Modulation of the Kv1.3 Potassium Channel by Receptor Tyrosine Kinases

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ABSTRACT The voltage-dependent potassium channel, Kv1.3, is modulated by the epidermal growth factor receptor (EGFr) and the insulin receptor tyrosine kinases. When the EGFr and Kv1.3 are coexpressed in HEK 293 cells, acute treatment of the cells with EGF during a patch recording can suppress the Kv1.3 current within tens of minutes. This effect appears to be due to tyrosine phosphorylation of the channel, as it is blocked by treatment with the tyrosine kinase inhibitor erbstatin, or by mutation of the tyrosine at channel amino acid position 479 to phenylalanine. Previous work has shown that there is a large increase in the tyrosine phosphorylation of Kv1.3 when it is coexpressed with the EGFr. Pretreatment of EGFr and Kv1.3 cotransfected cells with EGF before patch recording also results in a decrease in peak Kv1.3 current. Furthermore, pretreatment of cotransfected cells with an antibody to the EGFr ligand binding domain (α-EGFr), which blocks receptor dimerization and tyrosine kinase activation, blocks the EGFr-mediated suppression of Kv1.3 current. Insulin treatment during patch recording also causes an inhibition of Kv1.3 current after tens of minutes, while pretreatment for 18 h produces almost total suppression of current. In addition to depressing peak Kv1.3 current, EGF treatment produces a speeding of C-type inactivation, while pretreatment with the α-EGFr slows C-type inactivation. In contrast, insulin does not influence C-type inactivation kinetics. Mutational analysis indicates that the EGF-induced modulation of the inactivation rate occurs by a mechanism different from that of the EGF-induced decrease in peak current. Thus, receptor tyrosine kinases differentially modulate the current magnitude and kinetics of a voltage-dependent potassium channel.

KEY WORDS: epidermal growth factor • insulin • growth factor receptor • tyrosine phosphorylation • potassium channel modulation

INTRODUCTION

Receptor tyrosine kinases play essential roles in cell growth and differentiation, mitogenesis, and cell signaling (Schlessinger and Ullrich, 1992). These membrane receptors dimerize and/or alter their configuration upon ligand binding, resulting in kinase activation and the autophosphorylation of several COOH-terminal tyrosine residues. The phosphorylation of these residues facilitates the recruitment of specific proteins containing Src homology 2 domains, which in turn initiates downstream signaling cascades that affect a diverse group of cellular targets via both tyrosine and serine/threonine protein kinases. In particular, activation of the epidermal growth factor receptor (EGFr)[1] and the insulin receptor tyrosine kinases is known to induce activation of PLCγ, PLA$_2$, PI-3 kinase and the ras/

MAPK (mitogen-activated protein kinase) pathway (Clark and Dunlop, 1991; Schlessinger and Ullrich, 1992; Dikic et al., 1994). Tyrosine kinases, including the EGF and insulin receptors, are expressed at high levels in the nervous system, where they exhibit distinct and highly regionalized patterns of distribution both during development and in the adult (Hanley, 1988). In spite of this, little is known about the signaling functions of tyrosine kinases in neurons, although emerging evidence from several laboratories indicates that ion channels are among the targets of tyrosine kinases (reviewed by Siegelbaum, 1994; Jonas and Kaczmarek, 1996). For example, in spinal dorsal horn neurons, the activity of NMDA (N-methyl-D-aspartate) glutamate receptors is increased by tyrosine kinases and decreased by tyrosine phosphatases (Wang and Salter, 1994). In Aplysia bag cell neurons, a voltage-dependent cation channel is regulated by tyrosine phosphorylation, via a pathway involving serine/threonine kinases and phosphatases (Wilson and Kaczmarek, 1993), while a calcium and potassium channel, as well as neuropeptide secretion, are modulated by insulin (Jonas et al., 1996, 1997).

Voltage-dependent potassium (Kv) channels are important for the generation and regulation of electrical activity in neurons, as well as for maintaining the resting membrane potential in excitable and nonexcitable

cells (reviewed by Hille, 1992). Based on sequence homology, Kv channels can be divided into several subfamilies; the Kv1 subfamily exhibits sequence homology to the *Drosophila Shaker* potassium channel (Chandy, 1991; Chandy and Gutman, 1993). One important feature that distinguishes different Kv channels is their inactivation kinetics. Inactivation of Kv channels occurs by two distinct mechanisms. Fast or N-type inactivation generally occurs with a time constant of a few milliseconds via an open channel pore block by the NH_2-terminal region of the channel protein (Hoshi et al., 1990). C-type inactivation usually occurs with a time constant of hundreds or thousands of milliseconds (Choi et al., 1991), by a less well defined mechanism that probably involves a more global change in the channel protein conformation. Most Kv channels undergo C-type inactivation (although the time constant of inactivation can vary widely (Lopez-Barneo et al., 1993), while N-type inactivation is limited to those channels that possess the appropriate NH_2-terminal sequence or are associated with a β subunit containing a similar sequence (Rettig et al., 1994).

Studies during the last decade have made it evident that Kv channels are important targets for modulation by protein phosphorylation. Most work to date has focused on modulation by serine/threonine phosphorylation (reviewed in Levitan, 1994), but several Kv1 family members are also affected directly by tyrosine phosphorylation. In particular, Kv1.2 is suppressed by the tyrosine kinase PYK2 and EGFr (Huang et al., 1993; Lev et al., 1995; Peralta, 1995) and Kv1.5 is suppressed by Src (Holmes et al., 1996b), as well as by activation of several growth factor receptors (Timpe and Fantl, 1994). Kv1.3 is phosphorylated directly by nonreceptor tyrosine kinases, resulting in suppression of channel activity and modulation of channel kinetics (Holmes et al., 1996a; Fadool et al., 1997). Serine/threonine kinases can also be involved in the modulatory pathway leading to Kv channel modulation (Timpe and Fantl, 1994).

