Serine/Threonine Protein Phosphatases and Synaptic Inhibition Regulate the Expression of Cholinergic-Dependent Plateau Potentials

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Fraser, Douglas D., Daniel Doll, and Brian A. MacVicar. Serine/threonine protein phosphatases and synaptic inhibition regulate the expression of cholinergic-dependent plateau potentials. J Neurophysiol 85: 1197–1205, 2001. We previously identified cholinergic-dependent plateau potentials (PPs) in CA1 pyramidal neurons that were intrinsically generated by interplay between voltage-gated calcium entry and a Ca$^{2+}$-activated nonselective cation conductance. In the present study, we examined both the second-messenger pathway and the role of synaptic inhibition in the expression of PPs. The stimulation of m1/m3 cholinergic receptor subtypes and G-proteins were critical for activating PPs because selective receptor antagonists (pirenzepine, hexahydro-sila-difenidol hydrochloride, 4-diphenylacetyl-N-methylpiperidine methiodide) and intracellular guanosine-5'-O-(2-thiodiphosphate) prevented PP generation in carbachol. Intense synaptic stimulation occasionally activated PPs in the presence of oxytremorine M, a cholinergic agonist with preference for m1/m3 receptors. PPs were consistently activated by synaptic stimulation only when oxytremorine M was combined with antagonists at both GABA$\,\alpha$ and GABA$\,\beta$ receptors. These latter data indicate an important role for synaptic inhibition in preventing PP generation. Both intrinsically generated and synaptically activated PPs could not be elicited following inhibition of serine/threonine protein phosphatases by calyculin A, okadaic acid, or microcystin-L, suggesting that muscarinic-induced dephosphorylation is necessary for PP generation. PP genesis was also inhibited following irreversible thiophosphorylation by intracellular perfusion with ATP-$\gamma$S. These data indicate that the expression of cholinergic-dependent PPs requires protein phosphatase-induced dephosphorylation via G-protein–linked m1/m3 receptor(s). Moreover, synaptic inhibition via both GABA$\,\alpha$ and GABA$\,\beta$ receptors normally prevents the synaptic activation of PPs. Understanding the regulation of PPs should provide clues to the role of this regenerative potential in both normal activity and pathophysiological processes such as epilepsy.

INTRODUCTION

The cholinergic system has been implicated in pathological activities such as epileptogenesis (Lothman et al. 1991; Wasterlain et al. 1993). Elevations in endogenous acetylcholine are associated with seizure onset (Mizuno and Kimura 1996) and cholinergic agonists facilitate epileptogenesis in kindled animals (Buterbaugh et al. 1986). In tissue slice preparations, cholinergic receptor activation induces prolonged depolarizations (Bianchi and Wong 1994) and initiates ictal depolarizations (Nagao et al. 1996; Yaari and Jensen 1989). We have previously identified a novel plateau potential (PP) in hippocampal CA1 pyramidal neurons that has characteristics similar to ictal depolarizations (Fraser and MacVicar 1996a). This regenerative PP was observed in the presence of cholinergic (Haj-Dahmane and Andrade 1998; Klink and Alonso 1997) or metabotropic glutamate (Raggenbass et al. 1997; Svirskis and Hounsgaard 1998) agonists and requires interplay between calcium entry through high-voltage–activated (HVA) Ca$^{2+}$ channels and a Ca$^{2+}$-activated nonselective cation conductance (Congar et al. 1997; Crepel et al. 1994; Fraser and MacVicar 1996a). Direct enhancement of the Ca$^{2+}$-activated nonselective cation conductance by muscarinic receptor stimulation was suggested to underlie PP expression; however, neither the muscarinic receptor subtype(s) nor the signal transduction pathway have been identified. Elucidating both the ionic mechanisms and the second-messenger pathways underlying PPs could be essential for determining the role of this PP in hippocampal epileptogenesis.

