Protein Kinase Modulation of Dendritic K^+ Channels in Hippocampus Involves a Mitogen-Activated Protein Kinase Pathway

Li-Lian Yuan, J. Paige Adams, Michael Swank, J. David Sweatt, and Daniel Johnston

Division of Neuroscience, Baylor College of Medicine, Houston, Texas 77030

We investigated mitogen-activated protein kinase (MAPK) modulation of dendritic, A-type K^+ channels in CA1 pyramidal neurons in the hippocampus. Activation of cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC) leads to an increase in the amplitude of backpropagating action potentials in distal dendrites through downregulation of transient K^+ channels in CA1 pyramidal neurons in the hippocampus. We show here that both of these signaling pathways converge on extracellular-regulated kinases (ERK)-specific MAPK in mediating this reduction in dendritic K^+ current, which is confirmed, in parallel, by biochemical assays using phosphospecific antibodies against the ppERK and pKv4.2. Furthermore, immunostaining indicates dendritic localization of ppERK and pKv4.2. Taken together, these results demonstrate that dendritic, A-type K^+ channels are dually regulated by PKA and PKC through a common downstream pathway involving MAPK, and the modulation of these K^+ channels may be accounted for by the phosphorylation of Kv4.2 subunits.

Key words: dendrite; A-type K^+ channel; MAPK; PKA; PKC; hippocampus

Voltage-gated K^+ channels are the principal regulators of membrane excitability in the nervous system. Cell-attached patch recordings have revealed transient, A-type, and delayed rectifier-type K^+ channels in dendrites of hippocampal CA1 pyramidal neurons (Hoffman et al., 1997). The A-type K^+ channel, in particular, has a remarkable subcellular distribution, showing a more than fivefold increase in density from the soma to the distal dendrites and a relatively low range of voltage activation. The low-voltage activation and high density in dendrites suggest that these channels act to dampen active signal propagation and limit boosting of EPSPs that might otherwise occur because of the presence of voltage-gated Na^+ and Ca^{2+} channels (Magee and Johnston, 1995; Magee, 1998). Action potentials backpropagating in dendrites of CA1 pyramidal neurons become progressively smaller in amplitude the farther they travel from the soma (Spruston et al., 1995; Stuart et al., 1997; Magee, 1999). This amplitude decrement is primarily attributable to the increasing density of A-type K^+ channels, because blocking these channels leads to an increase in action potential amplitude (Hoffman et al., 1997). Evidence from dendritic patch recordings suggests that two protein kinases commonly found in the brain, protein kinase A (PKA) and protein kinase C (PKC), modulate the open probability of these A-type K^+ channels within the physiological voltage range (Hoffman and Johnston, 1998). Other modulators of these channels include neurotransmitters norepinephrine, acetylcholine, and dopamine, arachidonic acid, and auxiliary subunits (Colbert and Pan, 1999; Hoffman and Johnston, 1999; An et al., 2000).

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Correspondence should be addressed to Dr. Daniel Johnston, Division of Neuroscience, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. E-mail: dan@mossy.bcm.tmc.edu.

Although A-type K^+ channels play a key role in controlling membrane excitability and signal propagation in CA1 neurons, their molecular identity is not known. A variety of evidence suggests that *Shal* (Kv4) subunits contribute to forming these K^+ channels in hippocampal dendrites. Heterologously expressed *Shal* channels have similar biophysical and pharmacological properties to the dendritic K^+ channel (Serodio et al., 1996; Jan and Jan, 1997; Song et al., 1998; An et al., 2000). Immunohistochemical studies revealed segregation of two A-type K^+ channels in hippocampal CA1 pyramidal neurons. Kv1.4 is found mostly in axons, whereas Kv4.2 is found in the soma and dendrites (Sheng et al., 1992; Maletic-Savatic et al., 1995). *Shal* channels, especially Kv4.2, thus seem the best candidate for these transient K^+ channels. Kv4.2 possesses several interesting molecular features. Inspection of the amino acid sequence of Kv4.2 reveal several consensus sequences for PKA, PKC, Ca^{2+} and calmodulin-dependent protein kinase II (CaMKII), and mitogen-activated protein kinase (MAPK) phosphorylation sites, respectively, some of which are in the intracellular domains accessible to protein kinases (Baldwin et al., 1991; Adams et al., 2000; Anderson et al., 2000). Moreover, the very C terminus sequence of Kv4.2, S/TXL, which is an imperfect match for PSD-95/Dlg/ZO-1 (PDZ) binding consensus S/TXV, was also found at C termini of metabotropic GluR 1–3 and GluR 5, and they all interact with Homer, a dendritic protein containing only one PDZ domain (Brakeman et al., 1997). Such sequences may play a role in targeting Kv4.2 subunits in dendrites.

Accumulating evidence suggests that the extracellular-regulated kinases (ERK)-MAPKs play an important and essential role in induction and maintenance of certain types of neuronal plasticity and learning (English and Sweatt, 1996, 1997; Martin et al., 1997; Atkins et al., 1998; Winder et al., 1999; Watabe et al., 2000; Wu et al., 2001). Biochemical evidence suggests that ERK is downstream of both PKA and PKC, and coupling of PKA and PKC signaling to ERK leads to CREB protein phosphorylation in the hippocampus (Roberson et al.,

1999). In hippocampus, high-frequency stimulation of Schaffer collateral–CA1 pyramidal neuron synapses activates ERK, leading to nuclear signaling-dependent changes, and blocking ERK activation inhibits long-term potentiation (LTP) induction (English and Sweatt, 1996, 1997; Winder et al., 1999; Kanterewicz et al., 2000; Watabe et al., 2000; Dudek and Fields, 2001). Among these investigations, two studies indicated that, in addition to causing nuclear-signaling-dependent changes, the role of ERK in hippocampal LTP is at least in part through regulation of cellular excitability, although it was not at all clear what the mechanism of that regulation might be (Winder et al., 1999; Watabe et al., 2000).

We investigated the modulation of dendritic K^+ channels and backpropagating action potentials by neurotransmitter agonists and protein kinases in CA1 pyramidal neurons of adult rats. Our results suggest that the modulation of dendritic K^+ channels by PKA and PKC is mediated through the ERK MAPK pathway. In parallel biochemical studies using phosphorylation-site specific antibodies against ppERK and ppKv4.2, the results were consistent with the physiological data that ERK is phosphorylated and activated downstream of PKA and PKC and that Kv4.2 is at least one of the final effectors of ERK. This is further supported by the results from cell-attached patch experiments showing that dendritic transient K^+ channels are regulated by ERK phosphorylation (Watanabe et al., 2002). Understanding the interplay of PKA, PKC, MAPK, and dendritic K^+-channels should help illuminate the mechanisms of synaptic integration and synaptic plasticity in pyramidal neuron dendrites.

MATERIALS AND METHODS

Preparation of slices and solutions for electrophysiology. Hippocampal slices (350–400 μm) were prepared from 5- to 8-week-old Sprague Dawley rats following standard procedures (Hoffman and Johnston, 1998). A Zeiss Axioskop, fitted with a 40× water-immersion objective and differential interference contrast (DIC), was used to view slices. Light in the near infrared (IR) range (740 nm) was used in conjunction with a contrast-enhancing camera to visualize individual dendrites. For all recordings, the bath solution contained (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 2.0 $CaCl_2$, 1.0 $MgCl_2$, and 25 dextrose.