In situ hybridization experiments indicate that Kv1 family members are expressed widely in mammalian brain. One of these channels, Kv1.3, exhibits a particularly selective distribution in the olfactory bulb and olfactory cortex as well as in the dentate gyrus of the hippocampus (Kues and Wunder, 1992). Kv1.3 also is the major Kv channel in T lymphocytes, where it plays an essential role in cell proliferation and activation by antigens (Lewis and Cahalan, 1990, 1995). We have examined the modulation of Kv1.3 channels by the EGFr and insulin receptor tyrosine kinases. Activation of either receptor tyrosine kinase decreases the peak Kv1.3 current amplitude. In addition, EGF treatment produces a speeding of C-type inactivation kinetics, while insulin treatment does not alter C-type inactivation. Mutational analysis indicates that different molecular mechanisms mediate the effects of EGF on current amplitude and kinetics. Thus Kv channels are targets for complex modulation by growth factor receptor tyrosine kinases.

MATERIALS AND METHODS

Materials

Human recombinant EGF and erbstatin were purchased from Calbiochem Inc. (La Jolla, CA), while insulin and a neutralizing EGFr antibody were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Cell culture reagents were from Gibco Laboratories (Grand Island, NY). Other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Channel and Kinase Expression

The voltage-gated potassium channel Kv1.3 and the human EGFr were expressed transiently in HEK 293 cells. HEK 293 cells were maintained in MEM (12360; Gibco Laboratories) plus 2% penicillin/streptomycin (15140; Gibco Laboratories), and 10% fetal bovine serum (16000; Gibco Laboratories). Cells were grown to confluency (7 d), dissociated with trypsin-EDTA (Gibco Laboratories) and mechanical trituration, diluted in MEM to a concentration of ∼600 cells/μl, and replated on plastic dishes. Cells were allowed to recover for several days before transfection. Kv1.3 was expressed using the Invitrogen Corp. (San Diego, CA) vector pRC-CMV, and the EGFr was expressed in the GW1 vector (British Biotechnology Ltd., Oxford, UK). In both cases, the protein coding region was downstream from a cytomegalovirus promoter. The effect of stimulating endogenous insulin receptors was studied by singly transfecting HEK 293 cells with Kv1.3 and adding insulin to the medium.

cDNA was introduced into cells with a lipofectamine reagent (18324; Gibco Laboratories), using standard techniques (Hawley-Nelson et al., 1993). The lipofectamine and the cDNA(s) were mixed and allowed to complex for 30–40 min, after which the cells were transfected with the lipofectamine/cDNA complex for 4–6 h in serum-reduced OptiMEM (Gibco Laboratories) medium. For electrophysiology, cells at ∼30–50% density were transfected with a total of 2 μg DNA per 35-mm dish. For biochemistry, cells at ∼85–95% density were transfected with 10 μg per 60-mm dish (equal amounts of Kv1.3 cDNA and either EGFr or control vector cDNA). Expression levels after 48–72 h allow the recording of macroscopic currents from membrane patches and detection of proteins by immunoprecipitation and Western blotting.

Biochemistry

Kv1.3 protein expression was measured using Western blot methods as described previously (Holmes et al., 1996a). Cells were harvested 2 d after transfection by lysis in ice-cold lysis buffer. Lysate proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose blots. These Western blots were then probed with anti–Kv1.3 antiserum (Cai and Douglass, 1993), and bound antibody was visualized by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) to measure Kv1.3 protein expression. Film autoradiograms of Western blots were analyzed by densitometry using an imaging densitometer (GS-670; Bio-Rad Laboratories, Richmond, CA).

Electrophysiology

Macroscopic currents were recorded at room temperature from cell-attached membrane patches, using an Axopatch 200A (Axon

Instruments, Foster City, CA) patch clamp amplifier. To zero the cell resting membrane potential, the bath solution contained (mM): 150 KCl, 10 HEPES, 1 EGTA, 0.5 MgCl$_2$, pH 7.5. The pipette solution contained (mM): 30 KCl, 120 NaCl, 2 CaCl$_2$, 10 HEPES, pH 7.5. Pipettes (M15/10; Jencons Glass, Leighton Buzzard, UK) typically had resistances of 4–6 MΩ, and were coated near the tip with beeswax to reduce the capacitance of the glass. The standard pulse protocol consisted of voltage steps to +40 mV for 1 s, at a frequency of one per minute to avoid cumulative inactivation of Kv1.3 (Marom et al., 1993). Before application of any drugs, the current amplitude and inactivation rate were allowed to stabilize for 5 min after obtaining a seal. For drug treatment during patch recording, compounds were perfused directly into the bath through 2-μl capillary tubes. The amplifier output was filtered at 1–2 kHz, digitized at 2–5 kHz, and stored for later analysis. Data were collected and stored using software written by M. Bowlby and S. Marom, and hardware purchased from Microstar Laboratories, Inc. (Bellevue, WA). Inactivation of macroscopic current was fit to the sum of two exponentials by minimizing the sums of squares (Kupper et al., 1995).

Mutagenesis

Standard techniques of site-directed mutagenesis using the polymerase chain reaction were used to mutate channel tyrosine residues to phenylalanines. Briefly, the general approach was to carry out two successive PCR reactions. The first uses the channel cDNA as the template, with one upstream nonmutagenic oligonucleotide primer, and a downstream mutagenic primer with a one- to three-base mismatch. The product of this first PCR reaction is used as the upstream primer for the second PCR reaction, with the downstream primer being nonmutagenic. The amplified mutant DNA fragment is cut sequentially with flanking restriction enzymes, purified and ligated into the channel backbone (after the equivalent wild-type region has been removed). All mutants were sequenced to confirm the presence of the mutation and to detect any sequence errors introduced during the PCR reactions. Six tyrosine residues (at four locations) in Kv1.3 lie within tyrosine kinase consensus phosphorylation sequences and were targeted for mutation.

RESULTS

Expression of Kv1.3

Expression of Kv1.3 in HEK 293 cells produces large amounts of channel protein, thus allowing both the biochemical detection of channels using Western blotting techniques and the recording of macroscopic currents from membrane patches (Bowlby and Levitan, 1995; Holmes et al., 1996a). The expression of Kv1.3, as measured on a Western blot, is not altered by coexpression with the EGFr, when compared with coexpression of Kv1.3 and control vector (Fig. 1 *A*). Similarly, the expression of Kv1.3 is not affected by treatment with EGF (Fig. 1 *A*) or insulin (Fig. 1 *B*) for at least 60 min, although insulin treatment for 4 h or longer does reduce the expression of the channel (data not shown). Quantitative densitometric analysis of the Western blots (Table I) confirms these conclusions.

