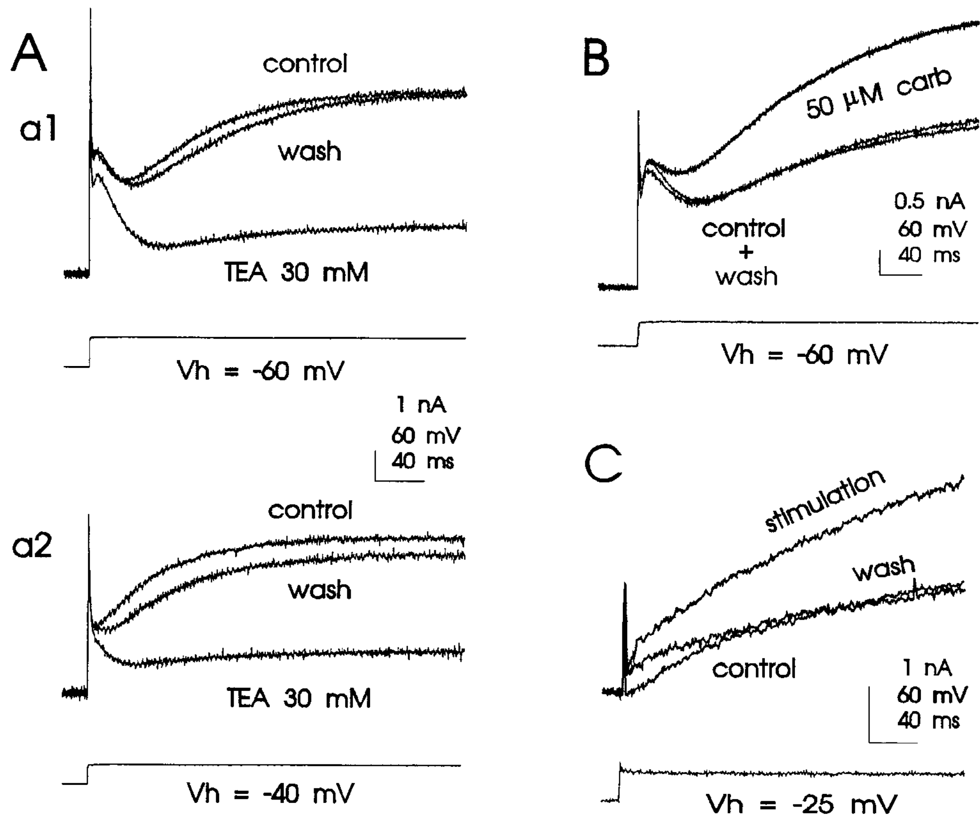

Figure 1. I_K blockade by TEA and potentiation by carbachol (*carb*) or synaptic stimulation. *A*, Outward currents evoked by depolarizing voltage steps from a CA1 neuron perfused with a medium containing 0.5 μM TTX, 5 mM EGTA, and zero added Ca^{2+}. The current traces consisted of a transient outward current (I_A) followed by a sustained component (I_K) at a holding potential (*Vh*) of −60 mV (*a1*), and I_K was the predominant current at a Vh of −40 mV (*a2*). Bath application of 30 mM TEA (4 min) blocked I_K at both holding potentials, but had only minor effects on I_A (*a1*). The TEA blockade vanished after about 15 min of washout. In *B*, currents were evoked from another neuron using the same paradigm as in *a1*. Note a selective increase in I_K magnitude after bath application of 50 μM carbachol. The carbachol-induced enhancement recovered after about 12 min of washout. In *C*, outward currents were evoked from another neuron at a Vh of −25 mV at 33°C, and the neuron was perfused with normal ACSF containing 1 μM eserine. A bipolar electrode was placed in the CA1 striatum radiatum, and a train of current pulses (duration 1 sec, 80 Hz, at submaximal intensity) was generated to stimulate synaptic afferents. The *top trace* represents the maximum enhancement at about 1.5 min after stimulation, and the *bottom trace* was taken 6 min after stimulation. Currents were evoked every 30 sec. Traces in *A* and *B* are averaged records from three to five measurements, and single measurements are illustrated in the traces of *C*.

