

# Insulin Activates Native and Recombinant Large Conductance $\text{Ca}^{2+}$ -Activated Potassium Channels via a Mitogen-Activated Protein Kinase-Dependent Process

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## ABSTRACT

Evidence is accumulating that, in addition to regulating peripheral energy metabolism, insulin is an important modulator of neuronal function. Indeed, high levels of insulin and insulin receptors are expressed in several brain regions including the hippocampus. We have shown previously that insulin inhibits aberrant synaptic activity in hippocampal neurons via activation of large conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  (BK) channels. In this study, we have examined further the effects of insulin on native hippocampal and recombinant (hS/o) BK channels expressed in human embryonic kidney (HEK) 293 cells. Pipette or bath application of insulin evoked a rapid increase in hippocampal BK channel activity, an action caused by activation of insulin receptors because insulin-like growth factor 1 (IGF-1) failed to mimic insulin action. In parallel studies, insulin, applied via the pipette or bath, also activated hS/o channels expressed in HEK293 cells. Although phosphoinositide 3-kinase is a key component of insulin and IGF-1 receptor signaling pathways,

activation of this lipid kinase does not underlie the effects of insulin because neither 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) nor wortmannin inhibited or reversed insulin action. However, specific inhibitors of mitogen-activated protein kinase (MAPK) activation, 2'-amino-3'-methoxyflavone (PD98059) or 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene (U0126), attenuated insulin action, indicating that a MAPK-dependent mechanism underlies this process. Furthermore, insulin activation of this pathway enhances BK channel activity by shifting the  $\text{Ca}^{2+}$ -sensitivity such that BK channels are active at more hyperpolarized membrane potentials. Because postsynaptic BK channels are important regulators of neuronal hyperexcitability, insulin-induced activation of BK channels, via stimulation of a MAPK-dependent pathway, may be an important process for regulating hippocampal function under normal and pathological conditions.

Insulin is an important hormone that regulates energy balance through its actions on peripheral glucose homeostasis. However, insulin can also enter the CNS and act in the hypothalamus to regulate energy balance (Schwartz et al., 2000). In contrast, there is limited evidence that insulin can stimulate glucose uptake in neurons (Heidenreich et al., 1988). However, neurons can secrete insulin in a calcium-dependent manner when depolarized (Clarke et al., 1986). Insulin, insulin receptors, and crucial elements of insulin signaling pathways are expressed in CNS regions not generally associated with energy homeostasis, including the hip-

poampus, cerebellum, olfactory bulb, and cortex (Folli et al., 1994). Insulin receptor expression exhibits a very distinct and highly regionalized pattern of distribution during development and in the adult (Hanley, 1988). Despite this, little is known about the precise role of insulin and its receptors in neuronal function.

In the hippocampus, insulin receptors may play a key role in associative memory processes (Wickelgren, 1998). Indeed, cognitive deficits have been reported in patients with diabetes mellitus (Gispén and Biessels, 2000). Insulin resistance may also underlie the pathophysiology and clinical symptoms associated with Alzheimer's disease (Watson and Craft, 2003). In rodents, impairments in spatial memory tasks have been observed in diabetic models (Gispén and Biessels, 2000),

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**ABBREVIATIONS:** CNS, central nervous system; IGF-1, insulin-like growth factor 1; PI 3-kinase, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; BK, large conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel; hS/o, recombinant BK channel; I-V, current-voltage;  $[\text{Ca}^{2+}]_i$ , intracellular calcium concentration; HEK, human embryonic kidney;  $\text{NfP}_o$ , average channel activity (Nf, the number of functional channels;  $P_o$ , open-state probability); ANOVA, analysis of variance; PD98059, 2'-amino-3'-methoxyflavone; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene; U0124, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; NS-1619, 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2*H*-benzimidazol-2-one.

and increases in insulin receptor mRNA and protein have been detected in hippocampal tissue after spatial memory tasks (Zhao et al., 1999; Zhao and Alkon, 2001). At the cellular level, insulin modulates glutamatergic receptor-mediated synaptic transmission at hippocampal synapses (Liu et al., 1995; Zhou et al., 2001), actions that may reflect alterations in the cell surface expression of *N*-methyl-D-aspartate (Skeberdis et al., 2001) or  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (Zhou et al., 2001) receptors. Voltage-dependent potassium channels are also a potential target for insulin receptors because insulin promotes suppression of Kv1.3-dependent currents in olfactory bulb neurons (Fadool and Levitan, 1998).

Insulin has a high affinity for both insulin and the insulin-like growth factor 1 (IGF-1) receptors. These receptors have overlapping sequence homology, and both can activate the insulin signaling cascade (Siddle et al., 2001). Insulin binding to either receptor leads to phosphorylation of insulin receptor substrate proteins which promote the recruitment of the Src homology 2 domain-containing lipid kinase, phosphoinositide 3-kinase (PI 3-kinase; Shepherd et al., 1998). PI 3-kinase catalyzes phosphorylation of phosphoinositides on the 3-position to produce phosphatidylinositol-3-phosphates, in particular, phosphatidylinositol (3,4,5)trisphosphate. Downstream of PI 3-kinase, phosphatidylinositol (3,4,5)trisphosphate is used by a variety of second messengers including mitogen-activated protein kinase (MAPK), stress-activated protein kinase 2, p38 MAPK, and protein kinase B (Shepherd et al., 1998). Insulin can also directly activate the Ras-Raf-MAPK pathway independently of insulin receptor substrate proteins. The Ras-Raf-MAPK signaling cascade can regulate many cellular activities ranging from gene expression to programmed death. Insulin receptor-driven MAPK stimulation is thought to underlie insulin modulation of hippocampal learning and memory because increases in tyrosine phosphorylation of Shc and MAPK were observed after water maze training (Zhao et al., 1999). We have also shown that insulin inhibits hippocampal epileptiform-like activity via MAPK-driven activation of  $K_{ATP}$  and BK channels (O'Malley et al., 2003).

BK channels are composed of two subunits, a pore-forming  $\alpha$  subunit and a modulatory  $\beta$  subunit (Toro et al., 1998). The  $\beta$  subunits alter the apparent  $Ca^{2+}$  and voltage sensitivity of the  $\alpha$  subunit, modify channel kinetics, and alter the pharmacological properties of the channel (Behrens et al., 2000; Uebele et al., 2000). Four distinct  $\beta$  subunits have been identified, with  $\beta_4$  being prevalent in the CNS (Behrens et al., 2000; Meera et al., 2000). In hippocampal neurons, BK channels are activated during an action potential by membrane depolarization together with a rise in intracellular  $Ca^{2+}$  levels ( $[Ca^{2+}]_i$ ). The resulting potassium current causes action potential repolarization and the generation of the fast after-hyperpolarization (Lancaster and Nicoll, 1987), which delays recovery of the membrane potential to firing threshold. Therefore, BK channel activity is vital in determining action potential firing rates and burst firing patterns. Thus, agents that modulate BK channel activity may regulate the level of neuronal excitability in the hippocampus.

In this study we have explored the effects of insulin on both native BK channels in hippocampal neurons and on cloned human  $\alpha$  (*Slo*) subunit expressed in HEK293 cells. Here, we show that insulin increases the activity of both native and cloned BK channels, via activation of the Ras-Raf-MAPK

signaling pathway. This process represents a novel mechanism for controlling hippocampal hyperexcitability.

## Materials and Methods

**Cell Culture.** Cultures of hippocampal neurons were prepared using standard procedures as described previously (Shanley et al., 2001) and were maintained in serum replacement medium (SR2; Sigma-Aldrich, St. Louis, MO). In brief, rat pups between 1 and 3 days old were sacrificed by cervical dislocation, and the hippocampi were removed and washed in standard HEPES-buffered saline comprising 135 mM NaCl, 5 mM KCl, 1 mM  $CaCl_2$ , 1 mM  $MgCl_2$ , 10 mM HEPES, and 25 mM D-glucose, pH 7.3. The cells were then treated with a mixture of protease types X and XIV (both at 0.5 mg/ml) for 30 min at room temperature. Dissociated cells were plated onto sterile culture dishes (Falcon 3001), pretreated with poly(L-lysine) (20  $\mu$ g/ml for 1–2 h). Cultures were maintained in a humidified atmosphere of 5%  $CO_2$  and 95%  $O_2$  for up to 2 weeks. Recordings were made from neurons of between 3 and 14 days in culture. To avoid any nonspecific actions of insulin in the sera, neurons were washed extensively with normal saline before recordings.

HEK293 cells, stably transfected with the  $\alpha$  (*hSlo*) subunit of the BK channel (Ahring et al., 1997), were grown in Dulbecco's modified Eagle's medium with sodium pyruvate and glucose, supplemented with 10% (v/v) fetal calf serum and 1% (v/v) penicillin-streptomycin at 37°C in a humidified atmosphere of 95%  $O_2$  and 5%  $CO_2$ . Cells were passaged every 3 to 4 days, plated onto 3.5-cm diameter Petri dishes (Falcon 3001), and used 1 to 4 days after plating. The expressed  $\alpha$  subunit was KCNMA1 (GenBank accession number U11717).

**Electrophysiological Recording Analysis.** Experiments were performed using cell-attached and inside-out patch-clamp recordings to examine single-channel responses as described previously (Shanley et al., 2002a). Currents and voltages were measured using an Axopatch 200B amplifier. Single-channel data were analyzed for current amplitude ( $I$ ) and average channel activity ( $NfP_o$ ), where  $Nf$  is the number of functional channels and  $P_o$ , the open-state probability. In the majority of single-channel recordings, one to two channels were observed under control (no drugs) conditions. Single-channel data were recorded onto digital audiotapes and replayed for illustration on a Gould chart recorder. All data are expressed as means  $\pm$  S.E.M., and statistical analyses were performed using a paired  $t$  test (two-tailed; 95% confidence interval) or a Student's  $t$  test for comparison of means, or two-way ANOVA (analysis of variance) for comparisons between multiple groups (unless otherwise stated).  $P$  values were obtained using the raw data.