Suppression of Kv1.3 Current by Activation of Growth Factor Receptors

Previous biochemical experiments have shown that Kv1.3 is phosphorylated robustly on tyrosine residues when the channel is coexpressed with the EGFr in HEK 293 cells (Holmes et al., 1996a). To determine whether acute EGFr activation produces changes in the functional properties of Kv1.3, cotransfected cells were exposed to EGF during a patch recording. The amplitude of Kv1.3 current in cell-attached patches is decreased
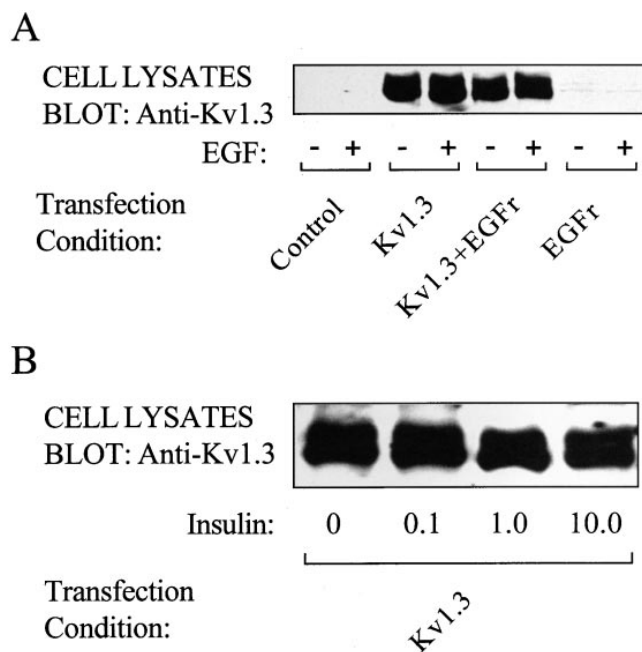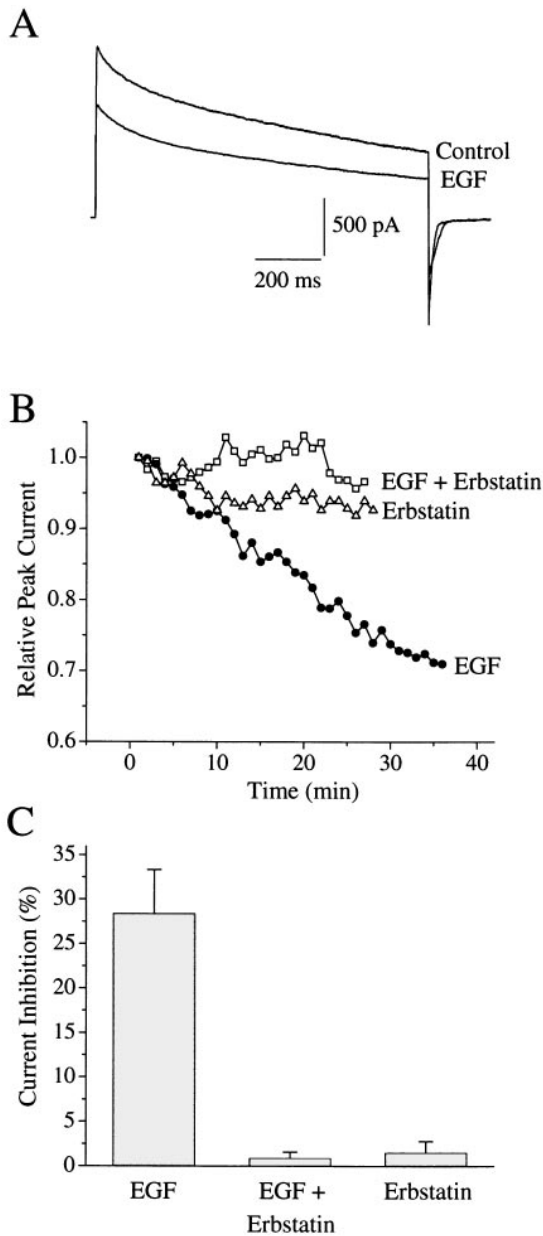
FIGURE 1. Coexpression of Kv1.3 and the EGFr, or treatment of Kv1.3-expressing cells with EGF or insulin, does not alter the expression of Kv1.3 protein. HEK 293 cells were transfected with vector DNA (*Control*), or cDNA encoding Kv1.3 and/or EGFr. Western blots of cell lysates were probed with an anti–Kv1.3 antiserum. (*A*) Expression of the EGFr, with or without EGF treatment (10 ng/ml, 60 min), does not alter Kv1.3 protein levels. (*B*) Insulin treatment of Kv1.3-expressing cells does not alter Kv1.3 protein levels. Cells were treated with 0.1–10 μg/ml insulin for 45 min.

TABLE I

Effects of Receptor Expression and Activation on Kv1.3 Protein Expression

Transfection condition	Treatment	Relative density units
		mean ± SEM
Kv1.3 alone	Vehicle	1.00 ± 0.20
Kv1.3 alone	EGF (10 ng/ml)	1.06 ± 0.19
Kv1.3 + EGFr	Vehicle	1.04 ± 0.13
Kv1.3 + EGFr	EGF (10 ng/ml)	0.92 ± 0.16
Kv1.3 alone	Vehicle	1.00 ± 0.14
Kv1.3 alone	Insulin (0.1 μg/ml)	1.25 ± 0.15
Kv1.3 alone	Insulin (1.0 μg/ml)	1.17 ± 0.08
Kv1.3 alone	Insulin (10 μg/ml)	1.25 ± 0.14

The density of the Kv1.3 band on Western blots (see Fig. 1) is expressed relative to that for Kv1.3 expressed alone and treated with vehicle. *n* = 4 for each experimental condition.