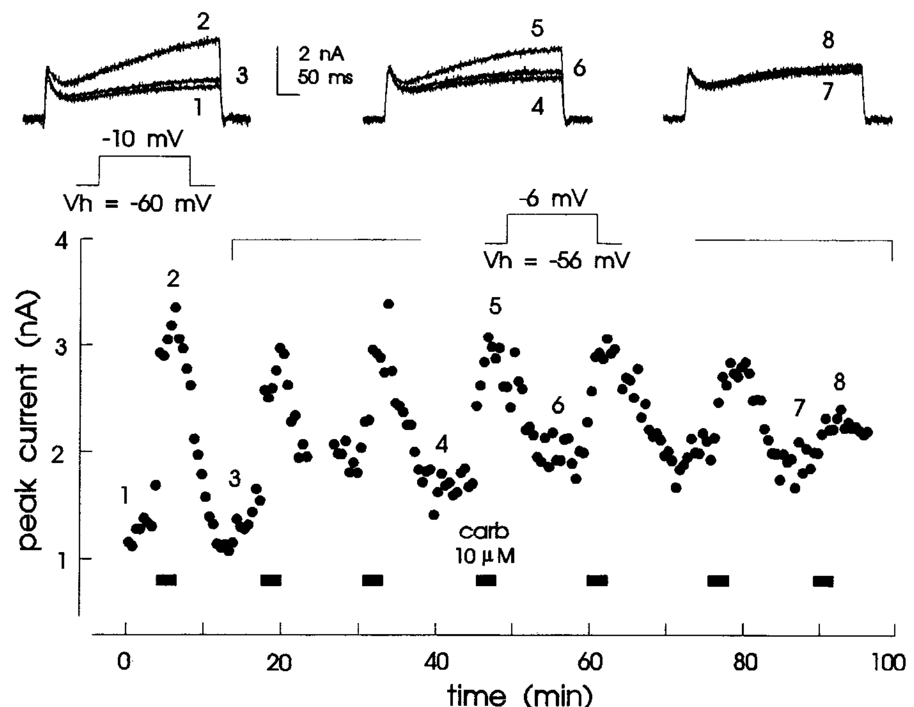

Figure 2. Potentiation of I_K by multiple applications of 10 μM carbachol (*carb*). Peak amplitude of sustained current versus time was plotted in the graph, and the data point at time zero was taken about 3 min after starting the whole-cell recording. Currents were evoked every 30 sec, and *traces above the graph* are averaged records from three or four measurements at the different times indicated. Recording paradigms are shown below the current traces, and Vh was −60 mV in *1–3* and −56 mV in *4–8*. Applications of carbachol (10 μM, 3 min) are indicated by *thick bars*. Recordings were performed in a medium containing 0.5 μM TTX, 2 mM $CoCl_2$, and zero added Ca^{2+}. During each washout of carbachol, the slice was perfused with normal ACSF for 2–3 min. Note the enhancement of the sustained current (I_K-type current) by multiple applications of carbachol, but no significant change in the transient current. The potentiation of I_K by carbachol was observed even after internal dialysis for over 70 min.

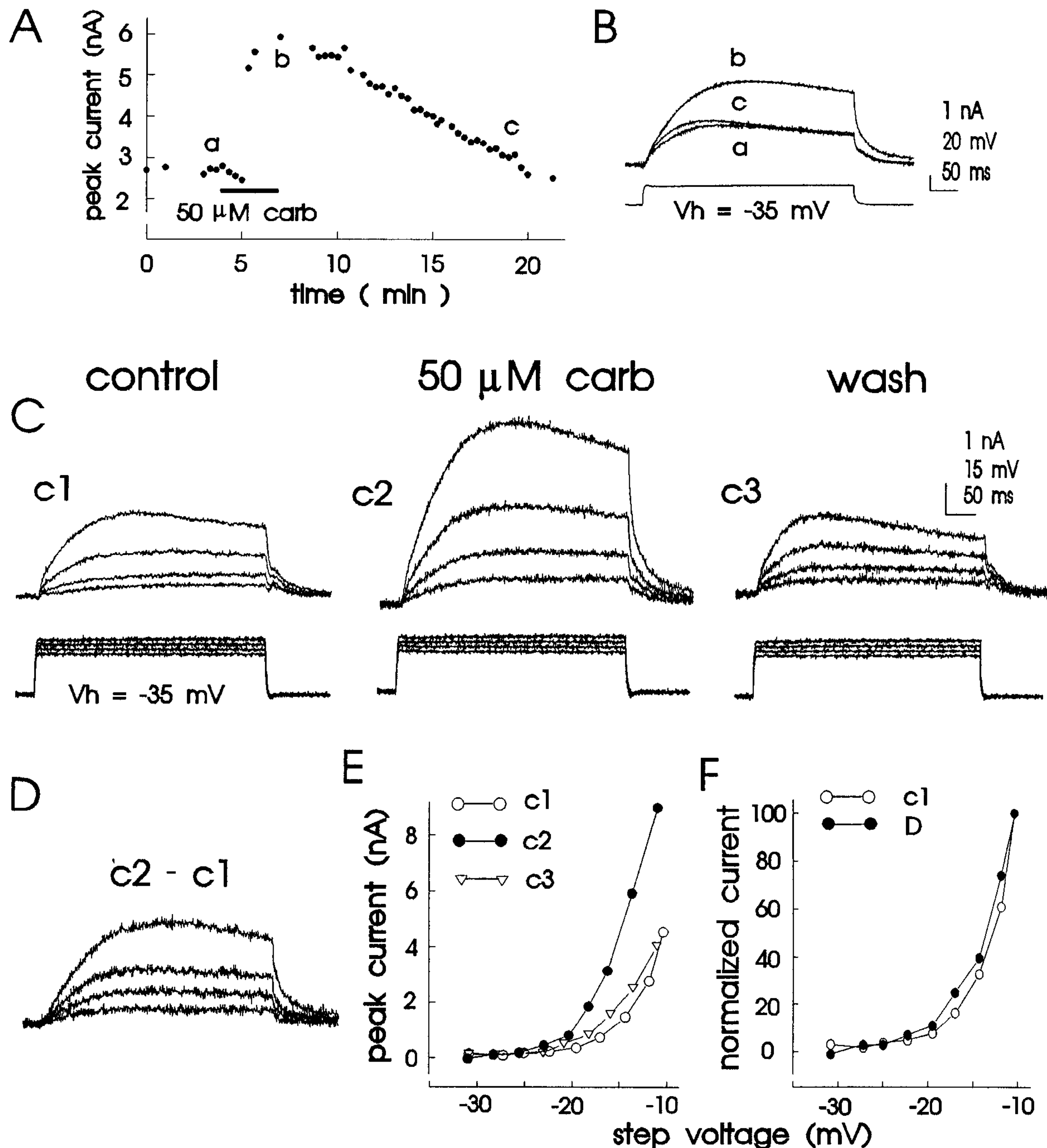

Figure 3. Carbachol-induced potentiation of I_K at Vh of −35 mV. All measurements were obtained from a neuron perfused with a medium containing 0.5 μM TTX, 5 mM EGTA, and zero added Ca^{2+}. In *A*, I_K was evoked by a depolarizing step to −5 mV every 20 sec and peak amplitude versus time was plotted. The data point at time zero was taken about 5 min after starting the whole-cell recording. Application of 50 μM carbachol (*carb*) is indicated by the *bar above the x-axis.* Current traces in *B* show currents recorded at the times indicated. Note the large, prolonged increase in I_K amplitude after carbachol application. Current traces in *C* represent I_K evoked by several depolarizing steps before (*c1*, near *a* indicated in graph *A*), during (*c2*, near *b*), and 15 min after carbachol application (*c3*, near *c*). At a step voltage of −10 mV, the activation time constant of I_K was 51 msec in *c1* and 63 msec in *c2* (*top traces*). Traces in *D* were obtained by subtracting the control currents (*c1*) from the currents recorded during carbachol application (*c2*), showing the net potentiated currents. The relationship of the peak I_K amplitude versus step voltage (*I–V*) for currents in *c1–c3* are plotted in *E*. Note the shift in the *I–V* relation to more negative potentials after carbachol application. In *F*, normalized *I–V* relations for the control currents in *c1* and the net potentiated currents in *D* are shown. The peak current evoked by a depolarizing voltage step of −10 mV was taken as maximal (100%). Note the similar *I–V* relation for the control and net potentiated currents.