Muscarinic receptor activation modulates several voltage- and ligand-gated ion channels (Halliwell 1990; Krmjevic 1993; McCormick 1993) through a number of second-messenger pathways. These include the phosphoinositide cascade (Nicoll et al. 1990), protein kinase C (PKC) (Cantrell et al. 1996; Figenschou et al. 1996; Marsh et al. 1995; Toselli and Lux 1989; Zhang et al. 1992), Ca$^{2+}$-calmodulin kinase II (CaMKII) (Muller et al. 1992; Pedarzani and Storm 1996), tyrosine kinase (Huang et al. 1993), and serine/threonine protein phosphatases (Herzig et al. 1995; Krause and Pedarzani 2000). Protein kinases catalyze the transfer of a phosphate from ATP to the side chain of an amino acyl residue, resulting in structural changes of the target protein (i.e., ion channels) (Hemmings et al. 1989). The degree of ion channel phosphorylation, however, depends not only on protein kinase activity, but also on protein phosphatases that catalyze dephosphorylation. Indeed, recent studies have demonstrated that the phosphorylation of ion channels results from the dynamic equilibrium between kinase and phosphatase activity (Bielefeldt and Jackson 1994; Pedarzani et al. 1998; Wang and Salter 1994; Wilson et al. 1998).

In the present study, we employed whole cell patch-clamp techniques in the hippocampal slice preparation to investigate...
Intracellular Ca$^{2+}$ deflection. The action potential threshold (AP TH) was measured as the membrane potential at the base of the action potential, whereas action potential amplitude (AP AMP) was measured as the voltage difference between the threshold and peak amplitude. Action potential duration (AP DUR) was measured at the threshold.

### METHODS

#### Hippocampal slice preparation and whole cell patch-clamp recording

These techniques have been described previously (Fraser and MacVicar 1996a). Sprague-Dawley rats, postnatal day 15–23, were decapitated and the brains immersed in chilled artificial cerebrospinal fluid containing (ACSF; in mM): 126 NaCl, 2.5 KCl, 2 MgCl$_2$, 2 CaCl$_2$, 1.25 Na$_3$HPO$_4$, 26 NaHCO$_3$, and 10 d-glucose; pH 7.3. The hippocampi were isolated, sectioned perpendicularly to their septotemporal axis (150–400 μM), and incubated in ACSF oxygenated with 5% CO$_2$/95% O$_2$ at room temperature.

Hippocampal slices were individually transferred to a recording chamber located on an upright microscope (Standard 14; Zeiss, Thornwood, NY) and submerged in rapidly flowing (1 ml/min) oxygenated ACSF (34–35°C). Patch electrodes (5–7 MΩ) were made from 1.5-mm OD thin-walled glass (150F-4, World Precision Instruments) and included EGTA, 0.1 CaCl$_2$, 10 HEPES, 2 Mg-ATP, and 0.3 Na-GTP, pH 7.2.

Intracellular Ca$^{2+}$ concentration was calculated to be 16 nM. Voltage recordings were obtained in bridge mode (Axoclamp-2A; Axon Instruments) and were low-pass filtered (4-pole Bessel) at 10 kHz (~3 dB). Capacitance neutralization was fully maximized, and series resistance was determined via a bridge circuit potentiometer by balancing the voltage drop across the patch in response to a negative current step (~30 pA; 10 ms). Data were digitized via a TI-1 A/D interface (Axon Instruments) and analyzed using computer software (pCLAMP or Axotape). All data are presented as means ± SE. To determine statistical significance, data groups were prescreened for normality (Kolmogorov-Smirnov) and compared using a Student’s paired t-test (SigmaStat, Jandel Scientific).

#### Chemicals

All salts were purchased from Fisher (Fair Lawn, NJ), Sigma (St. Louis, MO), or BDH (Toronto, ON). Carbachol (C-4832; Sigma), oxytremorine M (O-1140; Sigma), atropine (A-0257; Sigma), pirenzipine (P-114; RBI; Natick, MA), or BDH (Toronto, ON). Carbachol (C-4832; Sigma), and microcystin-LR were dissolved directly into the patch pipette solution and added to the ACSF from concentrated stocks. Also added to the patch pipette solution, but first dissolved in DMSO, were hexahydro-sila-difenidol (H-127; RBI), calyculin A (C-3987; LC Laboratories; Woburn, MA), and okadaic acid (O-2220; LC Laboratories). The final concentration of DMSO was always ≤0.1%; in control experiments, DMSO at these concentrations did not alter the cholinergic-dependent PP. Second-messenger inhibitors and analogues that were dissolved directly into the patch pipette solution included GDP-β-S (G-7637; Sigma), H-7 dihydrochloride (371955; Calbiochem; La Jolla, CA), ADP-β-S (A-8016; Sigma), 2,4-dinitrophenol (β-7004; Sigma), alkaline phosphatase (P-1153; Sigma), ATP-γ-S (A-1388; Sigma), and PP2B 476-501 (C-6481; LC Laboratories). Also added to the patch pipette solution, but first dissolved in DMSO (~0.1%), was microcystin-LR (M-173; RBI).