Recording electrodes were pulled from borosilicate glass capillaries and had resistances of 7 to 12 M $\Omega$  when filled with electrolyte solution comprising 140 mM KCl, 1 mM  $CaCl_2$ , 1 mM  $MgCl_2$ , 10 mM HEPES, pH 7.2. For cell-attached recordings, the bath solution was normal saline: 135 mM NaCl, 5 mM KCl, 1 mM  $MgCl_2$ , 1 mM  $CaCl_2$ , 10 mM HEPES, 10 mM D-glucose, pH 7.4, whereas for inside-out recordings, it contained 140 mM KCl, 1 mM  $MgCl_2$ , 7.97 mM  $CaCl_2$ , 10 mM EGTA, 10 mM HEPES (free  $[Ca^{2+}]$  of 1  $\mu$ M) or 140 mM KCl, 1 mM  $MgCl_2$ , 9.76 mM  $CaCl_2$ , 10 mM EGTA, 10 mM HEPES (free  $[Ca^{2+}]$  of 10  $\mu$ M). The free  $[Ca^{2+}]$  was calculated using the METLIG program (University of Bristol, Bristol, UK) and verified with a  $Ca^{2+}$ -sensitive electrode. All inside-out recordings were performed in the absence of ATP. All solution changes were achieved by superfusing the bath with a gravity feed system (stop-flow system) at a rate of 10 ml/min, which allowed complete bath exchange within 2 min. All onset times were corrected for exchange beginning after a dead time of 20 to 30 s. All experiments were performed at room temperature (22–25°C). The mean channel activity ( $NfP_o$ ) was calculated from a 2-min segment (or a minimum of 200 transitions) in cell-attached conditions and a 30-s section during inside-out recordings. To enable comparison of  $V_{1/2}$  (voltage for half-maximal activation) values under control conditions and after insulin treatment, cell-

attached recordings were initially performed in either the absence (control) or presence of insulin in the pipette solution. In both conditions, recordings were maintained for at least 15 min to ensure maximal activation of channels by insulin, before obtaining the inside-out configuration.

**Materials.** PD98059 was obtained from Tocris Cookson Inc. (Bristol, UK), whereas U0126, U0124, wortmannin, and LY294002 were obtained from Calbiochem (San Diego, CA). NS-1619 was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA), and H-7, genistein, and diadzein were obtained from Sigma-Aldrich. Human recombinant insulin (95–98% purity) and insulin-like growth factor-1 (IGF-1; >97% purity; Sigma-Aldrich) were prepared as stock solution in normal saline and were further diluted in normal saline containing 0.2% bovine serum albumin as a carrier.

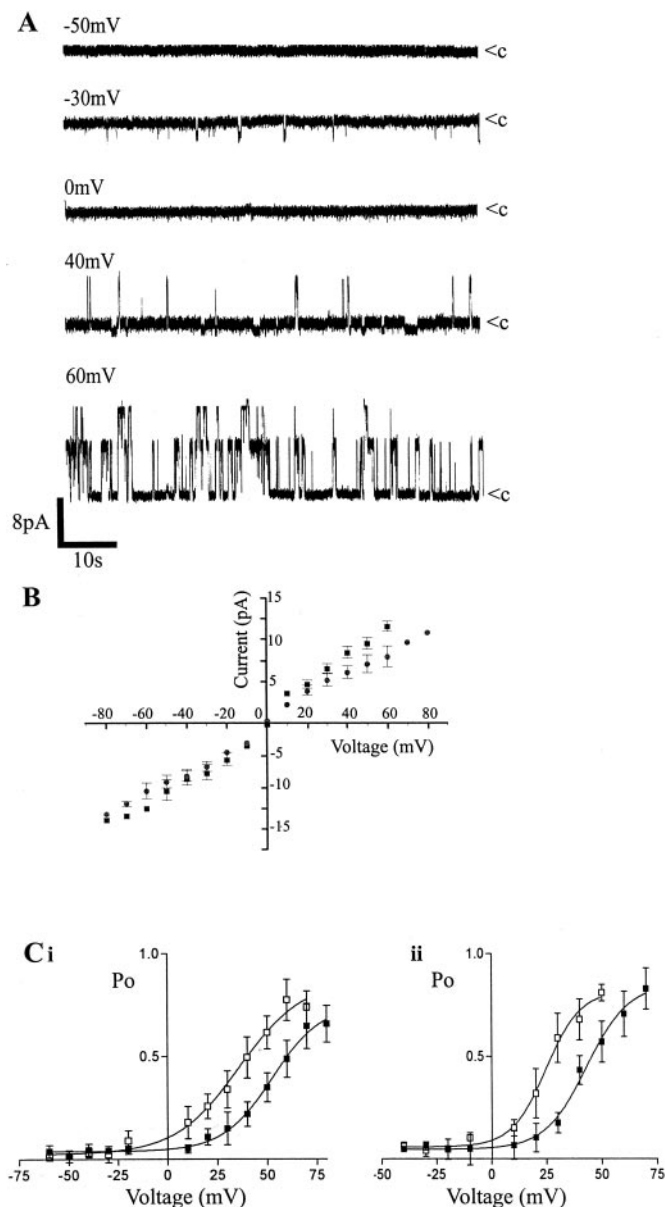
## Results

**BK Channels Are Expressed in Hippocampal Neurons.** In hippocampal neurons (3–14 days in culture), a large conductance channel was evident in most cell-attached recordings. In excised inside-out recordings performed in symmetrical 140 mM  $K^+$  conditions, this channel displayed a linear current-voltage (I-V) relationship ( $\pm 80$ mV), with a mean slope conductance of  $204 \pm 11.6$  pS ( $n = 5$ ; Fig. 1B), and as expected for a  $K^+$ -selective channel, the reversal potential was close to 0 mV. The channel also displayed sensitivity to both voltage and intracellular  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ), such that the open probability ( $P_o$ ) increased as the membrane potential was depolarized or the  $[Ca^{2+}]_i$  level increased. For example, the  $V_{1/2}$  values calculated from a Boltzmann plot were  $52.6 \pm 1.4$  mV at  $1 \mu M$   $Ca^{2+}$  ( $n = 4$ ) and  $36.1 \pm 2.9$  mV at  $10 \mu M$   $Ca^{2+}$  ( $n = 4$ ), respectively (Fig. 1Ci). Thus, on the basis of these findings, this large conductance  $K^+$  channel present in hippocampal neurons was identified as BK (Shanley et al., 2002a; Wann and Richards, 1994).

**Expression of *hSlo* in HEK293 Cells.** It is well known that BK channels are composed of a pore-forming  $\alpha$  subunit (*Slo*), with or without one of four regulatory  $\beta$  subunits (Toro et al., 1998). In the CNS, BK channels containing the  $\beta 4$  subunit are prevalent (Meera et al., 2000). However, neuronal BK channels that lack a  $\beta$  subunit also exist. Indeed, we have shown previously that BK channels, comprising the  $\alpha$  (*hSlo*) subunit alone, are the likely molecular target for leptin action in hippocampal neurons and HEK293 cells (Shanley et al., 2002a). Thus, to determine whether insulin was capable of activating recombinant BK channels comprising only *hSlo*, experiments were performed using a human embryonic kidney cell line stably expressing the  $\alpha$  subunit. In all cell-attached recordings, a large conductance potassium channel was evident ( $n = 164$ ). The channel displayed a linear I-V relationship ( $\pm 80$ mV) with a mean slope conductance of  $214 \pm 14.5$  pS ( $n = 6$ ) and reversal potential close to 0 mV, similar to the channel present in hippocampal neurons (Fig. 1A). Channel open probability ( $P_o$ ) increased with membrane potential depolarization or increasing  $[Ca^{2+}]_i$ , such that the  $V_{1/2}$  values obtained in  $1 \mu M$  and  $10 \mu M$   $Ca^{2+}$  were  $49.1 \pm 1.6$  mV ( $n = 5$ ) and  $34.7 \pm 1.6$  mV ( $n = 4$ , Fig. 1Ci), respectively. These properties are characteristic of BK channels.

**Insulin Activates BK Channels in Hippocampal Neurons and *hSlo* in HEK293 Cells.** In hippocampal neurons, bath application of insulin (50 nM) during cell-attached recordings (with +20 mV applied to the pipette) caused a

slowly developing increase in BK channel activity that was sustained in the continued presence of insulin for up to 45 min ( $n = 5$  of 6). There was a delay of 5 to 12 min (mean  $8.8 \pm 1.3$  min;  $n = 6$ ) before channel activity increased, such that  $NfP_o$  (mean channel activity) increased from  $0.04 \pm 0.01$  (at 2–4 min) to  $0.15 \pm 0.05$  (15 min after insulin application;  $n = 6$ ;  $P < 0.05$ ; Fig. 2Di). Application of insulin (10–50 nM) via the patch pipette resulted in a more rapid increase in BK channel activity (within 2–8 min; mean  $4.9 \pm 0.3$  min;  $n = 25$ ) in 25 of 29 cells. Thus, the mean channel activity increased from a control value of  $0.07 \pm 0.02$  (within 2–4 min of