In association with the potentiation of I_K, application of carbachol also decreased the outward holding current at holding potentials near −30 mV (by 82 ± 10 pA after application of 50 μM carbachol, from a control value of 244 ± 30 pA; $n = 12$), demonstrating an underlying carbachol-induced inward current. This inward current was also clearly seen when neurons were clamped near the resting potential (111 ± 24 pA, at Vh of −55 to −60 mV; $n = 10$) but was not observed in neurons pretreated with 1 μM atropine ($n = 4$). Interestingly, application of carbachol did not significantly affect the leak conductance measured using 5–10 mV hyperpolarizing voltage steps or a voltage ramp paradigm (−100 to −20 mV, duration of 1–1.4 sec). The

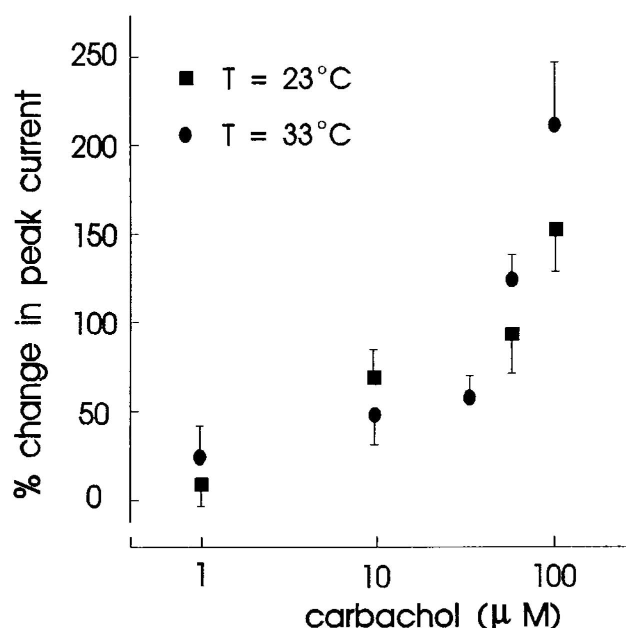

Figure 4. Dose–response relation of the carbachol-induced potentiation of I_K. Carbachol was bath applied for 3–4 min at concentrations of 1, 10, 20, 50, and 100 μM. The maximal I_K potentiation was obtained from neurons recorded at 33°C or at room temperature (23–24°C). Each data point represents mean potentiation ± SEM from 4–14 neurons. The data included represent only neuronal responses to first applications of carbachol.

mean slope conductance measured by the voltage ramp was 9.7 ± 1.7 nS in control and 9.6 ± 1.6 nS after application of 50 μM carbachol ($n = 7$).

The potentiation of I_K by carbachol was dose dependent, with significant potentiation occurring at a concentration of 10 μM at 33°C and 23°C. Overall, there was no significant effect of temperature on the carbachol enhancement of I_K in this study (Fig. 4). When I_K was depressed by bath application of 5 mM TEA (by 57 ± 8%; $n = 7$), application of 50 μM carbachol did not significantly change the peak amplitude of the residual I_K (by −15 ± 9%), but decreased the outward holding current by 84 ± 19 pA, suggesting a TEA blockade of the carbachol-sensitive portion of I_K current rather than a direct blockade of mAChRs by TEA. This observation coupled with the similar *I–V* relations seen in the control and potentiated currents (Fig. 3*F*) supports the idea that the muscarinic potentiation of I_K was due to a change in I_K itself rather than an enhancement of another type of outward K^+ current (see review by Nicoll et al., 1990; Tanaka et al., 1991).

G-protein–linked muscarinic modulation of I_K

Activation of mAChRs stimulates the turnover of inositol phospholipids through GTP-binding protein (G-protein)–dependent pathways (Cockcroft and Gomperts, 1985; Pfaffinger et al., 1988; Lechleiter et al., 1991). If carbachol-induced potentiation of I_K was similarly mediated, internal application of the nonhydrolysable GTP analog GTP-γS should mimic the carbachol-mediated enhancement of I_K and occlude any subsequent potentiation induced by carbachol (Pfaffinger, 1988; Brown et al., 1989; Lopez and Adams, 1989). Conversely, internal application of the GDP analog GDP-βS would be expected to abolish the carbachol-induced potentiation due to a permanent "deactivation" of G-proteins.

Indeed, in neurons recorded with patch pipettes containing 200 μM GTP-γS (Table 1, solution A), there was a progressive increase (by 98 ± 24%; $n = 10$) in I_K amplitude after forming the whole-cell recording that was associated with an increased outward holding current (by 167 ± 30 pA, from a control value of 220 ± 28 pA) and an increased leak conductance (by 9.2 ± 1.8 nS, from a control value of 12.9 ± 1.9 nS) at holding potentials near −30 mV. When I_K reached steady state (usually 10–15 min after starting the whole-cell recording), bath application of 50 μM carbachol caused no further potentiation of I_K in neurons loaded with GTP-γS (Fig. 5*A*).

On the other hand, in neurons recorded with patch pipettes containing 200 μM GDP-βS (Table 1, solution B), a decrease in I_K amplitude by 20 ± 7% was observed following membrane breakthrough ($n = 12$). In addition, application of 50 μM carbachol did not potentiate I_K in these neurons (Fig. 5*B*). Interestingly, in neurons loaded with either GTP-γS or GDP-βS, application of carbachol still caused a significant decrease in the outward holding current (by 117 ± 28 pA). These observations suggest that the activation of G-proteins is likely involved in the carbachol-induced enhancement of I_K, but not in the carbachol-induced decrease in the outward holding current.