During all pharmacological manipulations, control experiments were alternated with drug experiments to ensure the presence of cholinergic-dependent PPs in untreated matched slices. In addition, it was imperative that the protein phosphatase inhibitors okadaic acid and microcystin-LR were made fresh immediately before use, as these compounds demonstrated reduced inhibitory activity when taken from frozen concentrated stocks.

### RESULTS

The results in this paper were obtained from 207 CA1 pyramidal neurons in the hippocampal slice preparation. The whole cell patch-clamp method was used since this technique allows for both long-duration recordings and intracellular perfusion of second-messenger inhibitors and analogues. The average access resistance obtained in control neurons was 15.3 ± 0.4 MΩ (mean ± SE, range 7–20; n = 110); recordings with series resistance >20 MΩ were discarded. The resting membrane potential, input resistance, membrane time constant, and action potential characteristics of the control cells were similar to our previous reports (Table 1) (Fraser and MacVicar 1996a).

#### Concentration dependence of the cholinergic-dependent PP

We investigated the activation of PPs in varying carbachol concentrations. In all neurons tested under control conditions, depolarizing current injection (≥0.1 nA; 0.8 s) evoked repetitive action potential firing (Fig. 1A; n = 225), and afterward the membrane potential immediately returned to the prestimulation baseline. We tested the actions of different concentrations of carbachol, a nonhydrolyzable cholinergic agonist, on the afterpotentials following current-evoked action potential firing. Bath application at each dose lasted for >5 min. In 0.1 μM carbachol, as in control ACSF, the membrane potential returned to prestimulation level following cessation of the current-evoked action potentials (Fig. 1B; n = 6). However, in 0.3 μM carbachol, a slow afterdepolarization (sADP) of 4 ± 1

### Table 1. Membrane properties of CA1 pyramidal neurons following various pharmacological manipulations that abolished the cholinergic-dependent PP

<table>
<thead>
<tr>
<th></th>
<th>$R_{in}$, MΩ</th>
<th>$V_{th}$, mV</th>
<th>$V_{dur}$, ms</th>
<th>$\tau$, ms</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$-65 ± 0.3$</td>
<td>$146 ± 4$</td>
<td>$94 ± 1$</td>
<td>$-46 ± 0.4$</td>
<td>$1.6 ± 0.02$</td>
</tr>
<tr>
<td>GDP-β-S</td>
<td>$-58 ± 1.2$</td>
<td>$136 ± 10$</td>
<td>$101 ± 1$</td>
<td>$-40 ± 0.9$</td>
<td>$1.9 ± 0.07$</td>
</tr>
<tr>
<td>ATP-γ-S</td>
<td>$-65 ± 0.7$</td>
<td>$116 ± 11$</td>
<td>$93 ± 1$</td>
<td>$-47 ± 2.2$</td>
<td>$2.1 ± 0.09$</td>
</tr>
<tr>
<td>cal A</td>
<td>$-58 ± 1.5$</td>
<td>$152 ± 5$</td>
<td>$96 ± 2$</td>
<td>$-45 ± 1.8$</td>
<td>$1.9 ± 0.09$</td>
</tr>
<tr>
<td>OA</td>
<td>$-58 ± 1.7$</td>
<td>$143 ± 12$</td>
<td>$95 ± 2$</td>
<td>$-44 ± 1.2$</td>
<td>$1.8 ± 0.07$</td>
</tr>
<tr>
<td>m-LR</td>
<td>$-59 ± 3.5$</td>
<td>$137 ± 6$</td>
<td>$83 ± 6$</td>
<td>$-43 ± 1.7$</td>
<td>$1.7 ± 0.08$</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n$ is number of neurons. Input resistance ($R_{in}$) was calculated from a steady-state potential response, free of membrane rectification, to a hyperpolarizing current step. The membrane time constant ($\tau$) was calculated as the time necessary to reach $1 - e^{-1}$ (63%) of the maximum voltage deflection. The action potential threshold ($V_{th}$) was measured as the membrane potential at the base of the action potential, whereas action potential amplitude ($AP_{AMP}$) was measured as the voltage difference between the threshold and peak amplitude. Action potential duration ($AP_{DUR}$) was measured at the threshold potential, $rp$, resting membrane potential; cal A, calyculin A; OA, okadaic acid; m-LR, microcystin-LR.
Effects of muscarinic receptor antagonists