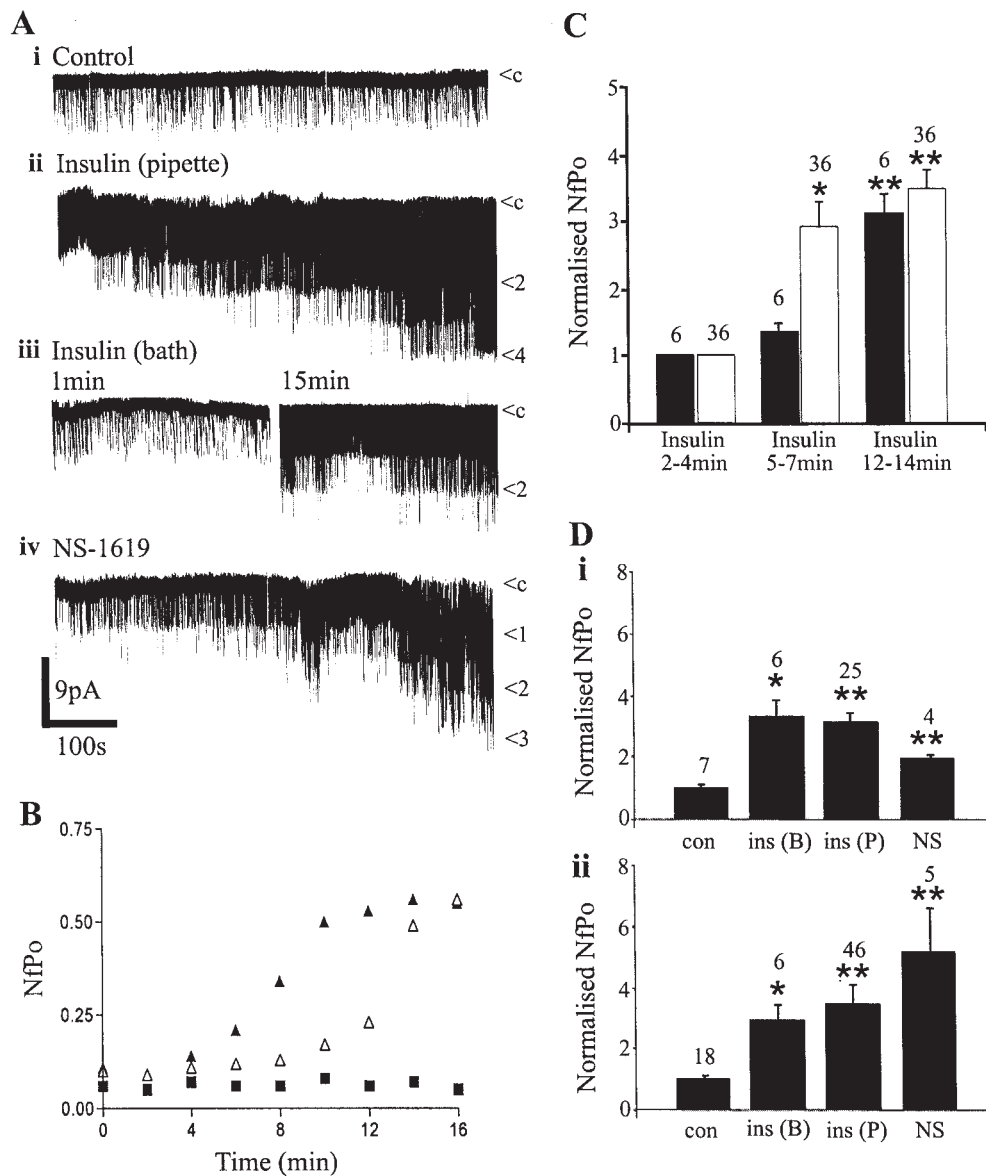


**Fig. 1.** Biophysical properties of native and recombinant BK channels. A, sample traces of inside-out recordings from hippocampal neurons performed in symmetrical (140 mM KCl) bathing conditions and in the presence of  $1 \mu M$   $Ca^{2+}$ . The probability of channel opening increases with membrane depolarization. B, pooled current-voltage relationship of the native (hippocampal) BK channel in  $1 \mu M$   $Ca^{2+}$  (●) and  $10 \mu M$   $Ca^{2+}$  (■). Ci and Cii, plot of the effects of membrane potential upon channel  $P_o$  as a function of intracellular  $Ca^{2+}$ . The fitted lines are the lines of best fit to a Boltzmann distribution for inside-out recordings in the presence of  $1 \mu M$   $Ca^{2+}$  (●) and  $10 \mu M$   $Ca^{2+}$  (□) for channels recorded in HEK293 cells (i) and hippocampal neurons (ii).

obtaining the cell-attached configuration) to  $0.18 \pm 0.04$ , 15 min after application of insulin ( $n = 25$ ,  $P < 0.001$ ). Control recordings (with no insulin in the bath or pipette) displayed no change in channel activity with time (Fig. 2Di). Thus, the mean  $NfP_o$  value at 2 to 4 min ( $0.057 \pm 0.016$ ) did not differ significantly from that obtained after 15 min of recording ( $0.056 \pm 0.018$ ;  $n = 7$ ;  $P > 0.05$ ). The increase in mean channel activity was caused by an increase in the mean open time ( $\tau_o$ ) of the channel because insulin (10 nM; pipette) increased  $\tau_o$  from  $0.76 \pm 0.11$  ms (at 2–4 min) to  $2.01 \pm 0.47$  ms (at 15–17 min;  $n = 7$ ;  $P < 0.05$ ), whereas there was no significant change in the mean channel closed time ( $\tau_c$ ) after exposure to insulin ( $n = 7$ ). Because BK channels are generally activated during an action potential, we also compared the actions of insulin in cell-attached recordings with +60 mV applied to the pipette (to mimic a neuron close to 0 mV, assuming the resting membrane potential of hippocampal neurons is around -60 mV). Under these conditions, application of insulin (10 nM; via the pipette) also resulted in an increase in BK channel activity (data not illustrated), such

that mean  $NfP_o$  values increased from  $0.086 \pm 0.04$  (at 2–4 min) to  $0.37 \pm 0.15$  after 15 to 17 min of exposure to insulin ( $n = 5$ ;  $P < 0.05$ ). Control recordings (with no insulin) at this applied potential also displayed no change in channel activity with time, such that the mean  $NfP_o$  values obtained at the 2- to 4-min and 15- to 17-min time points were  $0.197 \pm 0.12$  and  $0.186 \pm 0.12$ , respectively ( $n = 5$ ;  $P > 0.05$ ). Pipette application of the selective BK channel opener, NS-1619 (1  $\mu$ M), also caused an increase in mean channel activity from a control  $NfP_o$  value of  $0.03 \pm 0.01$  to  $0.07 \pm 0.02$  ( $n = 4$ ,  $P < 0.05$ ; Fig. 2Di).

Likewise, bath application of insulin (50 nM) during cell-attached recordings caused an increase in BK channel activity in HEK293 cells expressing *hSlo* ( $n = 6$ ), such that the  $NfP_o$  value at 2 to 4 min ( $0.052 \pm 0.02$ ) was increased to  $0.13 \pm 0.03$ , 15 min later ( $n = 6$ ,  $P < 0.05$ , Fig. 2, Aiii and Dii). Channel activation occurred within 5 to 11 min (mean  $8.2 \pm 0.7$  min) of insulin application ( $n = 6$ ; Fig. 2C). Application of insulin (1–50 nM) via the pipette also caused a rapid increase in channel activity ( $n = 42$  of 46 cells). Channel



**Fig. 2.** Insulin activates recombinant (*hSlo*) and native BK channels. A, representative traces of cell-attached recordings from individual HEK293 cells at +10 mV applied to the recording pipette. Ai, under control conditions, there was no change in BK channel activity with time. Aii and Aiii, application of insulin (10 nM) via the patch pipette (P; Aii) or bath (B; Aiii) caused an increase in channel activity that was maintained in the presence of insulin. Aiv, pipette application of the BK channel activator, NS-1619 (10  $\mu$ M), also readily increased the activity of BK channels. B, Plot of the corresponding mean channel activity ( $NfP_o$ ) versus time relations obtained from the experiments illustrated in A (i–iii). Pipette application of insulin (▲) activated BK channels faster than bath application did (△). Control recordings (■) displayed no change in channel activity with time. C, plot of pooled data from HEK293 cells illustrating the pooled normalized  $NfP_o$  values obtained 2 to 4 min after the start of recording, and 5 to 7 min and 12 to 14 min after insulin addition, for bath application (filled bars) compared with pipette addition (open bars). Asterisks represent  $P < 0.05$  (one-way ANOVA, repeated measures) relative to the control  $NfP_o$  values obtained 2 to 4 min after the initiation of recordings. D, plots of the pooled data obtained 12 to 14 min after insulin addition, showing the relative effects of insulin [bath (B; 50 nM) or pipette (P; 10 nM) application] and NS-1619 (NS; 1  $\mu$ M) on BK channel activity in hippocampal neurons (Di) and HEK293 cells (Dii). All values represent data normalized to a control period, 2 to 4 min after obtaining the cell-attached recording configuration. The  $n$  values for each experimental condition are shown above each histogram bar, and \* and \*\* represent  $P < 0.05$  and  $P < 0.01$  compared (paired  $t$  test) to the 2- to 4-min time point in the same cell.

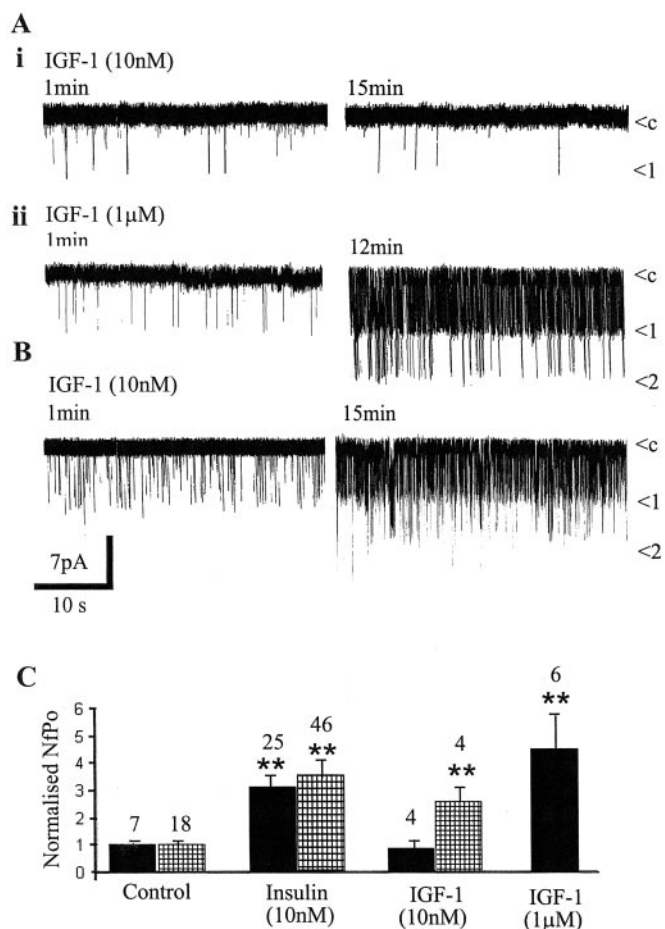
activation was seen within 2 to 8 min (mean  $5.3 \pm 0.3$  min;  $n = 35$ ), and  $NfP_o$  increased from  $0.09 \pm 0.02$  (2–4 min) to  $0.31 \pm 0.08$  ( $n = 46$ ,  $P < 0.001$ ) 15 min later (Fig. 2Aii). Control cell-attached recordings (with no insulin present in the bath or pipette) displayed no change in channel activity with time (Fig. 2Ai). Thus, the  $NfP_o$  values obtained at 2 to 4 min ( $0.113 \pm 0.045$ ;  $n = 18$ ) did not differ significantly from those values obtained after 15 min of recording ( $0.115 \pm 0.053$ ;  $n = 18$ ;  $P > 0.05$ ). Direct activation of BK channels by application of the BK channel opener, NS-1619 ( $1 \mu\text{M}$ ), had an effect comparable with that of insulin, increasing BK mean channel activity from a value of  $0.129 \pm 0.034$  (at 2–4 min) to  $0.499 \pm 0.061$  (at 12–14 min;  $n = 5$ ,  $P < 0.05$ ; Fig. 2, Aiv and Dii).