Involvement of intracellular Ca^{2+} in I_K *potentiation*

Stimulation of phosphoinositide turnover produces mainly two internal messengers, inositol trisphosphate (IP_3) and diacylglycerol. IP_3 releases Ca^{2+} from internal stores, and diacylglycerol activates PKC in a Ca^{2+}-dependent manner (Streb et al., 1985; Nishizuka, 1986; Huang, 1989; Rana and Hokin, 1990). It has been reported that stimulation of mAChRs causes Ca^{2+} release from intracellular stores (Pfaffinger et al., 1988; Lechleiter et al., 1991) and increases intracellular Ca^{2+} signals (Knöpfel et al., 1990; Müller and Connor, 1991). If raised intracellular Ca^{2+} by IP_3 is one of the factors required for the carbachol-induced I_K potentiation, one would expect that the I_K potentiation would be diminished either by blocking the IP_3-induced Ca^{2+} release or by strongly buffering internal Ca^{2+}.

This idea was tested in the following experiments. First, synthetic IP_3 (400 μM; Research Biochemicals Inc., Natick, MA) was added to the internal solution (Table 1, solution C) in an attempt to mimic the action of carbachol on I_K. However, we observed no significant increase in I_K in neurons internally dialyzed with IP_3 for 10–15 min ($n = 5$). Interestingly, subsequent application of 50 μM carbachol in these neurons produced only a minor increase in I_K amplitude (by 28.6 ± 6.8%) (Fig. 6*A,C,E*), which was significantly smaller than that observed in neurons dialyzed with the standard internal solution (*t* test, $p < 0.05$). These observations suggest that exogenously applied IP_3 alone is not sufficient to produce the enhancement of I_K. However, it seems to be involved in the I_K potentiation, as prolonged stimulation with IP_3 reduced the efficacy of carbachol, possibly by a depletion of IP_3-dependent Ca^{2+} stores.

Second, we examined the effects of heparin on the carbachol-induced I_K potentiation. In neurons dialyzed with internal solution containing heparin (3 mg/ml; Table 1, solution D), no potentiation of I_K was observed after application of 50 μM carbachol (Fig. 6*E*), but the holding current was still significantly decreased (by 105 ± 12 pA; $n = 10$). This blockade of I_K potentiation could be attributed to heparin-mediated antagonism of IP_3 receptors (cf. Bezprozvanny et al., 1991), although heparin may have other effects on intracellular messenger cascades, such

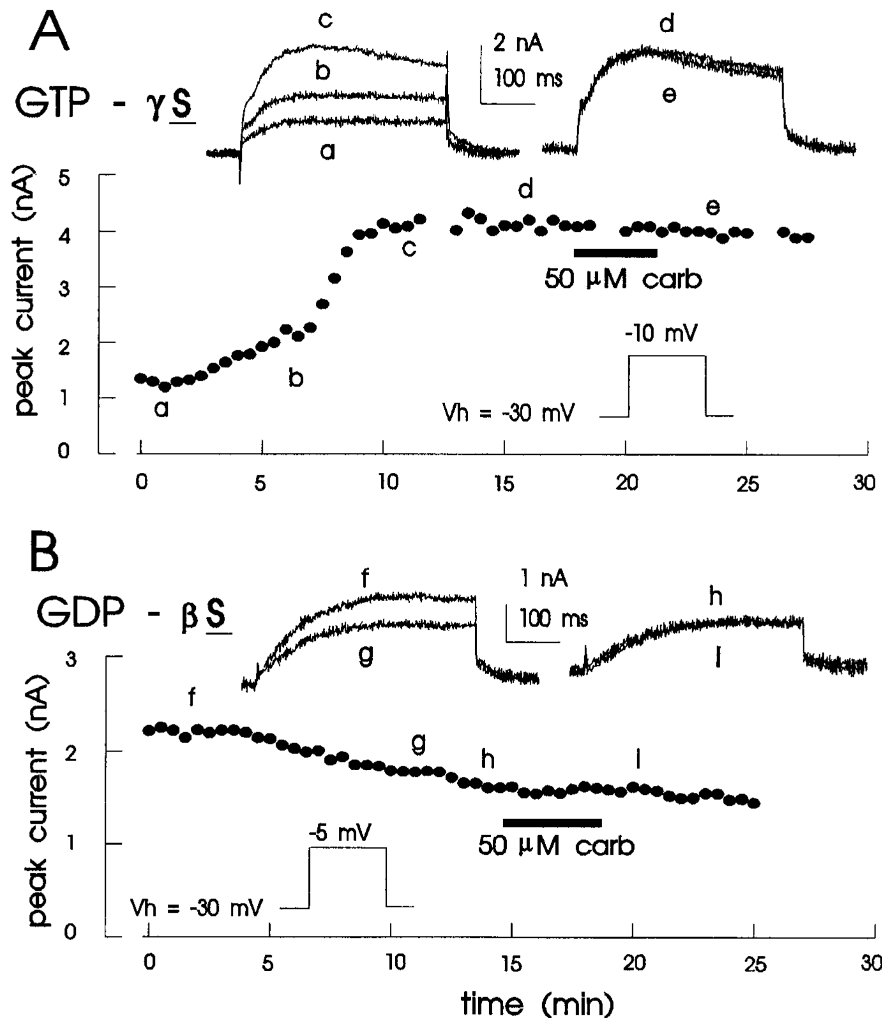

Figure 5. Effects of internal dialysis of GTP-γS or GDP-βS on I_K. Peak amplitude of I_K versus time is plotted in graphs *A* and *B*. The data points at time zero were taken about 2 min after starting the whole-cell recording. In *A*, a CA1 neuron was dialyzed with 200 μM GTP-γS. I_K was evoked every 30 sec by stepping to −10 mV from a Vh of −30 mV. Note a progressive increase in peak amplitude for the first 10 min of dialysis and no further increase in response to 50 μM carbachol (*carb*). *Superimposed traces above* show I_K recorded at the different times indicated in the graph. In *B*, another CA1 neuron was dialyzed with 200 μM GDP-βS. Note a steady decrease in I_K peak amplitude with time, and no potentiation of I_K after carbachol application.

as uncoupling mAChRs from G-proteins as suggested in heart cells (Ito et al., 1990).