As reported previously, the PPs were abolished following co-application of 1 μM atropine with 20 μM carbachol; although a small sADP was still elicited (2 ± 1 mV; 2.9 ± 0.5 s; n = 5/5) (Fraser and MacVicar 1996a). Since atropine is a nonselective antagonist of all muscarinic receptors, we tested the ability of relatively selective muscarinic receptor antagonists to suppress the PP elicited in the presence of 20 μM carbachol (Fig. 2). Coapplication of either 1 μM pirenzepine (n = 3), an antagonist with greater affinity for m1 over m3 receptors (Dorje et al. 1991), or 1 μM 4-diphenylacetoxy-N-methylpiperidine methiodide (n = 3; 4-DAMP), an antagonist with equal affinity to both m1 and m3 receptors (Michel et al. 1989; Thomas et al. 1992), abolished cholinergic-dependent PPs. Coapplication of 1 μM hexahydro-sila-difenidol hydrochloride (HHSiD; p-fluoro analog), an antagonist with greater affinity for m3 over m1 receptors (Lambrecht et al. 1989), also inhibited cholinergic-dependent PPs (n = 3/4; data not shown). These data implicate m1 and/or m3 receptors in cholinergic-dependent PP genesis.

G-protein involvement in the cholinergic-dependent PP

Muscarinic receptors are coupled to effector systems via G-proteins (Branl et al. 1993), and some studies have shown that G-proteins are involved in activation of hippocampal current (Crepe et al. 1994). However, other recent studies have excluded G-protein involvement in the muscarinic activation of a nonselective cation conductance in cultured neurons (Brown et al. 1993; Guerineau et al. 1995). To determine whether G-proteins are involved in the generation of the cholinergic-dependent PP, we substituted 2 mM guanosine-5’-O-(2-thiodiphosphate) (GDP-β-S) for GTP in the patch pipette solution. GDP-β-S is a hydrolysis-resistant guanine nucleotide that inhibits receptor-induced activation of G-proteins (Anadre 1994). Intracellular dialysis of hippocampal CA1 pyramidal neurons with this compound abolished both the initial membrane depolarization elicited by application of 20 μM carbachol (data not shown; n = 3/3) and the cholinergic-dependent PP (n = 3/3, Fig. 3). These data indicate that G-proteins are a necessary component of the cholinergic signal transduction pathway both in mediating the initial depolarization and in the expression of the PP.

Role of dephosphorylation and protein phosphorylation

The regulation of ion channels by phosphatases has recently been demonstrated (Bielefeldt and Jackson 1994; Wang and Salter 1994; White et al. 1991), suggesting that dephosphorylation may be important in the modulation of neuronal responses. To test whether dephosphorylation plays a role in PP genesis, ATP in the pipette solution was replaced with 5 mM adenosine-5’-O-(3-thiotriphosphate) (ATP-γ-S) (Astrand 1994). Intracellular dialysis of hippocampal CA1 pyramidal neurons with this compound abolished both the initial membrane depolarization elicited by application of 20 μM carbachol (data not shown; n = 3/3) and the cholinergic-dependent PP (n = 3/3, Fig. 3). These data indicate that G-proteins are a necessary component of the cholinergic signal transduction pathway both in mediating the initial depolarization and in the expression of the PP.