A

Control
EGF

500 pA
200 ms

B

EGF + Erbstatin
Erbstatin

EGF

Relative Peak Current (y-axis: 0.6 to 1.0)
Time (min) (x-axis: 0 to 40)

C

Current Inhibition (%) (y-axis: 0 to 35)
EGF, EGF + Erbstatin, Erbstatin

FIGURE 2. Acute treatment with EGF during patch recording suppresses Kv1.3 current. (*A*) Cell-attached patch recording from a cell expressing Kv1.3 and the EGFr. Traces are currents evoked before (*Control*) and 20 min after (*EGF*) beginning to perfuse 10 ng/ml human recombinant EGF into the bath. Voltage steps to +40 mV were applied once per minute. (*B*) Time course of suppression of peak current. Treatment with EGF (10 ng/ml) during patch recording suppresses Kv1.3 current within several minutes. Suppression is blocked when cells are treated for 1 h before and during recording with 25 μM erbstatin. No effect is observed when erbstatin alone is applied. Drugs were applied continuously after $t = 0$ min. (*C*) Mean (± SEM) inhibition of current in cells exposed to EGF (10 ng/ml), EGF + 25 μM erbstatin, or 25 μM erbstatin alone. $n = 10$ with EGF, 4 with EGF + erbstatin, and 3 with erbstatin.

when 10 ng/ml EGF is perfused over the cell (Fig. 2 *A*). The suppression by EGF begins within several minutes and generally continues for as long as the patch survives, although some patches could be held sufficiently long for a more-or-less steady state level of current to be reached (Fig. 2 *B*). This suppression by EGF applied during patch recording is blocked by addition of the tyrosine kinase inhibitor erbstatin, indicating that tyrosine kinase activation is necessary for current suppression. Application of erbstatin alone for up to 1 h has no effect (Fig. 2, *B* and *C*).

Longer term manipulation of EGFr activity also leads to modulation of Kv1.3. When cells are cotransfected with Kv1.3 and the EGFr, and exposed to EGF (10 ng/ml) before patch recording, Kv1.3 current is decreased compared with control cells transfected with Kv1.3 and vector (Fig. 3 *A*). On average, this protocol of coexpression and EGF pretreatment decreases the current recorded in a cell-attached patch by approximately half (Fig. 3 *B*), somewhat more than acute EGF treatment (Fig. 2). To test the specificity of this effect, we blocked EGFr activity with an EGFr antibody (α-EGFr) that selectively inhibits binding of EGF to its receptor and thus blocks subsequent receptor dimerization and kinase activation (Wu et al., 1989). Pretreatment of cells coexpressing Kv1.3 and the EGFr with α-EGFr for 4–12 h more than reverses the decrease in current (Fig. 3 *A*), while pretreatment with inactivated α-EGFr has no effect (Fig. 3 *B*).

It is noteworthy that the Kv1.3 current amplitude in cotransfected cells exposed to α-EGFr is more than six times greater than that in cotransfected cells that were exposed to EGF before recording (Fig. 3 *B*). In addition, the current in α-EGFr-treated cotransfected cells is greater than in untreated cells transfected with Kv1.3 and control vector (Fig. 3 *B*). This result indicates that an endogenous EGFr expressed in HEK 293 cells may be partially suppressing current, even in the absence of coexpressed EGFr or added EGF. Such endogenous EGFr can be detected in HEK 293 cell lysates, separated by SDS-PAGE/Western blots and probed with EGFr and phosphotyrosine-specific antibodies (data not shown).

Insulin treatment also produces a decrease in peak current amplitude in Kv1.3-transfected cells, presumably due to activation of an endogenous insulin receptor present in HEK 293 cells. Acute treatment of Kv1.3-transfected cells with 10 μg/ml insulin during a patch recording suppresses current recorded in cell-attached patches by approximately half after 10 min of perfusion (Fig. 4, *A* and *C*). Treatment of Kv1.3-transfected cells with insulin for 5–18 h before patch recording elicits an even larger decrease in current amplitude when compared with untreated Kv1.3-transfected cells (Fig. 4 *B*). Although short term treatment with insulin does not affect channel expression (Fig. 1, Table I), it should be

A

EGFr + α-EGFr
Control vector
EGFr + EGF

500 pA

200 ms

B

Normalized Peak Current

EGFr +
EGF

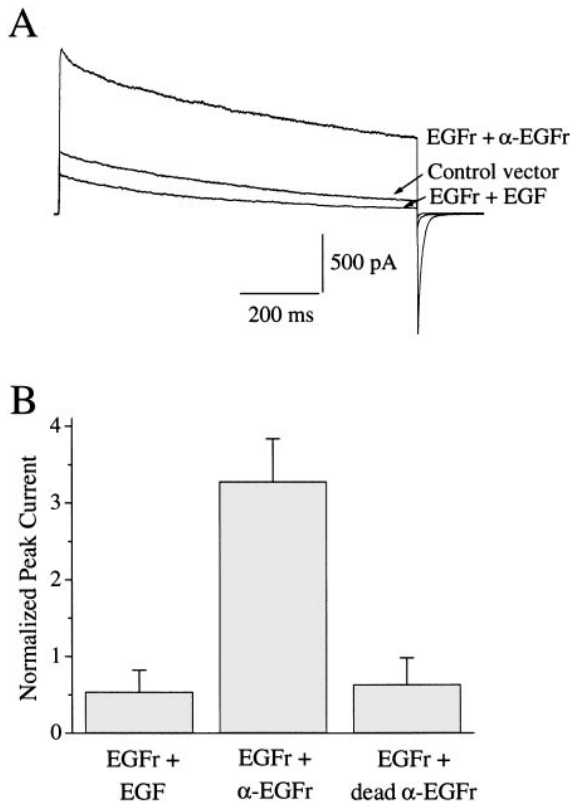EGFr +
α-EGFr

EGFr +
dead α-EGFr

FIGURE 3. Longer term manipulation of EGFr activity modulates Kv1.3 current. All cells were transfected with cDNA encoding Kv1.3. (*A*) Cell-attached patch recordings from two cells cotransfected with Kv1.3 and the EGFr, and one cell transfected with Kv1.3 and control vector. EGFr-cotransfected cells were pretreated either with 10 ng/ml EGF for 30 min, or overnight with 1 μg/ml α-EGFr, before patch recording. (*B*) Current amplitude from cell-attached patches under varying conditions, normalized to the average patch current recorded on the same day in cells transfected with Kv1.3 and control vector. EGF cells were pretreated with 10 ng/ml EGF for 30–60 min, while α-EGFr cells were pretreated with the antibody for 4–12 h, before patch recording. The α-EGFr was inactivated ("dead EGFr") by storing at 4°C for 6–8 wk; this antibody is stable at −20°C but its neutralizing activity is labile at 4°C (manufacturer's specifications, confirmed by us). $n = 4$ for each experimental condition; error bars represent the SEM.

