Protein kinase signalling requirements for metabotropic action of kainate receptors in rat CA1 pyramidal neurones

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Hippocampal pyramidal neurones display a Ca^{2+}-dependent K^+ current responsible for the slow afterhyperpolarization (I_{sAHP}), a prominent regulator of excitability. There is considerable transmitter convergence onto I_{sAHP} but little information about the interplay between the kinase-based transduction mechanisms underlying transmitter action. We have added to existing information about the role of protein kinase C (PKC) in kainate receptor actions by demonstrating that direct postsynaptic activation of PKC with either 1-oleoyl-2-acethyl-sn-glycerol (OAG) or indolactam is sufficient to inhibit I_{sAHP}. The physiological correlate of this action – activation of PKC by kainate receptors – requires $G\alpha_{i/o}$ proteins. The cAMP/PKA system is well documented to subserve the actions of monoamine transmitters. We have found an additional role for the cAMP/PKA system as a requirement for kainate receptor-mediated inhibition of I_{sAHP}. Inhibition of adenylyl cyclase with dideoxyadenosine or PKA with either H89 or RpcAMPs blocked kainate receptor-mediated actions but did not prevent the actions of direct PKC activation with either OAG or indolactam. We therefore propose that the PKA requirement is upstream from the actions of PKC. We additionally report a downstream link in the form of increased mitogen-activated protein (MAP) kinase activity, which may explain the long duration of metabotropic actions of kainate receptors on I_{sAHP}.

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Glutamatergic kainate receptors (KARs) are receptor-linked ionophores, yet an increasing body of evidence demonstrates that they also have metabotropic functions. This metabotropic action modulates hippocampal inhibitory (Rodriguez-Moreno *et al.* 1997) and excitatory postsynaptic currents (Frerking *et al.* 2001) as well as pyramidal cell excitability (Melyan *et al.* 2002, 2004). The metabotropic action of KARs on synaptic activity shows a consistent requirement for G proteins of the $G\alpha_{i/o}$ variety. Nothing is yet known about the mechanism whereby this ionotropic KAR is coupled to G proteins.

Activation of KARs has long been known to induce network oscillations and promote epileptiform seizure activity in the hippocampus (Prince, 1978; Ashwood *et al.* 1986; Traub *et al.* 2000). More recent data are starting to reveal the role played by different GluR subunits in this activity. GluR5−/− mutant mice show greater susceptibility to gamma oscillations and bursting activity, consistent with a role in regulating release from inhibitory terminals (Cossart *et al.* 1998; Fisahn *et al.* 2004). In contrast, the oscillatory and epileptogenic action of kainate is not observed in GluR6−/− mutant mice (Fisahn *et al.* 2004). This subunit dependence echoes the GluR susceptibility of the slow afterhyperpolarization current (I_{sAHP}). This current underlies spike frequency adaptation in hippocampal pyramidal neurones and is a convergent target for modulation by a wide variety of neurotransmitters including monoamines and glutamate (for review see Vogalis *et al.* 2003). Monoamines inhibit I_{sAHP} via the cAMP–protein kinase A (PKA) system (Madison & Nicoll, 1986; Pedarzani & Storm, 1993); in contrast the actions of KARs are blocked by calphostin C, suggesting the involvement of PKC (Melyan *et al.* 2002, 2004). Both pharmacological and knockout studies point to GluR6 as a crucial element for this metabotropic action (Melyan *et al.* 2002; Fisahn *et al.* 2005).

The current experiments dissect the signal transduction requirements for kainate-mediated metabotropic responses, to test necessity and sufficiency for the identified steps of the mechanism. We have tested the specific G protein requirements of the KAR action on pyramidal cell excitability and probed the subsequent protein kinase requirements in the signalling cascade underlying this

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metabotropic response. The results indicate that the metabotropic kainate response in CA1 neurones is linked to $G\alpha_{i/o}$, and reveal an unexpectedly diverse signalling requirement in which PKC is downstream of a requirement for PKA. The results have revealed an extra component to the kainate transduction mechanism in the form of MAP kinase, which is capable of a bidirectional regulation of I_{sAHP}.