**Insulin Activates Hippocampal BK Channels via Stimulation of Insulin Receptors.** Because insulin has an overlapping affinity for both insulin and IGF-1 receptors, the next series of experiments determined which receptor mediates insulin action on hippocampal BK channels. In the absence of selective antagonists for either receptor, a comparison of the potencies of insulin and IGF-1 for BK channel activation was used for receptor identification (Weiland et al., 1991). Application of insulin ( $10 \text{ nM}$ ) to the hippocampal neurons via the patch pipette caused  $NfP_o$  to increase from  $0.07 \pm 0.02$  (2–4 min after obtaining the cell-attached configuration) to  $0.18 \pm 0.04$ , 15 to 17 min later ( $n = 25$ ,  $P < 0.001$ ). In contrast, IGF-1 ( $10 \text{ nM}$ ) had little effect on BK channel activity such that the mean channel activity at 2 to 4 min ( $0.04 \pm 0.01$ ) did not differ significantly from that obtained 15 to 17 min later ( $0.03 \pm 0.01$ ;  $n = 4$ ,  $P > 0.05$ ; Fig. 3, A and C). However, at a concentration of  $1 \mu\text{M}$ , IGF-1 did result in an increase in channel activity from a control value of  $0.035 \pm 0.01$  to  $0.14 \pm 0.04$  ( $n = 6$ ,  $P < 0.05$ , Fig. 3, A and C). Because it is possible that differences in the potency of IGF-1 compared with insulin may be caused by differences in the voltage-dependence for channel activation, the effects of IGF-1 were also examined in cell-attached patches with  $+60 \text{ mV}$  applied to the pipette. At this voltage, application of IGF-1 ( $1 \mu\text{M}$ ; via the pipette) increased BK channel activity, such that the mean channel activity obtained 2 to 4 min after obtaining the cell-attached configuration and after 15 to 17 min of exposure to IGF-1 were  $0.05 \pm 0.01$  and  $0.115 \pm 0.03$ , respectively ( $n = 6$ ,  $P < 0.05$ ; data not illustrated). Together, these data indicate that in hippocampal neurons, insulin increases BK channel activity via activation of insulin receptors.

**Insulin Stimulates hSlo via Insulin or IGF-1 Receptor Activation in HEK293 Cells.** The potencies of IGF-1 and insulin on hSlo were also examined to determine the identity of the receptor underlying insulin action in HEK293 cells. In contrast to hippocampal neurons, pipette application of IGF-1 ( $10 \text{ nM}$ ) and insulin ( $10 \text{ nM}$ ) had comparable effects on BK channel activity. Thus, in the presence of insulin,  $NfP_o$  at 12 to 14 min was  $3.51 \pm 0.56$  ( $n = 46$ ,  $P < 0.01$ ), whereas  $NfP_o$  at 12 to 14 min after application of IGF-1 was  $2.74 \pm 0.37$  ( $n = 4$ ,  $P < 0.01$ , normalized to the control values at 2–4 min; Fig. 3, B and C). These data suggest that insulin modulation of BK channel activity is mediated by either insulin or IGF-1 receptor activation in HEK293 cells.

**BK Channel  $\text{Ca}^{2+}$ -Dependence, but Not Unitary Conductance, Is Altered after Insulin Action.** We have shown previously that the obese gene product, leptin, activates native BK channels in cultured hippocampal neurons

(Shanley et al., 2002a). After activation by leptin, the biophysical characteristics of these channels was altered such that there was a significant shift in the BK channel  $\text{Ca}^{2+}$ -sensitivity. Thus, in the next series of experiments, the effects of insulin on the properties of BK channels were investigated. After BK channel activation by insulin in the cell-attached configuration ( $10 \text{ nM}$ ; applied for 15 min via the patch pipette), subsequent inside-out recordings displayed a small, but significant shift in the BK channel apparent  $\text{Ca}^{2+}$ -sensitivity compared with control (no insulin) recordings. Thus, after exposure to insulin, the  $V_{1/2}$  values obtained in  $1 \mu\text{M}$   $\text{Ca}^{2+}$  and  $10 \mu\text{M}$   $\text{Ca}^{2+}$  were  $29.7 \pm 3.3 \text{ mV}$  ( $n = 5$ ) and  $13.1 \pm 4.9 \text{ mV}$  ( $n = 3$ ), respectively ( $P < 0.05$ ; Student's  $t$  test; Fig. 4C). This apparent alteration in the  $\text{Ca}^{2+}$ -sensitivity of the channel was not accompanied by any change in the chan-



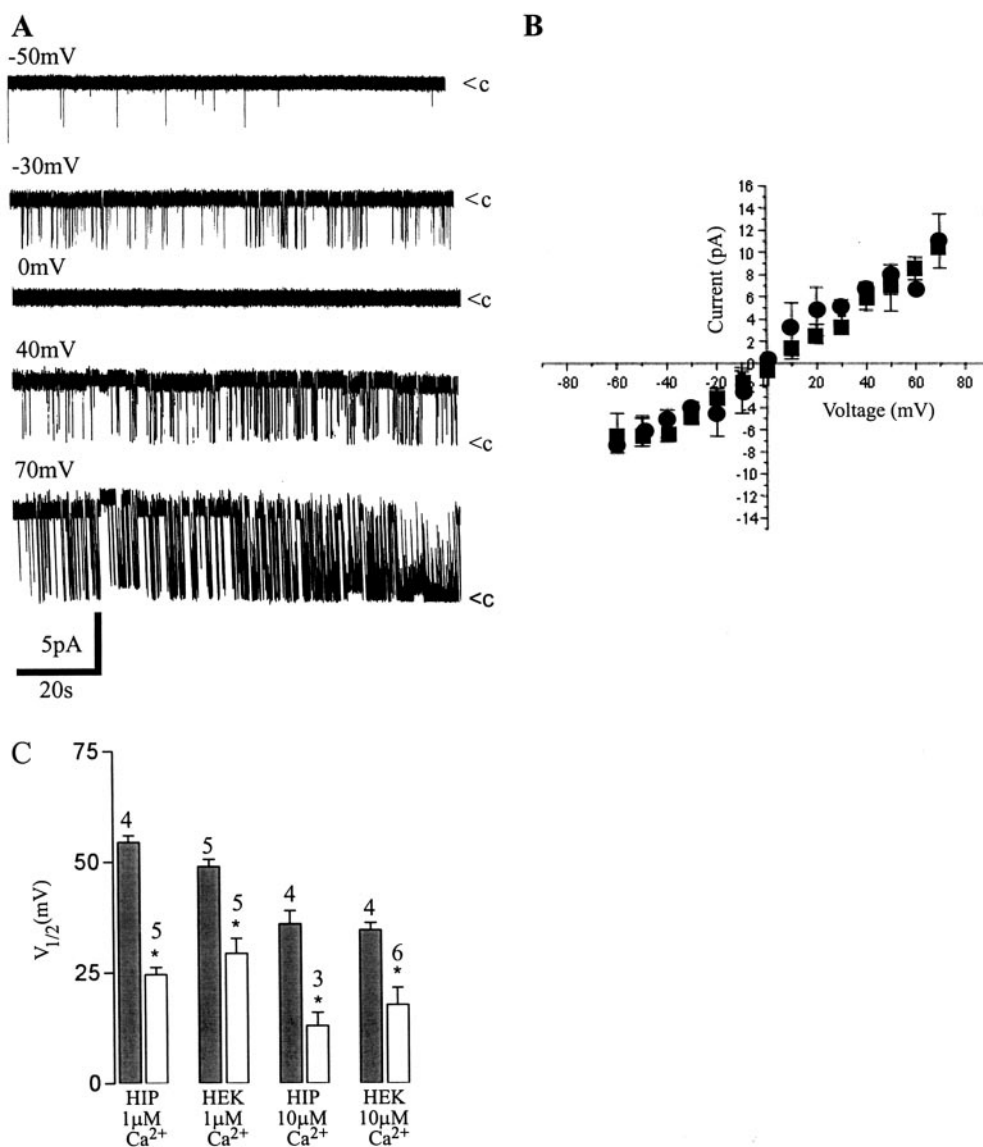
**Fig. 3.** Insulin and IGF-1 effects on native and recombinant BK channels. A, sample cell-attached recordings from hippocampal neurons with  $+20 \text{ mV}$  applied to the recording pipette. Pipette application of IGF-1 ( $10 \text{ nM}$ ) to hippocampal neurons had no effect on BK channels (Ai). In contrast, IGF-1 ( $1 \mu\text{M}$ ) evoked an increase in BK channel activity that was maintained in the presence of IGF-1 (Aii). B, Sample cell-attached recordings obtained from HEK293 cells at  $+10 \text{ mV}$  applied potential. In these cells, pipette application of IGF-1 ( $10 \text{ nM}$ ) mimicked insulin action such that an increase in BK channel activity was observed. C, pooled data obtained after 15 min of exposure to insulin or IGF-1, showing the normalized effects of insulin, IGF-1 ( $10 \text{ nM}$ ), and IGF-1 ( $1 \mu\text{M}$ ) on channel activity [normalized to the  $NfP_o$  values obtained at 2 to 4 min in the same cell in either hippocampal neurons (filled bars) or HEK293 cells (hatched bars)]. The  $n$  values for each experimental condition are shown above each histogram bar, and \* and \*\* represent  $P < 0.05$  and  $P < 0.01$ , respectively (paired  $t$  test) compared with the corresponding  $NfP_o$  values at the 2- to 4-min time point in the same cell.

nel slope conductance ( $207 \pm 9.6$  pS;  $n = 7$ ) calculated from the I-V relationships obtained in symmetrical 140 mM  $K^+$  conditions (Fig. 4B). Insulin treatment also failed to alter the I-V relations in asymmetrical (140 mM  $K^+$  in pipette and 3.5 mM  $K^+$  in bath) conditions (not illustrated).