We further examined the role of intracellular Ca^{2+} in the carbachol-induced potentiation of I_K using BAPTA, a fast Ca^{2+} chelator (Tsien, 1980). In neurons recorded with patch pipettes containing 20 mM BAPTA (Table 1, solution E), there was no potentiation of I_K after bath application of 50 μM carbachol, but the outward holding current was still decreased (by 122 ± 27 pA; $n = 15$) (Fig. 6*B,D,E*).

PKC activation-mediated I_K *potentiation*

Hippocampal pyramidal neurons have high PKC immunoreactivity (Huang, 1989). It has been suggested that stimulation of PKC may mediate a blockade of the I_{AHP} by muscarinic (Baraban et al., 1985; Malenka et al., 1986) or glutamatergic stimulation (Baskys et al., 1990) as well as modulate other K^+ currents (Doerner et al., 1988). We examined whether activation of Ca^{2+}-dependent kinases may be the additional factor involved in the carbachol-induced I_K potentiation. In neurons recorded with patch pipettes containing the standard internal solution, bath application of phorbol 12,13-dibutyrate (PDBu; 10 μM for 3–5 min), an activator of PKC (Madison et al., 1987; Huang, 1989), but not the inactive phorbol 12,13-didecanoate ($n = 10$) or 4α-phorbol 12,13-dibutyrate (10 μM; LC Services Corp., Woburn, MA) ($n = 4$), caused an increase in I_K amplitude in 6 of 10 neurons (by 132 ± 44%) (Fig. 7*A*). Moreover, no significant potentiation of I_K was observed after subsequent application of 50 μM carbachol (Fig. 7*A*). In four neurons dialyzed with 20 mM BAPTA (Table 1, solution E), an increase in I_K amplitude (by 15–160%) was still observed after application of 10 μM PDBu. These results are consistent with a previous finding that application of PDBu potentiates an I_K-type current in cardiac ventricular cells (Walsh and Kass, 1991).

In addition, in neurons dialyzed with the 200 μM H-7 (Table 1, solution F), a nonspecific kinase inhibitor (Malinow et al., 1989), there was no increase in I_K amplitude after application of 50 μM carbachol (by 4 ± 9%; $n = 5$). We also examined the effect of Ro-31-7549, a newly developed selective PKC inhibitor (Davis et al., 1989; kindly provided by Roche Products Limited, Welwyn, Garden City, UK). In 10 neurons dialyzed with 200 μM Ro-31-7549 (Table 1, solution F), bath application of 50 μM carbachol caused no significant increase in I_K amplitude, but a significant decrease in outward holding current (by 69 ± 8 pA). Bath application of 3 μM Ro-31-7549 for at least 10 min also prevented I_K potentiation induced by 50 μM carbachol ($n = 6$) (cf. Davis et al., 1989). Some I_K potentiation by carbachol could however be observed in Ro-31-7549–pretreated neurons following a prolonged washout (Fig. 7*B*).

Phosphorylation-dependent muscarinic potentiation of I_K

It is known that an I_K-type channel protein in mammalian skeletal muscle and brain contains several phosphorylation sites