Methods

Preparation of hippocampal slices was carried out in accordance with the UK Animals (Scientific Procedures) Act of 1986. Wistar rats (postnatal day 15–21) were deeply anaesthetized with isoflurane by inhalation, and the brains were removed following decapitation. Hippocampal slices (300 μm) were cut in ice-cold artificial cerebrospinal fluid (aCSF). The slices were maintained at room temperature; recordings were made from submerged, superfused slices at 28–30°C. The storage and perfusion solution contained (aCSF, mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1 NaH_2PO_4, 26 $NaHCO_3$, and 11 glucose, and was gassed with 95% O_2/5% CO_2. Recording pipettes contained (mM): 150 potassium methylsulphate, 10 KCl, 10 Hepes, 4 NaCl, 4 Mg_2ATP, and 0.4 Na_4GTP. Osmolarity was adjusted to 280–290 mosmol l^{-1} and pH to 7.35–7.4 with KOH. Chemicals were purchased from Sigma (Poole, UK), with the following exceptions: kainic acid (Ocean Products International, Shelburne, Nova Scotia, Canada; or Sigma); potassium methylsulphate (ICN Biomedicals, Aurora, OH, USA); 2′-5′-dideoxyadenosine (DDA), *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinoline-sulphonamide, 2HCl (H89) (Biomol, Plymouth Meeting, PA, USA); calphostin C, 1-oleoyl-2-acetyl-sn-glycerol (OAG), pertussis toxin (PTx), adenosine 3′5′-cyclic phosphorothionate-Rp (Rp-cAMPS), U0126, PD098059 and activated mitogen-activated protein (MAP) kinase (Calbiochem, Nottingham, UK); DL-AP5, (*S*)-MCPG, MSOP, 2-OH-saclofen, DPCPX, AM 251, naloxone, and NF023 (Tocris Cookson, Bristol, UK).

Electrophysiological data were obtained from 147 whole-cell patch-clamp recordings from pyramidal neurones in the CA1 region. Open pipette resistance was 3–4 MΩ, and access resistance during recordings was <20 MΩ. Membrane currents were recorded and the records were filtered at 1 kHz and sampled at 2 kHz (Axopatch 200B, Axon Instruments). Depolarizing current steps (80 ms, −60 to 0 mV) were used to evoke I_{sAHP}. The calcum influx during the depolarization triggers two calcium-dependent tail currents, which can be distinguished by their time course. An early component lasting <500 ms is due to SK channels; this is followed by I_{sAHP}, a slowly rising current which then decays over 2–3 s. Data are given as means ± S.E.M., and the significance was assessed with Student's *t* test. The concentration–response curve in Fig. 2 was derived from