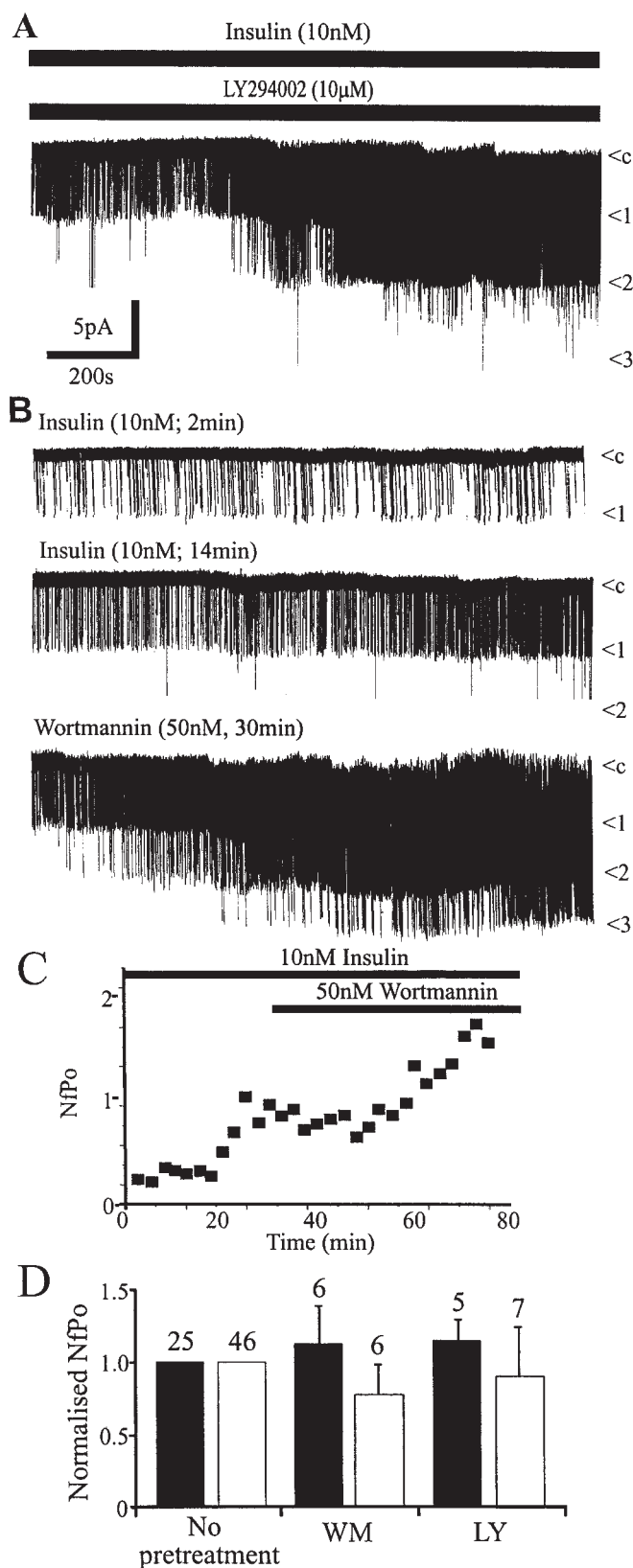
In parallel with native BK channels, the apparent  $Ca^{2+}$ -sensitivity of hSlo was altered after exposure to insulin, such that the  $V_{1/2}$  values were more negative at the same free  $Ca^{2+}$  concentrations. Thus, the  $V_{1/2}$  values obtained in 1  $\mu$ M and 10  $\mu$ M  $Ca^{2+}$  were  $36.4 \pm 4.5$  mV ( $n = 5$ ) and  $17.8 \pm 3.9$  mV ( $n = 6$ ), respectively ( $P < 0.05$ ; Student's  $t$  test; Fig. 4, A and C). This action of insulin was not accompanied by any change in BK single-channel conductance because the slope conductance obtained from the current-voltage plots in symmetrical conditions ( $206 \pm 10.7$  pS;  $n = 14$ ) did not differ significantly from control values ( $P > 0.05$ ; Student's  $t$  test; data not illustrated). Together, these data suggest that in a manner similar to that of leptin, insulin adjusts the apparent BK channel  $Ca^{2+}$ -sensitivity, without altering the single-channel unitary conductance.

**Activation of Native BK Channels by Insulin Involves a PI 3-Kinase-Independent Process.** It is well

established that PI 3-kinase is a key enzyme, associated with the signaling downstream of the insulin receptor (Shepherd et al., 1998). Furthermore, leptin receptor-driven activation of BK channels involves a PI 3-kinase-dependent process (Shanley et al., 2002a,b). Thus, the effects of two structurally unrelated PI 3-kinase inhibitors, LY294002 and wortmannin, on insulin action were examined. To determine the effects of PI 3-kinase blockade, hippocampal cultures were incubated with either LY294002 (10  $\mu$ M) or wortmannin (50 nM) for 30 min before obtaining the cell-attached recording configuration and subsequent application of insulin via the patch pipette. Neither application of wortmannin (50 nM) nor of LY294002 (10  $\mu$ M) had any effect on channel activity per se. Thus, during control cell-attached recordings (with no insulin present), neither agent significantly altered channel activity, such that the  $NfP_o$  values obtained after 15 min of incubation were  $0.28 \pm 0.003$  ( $n = 4$ ; wortmannin) and  $0.23 \pm 0.16$  ( $n = 5$ ; LY294002), compared with  $0.30 \pm 0.12$  ( $n = 4$ ; wortmannin) and  $0.25 \pm 0.19$  ( $n = 5$ ; LY294002) at 2 to 4 min, respectively ( $P > 0.05$ ). Furthermore, inhibition of PI 3-kinase activity failed to attenuate the actions of insulin (Fig. 5, A and D). Thus, in the presence of either wortmannin



**Fig. 4.** Characteristics of BK channels after insulin activation. A, sample traces of inside-out recordings from a HEK293 cell obtained in symmetrical 140 mM KCl conditions and in the presence of 1  $\mu$ M  $Ca^{2+}$ , after BK channel activation by insulin. Note that in this cell, a small conductance channel is evident at +40 and +70 mV. B, pooled current-voltage relationship of the channel obtained under control conditions (●) or after insulin stimulation (■; obtained 12 to 14 min after activation by insulin) in 1  $\mu$ M  $Ca^{2+}$ . C, pooled data illustrating the mean  $V_{1/2}$  values obtained in the presence of 1  $\mu$ M or 10  $\mu$ M  $Ca^{2+}$ , in cells under control conditions (filled bars), and in distinct cells after exposure to 10 nM insulin (open bars) in both hippocampal neurons (HIP) and HEK293 cells (HEK). The numbers above the histograms represent the  $n$  values, and \* represents  $P < 0.05$  (Student's  $t$  test) for insulin-treated cells compared with control (no insulin treatment) cells under similar conditions.



**Fig. 5.** Insulin activates BK channels via a PI 3-kinase-independent process. **A**, sample trace of a single cell-attached recording obtained from a hippocampal neuron with +20 mV applied to the recording pipette. Prior incubation with the PI 3-kinase inhibitor, LY294002 (10  $\mu$ M), for the time indicated had no effect on the ability of insulin to stimulate BK channel activity. **B**, cell-attached recordings (with +20 mV applied to the recording pipette) obtained from a hippocampal neuron 2 min and 14 min

or LY294002, insulin (10 nM) caused an increase in mean channel activity to  $3.34 \pm 0.59$  ( $n = 6$ ,  $P < 0.05$ ) and  $3.39 \pm 0.42$  ( $n = 5$ ,  $P < 0.01$ , normalized to control recordings at 2–4 min), respectively, after 15 min. Comparable levels of BK channel activation were also observed when insulin was applied alone ( $2.93 \pm 0.33$ ;  $n = 25$ ; paired  $t$  test;  $P < 0.001$ ). In addition, neither wortmannin nor LY294002 reversed the actions of insulin (Fig. 5, B and C). Thus, the mean channel activities under control conditions, after activation by insulin (15–17 min) and after 30 min of incubation with wortmannin (50 nM) were  $0.02 \pm 0.01$ ,  $0.05 \pm 0.02$ , and  $0.098 \pm 0.04$ , respectively ( $n = 5$ ; not significantly different,  $P > 0.05$ ; two-way ANOVA; Fig. 5, B and C), whereas the parallel  $NfP_o$  values obtained at similar time points before and after activation with insulin and 30 min after addition of LY294002 were  $0.04 \pm 0.02$ ,  $0.19 \pm 0.14$ , and  $0.51 \pm 0.41$ , respectively ( $n = 4$ ; not significantly different,  $P > 0.05$ ; two-way ANOVA). Together, these data indicate that the increase in BK channel activity in hippocampal neurons induced by insulin involves a PI 3-kinase-independent mechanism.