Whole-cell recording pipettes (7–12 MΩ) contained (in mM): 120 $KMeSO_4$ or 120 KGluconate, 20 KCl, 10 HEPES, 0.2 EGTA, 2 Mg_2Cl, 4 Na_2ATP, 0.3 Tris-GTP, and 14 phosphocreatine, pH 7.25, with KOH. The following were included in the external solution to block synaptic transmission (in μM): 50 D,L-APV, 10 CNQX, and 10 bicuculline, unless otherwise indicated for some experiments. Isoproterenol (1–2 μM) and 8-Br-cAMP (100 μM) were dissolved into the bath solution with the addition of 1 μM ascorbate right before use. U0126 and phorbol diacetate (PDA) were dissolved in DMSO to 10 mM, kept frozen until use, and then diluted to the appropriate concentrations. Forskolin was made in DMSO in 100 mM aliquots and diluted to a 50 μM final concentration with 100 μM Ro Z01724, a phosphodiesterase (PDE) inhibitor.

Recording techniques and data analysis. All neurons exhibited a resting membrane potential between −60 and −75 mV. Whole-cell recordings were made using an Axoclamp 2A (Axon Instruments, Foster City, CA) amplifier in "bridge" mode at 31–33°C and were analog filtered at 3 kHz. Series resistance was 15–30 MΩ. Antidromic action potentials (APs) were stimulated every 12–15 sec by 0.1 msec constant current pulses through tungsten electrodes placed in the alveus. Traces shown in figures are averages of 5–15 sweeps, but parameters such as amplitude and dV/dt are measured from each individual trace and averaged numbers are reported. Biocytin was dissolved in the intracellular solution at 0.4%, and the dye was allowed to enter into the cells by passive diffusion after a successful recording. The detailed morphological methods used are described in Esclapez et al. (1999). Significance ($p < 0.05$) was determined by paired or two-sample t tests. Error bars represent SEM.

Preparation of hippocampal slices for biochemistry. Hippocampal slices were prepared and maintained according the method of Roberson et al. (1999). Briefly, 400 μm hippocampal slices were prepared from the brains of 4- to 8-week-old male Sprague Dawley rats. The slices were cut in ice-cold cutting saline (in mM: 110 sucrose, 60 NaCl, 3 KCl, 1.25 NaH_2PO_4, 28 $NaHCO_3$, 5 D-glucose, 0.5 $CaCl_2$, 7 $MgCl_2$, and 0.6 ascorbate, saturated with 95% O_2 and 5% CO_2), then immediately transferred to a 1:1 mix of cutting saline and normal artificial CSF (ACSF) (in mM: 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 10 D-glucose, 2 $CaCl_2$, and 1 $MgCl_2$, saturated with 95% O_2 and 5% CO_2). After 20 min incubation, the slices were first transferred to room temperature (RT) ACSF for 1 hr, then to ACSF maintained at 32°C. The slices were then exposed to various pharmacological manipulations, after which they were immediately frozen on dry ice. The CA1 subregions were microdissected on dry ice and stored at −80°C until assayed. CA1 subregions from four to six slices were pooled for each experimental condition.

Sample preparation. The pooled CA1 subregions were sonicated according to Anderson et al. (2000). Briefly, slices were sonicated in buffer (in mM: 20 Tris pH 7.5, 1 EGTA, 1 EDTA, 1 $Na_4P_2O_7$, 4 pNPP, 1 Na_3VO_4; 25 μg/ml aprotinin, 25 μg/ml leupeptin, 100 μM PMSF). The sonicate was centrifuged for 3 min at 4°C at 3000 rpm to remove cellular debris. The supernatant was then centrifuged for 20 min at 4°C at 100,000 × g. The resulting supernatant (cytosolic fraction) was heated for 5 min at 95°C after addition of 4× sample buffer. The resulting pellet (membrane fraction) was resuspended in 10% SDS with 200 mM dithiothreitol (DTT), 10 μg/ml aprotinin, leupeptin, and pepstatin, 100 μM phenylmethylsulfonyl fluoride, and 1 μM microcystin-LR; subsequently, 4× sample buffer containing 200 mM DTT was added. Samples were loaded onto 10% SDS-polyacrylamide gels and resolved by standard electrophoresis (minigel apparatus; Bio-Rad, Hercules, CA). Total protein content was determined for each sample so that equal amounts of protein could be loaded in each gel lane.

Western blotting. Gels were blotted electrophoretically to Immobilon filter paper using a transfer tank maintained at 4°C. Transfer was completed overnight at 400 mA for membrane fraction samples; for cytosolic fraction samples, transfer was completed at 600 mA in 2 hr. Immobilon filters were blocked for 1 hr at RT in a blocking solution containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, 5% milk, 0.01% thimerosol, and 1 mM microcystin-LR. The filters were incubated at RT sequentially with primary antibody for 1 hr, followed by an HRP-conjugated secondary antibody (1:20,000) for 45 min. All blots were washed extensively in TTBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) after incubations with the primary and secondary antibodies. All blots were developed using enhanced chemiluminescence (ECL). Blots were probed first with purified antibody JPA170 (1:500). After several washes in TTBS, the blots were probed with anti-dual-phospho-ERK (either monoclonal or polyclonal; 1:1000). Blots were then stripped in 0.2 M NaOH and reprobed with anti-total ERK (1:1500).

Immunostaining of ppERK and pKv4.2/confocal imaging. Hippocampal slices (300–350 μm) were processed as described (Winder et al., 1999). Primary antibody concentrations: mouse antibody (Ab) for ppERK (1:1000), rabbit Ab for ERK-triply phosphorylated Kv4.2 (1:500) (JAP 170). Secondary antibodies: Cy3-conjugated anti-mouse or anti-rabbit IgG (1:200). Slices were imaged on a Zeiss laser-scanning confocal microscope equipped with an argon–krypton laser.

Materials. Antibody JPA170 was developed by J. P. Adams (Adams et al., 2000). Polyclonal anti-dual-phospho-ERK, monoclonal anti-dual phospho-ERK, anti-total ERK1/2, and HRP-conjugated secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA), Cy3-conjugated anti-mouse or anti-rabbit IgG from Jackson ImmunoResearch (West Grove, PA), ECL Western blotting detection reagents from Amersham Biosciences (Piscataway, NJ), isoproterenol (iso), forskolin (FSK), and PDA from Sigma (St. Louis, MO), U0126 from Promega (Madison, WI), PD 98059 from Calbiochem (La Jolla, CA), and Ro-201754, a PDE inhibitor, from Biomol (Plymouth Meeting, PA).