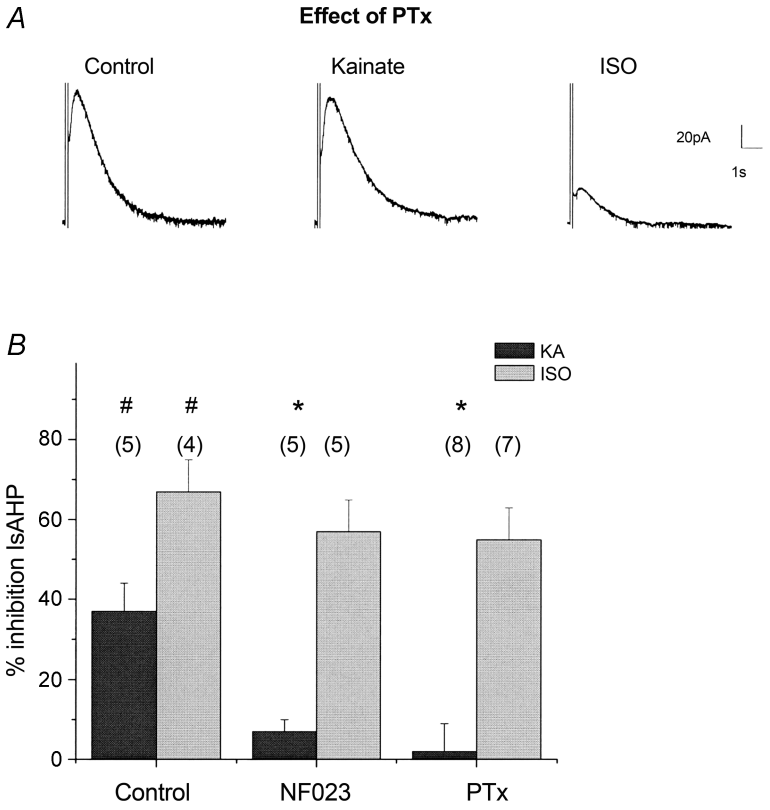

Figure 1. Effect of $G\alpha_{i/o}$ protein blockers on the amplitude of I_{sAHP}
A, sample traces recorded during intracellular dialysis of PTx (2.5 μg ml^{-1}, >10 min) before (left) and after exposure with kainate (middle trace) or isoproterenol (right). *B*, summary histogram to compare combined effects of kainate (black bars, 200 nM) and isoproterenol (grey bars, 5 μM) on the amplitude of I_{sAHP} in control conditions and during intracellular dialysis (>10 min) with NF023 (10 μM) or PTx (2.5 μg ml^{-1}). The number of recorded cells for each group is in parentheses. #Significant difference ($P < 0.005$) over the control group, *significant difference compared to the control action of KA.

$I/I_{control} = C^n/(C^n + EC_{50}{}^n)$, where C is the concentration of (−)indolactam V, and n the hill coefficient (presumed = 1).

Western blot method

Hippocampal slices were prepared as described above. Slices were incubated in aCSF for 1 h, then bathed in the cocktail of antagonists for 1 h (DL-AP5, GYKI, picrotoxin (*S*)-MCPG, MSOP, 2-OH-saclofen, DPCPX, AM 251, naloxone, atropine, at concentrations described in Melyan *et al.* 2002). The slices were then treated for 15 min with solutions containing kainate or with the MAP kinase kinase (MEK) inhibitor U0126 in addition to kainate. Slices were washed with normal aCSF for increasing periods up to 60 min after drug treatment before being homogenized (10 w/v) in buffer (mM: 25 NaCl, 2 EDTA, 0.5 DTT, 20 Hepes, 20 β-glycerolphosphate) containing a protease inhibitor cocktail (Complete, Roche). Protein concentrations were assayed using a Bio-Rad DC protein assay kit. Homegenates (25 μg) were subjected to Western blotting by resolving samples by SDS-PAGE before transfer to nitrocellulose (Pellegrini *et al.* 1995). Nitrocellulose was probed with phospho p42/44 MAP kinase and p42/44 MAP kinase antibodies as prescribed by the manufacturer (Cell Signalling). The immunoreactivity of the primary antibodies was detected by incubating nitrocellulose AlexaFluor680-conjugated goat antirabbit secondary antibody for 2 h in the dark in TBS-T-containing 3% Marvel milk. After subsequent washing, immunoreactivity was imaged and the relative fluorescence intensity quantified using a Licor Odyssey system (laser setting at 700 nm). The fluorescence intensity seen in kainate and U0126 treatments was normalized to