**Insulin Activates *hSlo* via a PI 3-Kinase-Independent Process.** Because the signaling pathways used by IGF-1 receptors parallel those activated by insulin receptors (Siddle et al., 2001), we also examined the role of PI 3-kinase in insulin action in HEK293 cells. Incubation of HEK293 cells with the PI 3-kinase inhibitors, LY294002 (10  $\mu$ M) or wortmannin (10–50 nM), had no effect on BK channel activity per se, so that the mean  $NfP_o$  values obtained within 2 to 4 min of achieving the cell-attached configuration were  $0.05 \pm 0.02$  (LY294002 control;  $n = 4$ ) and  $0.12 \pm 0.06$  (wortmannin control;  $n = 4$ ), whereas after 15 min of incubation with either LY294002 or wortmannin (after achieving the cell-attached mode), the  $NfP_o$  values were  $0.04 \pm 0.02$  ( $n = 4$ ,  $P > 0.05$ ) and  $0.11 \pm 0.06$  ( $n = 4$ ,  $P > 0.05$ ), respectively. However, exposure of the HEK293 cells to either PI 3-kinase inhibitor, for at least 30 min, failed to inhibit the ability of insulin to activate BK channels. Thus, the normalized  $NfP_o$  values obtained after incubation with LY294002 or wortmannin and after 15 min of pipette application of insulin (10 nM) were  $3.45 \pm 0.81$  ( $n = 7$ ,  $P < 0.05$ ) and  $2.89 \pm 0.71$  ( $n = 6$ ,  $P < 0.01$ ), respectively, compared with a control  $NfP_o$  value of  $3.76 \pm 0.68$  ( $n = 46$ ,  $P < 0.01$ ; Fig. 5D), obtained after exposure to insulin (10 nM) in the absence of either PI 3-kinase inhibitor. Furthermore, neither LY294002 nor wortmannin was able to reverse insulin-induced BK channel activation in this cell line. Thus, pipette application of insulin (10 nM) increased BK channel activity from a control value of  $0.076 \pm 0.04$  (at 2–4 min) to  $0.20 \pm 0.11$  (15–17 min after insulin addition). Subsequent addition of LY294002 (10  $\mu$ M)

after insulin addition, and after 30 min of incubation with wortmannin (and 50 min after insulin addition). Application of wortmannin after insulin-induced BK channel activation failed to reverse insulin action. **C** illustrates the corresponding diary plot of  $NfP_o$  against time for this experiment. **D**, pooled data, obtained after 15 to 17 min of exposure to insulin and after at least 30 min of incubation with either PI 3-kinase inhibitor, showing the normalized effects of prior treatment of either wortmannin or LY294002 on insulin-induced BK channel activation (normalized to control stimulation of BK channel activity by insulin in distinct cells) in hippocampal neurons (filled bars) and HEK293 cells (open bars). There was no significant difference in the ability of insulin to stimulate BK channels under control conditions (no pretreatment), or in the presence of either wortmannin or LY294002 (one-way ANOVA plus Tukey's post hoc test). The  $n$  values for each experimental condition are depicted above the histogram bars.

failed to reverse BK channel activity to preinsulin levels but may have, in fact, enhanced channel activity because the  $NfP_o$  value obtained after 30 min of exposure to LY294002 was  $1.02 \pm 0.85$  ( $n = 4$ ; not significantly different,  $P > 0.05$ ; two-way ANOVA). Likewise, wortmannin failed to reverse insulin-induced BK channel activation such that the  $NfP_o$  values obtained under control (2–4 min) conditions, after 15 min of pipette application of 10 nM insulin and after 30 min of incubation with wortmannin (50 nM) were  $0.080 \pm 0.035$ ,  $0.16 \pm 0.06$ , and  $0.98 \pm 0.64$ , respectively ( $n = 4$ ; not significantly different,  $P > 0.05$ ; two-way ANOVA; not illustrated). Thus, these data indicate that insulin modulation of BK channel activity in HEK293 cells involves a signaling mechanism that is independent of PI 3-kinase.

**A MAPK-Dependent Pathway Underlies Insulin-Induced Activation of Native and Recombinant BK Channels.** The Ras-Raf-MAPK pathway is activated in parallel with PI 3-kinase and offers an alternative signaling cascade for insulin receptors. To address the role of the Ras-Raf-MAPK pathway in insulin action, the effects of specific inhibitors of MAPK activation, PD98059 and U0126, were investigated. In the absence of insulin, application of either agent had no effect on channel activity per se such that the normalized  $NfP_o$  values obtained after 15 min of incubation with PD98059 (10  $\mu$ M) or U0126 (1  $\mu$ M) were  $0.99 \pm 0.15$  ( $n = 4$ ;  $P > 0.05$ ) and  $0.83 \pm 0.27$  ( $n = 4$ ;  $P > 0.05$ ), respectively. However, unlike the PI 3-kinase inhibitors, preincubation with either PD98059 or U0126 attenuated the actions of insulin. Thus, in hippocampal neurons the  $NfP_o$  values obtained after 30 min of exposure to PD 98059 (10  $\mu$ M) and 2 to 4 min after obtaining cell-attached recordings ( $0.019 \pm 0.004$ ) did not differ significantly from those obtained 15 to 17 min later ( $0.021 \pm 0.008$ ;  $n = 4$ ;  $P > 0.05$ ; Fig. 6E). Likewise, U0126 (1  $\mu$ M) occluded insulin action with the corresponding  $NfP_o$  values of  $0.050 \pm 0.013$  (2–4 min;  $n = 5$ ) and  $0.050 \pm 0.009$  (15–17 min;  $n = 5$ ,  $P > 0.05$ ; Fig. 6E), respectively. Furthermore, incubation with the inactive analog of U0126, U0124 (1  $\mu$ M), had no inhibitory effect on the action of insulin because insulin (10 nM) increased channel activity to  $4.77 \pm 0.97$  ( $n = 5$ ,  $P < 0.05$ ; Fig. 6E) from a control value of  $1.3 \pm 0.33$  ( $n = 4$ ,  $P > 0.05$ , normalized to control values at 2–4 min). After insulin-induced activation of BK channels ( $NfP_o$  increased from  $0.095 \pm 0.06$  to  $0.20 \pm 0.14$ ) in cell-attached recordings, application of PD98059 (10  $\mu$ M) caused BK channel activity to return to preinsulin levels ( $0.035 \pm 0.002$ ,  $n = 4$ ; not significantly different,  $P > 0.05$ ; two-way ANOVA; Fig. 6, C–E). Together, these data indicate that the Ras-Raf-MAPK signaling pathway links insulin receptor activation with BK channel stimulation in hippocampal neurons.

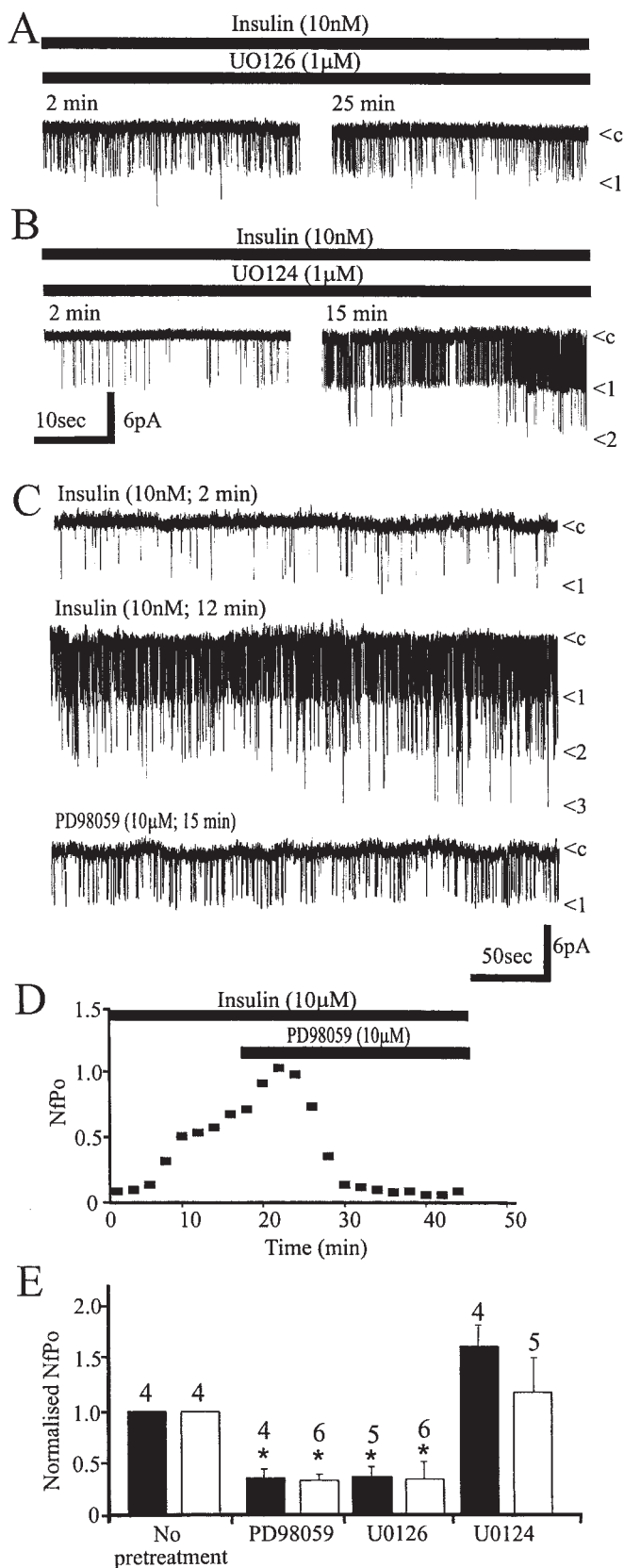
Because MAPK is required for insulin activation of native BK channels, the role of MAPK in insulin activation of hSlo was also examined. Application of either PD98059 (10  $\mu$ M) or U0126 (1  $\mu$ M) for 30 min had no effect on channel activity per se, such that the  $NfP_o$  values obtained under control conditions (2–4 min) and after 15 min of incubation with PD98059 were  $0.19 \pm 0.1$  and  $0.16 \pm 0.08$ , respectively ( $n = 4$ ,  $P > 0.05$ ). Likewise, the  $NfP_o$  values obtained in the absence and presence of U0126 (1  $\mu$ M) were  $0.10 \pm 0.06$  and  $0.096 \pm 0.06$ , respectively ( $n = 3$ ,  $P > 0.05$ ). However, prior exposure of HEK293 cells to either inhibitor of MAPK activation attenuated insulin action. Thus, after 30 min incubation with PD98059 or U0126, the  $NfP_o$  values obtained 2 to 4 min after

achieving the cell-attached configuration were  $0.09 \pm 0.03$  (PD98059) and  $0.099 \pm 0.05$  (U0126), respectively, and after 15 to 17 min, addition of insulin (10 nM) via the pipette resulted in values of  $0.10 \pm 0.02$  (PD98059;  $n = 6$ ,  $P > 0.05$ ) and  $0.13 \pm 0.06$  (U0126;  $n = 6$ ,  $P > 0.05$ ; Fig. 6, A and E), respectively. In contrast, the inactive analog of U0126, U0124 (1  $\mu$ M), did not attenuate the actions of insulin because the mean channel activities obtained in the presence of U0124 and after the 2- to 4-min and 15- to 17-min exposure to insulin were  $0.05 \pm 0.02$  and  $0.19 \pm 0.06$ , respectively ( $n = 5$ ,  $P < 0.05$ ; Fig. 6, B and E). Furthermore, after hSlo activation by insulin ( $NfP_o$  increased from  $0.06 \pm 0.02$  to  $0.19 \pm 0.06$ ) in cell-attached recordings, application of PD98059 (10  $\mu$ M) caused the channel activity to return to preinsulin levels ( $0.06 \pm 0.03$ ;  $n = 4$ ; not significantly different,  $P > 0.05$ ; two-way ANOVA). Thus, together, these data indicate that the ability of insulin to activate hSlo channels expressed in HEK293 cells involves a MAPK-dependent process.