RESULTS

The amplitude of the backpropagating action potential decreases with distance from the soma

APs triggered by alvear stimulation are initiated in the axon and propagated retrogradely (backpropagated) into the apical dendrites of hippocampal CA1 pyramidal neurons (Spruston et al., 1995; Colbert and Johnston, 1996; Colbert et al., 1997). Whole-cell recordings from the apical dendrites of these neurons revealed a decreasing amplitude of the AP with increasing distance from the soma (Callaway and Ross, 1995; Spruston et al., 1995)

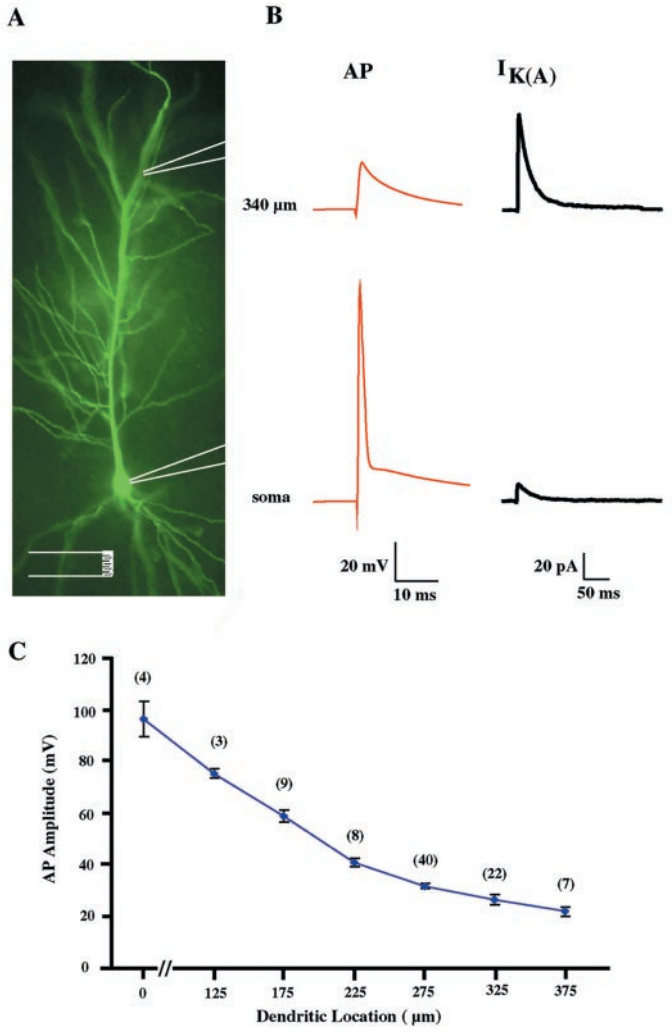

Figure 1. Examples of CA1 pyramidal neurons and whole-cell and cell-attached recordings from soma and dendrites. *A,* A pyramidal neuron in hippocampal CA1 region filled with biocytin through a recording electrode patched in the dendrites. Locations for patch electrodes are indicated. *B,* Representative traces for action potentials (*AP*) and outward transient K^+ currents ($I_{K(A)}$) from the soma and a dendrite 340 μm from the soma (current traces from Hoffman et al., 1997). In the soma, the AP amplitude is ~120 mV, whereas I_A is ~11 pA. In the dendrites the amplitude of the AP has declined to ~26 mV, whereas I_A is almost sixfold bigger than in the soma. *C,* Summary data for AP amplitude as a function of recording distance from the soma (recording data are binned to 50 μm segments). The number of cells for each group is in *parentheses*.

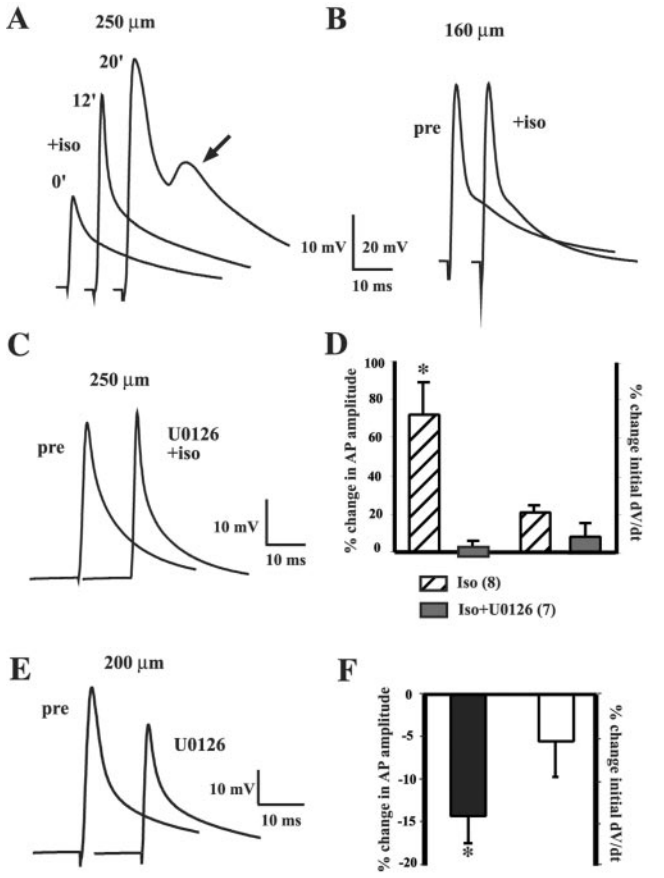

Figure 2. Isoproterenol application produced a time- and kinase-dependent increase in action potential amplitude in distal dendrites. *A,* At 32°C, iso application (1 μM) resulted in a 105% increase in amplitude of an AP recorded 250 μm from the soma. Prolonged application sometimes resulted in a broad, presumably Ca^{2+}-dependent AP (*arrow*) after the Na^+ AP. *B,* In a more proximal recording location (160 μm from the soma), iso had no effect, even at 32°C. *C,* Preincubation of the slices in U0126 (20 μM) for 30 min blocked the effect of iso on AP amplitude in a dendrite 250 μm from the soma. *D,* Summary data. Percentage of changes in action potential amplitude and initial rate of rise were measured when responses got stable, usually between 15 and 25 min. The number of cells for each group is in *parentheses*. Recordings were made from distal dendrites (250–350 μm). *E,* Wash-in of 20 μM U0126 for 30 min caused a reduction of AP amplitude at a 200 μm dendritic location. *F,* Summary data. Percentage of changes in AP amplitude and initial rate of rise are indicated from recordings made from 150–225 μm on dendrites ($n = 5$).

(Fig. 1*B,C*). AP amplitude was 60.3 ± 5.0 mV ($n = 4$), 34.6 ± 1.3 mV ($n = 25$), and 25.7 ± 1.0 mV ($n = 17$) at dendritic distances of 200, 250, and 300 μm from the soma, respectively (Fig. 1*C*), compared with 96.6 ± 6.8 mV ($n = 4$) in the soma under similar conditions (Fig. 1*B*). The smaller amplitude in the dendrites is primarily determined by the high density of transient K^+ current, which is almost six times greater at 340 μm compared with that in the soma (Hoffman et al., 1997, their Fig. 1*B, right*).