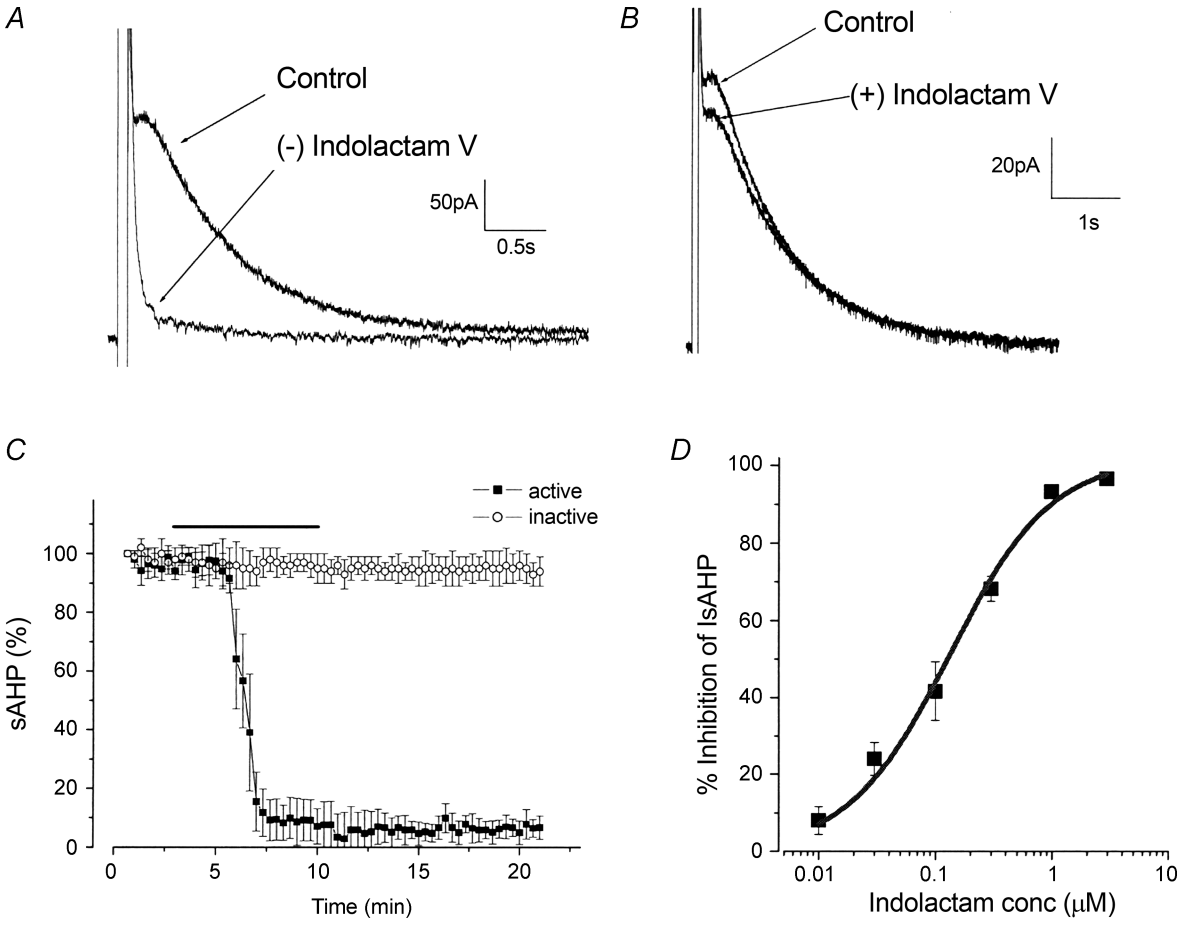

Figure 2. Effect of indolactam on the amplitude of I_{sAHP}
A, samples of superimposed traces demonstrate that 10 min extracellular application of 1 μM (−)indolactam V completely inhibited the amplitude of I_{sAHP}. *B*, sample traces of I_{sAHP} demonstrating that 10 min application of 10 μM (+)indolactam V (the inactive analogue) did not affect I_{sAHP}. *C*, summary of time course of I_{sAHP} in response to extracellular application of active and inactive forms of indolactam; (−)indolactam V (1 μM, $n = 8$) and (+)indolactam V (10 μM, $n = 5$), respectively. *D*, concentration–response curve demonstrating potency of (−)indolactam V to inhibit the amplitude of I_{sAHP}. Each point is the average from at least five cells.

phospho-MAP kinase signal obtained in slices without such treatment but incubated with the background antagonist cocktail, and the results compared using Student's *t* test.

Results

The role of $G\alpha_{i/o}$

The involvement of pertussis toxin-sensitive G proteins in kainate action on I_{sAHP} was suggested by the previously described sensitivity to the reagent NEM (Melyan *et al.* 2002). To test the role of G proteins more directly, we tested kainate action on the I_{sAHP} in CA1 neurones that were dialysed with PTx (2.5 μg ml^{-1}, >10 min; Katada & Ui, 1982) through the recording electrode. In the presence of PTx, the inhibitory effect of kainate, but not isoproterenol, was abolished (inhibition 2% ± 7%, $n = 8$ and 55% ± 8%, $n = 7$, respectively, Fig. 1). This result was supported by another series of experiments in which a different type of $G\alpha_{i/o}$ inhibitor, NF023, was used (Freissmuth *et al.* 1996). Intracellular pretreatment of CA1 neurones with NF023 (10 μM) was much more effective against the action of kainate (inhibition 7% ± 3%; $n = 15$, Fig. 1) than the inhibitory effect of isoproterenol on the amplitude of I_{sAHP} (57% ± 8%, $n = 7$). These results indicate that $G\alpha_{i/o}$ subunits are involved in the transduction mechanism coupled to kainate receptors but not noradrenergic β receptors.