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irreversibly thiophosphorylate substrate proteins. Neurons were internally perfused with this compound for a minimum of 20 min prior to data collection. Following this intracellular perfusion, the initial depolarization induced by carbachol activation was significantly reduced to 48 ± 14% of control (P ≤ 0.038, n = 6). Furthermore, expression of the cholinergic-dependent PP was abolished (Fig. 4, A and B; n = 6/6). These data indicate that dephosphorylation was necessary for expression of cholinergic-dependent PPs.

We tested whether inhibitors of protein phosphatases could block the expression of cholinergic PPs because the above data implicated that dephosphorylation was necessary. Several approaches were used to test for the involvement of serine/threonine protein phosphatases. First, hippocampal slices (150–450 μm) were incubated in either calyculin A (cal A) or okadaic acid (OA) at concentrations of 1–2 μM for >3 h. This experimental approach has been used successfully in previous studies, indicating that these inhibitors permeate neurons in tissue slices (Mulkey et al. 1993; Muller et al. 1992). Second, the cell-impermeable protein phosphatase inhibitor microcystin-LR (m-LR), was applied internally via the patch pipette at a concentration of 10 μM (Mulkey et al. 1993). In this latter experiment, data collection began only after 20 min of internal perfusion. The blockade of serine/threonine protein phosphatases, by either calyculin A, okadaic acid, or microcystin-LR, resulted in a depolarized resting membrane potential (Table 1) and a reduction in the depolarization induced by 20 μM carbachol (cal A, decreased to 33 ± 21% of control, P < 0.001, n = 7; OA, decreased to 44 ± 9% of control, P ≤ 0.003, n = 7; m-LR, decreased to 47 ± 25% of control, P ≤ 0.008, n = 6). Finally, the cholinergic-dependent PP could not be evoked in most neurons recorded from slices incubated in either cal A (Fig. 4C; n = 12/14 slices) or OA (Fig. 4D; n = 6/7 slices). The cholinergic-dependent PP was also abolished in 67% of neurons intracellularly loaded with m-LR (Fig. 4E; n = 4/6).

To examine the involvement of other protein phosphatase subtypes, we also tested specific inhibitors of either protein phosphatase 2B (calcineurin) or tyrosine phosphatase. To inhibit calcineurin, the selective peptide inhibitor 476-501 (100 μM) (Hendey et al. 1992) was internally perfused via the patch-clamp electrode. At 100 μM, peptide 476-501 does not...
inhibit serine/threonine protein phosphatases (Hendey et al. 1992). Dialysis of neurons for 20 min with this peptide inhibitor did not significantly depress the initial cholinergic-induced depolarization (decreased to 90 ± 24% of control, n = 4) and was also ineffective in inhibiting the cholinergic-dependent PP (data not shown, n = 4/4). To test for the involvement of tyrosine phosphatase, hippocampal slices were first incubated in 100 µM Na-orthovanadate for >2 h, and this same concentration of inhibitor was included in the patch pipette solution. At 100 µM, Na-orthovanadate has virtually no dephosphorylating effect on serine-threonine phosphoproteins (Swarup et al. 1982). This inhibitor neither affected the resting membrane potential nor significantly depressed the initial cholinergic-induced depolarization (decreased to 94 ± 16% of control, n = 4). In addition, the cholinergic-dependent PP was consistently elicited (data not shown, n = 4/4), suggesting that tyrosine phosphatase is not a component of this signal transduction cascade. Hence these data exclude the involvement of calcineurin or tyrosine phosphatase in expression of the cholinergic-dependent PP.

Lack of effect of protein kinase inhibition

Our experiments with ATP-γ-S and serine/threonine protein phosphatase inhibitors indicated that dephosphorylation was critical for the expression of cholinergic-dependent PPs. Protein kinases are reported to activate protein phosphatases directly in some signal transduction pathways (Surmeier et al. 1995; Wilson and Kaczmarek 1993). Therefore we tested whether internal perfusion of 300 µM H-7, a potent, but nonselective kinase inhibitor (Hidaka et al. 1991; Ruegg and Burgess 1989), could abolish PPs elicited in 20 µM carbachol (Fraser et al. 1993; Malenka et al. 1989; Malinow et al. 1989; Zhang et al. 1992). The initial cholinergic-induced depolarization was significantly reduced by H-7 in the patch pipette solution (decreased to 64 ± 8% of control; P ≤ 0.032, n = 4). In contrast, H-7 did not block the expression of cholinergic-dependent PPs elicited by action potential firing (Fig. 5A; n = 4/4). These data imply that, although protein kinase(s) mediated some of the initial cholinergic-induced depolarization, there is no apparent involvement of protein kinases in expression of the cholinergic-dependent PP.