Isoproterenol increases AP amplitude in a PKA-, temperature-, and location-dependent manner

Previous work has shown that activation of either PKA or PKC increases dendritic AP amplitude through downregulation of dendritic K^+ channels (Hoffman and Johnston, 1998, 1999). Physiologically, an increase in intracellular PKA and PKC would be expected after activation of neurotransmitter systems such as norepinephrine, ACh, dopamine, 5-HT, and neuroactive peptides (Kaczmarek and Levitan, 1987; Chetkovich et al., 1993). In the present experiments we found that, in addition to a small membrane potential depolarization (~5 mV), bath application of 1–2 μM of the β-adrenergic receptor agonist isoproterenol (iso) increased AP amplitude by 71.7 ± 16.9% ($n = 8$; $p < 0.005$) in distal dendrites (250–350 μm from the soma) without a change in the initial rate of rise. The latter suggests that the boosting of the AP amplitude is not through Na^+ channel regulation (Colbert and Johnston, 1998; Hoffman and Johnston, 1998). Extended application of iso sometimes resulted in the occurrence of prolonged, presumably Ca^{2+}-dependent, APs and dendritic bursting (Magee and Carruth, 1999) (Fig. 2*A*). This boosting of the AP by iso was not uniform along the dendrites. The same concentration of iso had little or no effect on APs recorded at more proximal locations

(<200 μm) (Fig. 2*B*). The boosting of AP amplitude in distal dendrites by iso was also temperature dependent. At RT (22°C), APs in distal locations were larger in amplitude than those recorded at higher temperatures (76 ± 20.7% increase, paired data $n = 3$; 225–280 μm), (the averaged AP amplitude from 225 to 300 μm at RT was 54.6 ± 3.6mV, $n = 7$ vs 31.5 ± 0.8 mV, $n = 75$ at higher T). The same concentration of iso had little effect on AP amplitude at distal (>250 μm) locations at room temperature (4.7% increase; $n = 3$; data not shown)

MAPK inhibition blocks PKA and PKC boosting of dendritic APs

Biochemical experiments have shown that there are MAPK phosphorylation sites on the cytoplasmic domain of Kv4.2 subunits and furthermore that ERK–MAPK serves as a possible target for PKA and PKC. We therefore tested the effect of ERK blockade on the boosting of APs by iso. Because of the lack of a specific blocker on ERK, we have used U0126, a specific inhibitor on MEK, a kinase one step upstream of ERK. Preincubation of slices for at least 20 min with 20 μM of the MEK inhibitor U0126 blocked the boosting of APs by iso ($n = 7$) (Fig. 2*C*), suggesting that ERK is involved in the signaling pathway evoked by activation of β-adrenergic receptors. To control for the specificity of U0126, we also tried another MEK inhibitor, PD 98059. Under similar conditions, 50 μM PD 98059 preincubation for at least 30 min also blocked the increase in AP amplitude by iso (2.1% increase; $n = 2$; data not shown). We also tested the effects of U0126 alone on AP amplitude. We found that wash-in of U0126 for 20–30 min caused a small but significant decrease in AP amplitude (14.2 ± 3.1%; $p < 0.05$; $n = 5$) in dendrites without much change in initial rate of rise (5.5 ± 4.1%) (Fig. 2*E,F*) and without changing membrane potentials. This can be explained by the experiments using cell-attached patch recordings from CA1 dendrites, which found that wash-in or preincubation of MEK inhibitors U0126 or PD98059 caused a leftward shift of the activation curve for dendritic transient K^+ currents (Watanabe et al., 2002).

We then tested whether the block by U0126 could be overcome by the direct addition of the cAMP activators FSK or 8-Br-cAMP. At distal dendrites (>250 μm) 50 μM FSK caused a small membrane depolarization, and also increased AP amplitude by 41.2 ± 4.7% ($n = 5$; $p < 0.01$) (Fig. 3*A*). If the slices were preincubated in 20 μM U0126 for 30 min, however, the same concentration of FSK had no significant effect on AP amplitude or dV/dt ($n = 4$) (Fig. 3*B,E*). The blocking effect of U0126 on FSK-induced AP increase was significant as tested by a two-sample *t* test ($p < 0.005$). Similarly, 100 μM 8-Br-cAMP, a membrane-permeable analog of cAMP, increased AP amplitude by 21.6% ($n = 3$). 8-Br-cAMP + U0126, however, again resulted in no change (6.0%; $n = 2$; data not shown).

Protein kinase C activation has also been shown to downregulate dendritic, A-type K^+ channels (Hoffman and Johnston, 1998) as well as reduce steady-state inactivation of Na^+ channels (Tsubokawa and Ross, 1997; Colbert and Johnston, 1998). In addition, PKC activation by phorbol esters has been shown to have presynaptic effects and increase neurotransmitter release (Malenka et al., 1986). In the absence of neurotransmitter receptor antagonists (see Materials and Methods), we observed five types of changes after bath-applying PDA (10 μM). These were: (1) the resting membrane potential depolarized by 15–20 mV ($n = 7$); (2) the frequency of spontaneous EPSPs increased (Fig. 4*A*) ($n = 7$); (3) the cell started firing spontaneous, single, and bursts of APs and occasionally displayed what appeared to be Ca^{2+}-dependent APs (Fig. 4*B*) ($n = 3$); (4) the amplitudes of APs in distal dendrites during a train displayed less decrement ($n = 5$) (Fig. 4*C*) (Tsubokawa and Ross, 1997; Colbert and Johnston 1998); and (5) the amplitude of single backpropagating APs increased (Fig. 3*C*, see below).

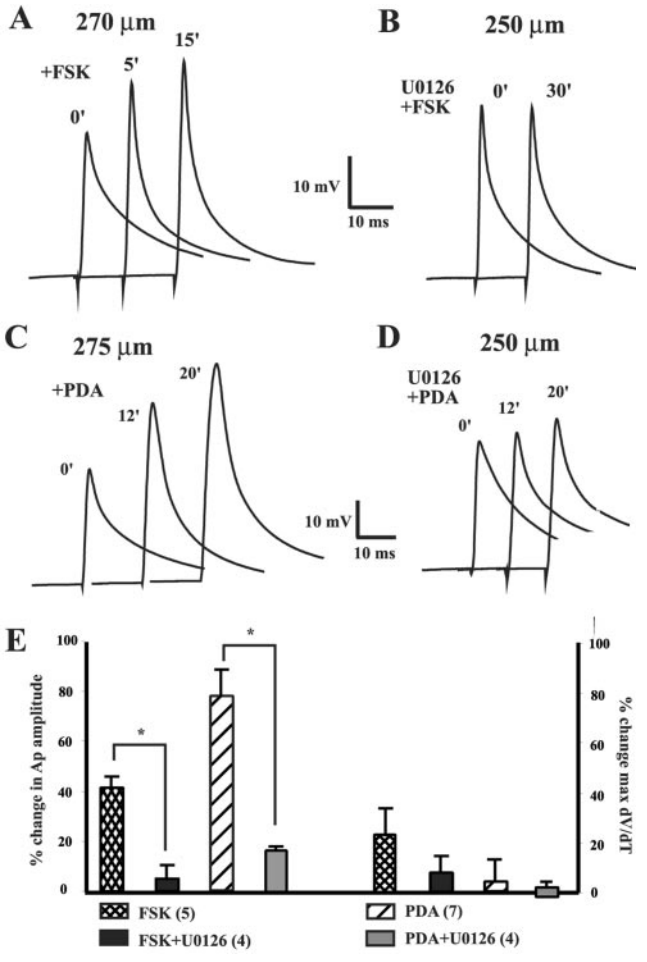

Figure 3. Effects of direct activation of PKA or PKC were blocked by MEK inhibitors. *A,* Antidromically initiated APs (recorded 270 μm from the soma) at various time points before and after the application of 50 μM forskolin (*FSK*). FSK increased the AP amplitude in distal dendrites by 77.9 ± 10.5% ($n = 7$). *B,* Previous incubation with MEK inhibitor U0126 (20 μM) blocked the effect of FSK ($n = 4$) (different cell, 250 μm from the soma). *C,* APs recorded 275 μm from the soma before and during the presence of PKC activator PDA (10 μM). Cells were held hyperpolarized to −80 mV to remove Na^+ channel inactivation before antidromic stimulation. *D,* Another recording 250 μm from the soma in the slice preincubated in U0126 for 30 min, AP amplitudes are shown at various time points of PDA application. Cell was held −80 mV before antidromic stimulation. *E,* Summary data. Percentage of changes in action potential amplitude and maximum rate of rise were measured when responses got stable, usually between 15 and 25 min, and the number of cells for each group is in *parentheses*. Recordings were made from distal dendrites (250–350 μm). Significant difference revealed by a two-sample *t* test is designated by an *asterisk*.