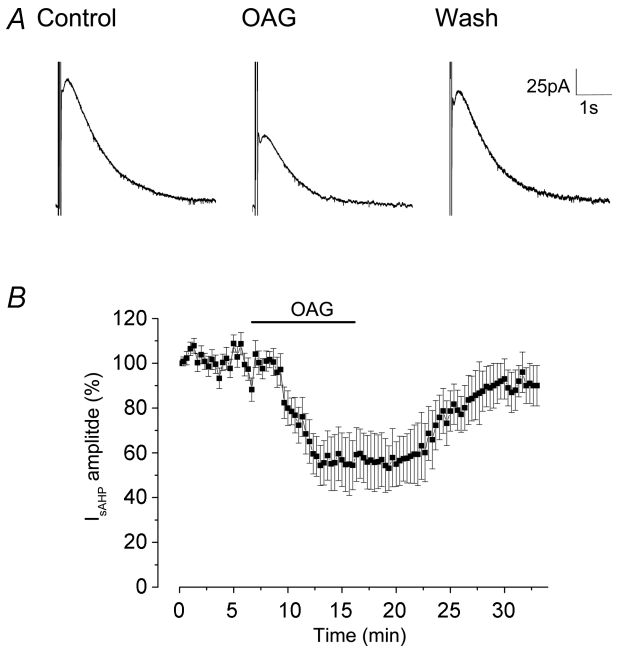

Figure 3. Effect of the PKC activator OAG on I_{sAHP}
A, sample of traces of I_{sAHP} obtained in control conditions (left) after extracellular application of 50 μM OAG (middle trace) and after washout (right). *B*, summary data ($n = 5$) for the time course of OAG action on I_{sAHP}.

PKC activity is sufficient to modulate I_{sAHP}

The involvement of PKC in the actions of KARs has been inferred from the sensitivity to the PKC inhibitor calphostin C (Melyan *et al.* 2002). In light of this observation, we tested specifically whether the activity of PKC alone was sufficient to inhibit I_{sAHP}. Both extracellular and intracellular application of the PKC activator (−)indolactam V (1 μM; $n = 12$ and $n = 10$, respectively; Shieh *et al.* 1995) caused almost complete inhibition of I_{sAHP} (mean 92% ± 7% and 96% ± 3% inhibition, respectively; Fig. 2). The effect of extracellular application of (−)indolactam V was concentration dependent, with an $IC_{50} = 110$ nM (Fig. 2*D*). The specificity of (−)indolactam V was tested by using the inactive analogue ((+)indolactam V, 10 (μM), which did not significantly affect the amplitude of I_{sAHP} (91% ± 7%, $n = 5$, Fig. 2*C*). Extracellular application of another PKC activator, the cell-permeable diacylglycerol analogue OAG (50 μM; Kaibuchi *et al.* 1983), partially inhibited the amplitude of I_{sAHP} (36% ± 6%, $n = 5$), however, in contrast to (−)indolactam V, the effect of OAG was reversible (Fig. 3).

An upstream requirement for PKA

A striking characteristic of the slow AHP is the convergent action of many transmitters to inhibit this current. Monoamines including noradrenaline inhibit the I_{sAHP} via activation of the adenylyl cyclase–PKA cascade (Madison & Nicoll, 1986; Pedarzani & Storm, 1993). To assess the presumed independence of the various transmitter mechanisms, we used the PKA inhibitors Rp-cAMPS (50 μM) and H89 (10 μM) included in the recording pipettes (Chijiwa *et al.* 1990; Dostmann *et al.* 1990). As expected from previous work (Pedarzani & Storm, 1993), both compounds effectively prevented the inhibitory action of β-receptor agonists on the amplitude of I_{sAHP} (inhibition was 5% ± 7%, $n = 8$ and 9% ± 7%, $n = 5$, respectively). In light of previous work highlighting a role for PKC we were, however, surprised to find that the action of kainate was also sensitive to both Rp-cAMPS, and H89; inhibition was reduced to 8% ± 8%, $n = 7$ and 5% ± 7%, $n = 5$, respectively (Fig. 4*B*).