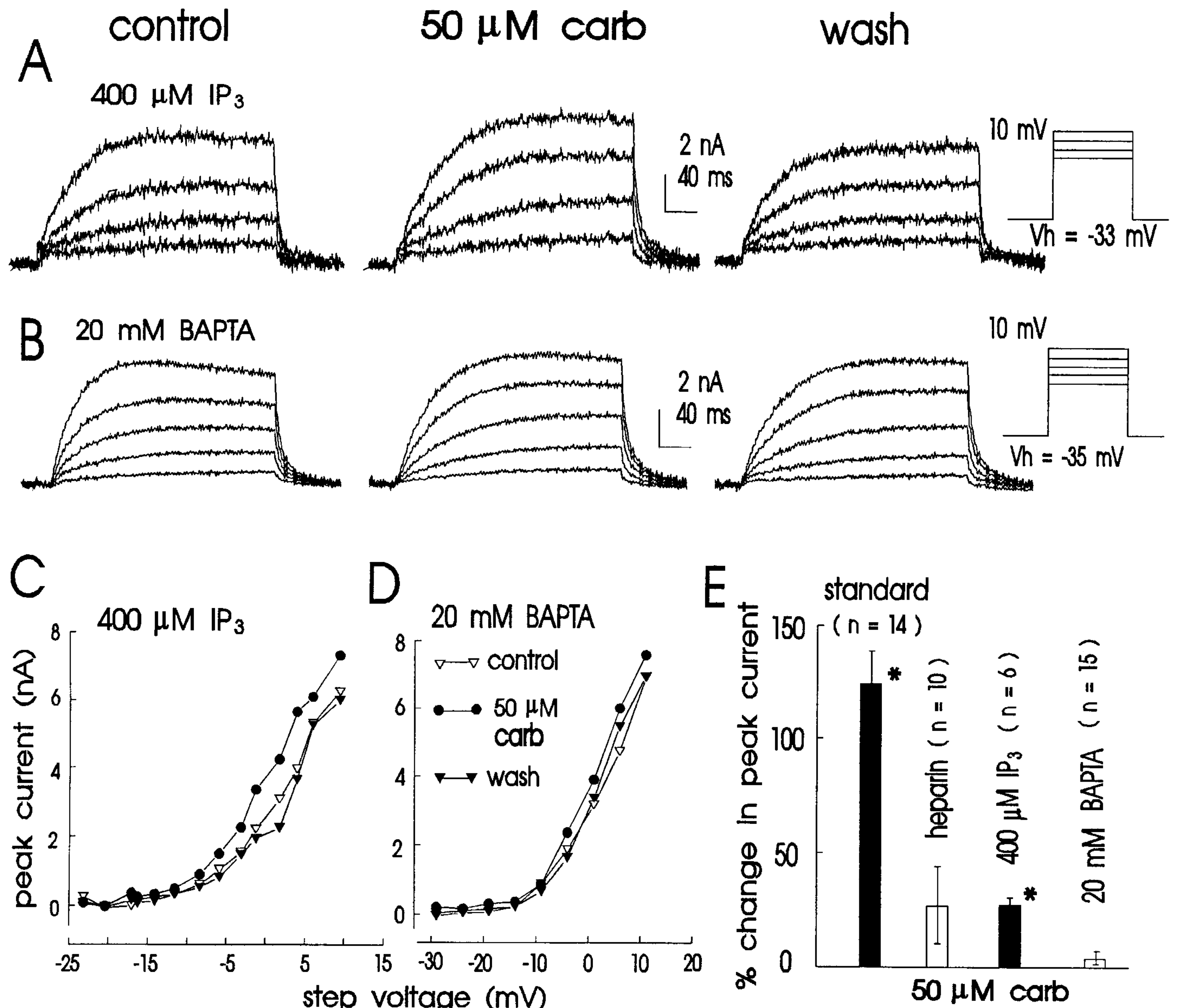

Figure 6. Internal dialysis with 400 μM IP_3 (*A*) or 20 mM BAPTA (*B*) attenuated the carbachol-induced I_K potentiation. In both *A* and *B*, controls were taken after internal dialysis for 11 and 15 min, respectively. Note only a slight increase in I_K amplitude in *A* and *B* after carbachol (*carb*) application (50 μM, for 3 min). The corresponding *I–V* relations during control, carbachol application and washout are plotted in *C* and *D*, respectively. In *E*, a bar graph summarizes effects of 50 μM carbachol on neurons dialyzed with different internal solutions. There is a significant percentage increase (*, Student's *t* test, two tailed, $p < 0.05$) in I_K amplitude in neurons loaded with the standard internal solution or IP_3-containing solution, but not in those loaded with either 20 mM BAPTA or heparin. However, the potentiation of I_K in neurons dialyzed with IP_3 is significantly smaller than that seen in neurons loaded with the standard internal solution ($p < 0.05$).

(Rehm and Tempel, 1991) and that Mg-ATP- and kinase-dependent phosphorylation leads to an enhancement of an I_K-type current in squid axons (Augustine and Bezanilla, 1990). To test the role of phosphorylation in the muscarinic potentiation of I_K, neurons were dialyzed with internal solutions without Mg-ATP or with the ATP analogs AMP-PNP or ATP-γS (Table 1), and the effect of carbachol on I_K was then examined. It is generally believed that AMP-PNP provides a phosphate that is not available for kinases and that ATP-γS irreversibly thiophosphorylates substrates (Yount, 1975). In neurons dialyzed with the solution containing zero added Mg^{2+} (Table 1, solution G), I_K currents were stable, but the carbachol-induced potentiation of I_K (50 μM, 3–4 min) was quite variable, being observed in only 6 of 11 neurons tested, with a mean increase of 49 ± 26%. In neurons dialyzed with internal solution containing 2 mM AMP-PNP with or without 2 mM $MgCl_2$ ($n = 4$ for each group) (Table 1, solution H), I_K showed a consistent decrease in amplitude with time (Fig. 8). The peak I_K amplitude was reduced by 49 ± 10% in about 5–7 min after forming the whole-cell recording, from an initial value of 3.9 ± 0.5 nA ($n = 8$). This decrease in I_K amplitude was not associated with any significant change in leak conductance or holding current. Once I_K reached a depressed steady state, application of carbachol (50 μM for 3–5 min) caused no potentiation, but rather a small depression (by −23 ± 5%; $n = 7$) in I_K amplitude (Fig. 8).