When cells were held at resting membrane potentials (−65 to −70 mV), backpropagating AP amplitude increased by 87.1 ± 16.8% ($n = 5$; $p < 0.005$) with PDA, and, although as a group there was no significant increase in rate of rise (15.0 ± 5.8%; $n = 5$; $p = 0.08$), three of the five cells showed quite large increases in dV/dt (23.4 ± 6.9%; $p < 0.008$; $n = 3$). To reduce the effect of

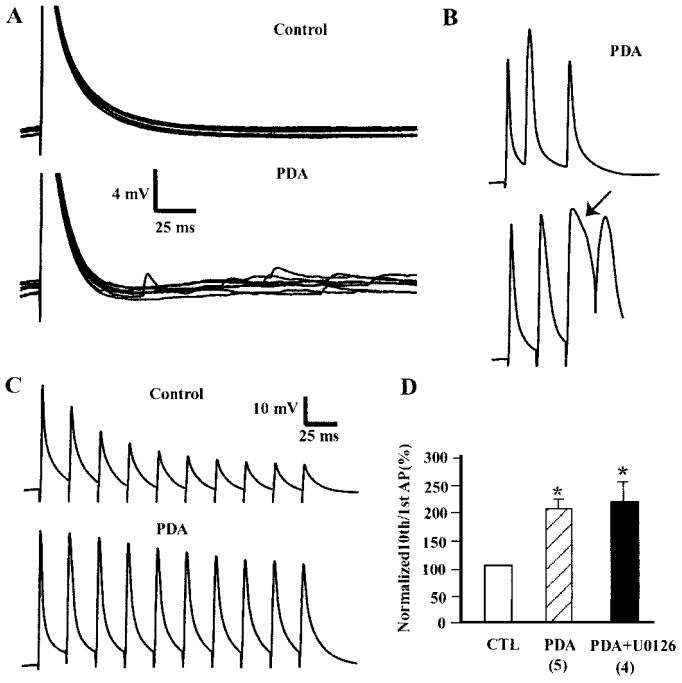

Figure 4. Effects of bath application of PDA. *A,* Responses recorded from a 250 μm dendritic location in the absence (*top traces*) and in the presence of PDA (20 μM) (*bottom traces*) were superimposed. PDA application increased frequency of spontaneous EPSPs recorded after the antidromic AP (truncated). *B,* In PDA, in response to single antidromic stimulation, sometimes more than one AP (*top trace*) and possible Ca^{2+}-dependent AP (*bottom trace, arrow*) can be observed. *C,* A train of antidromic APs was evoked at a rate of 50 Hz. PDA greatly decreased the attenuation of dendritic APs during a train. *D,* Summary data for *C*. Percentage of changes of normalized 10th/first AP in a train of antidromic APs are compared between control, PDA ($n = 5$), and PDA + U0126 ($n = 4$).

PDA on Na^+ channel inactivation (Jung et al., 1997; Tsubokawa and Ross, 1997; Colbert and Johnston, 1998), we did further experiments with the cells hyperpolarized from rest. When the same cells were held at −80 mV, PDA increased distal dendritic AP amplitude by 77.9 ± 10.5% ($n = 7$) with little or no change in maximal rate of rise in any of the cells (3.95 ± 8.7%; $n = 7$) (Fig. 3*C,E*). These results support the conclusion that the increase in AP amplitude with cells at −80 mV is attributable primarily to decreases in K^+ channel activation. Preincubation of slices with U0126 blocked the increase in AP amplitude by PDA with cells held at −80 mV (15.4 ± 1.34%) ($n = 4$) (Fig. 3*D*), but not with the cells at the resting potential (74.4 ± 14.5%; $n = 5$; $p < 0.05$; data not shown). The blocking effect of U0126 on PDA-induced AP increase when V_m was held at −80 mV was significant as tested by a two-sample t test ($p < 0.05$) between PDA and PDA+U0126. Moreover, neither the effect of PDA on AP amplitude during a train (Fig. 4*D*) nor the small depolarization of the resting potential was blocked by U0126, suggesting that MAPK is not downstream of PDA modulation of Na^+ channels. Although most of the PDA experiments were conducted with neurotransmitter receptor antagonists in the bath (see Materials and Methods), in a few experiments we also observed similar increases in spontaneous firing and spontaneous EPSPs with PDA and U0126 ($n = 2$; data not shown) as with PDA alone.

Western blot analysis of Kv4.2 phosphorylation by ERK

In addition to our physiological results examining the downregulation of dendritic transient K^+ channels by ERK, we also used biochemical methods to examine the phosphorylation of Kv4.2 by ERK. To assess the extent and modulation of phosphorylation of Kv4.2 by ERK, we used an antibody that recognizes Kv4.2 when phosphorylated by ERK (Adams et al., 2000). This antibody, JPA170, was generated against a short peptide consisting of amino acids 598–620 of the Kv4.2 C terminus, with a phosphothreonine or phosphoserine substituted at sites previously identified as ERK phosphorylation sites in *in vitro* experiments (T602, T607, and S616). In our previous characterization of JPA170, we found that it is selective for the ERK-phosphorylated form of Kv4.2 (Adams et al., 2000).

β-adrenergic receptor activation leads to Kv4.2 phosphorylation via ERK

As Roberson et al. (1999) have previously reported, a short application (2 min) of the β-adrenergic receptor agonist isoproterenol (10 μm) to hippocampal slices leads to increased ERK activation in hippocampal area CA1, as assessed by an increase in the immunoreactivity of the dual-phospho-ERK antibody, α-ppERK. This same treatment resulted in increased phosphorylation of Kv4.2 by ERK in area CA1 (Fig. 5*A*), as assessed using a phospho-Kv4.2 selective antiserum. The iso-induced increase in Kv4.2 immunoreactivity was blocked by conjunctive application of the MEK inhibitor U0126 (20 μM, 60 min), as was the iso-induced increase in ERK activation (Fig. 5*A*). In this and all subsequent experiments, all blots were reprobed with an antibody that recognizes total ERK1/2 (p44/P42), and total ERK immunoreactivity did not change with experimental manipulation for any experiment (data not shown). Overall, we conclude that activation of β-adrenergic receptors in the hippocampus leads to activation of ERK and subsequent phosphorylation of Kv4.2 by ERK in area CA1.