We next determined the potential role for phosphorylation in the expression of PPs elicited in 20 µM carbachol by internally perfusing a phosphorylation-inhibiting cocktail (PIC) containing 0 Mg-ATP, 5 mM ADP-β-S, 50 µM dinitrophenol, and 1 mg/ml alkaline phosphatase (Chen and Smith 1992; Chen et al. 1990; Hescheler et al. 1987; Shuba et al. 1990). The resting membrane potentials of neurons loaded with PIC were significantly more depolarized than control neurons (PIC −59 ± 2 mV, n = 9; see Table 1 for control values), and the initial cholinergic-induced depolarization was significantly depressed in neurons containing PIC (decreased to 40 ± 14% of control, P ≤ 0.017, n = 4). In contrast, the cholinergic-dependent PP was consistently elicited by evoked action potential firing in the presence of 20 µM carbachol (Fig. 5B; n = 4/4). This experiment further indicates that phosphorylation is unnecessary for the expression of cholinergic-dependent PPs.

Synaptic activation of PPs and GABA antagonists

The activation of PPs by current-evoked action potentials is an all-or-none event (Fraser and MacVicar 1996a). We investigated whether synaptic activation could also evoke all-or-none PPs in CA1 pyramidal neurons by stimulating the Schaffer collaterals while bath applying a cholinergic agonist (carbachol or oxotremorine M). Oxotremorine M was sometimes used because it has slight preference for m1/m3 as compared with m2 cholinergic receptors (Brann et al. 1993), and we showed above that m1/m3 receptors mediate PP genesis. In the presence of either carbachol (20 µM) or oxotremorine M (15 µM) alone, synaptic stimulation rarely activated PPs (n = 2/6). In contrast, intracellular current injection always evoked PPs in these same neurons (n = 6/6). Synaptic stimulation did not evoke PPs even though the synaptic-induced depolarization was equivalent to the depolarization elicited by intracellular current injection that resulted in PP genesis (Fig. 6A). As inhibitory circuits are also activated by synaptic stimulation, we perfused GABAergic antagonists to determine whether synaptic stimulation would elicit PPs when inhibition was depressed. Antagonists of GABA_A receptors (30 µM bicuculline) and GABA_B receptors (50 µM CGP 35348) alone did not unmask synthetically stimulated PPs (n = 0/6; Fig. 6B). Coapplication of oxotremorine M with either the GABA_A antagonist bicuculline (30 µM; n = 5), or with the GABA_B receptor antagonist CGP 35348 (50 µM; n = 6) also did not result in PP genesis with synaptic stimulation (data not shown). However, PPs were consistently evoked under these conditions.
PPs when the application of both GABA A and GABA B receptor antagonists stimulation. Both intrinsic and synaptic stimulation failed to unmask PP genesis by either intrinsic or synaptic stimulation. Application of the Schaffer collaterals failed to trigger PP genesis. Both intrinsic and synaptic stimulation consistently evoked PPs (n = 15/16; data not shown). In contrast, when both GABA A and GABA B antagonists (bicuculline 30 μM and CGP 35348 50 μM) were bath applied in conjunction with oxotremorine M, synaptic stimulation consistently evoked PPs (n = 21/24; Fig. 6C). These results indicate that depression of both GABA A- and GABA B-mediated synaptic inhibition and activation of muscarinic receptors was necessary for the expression of synaptic-driven PPs. In any one cell, the waveform of the synaptically evoked PP was remarkably similar to the PP evoked intrinsically by current injection (Fig. 6D). Similar to our previous report on intrinsically generated PPs (Fraser and MacVicar 1996a), PPs were not evoked by synaptic stimulation in oxotremorine M, bicuculline, and CGP 34358 when 10 mM bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid was included in the patch pipette to chelate intracellular calcium (n = 0/4; data not shown). Therefore synaptic stimulation appears to evoke an intrinsically generated all-or-none PP when muscarinic stimulation is combined with depression of synaptic inhibition.