In contrast, in five neurons dialyzed with an internal solution

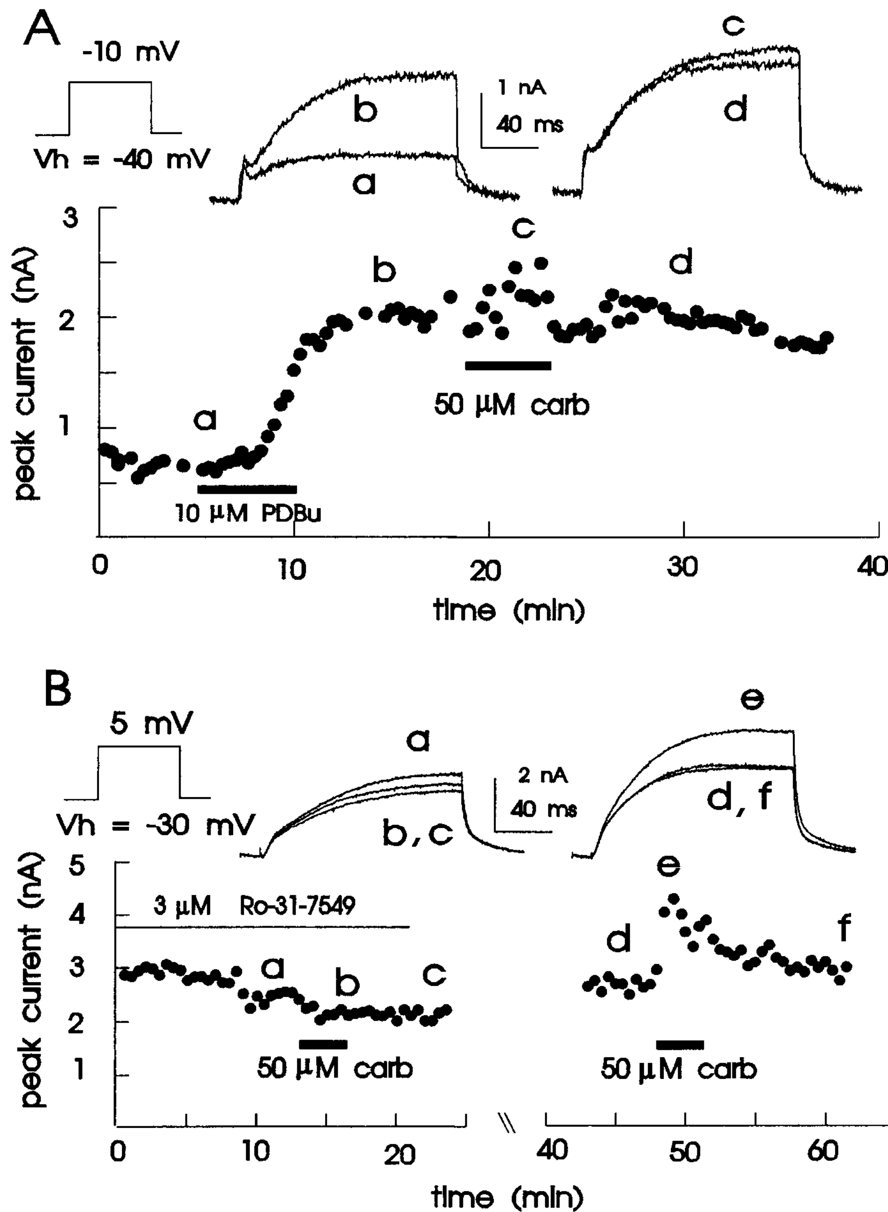

Figure 7. PKC-mediated I_K potentiation. *A*, Bath application of 10 μM PDBu potentiated I_K in a CA1 neuron loaded with the standard internal solution. I_K was evoked repetitively every 20 sec. Peak current amplitude versus time is plotted in the *graph below.* The current at zero time was evoked immediately after forming the whole-cell clamp configuration. *Insets above* show individual currents evoked at the different times indicated in the graph. Note the large increase in current amplitude after application of 10 μM PDBu and only a minor increase following subsequent application of 50 μM carbachol (*carb*). *B*, Bath application of 3 μM Ro-31-7549 was started 5 min before forming the whole-cell clamp configuration and was continued for another 20 min as indicated in the graph. The currents were evoked every 30 sec. Note that there was no potentiation of I_K by 50 μM carbachol in the presence of Ro-31-7549, but after washout of Ro-31-7549 for about 25 min, I_K amplitude could be potentiated by 50 μM carbachol.

containing ATP-γS (Table 1, solution I), I_K currents showed a time-dependent increase in amplitude by 185 ± 55%, in association with an increase in leak conductance and outward holding current. Application of 50 μM carbachol did not cause further potentiation of I_K in two neurons tested. Since both AMP-PNP and ATP-γS are tetralithium salts, we tested the effects of adding 8 mM LiCl to the standard internal solution. In six neurons dialyzed with the Li^+-containing solution for 10 min, no change in I_K, holding current, or leak conductance was observed.

Discussion

It was originally proposed by Krnjević et al. (1971) that ACh (muscarine) acts as a modulatory neurotransmitter in mammalian central neurons. Multiple postsynaptic actions of mAChR stimulation have been reported in hippocampal neurons, including a depression of voltage-activated K^+ currents (such as I_M, I_A, and I_{AHP}; Benardo and Prince, 1982; Halliwell and Adams, 1982; Cole and Nicoll, 1983; Nakajima et al., 1986; Storm, 1989), an enhancement of the response to NMDA (Markram and Segal, 1990), facilitation of the induction of long-term potentiation (Blitzer et al., 1990; Burgard and Sarvey, 1990), and an induction of slow rhythmical activity (MacVicar and Tse, 1989). These multiple actions likely involve different mAChR subtypes and underlying internal second messenger systems (cf. Nicoll et al., 1990). For example, the muscarinic blockade of the I_{AHP} is independent of changes in intracellular Ca^{2+} signals (Knöpfel et al., 1990; Müller and Connor, 1990), but can be mimicked by stimulating PKC with phorbol esters (Baraban et al., 1985; Malenka et al., 1986). On the other hand, application of phorbol esters fails to block I_M, but intracellular injection of IP_3 inhibits this current (Dutar and Nicoll, 1988).

The present study demonstrates that stimulation of mAChRs can also potentiate a voltage-activated I_K-type current. We have shown, through the use of GTP-γS and GDP-βS, that a G-protein(s) couples the activation of mAChRs to the potentiation of I_K. Since internal application of BAPTA prevents the carbachol-

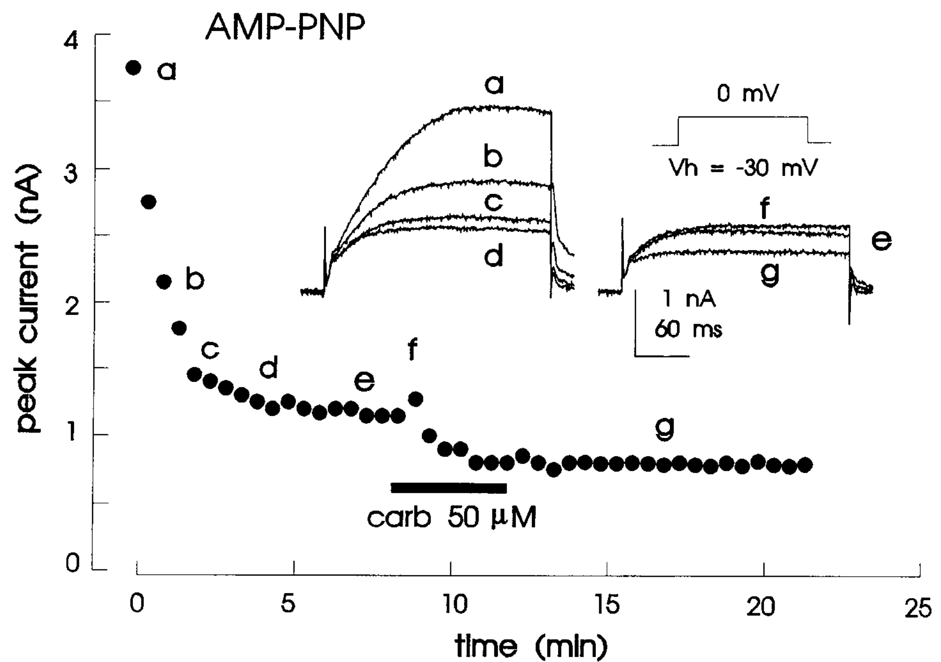

Figure 8. Depression of I_K by internal dialysis of 2 mM AMP-PNP (with 2 mM $MgCl_2$). I_K currents were evoked every 30 sec and peak current versus time was plotted. The current at time zero was evoked about 2 min after breaking through the membrane. Inset traces were taken at the different times indicated. The recording paradigm is illustrated above the current trace. Note the decrease of I_K with time and no substantial potentiation after application of carbachol.