Activation of the PKA cascade leads to Kv4.2 phosphorylation via ERK

Previous results have demonstrated that activation of the PKA cascade by forskolin treatment of hippocampal slices leads to activation of ERK2 in area CA1 (Martin et al., 1997; Roberson et al., 1999). Here we found that application (30–45 min) of forskolin (50 μM/150 μM Ro-201754) to hippocampal slices led to increased ERK activation as well as increased phosphorylation of Kv4.2 by ERK in area CA1 (Fig. 5*B*). The immunoreactivity of antibody JPA170 was significantly increased in FSK-treated slices relative to vehicle-treated slices, and the FSK-induced increase in immunoreactivity was blocked by conjunctive application of the MEK inhibitor U0126 (20 μM, 60 min). ERK activation in response to FSK application was also blocked by application of U0126. Thus, activation of the PKA cascade in the hippocampus leads to activation of ERK and subsequent phosphorylation of Kv4.2 by ERK in area CA1.

Activation of PKC leads to Kv4.2 phosphorylation via ERK

We have established that activation of PKC by application of a phorbol ester to hippocampal slices leads to activation of ERK2 in area CA1 (English and Sweatt, 1996; Roberson et al., 1999). Here we similarly found that application (5–7 min) of PDA (10 μm) to hippocampal slices leads to increased ERK activation, and we also observed an increase in phosphorylation of Kv4.2 by ERK in area CA1 (Fig. 5*C*). Relative to vehicle-treated slices, the PDA-treated slices showed significantly increased immunoreactivity with antibody JPA170. Conjunctive application of U0126 (20 μM, 60 min) blocked the PDA-induced increase in immuno-

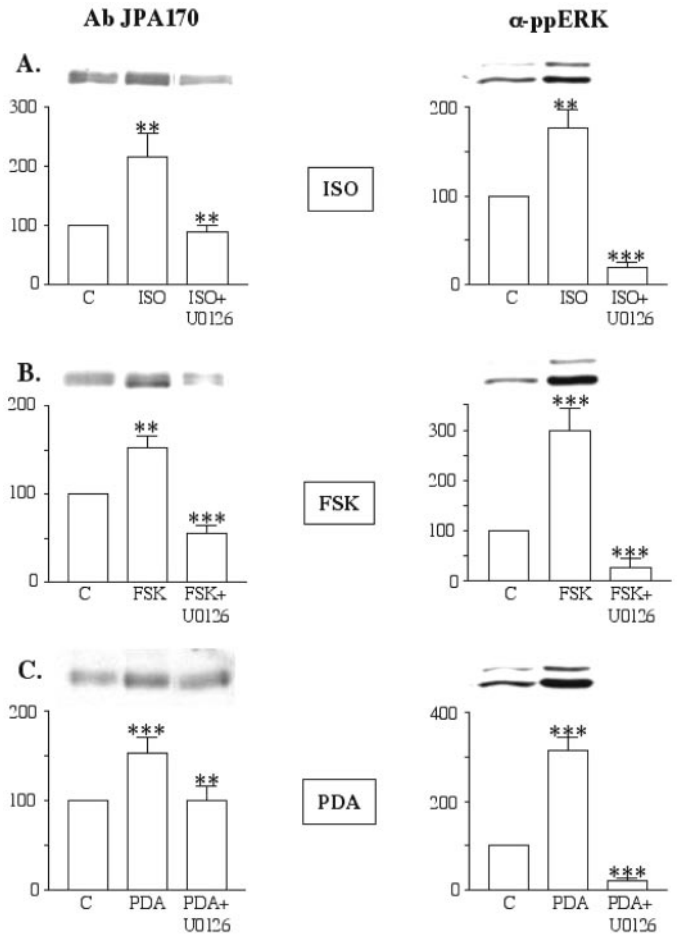

Figure 5. Stimulation of β-adrenergic receptors, the PKA cascade, or PKC leads to increased activation of ERK2 and increased ERK phosphorylation of Kv4.2 in hippocampal area CA1. *A,* β-adrenergic receptor activation by isoproterenol (*ISO*) application leads to Kv4.2 phosphorylation by ERK. Slices were exposed to vehicle [control (*C*)] or iso, with or without the MEK inhibitor U0126. Shown are representative blots of a membrane fraction prepared from area CA1 tissue, probed with JPA170 (*top, left*) or α-ppERK (*top, right*). Two bands revealed by α-ppERK represent ERK1/2 (p42 and p44). Below the blots are summary densitometric analyses, showing increased immunoreactivity following stimulation with iso, as detected with antibody JPA170 (215.3 ± 41.0% of vehicle-treated control; $n = 11$; $p < 0.01$) or α-ppERK (177.2 ± 20.2%; $n = 20$; $p < 0.001$). Conjunctive application of U0126 blocked the isoproterenol-induced increase in ERK activation and phospho-Kv4.2 immunoreactivity (JPA170: 88.5 ± 11.6%, $n = 11$, $p < 0.01$; α–ppERK: 19.8 ± 4.7%, $n = 20$, $p < 0.001$). *B,* Activation of the PKA cascade by forskolin (*FSK*) leads to Kv4.2 phosphorylation by ERK. Slices were exposed to vehicle (*C*) or FSK, with or without the MEK inhibitor U0126. Shown are representative blots of a membrane fraction prepared from area CA1 tissue, probed with JPA170 (*middle, left*) or α–ppERK (*middle, right*). Below the blots are summary densitometric analyses, showing increased immunoreactivity after stimulation with FSK, as detected with antibody JPA170 (152.1 ± 14.0% of vehicle-treated control; $n = 8$; $p < 0.01$) or α-ppERK (298.2 ± 44.0%; $n = 13$; $p < 0.001$). Conjunctive application of U0126 blocked the FSK-induced increase (JPA170: 55.1 ± 8.6%, $n = 8$, $p < 0.001$; α-ppERK: 24.5 ± 16.0, $n = 13$, $p < 0.001$). *C,* PKC activation by phorbol ester application leads to Kv4.2 phosphorylation by ERK. Slices were exposed to vehicle (*C*) or phorbol 12,13-diacetate (PDA), with or without the MEK inhibitor U0126. Shown are representative blots of a membrane fraction prepared from area CA1 tissue, probed with JPA170 (*bottom, left*) or α-ppERK (*bottom, right*). Below the blots is summary densitometric analysis, showing increased immunoreactivity following stimulation with PDA, as detected with antibody JPA170 (153.0 ± 18.3% of vehicle-treated control; $n = 9$; $p < 0.01$) or α-ppERK (316.5 ± 28.9; $n = 13$; $p < 0.001$). Conjunctive application of U0126 blocked the PDA-induced increase relative to U0126-treated control (JPA170: 100.9 ± 14.9%, $n = 9$, $p < 0.05$; α-ppERK: 19.4 ± 8.2%, $n = 13$, $p < 0.001$). For all experiments, the immunoreactivity of the dual-phospho-ERK antibody, α-ppERK, was increased in both the membrane and cytosolic fractions (data not shown) in response to activator application. There was no JPA immunoactivity detected in control experiments for cytosolic fraction. Activator + U0126 immunoreactivity was normalized to U0126-alone control.