These results indicate that PKA is required for the action of both kainate and isoproterenol. A requirement for PKA suggests that there should be a co-requirement for cAMP production. To examine the contribution of adenylyl cyclase, 10 μM DDA (an adenylyl cyclase inhibitor, Holgate *et al.* 1980) was dialysed into the cell from the recording pipette. This manipulation also resulted in full attenuation of the effects of kainate and isoproterenol on I_{sAHP} (inhibition 5% ± 5%, $n = 7$ and 7% ± 9%, $n = 7$, Fig. 4*B*). Taken together the data indicate that activation of adenylyl cyclase–PKA is a common requirement for

metabotropic actions of KARs and β-adrenergic receptors; however, the involvement of $G\alpha_{i/o}$ and PKC is specific to KARs.

Given a co-requirement for PKA and PKC in kainate action, we performed experiments to establish the relative positions of these kinases in the signal transduction pathway. Experiments used the PKA inhibitor Rp-cAMPS in tandem with the PKC activators (−)indolactam V or OAG. We included Rp-cAMPS (0.1 mM) in the recording pipette and applied (−)indolactam V (1 μM; $n = 4$) or OAG (50 μM; $n = 5$) in the perfusate. The degree of inhibition produced by indolactam V or OAG alone ($92 \pm 7\%$ and $36 \pm 6\%$) was unaffected by Rp-cAMPs ($89 \pm 6\%$ and $35 \pm 8\%$, respectively). The result indicates that PKA has no significant effect on the outcome of indolactam or OAG application on the amplitude of I_{sAHP} and that PKC inhibits the I_{sAHP} acting either independently or downstream to PKA.

Actions of kainate and β-receptors on I_{sAHP}

The inhibition of I_{sAHP} following KAR activation is particularly long-lasting and shows no clear reversal during the time of whole-cell recording, in some instances >1.5 h. Figure 5*B* shows that bath application of 200 nM kainate for up to 15 min resulted in a gradual (5–15 min) inhibition of I_{sAHP} (inhibition $37\% \pm 7\%$ of control, $n = 5$). This is similar to the data of Melyan *et al.* (2002), which showed that even brief (5 min) kainate applications exhibited this long-lasting effect. Conceivably this might be a unique property of kainate receptors or the particular elements of the transduction pathway.

One downstream effector mechanism with suitable properties is the MAP kinase cascade. This has long-lasting actions because it is known to be required for late-stage long-term potentiation (LTP) (English & Sweatt, 1996; Coogan *et al.* 1999; Winder *et al.* 1999); furthermore in the CA1 region both PKC and β-adrenergic receptors,