**DISCUSSION**

We have previously identified a novel cholinergic-dependent PP in CA1 pyramidal neurons, which relies on the interaction between HVA Ca 2+ channels and the Ca 2+ -activated nonselective cation conductance (Fraser and MacVicar 1996a). This PP is observed in the presence of cholinergic (or metabotropic glutamate) agonists and is similar to ictal depolarizations observed during cholinergic-induced seizures (Nagao et al. 1996; Yaari and Jensen 1989). In this paper, we have elucidated the muscarinic receptors and various components of the signal transduction pathway underlying the expression of cholinergic-dependent PPs. Our evidence suggests that m1/m3 receptors are coupled to serine/threonine protein phosphatases, either directly or indirectly via G-proteins. The expression of cholinergic-dependent PPs therefore requires phosphatase-induced dephosphorylation. We have also demonstrated that, similar to the generation of ictal depolarizations, the synaptic activation of the PP is facilitated by depression of inhibition.

The expression of the cholinergic-dependent PP appears to be mediated by m1 and/or m3 receptors. The presence of both m1/m3 receptor subtypes in hippocampal tissue is well-documented by anatomical studies (Vilaro et al. 1993), and m1/m3 receptor stimulation is implicated in the modulation of cellular excitability (Auerbach and Segal 1996; Cox et al. 1994). The binding of m1/m3 receptor antagonists, [3H]pirenzepine and [3H]4-DAMP, is maximal in the rat hippocampus relative to other structures (Aubert et al. 1996), and these antagonists inhibit phosphoinositide metabolism, suggesting that stimulation of m1/m3 receptors liberate this second messenger (Can- duра et al. 1995). The phosphoinositide signal transduction cascade activated by m1/m3 receptors is mediated by a G-protein (Gm11) (Vilaro et al. 1993), and in agreement with this we have found that the effects of cholinergic stimulation were occluded by GDP-β-S. Indeed, several studies have demonstrated that muscarinic or metabotropic glutamate receptor stimulation activates phospholipase C via G-proteins, resulting in IP 3 production and elevated intracellular Ca 2+ (Kostyuk and Verkhratsky 1994). Intracellular Ca 2+ elevated by these neurotransmitters stimulates the Ca 2+ -activated nonselective cation conductance, thereby depolarizing the membrane potential (Congar et al. 1997; Crepel et al. 1994).

We have demonstrated critical roles for protein phosphatases and dephosphorylation in the expression of cholinergic-dependent PPs. The involvement of dephosphorylation was first tested using intracellular perfusion of ATP-γ-S, which irreversibly phosphorylates substrate proteins. Intracellular dialysis of this compound depressed the initial cholinergic-induced depolarization and abolished the PPs elicited in carbachol. As dephosphorylation is mediated by protein phosphatases, we then investigated whether inhibitors of protein phosphatases prevented PP genesis. Both membrane-permeable (calyculin A and okadaic acid) and -impermeable (m-LR) inhibitors of serine/threonine protein phosphatases abolished the cholinergic-dependent PP. The effectiveness of these inhibitors in our study is similar to a previous study of protein phosphatases in hippocampal slices (Mulkey et al. 1993). Inhibitors of calcineurin (peptide 476-501) or tyrosine phospho-
tase (Na⁺-orthovanadate) did not, however, affect PP genesis. The expression of cholinergic-dependent PPs therefore required the activation of serine/threonine protein phosphatases, either directly or indirectly via G-proteins. A direct activation of protein phosphatases by G-proteins has been suggested previously (Bielefeldt and Jackson 1994). Recently, muscarinic modulation of calcium-activated potassium conductances has also been shown to rely on protein phosphatase activation (Krause and Pedarzani 2000).