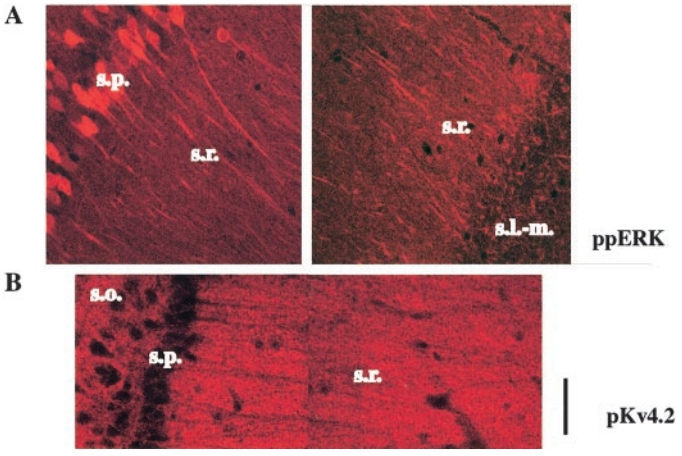

Figure 6. Distribution of ppERK and pKv4.2 in adult rat hippocampal slices. *A,* ppERK staining at proximal (*top, left*) and distal dendrites (*top, right*) in stratum pyramidal (*s.p.*), stratum radiatum (*s.r.*), but not in stratum oriens (*s.o.*) and stratum lacunosum-moleculare (*s.l.-m.*) of CA1 region of hippocampus. Scale bar, 50 μm. *B,* pKv4.2 staining in the same region of hippocampus. Scale bar, 50 μm.

reactivity. Similarly, the immunoreactivity of the dual-phospho-ERK antibody, α-ppERK, was increased in area CA1 after PDA application, and this increase was blocked by application of U0126. Thus, we conclude that activation of PKC in the hippocampus leads to activation of ERK and subsequent phosphorylation of Kv4.2 by ERK in area CA1.

Localization of ppERK and pKv4.2

Previous studies have revealed the gross distribution of total ERK (Dudek and Fields, 2001), total Kv4.2 (Maletic-Savatic et al., 1995), and Kv4.2 in differential phosphorylation states, such as by ERK and by PKC (Varga et al., 2000). Data presented above from both physiological recordings and *in vitro* protein assays suggest the existence of basal phosphorylation of ERK and Kv4.2 and their functional coupling in the dendrites of hippocampal CA1 pyramidal neurons. In a final series of studies, we used immunolocalization to confirm that both phospho-ERK and ERK-phosphorylated Kv4.2 are present in dendrites. We focused our study on the adult hippocampal CA1 region at the subcellular level. ppERK was detected in almost all compartments of CA1 pyramidal neurons, including somata and apical and basal dendrites (Fig. 6*A*). Staining for pKv4.2 revealed by JPA 170 antibody showed an overlapping but distinct distribution from that of ppERK. In CA1, as reported before (Varga et al., 2000), pKv4.2 expressed predominantly in stratum radiatum and stratum oriens, that is, in distal apical and basal dendrites with the minimal staining in the soma and proximal dendrites (Fig. 6*B*).

DISCUSSION

From the results of the present set of experiments along with previous studies on the modulation of dendritic, A-type K^+

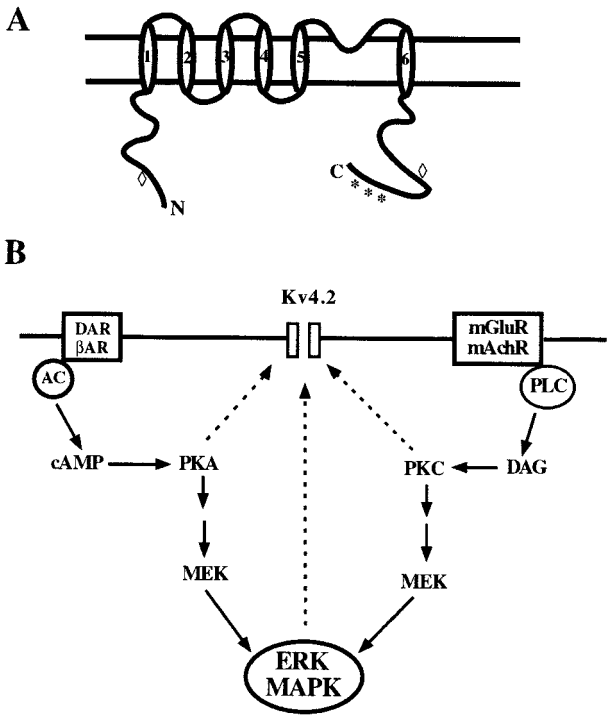

Figure 7. Kv4.2 subunit is the target of MAPK signaling pathway. *A,* Schematic diagram of Kv4.2 subunit, showing six transmembrane domains and the intracellular N and C termini domains. Approximate locations of known ERK (*) and PKA (◇) phosphorylation sites are highlighted. *B,* Our work suggests the following signal transduction pathways leading to Kv4.2 phosphorylation. PKA and PKC activation by neurotransmitters converge onto MAPK, which phosphorylates Kv4.2, resulting in changes in channel biophysical properties. PKA and PKC can also phosphorylate Kv4.2 directly without going through MAPK pathway, possibly serving as channel targeting mechanisms.

channels (Hoffman and Johnston, 1998, 1999; Adams et al., 2000; Anderson et al., 2000), it appears that endogenous neurotransmitters exerting their effects through PKA and PKC can converge on MAPK in hippocampal CA1 region to phosphorylate dendritic K^+ channels and alter their function (Fig. 7). These results are consistent with biochemical evidence that ERK1/2 is activated secondary to activation of either PKA or PKC. Both the physiological recordings and the protein assays also provide strong support for the hypothesis that Kv4.2 is the target of MAPK phosphorylation in dendrites of CA1 pyramidal neurons. The same procedures that led to phosphorylation of Kv4.2 also resulted in downregulation of dendritic, A-type K^+ currents resulting in increased AP amplitude and increased dendritic membrane excitability. Nonetheless, it is difficult to assign a molecular species to a native current (channel) observed in intact neurons with absolute certainty. We thus cannot rule out that another channel subunit besides Kv4.2 contributes to the effects we observe or that Kv4.2 needs other auxiliary subunits to resemble the native channels in dendrites. Our hypothesis of modulation of Kv4.2, however, is clearly the most parsimonious interpretation of the available data.

Certainly not all effects of PKA or PKC activation are mediated through MAPK. Good examples for this are our observations that MAPK inhibition did not block the effects of PDA on the decreasing amplitudes of APs during a train, on the small depolarization of the resting potential, or on the increase in spontaneous EPSPs. Clearly both PKA and PKC activity lead to phosphorylation of multiple target proteins having multiple functions on neural activity. The results presented here and elsewhere (Adams et al., 2000; Anderson et al., 2000), however, provide strong support for at least one consequence for these kinase activities being changes in K^+ channel function mediated through the MAPK pathway.

Location- and temperature-dependent boosting of dendritic APs by iso

Isoproterenol exerts its effect on backpropagating APs along the dendrites in a location-dependent manner. Iso had little effect at proximal locations (<200 μm) while having its largest effects at the more distal locations. This is similar to what was observed previously with direct application of cAMP analogs (Hoffman and Johnston, 1998) and is in keeping with the conclusion that this nonuniformity is attributable to the gradient in density of transient K^+ channels rather than that of β-adrenoceptors. Similarly, we have observed that the amplitude of the AP in dendrites is significantly greater at room temperature, which might be explained by the fact that both activation and inactivation of K^+ channels are highly temperature-sensitive (Hille, 1984; Singleton et al., 1999). For this reason, the lack of effect of iso at room temperature is to be expected, because the AP amplitude at lower temperatures is less influenced by K^+ channels under control conditions and therefore would be less subject to modulation by iso.