noted that insulin treatment for 4 h or longer reduces Kv1.3 protein levels as detected by Western blots (data not shown).

Modulation of C-Type Inactivation of Kv1.3

The kinetics of C-type inactivation of Kv1.3 are altered markedly by EGFr coexpression and activation. When cells cotransfected with Kv1.3 and the EGFr are treated with EGF during patch recording, the inactivation rate of the current increases (Fig. 5, *A* and *B*). No consistent change in the activation or deactivation rates are found (data not shown). The change in inactivation rate induced by EGF treatment is blocked when the cell is pre-
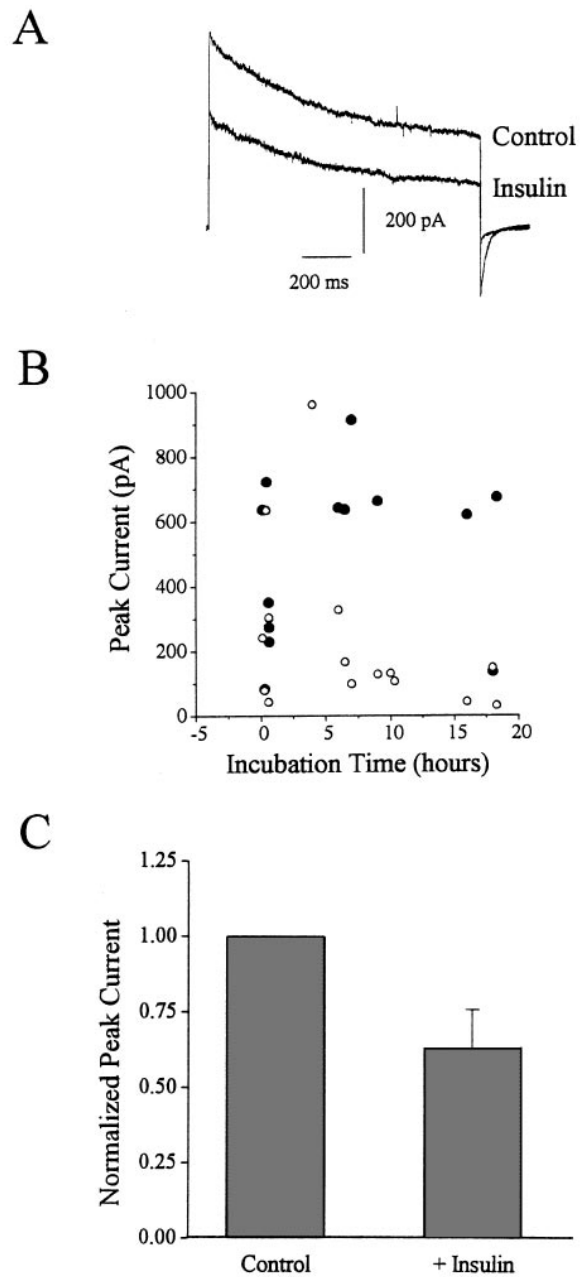
A

Control
Insulin

200 pA

200 ms

B

Peak Current (pA)

Incubation Time (hours)

C

Normalized Peak Current

Control   + Insulin

FIGURE 4. Insulin treatment suppresses Kv1.3 current. (*A*) Cell-attached patch recording from a cell expressing Kv1.3. Traces are before (*Control*) and 10 min after (*Insulin*) beginning to perfuse 10 μg/ml insulin into the bath. (*B*) Suppression by insulin pretreatment before patch recording. Filled circles are peak current amplitudes in untreated control cells, while open circles are values for insulin-pretreated cells. (*C*) Mean current amplitude for patches from cells treated with insulin for 4 h or less, normalized to the values from untreated control cells recorded on the same day.