Protein phosphatases are directly activated by protein kinases in some systems (Surmeier et al. 1995; Wilson and Kaczmarek 1993), and a variety of protein kinases are activated by muscarinic stimulation (PKC, Figenschou et al. 1996; Marsh et al. 1995; Zhang et al. 1992; CaMKII, Muller et al. 1992; Pedarzani and Storm 1996; tyrosine kinase, Huang et al. 1993). Although the nonselective protein kinase inhibitor H-7 depressed the initial cholinergic-induced depolarization in the present study, no inhibition of the cholinergic-dependent PP was observed. This finding suggested that PP genesis may be independent of protein kinase activity. To further test this possibility, we internally perfused neurons with PIC, a phosphorylation-inhibiting cocktail (Chen and Smith 1992; Chen et al. 1990; Hescheler et al. 1987; Shuba et al. 1990). Similar to the H-7 experiments, the initial cholinergic-induced depolarization was depressed by PIC. The cholinergic-dependent PP was still generated, however, again demonstrating that phosphorylation is not involved in PP genesis.

What is the target for phosphatase-induced dephosphorylation? We have previously demonstrated that the PP relies on interactions between calcium entry through HVA Ca²⁺ channels and the Ca²⁺-activated cation conductance (Fraser and MacVicar 1996a). We further postulated that this latter conductance is directly enhanced by cholinergic stimulation. Based on the data presented here, we postulate that the target for dephosphorylation is a serine/threonine site on the Ca²⁺-activated nonselective cation channel. This theory is consistent with previous reports demonstrating that the open channel time and Ca²⁺ sensitivity of nonselective cation channels are greatly reduced by PKA-induced phosphorylation in both invertebrate (Partridge et al. 1990) and vertebrate neurons (Razani-Boroujerdi and Partridge 1993). Elevations in the activity of either cAMP or PKA also depress the Ca²⁺-activated nonselective cation channel recorded from cocklea (Van den Abbeele et al. 1996) or insulinoma (Reale et al. 1995) cells. Consistent with these findings, we have shown that the PP could not be generated in the presence of ATP-γ-S or inhibitors of serine/threonine phosphatases, indicating the importance of dephosphorylation in PP genesis. Interestingly, the conditions that reduce PP generation also enhance activity of HVA Ca²⁺ channels. For example, L-type Ca²⁺ currents in hippocampal neurons were enhanced by elevations in intracellular PKA activity (Chetkovich et al. 1991; Hell et al. 1995) or by protein phosphatase inhibitors (Mironov and Lux 1991). Given that PPs rely on both HVA Ca²⁺ channels and the Ca²⁺-activated nonselective cation conductance, and that Ca²⁺ influx was probably augmented by protein phosphatase inhibition, it is possible that the Ca²⁺-activated nonselective cation conductance was depressed to a much greater extent than is evident from our observations.

We have previously suggested that the PP is an excellent candidate for an intrinsic mechanism underlying ictal depolarizations observed during both experimental (Nagao et al. 1996; Yaari and Jensen 1989) and clinical (Lothman et al. 1991) epileptogenesis. We have found that synaptic stimulation can elicit PPs, but only when synaptic inhibition is depressed by GABA receptor antagonists. Depression of synaptic inhibition is also an important trigger for eliciting seizures in vivo (Lothman et al. 1991). Our results support the hypothesis that the ictal depolarization of epilepsy is an all-or-none PP that can be triggered by spike activity or synaptic inputs (Fraser and MacVicar 1996a). In addition, it has recently been demonstrated that a clinically relevant anticonvulsant (10–100 μM topiramate) depresses the cholinergic-dependent PP in subicular neurons (Palmieri et al. 2000). During interctal bursting, elevated levels of ACh and glutamate could stimulate muscarinic and metabotropic receptors, respectively. Activation of the protein phosphatase pathway via these receptors, in conjunction with increased intracellular calcium, may lead to PP genesis as described in this study. Recurrent seizures can, as a consequence, induce profound hypoglycemia (Wasterlain et al. 1993) and precipitous decreases in ATP and phosphocreatinine levels (DeFrance and McCandless 1991; Fujikawa et al. 1988). These conditions would favor dephosphorylation and possibly exacerbate existing seizure activity. As the PP represents a feed-forward regenerative potential that results in prolonged depolarization and maintained Ca²⁺ influx, these mechanisms may also represent a crucial component of excitotoxicity (Chen et al. 1997).

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