**Insulin Activation of BK Channels Involves Tyrosine Dephosphorylation.** Because protein kinases are capable of phosphorylating serine/threonine residues of a range of proteins and the activity of BK channels can be modulated by protein kinases and phosphatases, we examined the role of serine/threonine kinases in insulin action using the broad-spectrum inhibitor, H-7. At the concentration examined (10  $\mu$ M), H-7 inhibits protein kinases A, C, and G. Application of H-7 (10  $\mu$ M) to the bath during cell-attached recordings (in the absence of insulin) had no effect on BK channels per se, such that the  $NfP_o$  values obtained under control conditions and after 15 to 17 min of exposure to H-7 were  $0.04 \pm 0.003$  and  $0.04 \pm 0.002$ , respectively ( $n = 5$ ,  $P > 0.05$ ). However, application of insulin (10 nM) via the patch pipette, after a prior bath incubation with H-7 (10  $\mu$ M) for at least 30 min, resulted in an increase in BK channel activity such that the mean channel activity obtained 2 to 4 min after obtaining the cell-attached configuration and after 15 to 17 min of exposure to insulin were  $0.04 \pm 0.001$  and  $0.082 \pm 0.02$ , respectively ( $n = 7$ ,  $P < 0.05$ ; Fig. 7A). Thus, blockade of serine/threonine-specific protein kinases with H-7 neither mimics nor occludes insulin activation of native BK channels.

To determine whether tyrosine phosphorylation underlies the actions of insulin, the effects of the broad-spectrum tyrosine kinase inhibitor, genistein, were investigated. Application of genistein (10–20  $\mu$ M) to the bath during cell-attached recordings resulted in activation of BK channels ( $n = 11$ ; Fig. 7B). Thus, after incubation with genistein (10–20  $\mu$ M) for 15–17 min, the mean channel activity had increased to  $0.298 \pm 0.41$  from a control value of  $0.148 \pm 0.199$  ( $n = 11$ ). Furthermore, application of insulin (either 10 nM via the pipette or 50 nM in the bath) after at least 20 min of incubation with genistein (10–20  $\mu$ M) failed to increase BK channel activity further. Thus, the  $NfP_o$  values obtained after incubation with genistein ( $0.067 \pm 0.06$ ) did not differ significantly from those obtained after subsequent 15- to 17-min exposure to insulin ( $0.075 \pm 0.06$ ;  $n = 12$ ,  $P > 0.05$ ; Student's  $t$  test). In contrast, application of daidzein (10  $\mu$ M); the inactive analog of genistein) to the bath during cell-attached recordings had no effect on BK channel activity per se ( $n = 4$ ; not illustrated). Thus, after incubation with daidzein for 15 to 17 min, the mean channel activity ( $0.03 \pm 0.01$ ;  $n = 4$ ) was not significantly different from that obtained in the absence of this agent ( $0.028 \pm 0.01$ ;  $n = 4$ ,  $P > 0.05$ ; paired  $t$  test).





**Fig. 6.** Insulin stimulates BK channel activation via a MAPK-dependent process. **A**, sample traces of a single cell-attached recording obtained from a hippocampal neuron with +20 mV applied to the recording pipette and obtained 2 min and 25 min after the start of the experiment. Prior incubation with the MAPK inhibitor, UO126 (1 μM), occluded the ability

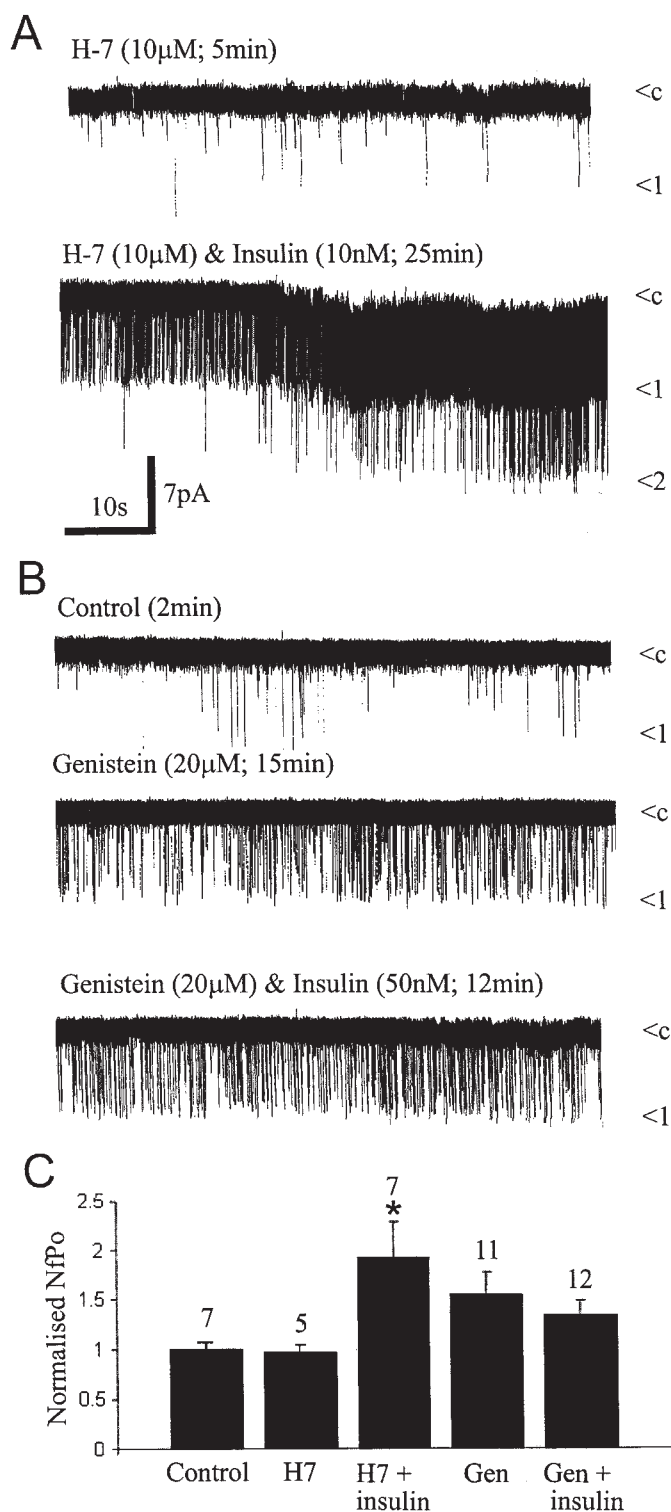
Furthermore, daidzein had no effect on the ability of insulin to increase BK channel activity ( $n = 4$ ; not illustrated). Thus, the  $NfP_o$  values obtained after at least 20 min of incubation with daidzein were increased from  $0.03 \pm 0.01$  to  $0.09 \pm 0.03$  after 15- to 17-min exposure to insulin ( $n = 4$ ,  $P < 0.05$ ; paired  $t$  test). These data indicate that insulin activation of BK channels is mimicked by a tyrosine kinase inhibitor and that activation of BK channels by insulin may require dephosphorylation of some unidentified component of the pathway.

## Discussion

It is well established that insulin, through both its peripheral and central actions, is an important regulator of energy balance. However, evidence is accumulating that insulin plays a key role in various CNS functions. Indeed, insulin can modify certain behaviors, including hippocampal learning and memory processes (Wickelgren, 1998; Zhao et al., 1999; Zhao and Alkon, 2001). Insulin can also modulate glutamatergic synaptic transmission (Liu et al., 1995; Zhou et al., 2001) and cause suppression of  $Kv1.3$  current in the olfactory bulb (Fadool and Levitan, 1998), as well as regulate the cell surface expression of  $GABA_A$  (Wan et al., 1997),  $N$ -methyl-D-aspartate (Skeberdis et al., 2001), and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (Zhou et al., 2001) receptors. In this study we have demonstrated that insulin increases the activity of neuronal BK channels in hippocampal cultures. This process requires insulin receptor-driven stimulation of a MAPK-, but not PI 3-kinase-dependent process. In parallel studies, insulin activates recombinant (hSlo) BK channels, also, via activation of a MAPK-dependent process. This insulin receptor-driven process may be a novel mechanism for regulating hippocampal excitability.