treated with the tyrosine kinase inhibitor erbstatin, and then exposed to EGF together with erbstatin during the patch recording (Fig. 5 *C*). Cells cotransfected with Kv1.3 and the EGFr, and pretreated with EGF (10 ng/ml) before patch recording, also show a faster rate of inactiva-
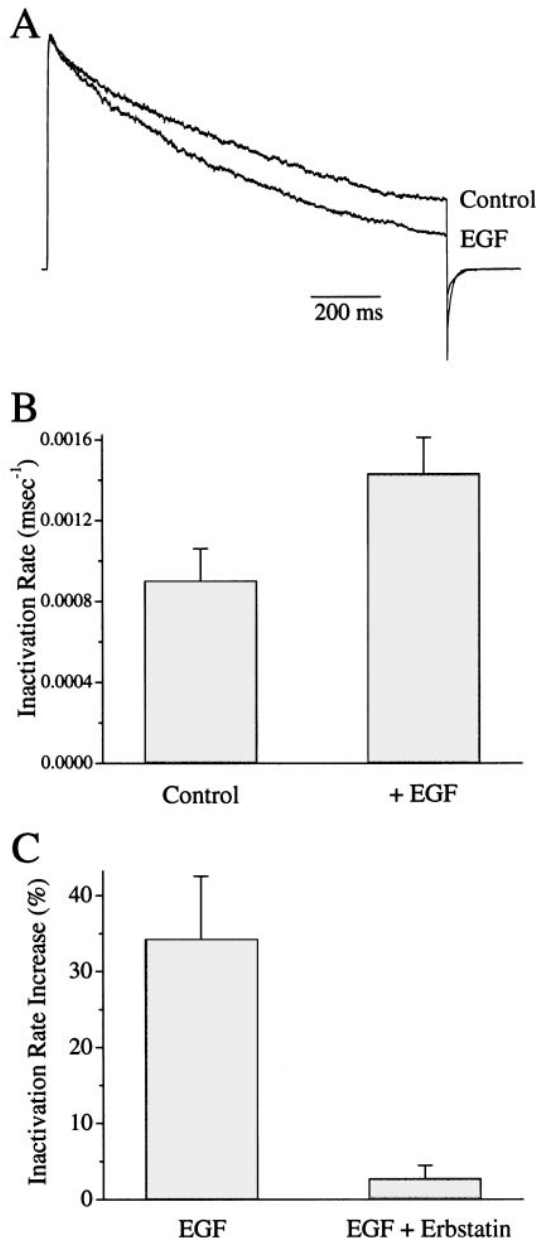
FIGURE 5. Application of 10 ng/ml EGF during patch recording speeds C-type inactivation of Kv1.3. Cells were transfected with Kv1.3 and EGFr. (*A*) Traces before (*Control*) and 30 min after (*EGF*) addition of 10 ng/ml EGF to the bath, normalized to the peak current to better observe the inactivation kinetics. (*B*) Mean inactivation rates before and after EGF application. Inactivation was fit with a weighted double exponential and the two components were combined ($n = 10$). (*C*) Mean percent increase in the inactivation rate for cells treated with EGF (10 ng/ml; $n = 10$) or EGF and erbstatin (25 μM; $n = 4$). Erbstatin-treated cells were pretreated with the kinase inhibitor for 1–3 h before recording, as well as during recording.

tion when compared with Kv1.3 and vector-transfected controls (Fig. 6, *A* and *B*). Pretreatment of these cotransfected cells for 4–14 h with α-EGFr reverses this effect, and even slows the inactivation rate beyond that of the

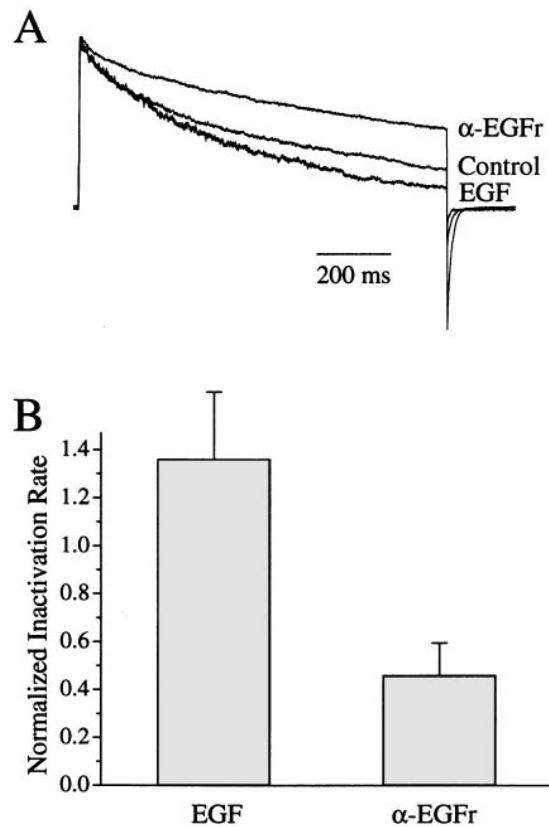FIGURE 6. Blocking the EGFr with α-EGFr slows the inactivation rate. (*A*) Normalized traces from three cells cotransfected with Kv1.3 and the EGFr and pretreated with no drug (*Control*), 10 ng/ml EGF for 30 min, or 1 μg/ml α-EGFr for 12 h. (*B*) Inactivation rates of Kv1.3 in cells pretreated as in *A*, normalized to control cells recorded on the same day. $n = 4$ for each treatment.

control (Fig. 6, *A* and *B*). Thus, interruption of tyrosine kinase activity or EGF binding blocks the EGFr-induced alteration of the inactivation rate.

In contrast, neither short (minutes) nor longer (up to 18 h) term insulin treatment of Kv1.3-transfected cells has a significant effect on the rate of C-type inactivation of the channel (Fig. 7, *A* and *B*). This difference in the physiological effects of EGFr and insulin receptor stimulation implies that activation of the two receptor tyrosine kinases results in different patterns of phosphorylation of Kv1.3.

Two Distinct Actions of EGF on Kv1.3

The speeding of the inactivation rate observed with EGF perfusion usually occurs simultaneously with the decrease in current amplitude, although in several experiments the inactivation rate changed before the current amplitude decreased, or only the inactivation rate increased. This led us to hypothesize that the two effects of EGF might result from distinct molecular events. The Kv1.3 channel has six putatively internal tyrosine residues that reside in consensus sequences for
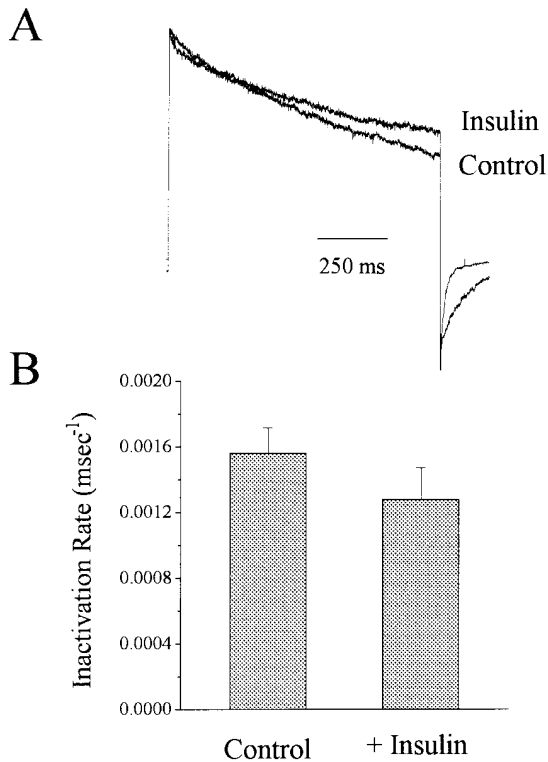
A