induced potentiation of I_K, but does not fully block the enhancement of I_K by the active phorbol ester PDBu, we favor the idea that a rise in intracellular Ca^{2+} after stimulation of mAChRs plays a role in the potentiation of I_K. Our data suggest, albeit not definitively, that IP_3-dependent Ca^{2+} release is likely involved in this potentiation. Furthermore, this potentiation can be mimicked by directly stimulating PKC with PDBu and is blocked by PKC inhibitors. Given existing biochemical evidence that several mAChR subtypes are coupled to phosphoinositide hydrolysis, and that a rise in intracellular Ca^{2+} and PKC activation is the natural consequence of phosphoinositide turnover in many preparations (Huang, 1989; Rana and Hokin, 1990), our data suggest a functional role for activation of this pathway in mammalian CNS neurons. A similar pathway may also be involved in the muscarinic generation of hippocampal rhythmic activity (Tse and MacVicar, 1989), and may play a role in mAChR-mediated enhancement of NMDA responses (Markram and Segal, 1990), thereby facilitating long-term potentiation (Blitzer et al., 1990; Burgard and Sarvey, 1990).

Muscarinic stimulation has also been reported to potentiate other types of voltage-activated K^+ currents, such as a transient I_A in neostriatal neurons (Akins et al., 1990) and a sustained K^+ current in sympathetic ganglion neurons (Pfaffinger, 1988). In sympathetic ganglion neurons, I_M shows an initial depression and a delayed enhancement after muscarinic stimulation. The delayed enhancement of I_M requires internal Ca^{2+} (Marrion et al., 1991), suggesting mediation by second messenger mechanisms.

Generally, Mg^{2+} and ATP are required for phosphorylation-dependent processes (cf. Augustine and Bezanilla, 1990). In the present experiments, intracellular dialysis with zero added Mg^{2+} or AMP-PNP, a nonhydrolyzable ATP analog whose phosphate is not available for kinases (Yount, 1975), caused an inconsistency or failure of carbachol-induced I_K potentiation. In addition, in the presence of AMP-PNP, I_K showed a time-dependent decrease in amplitude. In contrast, intracellular dialysis with ATP-γS, an ATP analog that irreversibly thiophosphorylates substrates (Yount, 1975), potentiated I_K, and occluded further potentiation by carbachol. These results favor the idea that phosphorylation-dependent processes are involved in the maintenance of I_K as well as the potentiation of this current by muscarinic receptor stimulation.

It should, however, be noted that depletion of intracellular Mg^{2+} may affect not only phosphorylation, but also other steps in internal messenger cascades. For example, in heart cells, lowering internal Mg^{2+} weakens the coupling of G-proteins to muscarinic K^+ channels (Horie and Irisawa, 1989). In addition, since I_K was not stable in the presence of AMP-PNP, the failure of carbachol to enhance I_K under these recording conditions cannot be taken as conclusive evidence for a role of phosphorylation in the muscarinic potentiation of I_K. There are also reports that ATP-γS can mimic GTP-γS to activate cardiac muscarinic K^+ channels via a conversion of ATP-γS to GTP-γS (Otero et al., 1988), and that ATP-γS reduces neuronal L-type Ca^{2+} current via a kinase-independent, pertussis toxin–sensitive process (Gross et al., 1990). In the present study, the effects of ATP-γS, that is, enhancement of I_K in association with an increase in outward holding current and leak conductance, were very similar to those induced by internal application of 200 μM GTP-γS. It is possible that direct stimulation of G-proteins by ATP-γS may have been at least partly responsible for the increase in I_K.

The properties of the carbachol-induced inward current are not defined in the present experiments. Muscarine-induced membrane depolarization or inward current has been reported previously and has been attributed to a blockade of I_M and a muscarine-sensitive K^+ leak conductance (Halliwell and Adams, 1982; Gähwiler and Brown, 1985; Madison et al., 1987). It has also been reported that replacement of extracellular Ca^{2+} with Mn^{2+} diminishes an initial, but not sustained muscarinic depolarization (Pitler et al., 1988). Considering the fact that I_M (and possibly the muscarine-sensitive leak conductance) runs down in the whole-cell configuration, and that recordings were performed in a medium without added Ca^{2+} and with Co^{2+} or EGTA, it is possible that the carbachol-induced inward current

might be generated at electrotonically remote dendrites, and therefore the associated conductance change would hardly be detected from the somatic recording site (Knöpfel et al., 1990).

Carbachol may have improved the space clamp through muscarinic blockade of other K^+ currents (see above). This may have resulted in some enhancement of I_K. However, no change in leak conductance was observed following carbachol application in the present experiments. In addition, I_K was potentiated in neurons treated with GTP-γS and an active phorbol ester in association with an increase in leak conductance. In the presence of internal BAPTA or heparin, application of carbachol did not potentiate I_K, but still induced the inward current. Thus, although an inward current is associated with mAChR stimulation, this inward current cannot be a major contributor to the potentiation of I_K.

The facts that I_K could be enhanced by repetitive stimulation of synaptic afferents, and that this potentiation was blocked by atropine, suggest a functional role for muscarinic activation of I_K. Generally, I_K plays a minor role in the repolarization of the action potential owing to its slow activation. However, if I_K was signicantly enhanced, it may then make a greater contribution to neuronal repolarization (cf. Storm, 1990), particularly during sustained depolarizations in which other transient, voltage-activated K^+ currents are inactivated such as those associated with seizure activity (review by Lothman et al., 1990) and hypoxia (Leblond and Krnjević, 1990).

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