Our previous studies have demonstrated that insulin, via activation of BK- and ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channels, potentially inhibits the aberrant synaptic activity induced by  $Mg^{2+}$  removal in hippocampal neurons (O'Malley et al., 2003). In this study, we examined the effects of insulin on native and recombinant (hSlo) BK channels expressed in hippocampal and HEK293 cells, respectively. During cell-attached recordings, pipette application of insulin, at concentrations comparable with those circulating in the plasma (Schechter et al., 1992), caused a 3- to 4-fold increase in the activity of native and recombinant BK channels. This action of insulin was rapid, occurring within minutes, and was maintained, in the presence of insulin, for the duration of

of insulin (via the pipette) to stimulate BK channel activity. **B**, in contrast, the inactive analog, U0124 (1 μM), failed to prevent insulin action. Cell-attached recordings were obtained from a hippocampal neuron with +20 mV applied to the recording pipette. **C**, sample cell-attached recordings from a HEK293 cell (with +10 mV applied potential) 2 to 4 min after obtaining the cell-attached configuration, 12 min after insulin addition, and after 30 min of incubation with PD98059. Application of PD98059 after BK channel activation reversed the actions of insulin. **D** illustrates the corresponding diary plot of  $NfP_o$  against time for this experiment. **E**i and **E**ii, pooled data, obtained after 15 to 17 min of incubation with insulin, showing the normalized effects of prior treatment (for 30 min) with PD98059, U0126, or U0124 on insulin-induced BK channel activation in hippocampal neurons (filled bars) and HEK293 cells (open bars). The numbers above the histogram bars represent the  $n$  values for each experimental condition, and \* represents  $P < 0.05$  (one-way ANOVA plus Tukey's post hoc test) compared with control insulin response (obtained from distinct cells).



**Fig. 7.** Insulin-induced BK channel activation involves tyrosine dephosphorylation. **A**, sample traces of cell-attached recordings from a hippocampal neuron with +20 mV applied potential. Upper trace, incubation with the broad-spectrum serine/threonine kinase inhibitor, H-7 (10  $\mu$ M) had no effect on BK channel activity per se. Sample recording was taken 2 min after obtaining the cell-attached configuration, but after a 30-min prior incubation with H-7 in the bath. Lower trace, in the same cell-attached recording, subsequent exposure (15 min) to insulin (10 nM; via the patch pipette) resulted in BK channel activation. **B**, sample traces of representative cell-attached recordings (with +20 mV applied potential) obtained from an individual hippocampal neuron. Bath application of the tyrosine kinase inhibitor, genistein (20  $\mu$ M), resulted in an increase

recordings (up to 45 min). Bath application of higher concentrations of insulin also increased the activity of native and hSlo BK channels, albeit at a slower rate, indicating that this process is not limited by the membrane. It is interesting that insulin activation of hypothalamic  $K_{ATP}$  channels is not limited to channels located within the membrane microdomain associated with the insulin receptor (Spanswick et al., 2000). The ability of insulin to modify BK channel activity when applied either in the pipette or the bath suggests that an indirect process underlies this action of insulin. In hippocampal neurons, the ability of insulin to stimulate BK channels is most probably a result of insulin receptor activation because comparable concentrations of IGF-1 failed to mimic insulin action, although IGF-1 did promote channel activation at 100-fold higher concentrations. The differential sensitivity of native BK channels to IGF-1 and insulin was not attributable to differences in the voltage-dependence for BK channel activation because both IGF-1 and insulin were capable of stimulating BK channels in cell-attached patches with +60 mV applied voltage. It is noteworthy that in olfactory bulb neurons, insulin, but not IGF-1 or IGF-11, is capable of down-regulating native Kv1.3 channels (Fadool et al., 2000). In contrast, low concentrations of IGF-1 did recapitulate insulin action in the HEK293 cells, indicating that either insulin or IGF-1 receptors can mediate the actions of insulin in this system. The BK channel opener, NS-1619, also increased the activity of native and cloned BK channels. However, NS-1619 was more effective at increasing hSlo activity compared with native channels, suggesting that the molecular composition of native channels may be distinct from hSlo. On the other hand, because human and rat Slo display 93% sequence homology, the potency differences may reflect species differences in the effectiveness of NS-1619 at opening BK channels.

The insulin and IGF-1 receptors belong to the same subfamily of receptor tyrosine kinases that share a high similarity of structure and intracellular signaling capacity (Siddle et al., 2001). Binding of insulin to either receptor induces receptor tyrosine kinase activity, which leads to activation of multiple signaling cascades downstream (Shepherd et al., 1998). One of the main mediators of insulin action is the lipid kinase, PI 3-kinase (Shepherd et al., 1998). Indeed, insulin activation of hypothalamic  $K_{ATP}$  channels involves PI 3-kinase (Spanswick et al., 2000). Furthermore, a PI 3-kinase-driven process underlies leptin coupling to hippocampal BK channels (Shanley et al., 2002). However, in this study, insulin modulation of native and cloned BK channels was neither attenuated nor reversed by two different PI 3-kinase inhibitors, LY294002 and wortmannin, indicating that it is likely that a PI 3-kinase-independent signaling pathway underlies insulin action. But after insulin-induced channel activation, application of the PI 3-kinase inhibitors enhanced channel activity, suggesting that insulin or IGF-1 receptor-

in BK channel activity (**B**, middle trace) relative to control (**B**, upper trace). Subsequent addition of insulin (50 nM; via the bath), after at least 30 min of incubation with genistein, failed to further increase channel activity (**B**, lower trace). **C**, pooled data showing the normalized effects of prior treatment of either H-7 (for 30 min) or genistein (for 20 min) on the ability of insulin (at 15–17 min after insulin exposure) to stimulate BK channel activity in hippocampal neurons. The *n* values are depicted above the histogram bars, and \* represents  $P < 0.05$  (paired *t* test) compared with control conditions at 2 to 4 min in the same cell.

driven PI 3-kinase activation may act as a negative feedback mechanism to regulate insulin action.

Because insulin-induced attenuation of hippocampal epileptiform-like activity requires MAPK stimulation (O'Malley et al., 2003), the Ras-Raf-MAPK pathway is another possible signaling candidate underlying insulin action. Indeed, prior treatment with either PD98059 or U0126 significantly reduced the ability of insulin to stimulate native and recombinant channels. In contrast, the inactive analog U0124 failed to attenuate insulin action. Moreover, PD98059 reversed the actions of insulin, indicating that activation of a MAPK-dependent process is required for insulin action. In support of a role for the Ras-Raf-MAPK signaling pathway, increases in insulin receptor mRNA and protein as well as tyrosine phosphorylation of Shc and MAPK have been detected in hippocampal tissue from rodents trained in spatial memory tasks (Zhao et al., 1999; Zhao and Alkon, 2001).

BK channels consist of a pore-forming  $\alpha$  subunit, with or without a regulatory  $\beta$  subunit, and in the CNS, the  $\beta 4$  subunit is widely expressed (Behrens et al., 2000; Meera et al., 2000). Expression of this subunit significantly reduces the sensitivity of BK channels to iberiotoxin and charybdotoxin (Behrens et al., 2000; Uebele et al., 2000). However, in the present study, insulin is capable of activating recombinant BK channels comprising hSlo alone. Moreover, insulin inhibition of hippocampal neurons is blocked by low nanomolar concentrations of these toxins (O'Malley et al., 2003). Thus, it is unlikely that the hippocampal BK channel targeted by insulin requires the presence of the  $\beta 4$  subunit for activation. This finding parallels our previous work that indicated that leptin is unlikely to stimulate BK channels comprising the brain  $\beta 4$  subunit (Shanley et al., 2002a). Because hippocampal BK channels are located at both pre- and postsynaptic sites, it is feasible that channels of differing molecular composition are expressed at presynaptic terminals and somatodendritic regions. Indeed, postsynaptic BK channels are the predominant target underlying leptin action on hippocampal epileptiform-like activity (Shanley et al., 2002b). Thus, like leptin, insulin may primarily activate postsynaptic BK channels comprising the  $\alpha$  (Slo) subunit. However, further experiments are required to determine the precise molecular identity and subcellular location of the hippocampal BK channels targeted by insulin.

It is well established that BK channel activity is tightly controlled by  $[Ca^{2+}]_i$  at any given membrane potential (Latorre, 1989). Thus, alterations in the somatic levels of  $[Ca^{2+}]_i$ , induced by insulin or IGF-1 receptor stimulation, could potentially stimulate BK channels. This is unlikely, however, because insulin is unable to increase the basal levels of  $[Ca^{2+}]_i$  in hippocampal neurons (O'Malley et al., 2003). It is well established that native BK channels can be up- or down-regulated by protein phosphorylation. In hippocampal neurons, BK channels are inhibited by protein phosphorylation (Pedarzani and Storm, 1995), whereas protein dephosphorylation increases channel activity (Abdul-Ghani et al., 1996). Our present data are consistent with protein dephosphorylation increasing channel activity, because the tyrosine kinase inhibitor, genistein, but not the inactive analog daidzein, stimulated hippocampal BK channel activity. This up-regulation seems to be specific for tyrosine residues, because the serine/threonine kinase inhibitor, H-7, had no effect on BK channel activity per se. In

contrast, recombinant murine BK channels are up-regulated by Src tyrosine kinase (Ling et al., 2000), suggesting that there may be species-dependent differences in the regulation of BK channels by tyrosine phosphorylation. In the present study, insulin-induced stimulation of BK channels is likely to involve tyrosine dephosphorylation because genistein prevented further activation by insulin. In contrast, H-7 failed to prevent insulin action. The ability of insulin to stimulate BK channels via protein dephosphorylation parallels somatostatin activation of BK channels in the GH<sub>4</sub>C<sub>1</sub> cell line (White et al., 1991). In this cell line, dephosphorylated BK channels also display an increased sensitivity to intracellular Ca<sup>2+</sup> (Hall and Armstrong, 2000), which parallels the apparent shift in the BK Ca<sup>2+</sup>-sensitivity observed after insulin in this study.

In hippocampal neurons, postsynaptic BK channels regulate action potential firing rates and burst firing patterns (Lancaster and Nicoll, 1987). Because unregulated excitability in the hippocampus may be one underlying cause of temporal lobe epilepsy, activation of BK channels by insulin is one potential way of ameliorating this hyperexcitable state. Indeed, BK channel activation contributes to insulin inhibition of epileptiform-like activity evoked in hippocampal neurons (O'Malley et al., 2003). Thus, functionally, insulin receptor-driven BK channel activation may be important in diseases such as epilepsy. Evidence is accumulating that insulin plays a pivotal role in numerous neuronal functions that may be related to its peripheral actions. For instance, during periods of hyperglycemia there is a higher incidence of strokes and seizures in diabetic patients. It is also apparent that, in addition to the metabolic and vascular changes associated with hyperglycemia, deficiencies in the central actions of insulin contribute to the pathogenesis of diabetes. Thus, a greater understanding of insulin receptor-driven signaling pathways and the potential CNS targets for insulin may provide novel therapeutic targets for treating diabetes.

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