Insulin

Control

250 ms

B

FIGURE 7. Insulin treatment does not affect the inactivation rate. Cells were transfected with Kv1.3. (*A*) Normalized traces from a cell before (*Control*) and 10 min after (*Insulin*) addition of 10 μg/ml insulin. (*B*) Mean inactivation rates of control cells and cells pretreated for 10 min to 9 h with 10 μg/ml insulin. $n = 13$ with control cells and 10 with insulin-pretreated cells.

phosphorylation by tyrosine kinases (Songyang et al., 1994). Each of these tyrosine residues was mutated to phenylalanine and the mutant channels were tested for their responses to EGF. Three of these tyrosines are adjacent to one another at positions 111, 112, and 113, and thus they were treated as a unit and mutated together (YYY111–113FFF). Acute EGF treatment of cells expressing the Kv1.3, Y137F, and Y449F mutant channels elicits a decrease in peak current and a speeding of inactivation similar to that of the wild-type channel (Fig. 8, *A* and *B*). A combination mutant of Y449F and YYY111–113FFF also behaves like the wild-type Kv1.3 with respect to its response to EGF (Fig. 8 *C*). In contrast, Kv1.3 Y479F does not exhibit a change in peak current in response to EGF, although the effect of EGF on the inactivation rate is still intact (Fig. 8 *D*). The average EGF-induced inhibition of current is significant (paired *t* test, $P < 0.05$) for all the mutant channels except for Kv1.3 Y479F (Fig. 8 *E*), while the increase in the inactivation rate is significant (paired *t* test, $P < 0.05$) for all the mutant channels (Fig. 8 *F*). Thus, the EGF-elicited changes in peak current and inactivation rate are dissociable and are probably caused by different molecular mechanisms.

DISCUSSION

We have shown previously that expression and activation of an EGFr results in increased tyrosine phosphorylation of Kv1.3 in HEK 293 cells (Holmes et al., 1996*a*). The present results demonstrate that coexpression of the EGFr and Kv1.3, followed by EGF treatment, produces a decrease in peak current levels and a speeding of C-type inactivation. Both of these changes are blocked by pretreatment with α-EGFr, suggesting that they require receptor dimerization and tyrosine kinase activation. Insulin treatment produces a similar decrease in peak current, but no change in C-type inactivation kinetics, suggesting that the actions of receptor tyrosine kinases on current amplitude and inactivation kinetics are distinct and separable. EGF treatment of cotransfected cells suppresses Kv1.3 current and speeds the inactivation within tens of minutes; both of these effects are tyrosine kinase dependent because they are blocked by a tyrosine kinase inhibitor. These two effects of EGF are also dissociable. Suppression of current by EGF is dependent upon the presence of Y479 in the channel, while the change in inactivation kinetics is not altered by mutating any of the likely targets of tyrosine kinases. Changes in Kv1.3 amplitude and kinetics induced by the nonreceptor Src tyrosine kinase also can be attributed to phosphorylation of different tyrosine residues (Fadool et al., 1997). Interestingly, Y479 is not required for the suppression of Kv1.3 current by Src (Fadool et al., 1997), suggesting that suppression can be achieved by different molecular mechanisms.

It is clear that ion channels are targets of tyrosine kinases. Tyrosine phosphorylation has a variety of physiological effects on channels, including activation of calcium channels (Peppelenbosch et al., 1991, 1992), potentiation of NMDA receptor-mediated glutamate currents (Wang and Salter, 1994), stabilization of the assembly of acetylcholine receptors (Huganir et al., 1984; Wallace et al., 1991; Wallace, 1995), and both potentiation and inhibition of potassium channel activity (Peppelenbosch et al., 1991; Huang et al., 1993; Lev et al., 1995; Holmes et al., 1996*a*, 1996*b*; Jonas et al., 1996; Fadool et al., 1997). Modulation of ion channels by growth factor tyrosine kinases often occurs at the level of gene expression and protein synthesis, as several studies have reported effects on mRNA levels over several days (Fanger et al., 1995; Lesser and Lo, 1995; Levine et al., 1995). Suppression of Kv1.5, however, occurs rapidly upon exposure of cells to platelet-derived growth factor or fibroblast growth factor (Timpe and Fantl, 1994), and similar acute exposure to EGF suppresses Kv1.2 (Peralta, 1995). In both of these examples, PLCγ activation is required for suppression, although it is not known whether phosphorylation (tyrosine or otherwise) of the channel is involved.