Specificity of ERK effects on transient K^+ channels and not on Na^+ channels in dendrites

A series of reports have investigated kinase modulation of Na^+ channels in CA1 pyramidal neurons in hippocampus and found both PKA and PKC activation led to reduction of the peak Na^+ current (Cantrell et al., 1996, 1997). Moreover, PKC activation decreases slow inactivation of dendritic Na^+ channels (Tsubokawa and Ross, 1997; Colbert and Johnston, 1998), making them behave similarly to somatic Na^+ channels. In our experiments, we have also observed PKC effects on Na^+ channel inactivation isolated by holding cells at different membrane potentials (−65 vs −80 mV). When cells were held at approximately −65 mV, an increase of dendritic AP amplitude by PDA is caused by both decreases in K^+ channel activation and Na^+ channel inactivation, as evidenced by increases in the initial rate of rise. The effect of PDA on Na^+ channel inactivation has been demonstrated previously (Colbert and Johnston, 1998), but the effects on K^+ channels and Na^+ channels can be separated by previous hyperpolarization to remove any residual Na^+ inactivation at rest. With this previous hyperpolarization, increases in spike amplitude occur without any change in the initial slope. The fact that MEK inhibitors have no effect on the previously reported PDA-induced reduction in Na^+ channel slow inactivation further supports the hypothesis that only the effect on K^+ channels is mediated through MAPK. Although not the main focus of these experiments, several other reported effects of phorbol esters on hippocampal slices, such as increased spontaneous EPSPs, depolarization of the membrane potential, and spontaneous firing were also not blocked by U0126. These results thus suggest that although PKC activation in hippocampus leads to a number of electrophysiological changes, only the effects on K^+ channels appear to be mediated by MAPK.

PKA and PKC phosphorylation of Kv4.2 that are not mediated through MAPK pathways

Our data support the hypothesis that PKA- and PKC-mediated signaling pathways can converge onto MAPK and that MAPK phosphorylation of dendritic K^+ channels or Kv4.2 subunits results in changes in channel biophysics and fine-tuning of membrane excitability. Phosphorylation consensus sites for PKA and PKC on Kv4.2 have been identified, however (Adams et al., 2000; Anderson et al., 2000), suggesting that PKA and PKC could affect Kv4.2 independently of the MAPK pathway. Several possibilities may explain why we did not see MAPK-independent modulation of K^+ channels in the present studies. For example, in the pyramidal neuron PKA may be constrained in its ability to modulate Kv4.2 because of the presence or absence of additional local protein cofactors, which may not be present in distal dendrites. An additional interesting possibility is that Kv4.2 may be serving as a signal integration molecule, and that PKA (or PKC) modulation may require concomitant phosphorylation at both the PKA and MAPK sites. Alternatively, Varga et al. (2000) reported that in hippocampus, the differentially phosphorylated Kv4.2 subunits can localize to specific pathways, indicating that Kv4.2 phosphorylation may be synaptic-input specific. One intriguing implication of this differential pattern is that targeting of Kv4.2 within hippocampus might be dependent on its phosphorylation state, and thus direct PKA phosphorylation may be more involved in subcellular localization than in modulation of channel biophysical properties. It has been shown recently that in the hippocampus, the targeting of another K^+ channel subunit, Kv2.1, is dependent on a 26-AA targeting signal (Lim et al., 2000). Within this signal, three of four residues shown to be critical by mutagenesis were serines, perhaps implicating a relationship between phosphorylation of these serine residues and localization of Kv2.1. It would be of interest to address the role of phosphorylation for localization within a single neuron transfected with Kv4.2 in which the ERK, PKA, or PKC phosphorylation sites have been eliminated.

Specificity of MEK inhibitors

The major MEK inhibitors we chose to use in this study were U0126 and PD 98059, and much work by ourselves and others indicates that these two inhibitors are quite selective for this kinase. Overall these inhibitors have been tested for selectivity versus a wide variety of other protein kinases, and we have specifically confirmed their lack of effects on PKA, PKC, and CaMKII (English and Sweatt, 1997; Roberson et al., 1999). MEK is a very unusual kinase in that it has the capacity to phosphorylate both Tyr and Ser/Thr residues; this apparently allows for a greater selectivity by inhibitors of this enzyme than is typical for the general category of kinase inhibitors. Interestingly, in a recent study Giovannini et al. (2001) showed a block of LTP-associated CaMKII activation by MEK inhibitors (as also was observed by Liu et al., 1999) that is caused by a block of an upstream, LTP-triggering event (Giovannini et al., 2001). The results we present here are of course consistent with this result and interpretation and suggest that MAPK regulation of K^+ channels may be one mechanism contributing to downstream CaMKII activation in LTP.

Basal levels of ppERK and pKv4.2

Several lines of evidence suggest the existence of basal phosphorylation of dendritic, A-type K^+ channels and Kv4.2 subunits in hippocampus. First, we found a small but significant decrease in AP amplitude by U0126 alone. This is entirely consistent with the results from cell-attached patch experiments showing that wash-in or preincubation of MEK inhibitor U0126 or PD98059 caused a leftward shift of the activation curve for dendritic A currents, suggesting the existence of basal MAPK phosphorylation of dendritic A type K^+ channels (Watanabe et al., 2002). Second, Western blot analysis revealed a considerable level of endogenous phosphorylation for both ppERK and pKv4.2 in CA1 region. Third, immunohistochemical staining using the antibodies against pKv4.2 and ppERK along with previous studies (Varga et al., 2000) indicate that there exists basal phosphorylation of Kv4.2 subunits and ERK in distal dendrites of CA1 pyramidal neurons.

Kv4.2, MAPK, and long-term synaptic plasticity

There is good evidence for PKA, PKC, and MAPK involvement in certain forms of LTP, learning, and memory (for review, see Sweatt, 1999). The downstream targets for these kinases with respect to their roles in synaptic plasticity or learning, however, are not clear. The results from the present experiments raise the possibility that dendritic, A-type K^+ channels could be involved, perhaps at different time points, during the induction or expression of these neural events. For example, pairing postsynaptic APs with synaptic input leads to boosting of dendritic APs and LTP induction (Magee and Johnston, 1997; Watanabe et al., 2002). This boosting of AP amplitude is similar to what we observed here with isoproterenol, forskolin, and phorbol esters, suggesting that neurotransmitters that activate one or more of these kinases could enhance the induction of synaptic plasticity using certain stimulus paradigms (Blitzer et al., 1995; Huerta and Lisman, 1995; English and Sweatt, 1996, 1997; Thomas et al., 1996; Blitzer et al., 1998; Winder et al., 1999; Watabe et al., 2000). Moreover, PKA, PKC, and MAPK are activated for different durations during stimulus protocols that induce LTP (Sweatt, 1999). Finally, recordings from awake and freely moving animals revealed that experience-dependent spike amplitude reduction may reflect regulation of dendritic backpropagating action potentials in hippocampal pyramidal neurons (Quirk et al., 2001). It is therefore possible that changes in K^+ channel function mediated by the MAPK pathway described here could accompany the expression of certain forms of LTP. The molecular mechanisms for synaptic integration, synaptic plasticity, and learning are of great contemporary interest to neuroscientists, and the results from the present experiments suggest that dendritic K^+ channels and the MAPK pathways are important components of these phenomena.

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