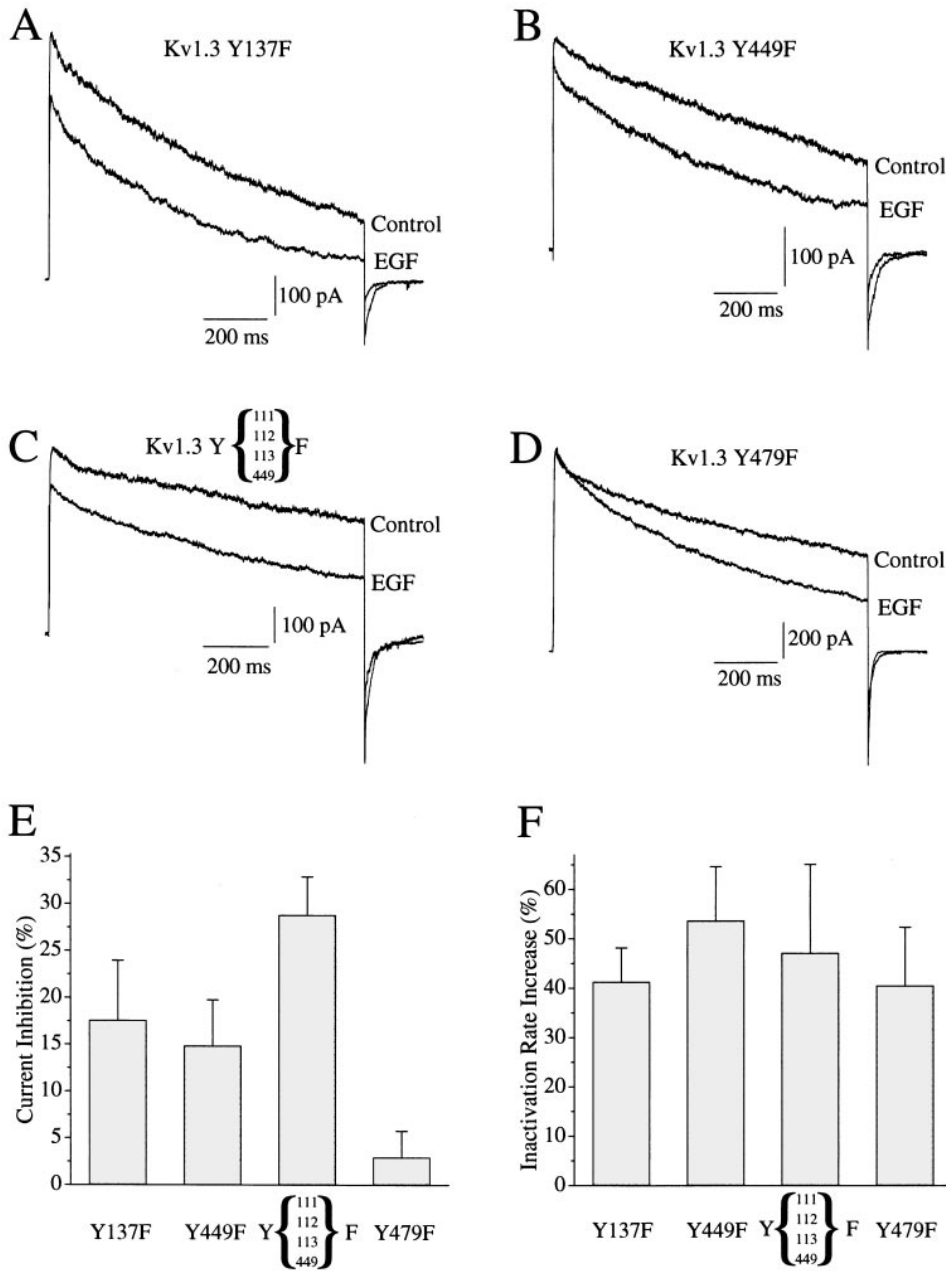Some of the effects of activation of the EGFr and the

FIGURE 8. EGF effects on peak current amplitude and inactivation rate are dissociable. Traces from cell-attached patches from cells expressing (A) Kv1.3 Y137F, (B) Kv1.3 Y449F, (C) Kv1.3 YYY111-113FFF and Y449F, or (D) Kv1.3 Y479F before (*Control*), and 25 min after (*EGF*) acute treatment with EGF (10 ng/ml) during patch recording. In each case, the mutant channel was coexpressed with the EGFr. (E) Mean decrease in current amplitude evoked by EGF for the four channel mutants shown in A–D. $n = 4$ for each mutant. (F) Mean increase in the inactivation rate evoked by EGF for the four channel mutants shown in A–D. $n = 4$ for each mutant.

insulin receptor on Kv1.3 current levels reported here may be due either to a change in channel activity or to a reduced number of channels on the cell surface, or both. Growth factor receptors internalize soon after activation (reviewed by Schlessinger and Ullrich, 1992), and this conceivably might result in nonspecific internalization of other membrane proteins, such as ion channels. The decrease in current observed with growth factor activation could be due in whole or in part to such internalization of the channel. Similarly, the reversal of current suppression by α-EGFr may reflect a larger amount of channel protein on the cell surface. These putative internalization effects may not be detectable on Western blots (unless internalization is cou-

pled with protein degradation), as this method measures protein in all compartments of the cell. However, the acute effects of EGF and insulin that occur during a patch recording are probably due to phosphorylation of the channel and not to internalization since: (*a*) the cell-attached patch is not exposed directly to EGF or insulin and hence receptors in the patch, immediately adjacent to the channels, do not bind ligand; and (*b*) internalization of membrane from the patch probably would disrupt the patch and interrupt the recording.

The effects of EGF and insulin may not all be due to direct phosphorylation of the channel by the receptors, especially given the fact that the patch of membrane containing the channels is isolated from direct contact

with the ligand by the recording electrode. The modulatory process therefore is likely to be dependent on intracellular factors in an endogenous signal transduction cascade. In the case of EGF, this cascade presumably results in phosphorylation of Y479, which causes the decrease in current amplitude, although serine/threonine protein kinases may also be involved in the pathway. In contrast to the effects on peak current, the change in inactivation kinetics induced by the EGFr may not be due to tyrosine phosphorylation of the channel, as the inactivation change still occurs when each of the tyrosine residues in good consensus sequences for tyrosine kinases is mutated. Changes in inactivation may be caused by phosphorylation of serine/threonine residues or other tyrosine residues, or by other non–phosphorylation-dependent mechanisms (e.g., Bowlby and Levitan, 1995).

The EGFr is expressed widely, and its activation is critical for signaling in many cell types (Threadgill et al., 1995), including neurons (Morrison et al., 1987; Kornblum et al., 1990). Activation of the EGFr in over-expressing cell lines leads to activation of phospholipase A2, activation of a voltage-independent calcium channel, and calcium influx (Peppelenbosch et al., 1991, 1992). This calcium influx leads, in turn, to activation of calcium-dependent potassium channels and subsequent hyperpolarization of the cell resting potential (Pandiella et al., 1989; Magni et al., 1991). Fundamental aspects of cell physiology are affected greatly by such fluctuations in the resting membrane potential. The modulation of Kv1.3 current by EGFr or insulin receptor activation will generally increase cell excitability and oppose any hyperpolarization-induced decrease in excitability. Thus, complex integration of signals at the level of individual ion channels could lead to changes in growth rates, calcium influx, and a host of other cellular phenomena.

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