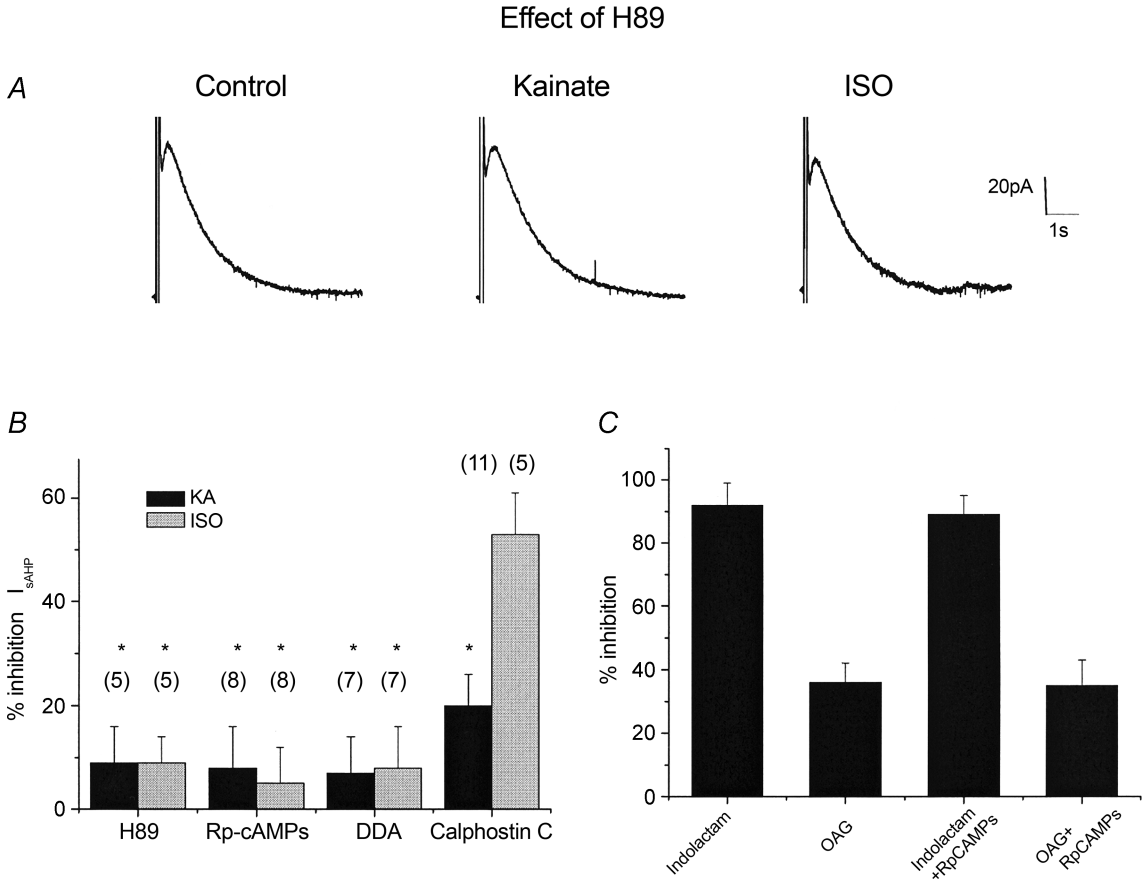

Figure 4. Action of kinase inhibitors on I_{sAHP}
A, sample traces of I_{sAHP} from H89 recordings obtained in control conditions (left) after extracellular application of 200 nM kainate (middle trace) and 5 μM isoproterenol (right). *B*, summary histogram to compare combined effects of kainate (black bars, 200 nm) and isoproterenol (grey bars, 5 μM) on the amplitude of I_{sAHP} after intracellular dialysis (>10 min) with one of following substances: H89 (10 μM), Rp-cAMPs (10 μM), DDA (10 μM), calphostin C (1 μM). The number of recorded cells for each group is in parentheses. *Significant difference over the normal effect of KA or isoproterenol. *C*, summary histogram of pooled data illustrates that extracellular superfusion of PKC activators indolactam V (2 μM) or OAG (50 μM) inhibit the amplitude of I_{sAHP} and these effects are not attenuated by intracellular dialysis of the PKA inhibitor Rp-cAMPS (0.5 mM).

via PKA, can engage this pathway (Roberson *et al.* 1999). Activation of the MAP kinase pathway by isoproterenol is both time and concentration dependent (Schmitt & Stork, 2000). In light of this, we tested the effect on I_{sAHP} of longer (10 min) isoproterenol applications to match those used previously (Roberson *et al.* 1999) and compared them with the 2–3 min application we used normally. Short applications of isoproterenol (5 μM for <3 min, Fig. 5*A* and *C*, ■) caused 69% ± 8% inhibition of I_{sAHP} ($n = 4$), which was fully reversible. However, the longer exposure to isoproterenol (10 min, ●) caused inhibition of the I_{sAHP}, which showed little recovery during the recording ($n = 4$, Fig. 5*C*) similar to the effects of kainate receptor activation (Fig. 5*B*).

These results raised the possibility that MAP kinase activity might also explain the persistent action of KARs. This was tested initially using an *in vitro* assay for phosphorylatedMAP kinase (ppMAP) that recognizes both p42 and p44 forms. Incubation in 200 nM kainate for 15 min caused a significant increase in ppMAP kinase activity, which recovered back to stable control levels in 30 min (Fig. 6). In contrast, when kainate was co-applied with the MEK inhibitor U0126 (10 μM) the action of kainate was obliterated by a reduction in overall ppMAP kinase activity (Fig. 6).

To test whether MEK, and by implication ppMAP kinase, was required for the action of kainate on I_{sAHP}, we included within the recording pipette one of two structurally distinct MEK inhibitors; U0126 and PD098059 (10 μM). Recordings were performed at least 10 min after establishing the whole-cell configuration. Both compounds completely prevented the inhibition of I_{sAHP} by bath application of 200 nM kainate, while in the same cells a short application of isoproterenol (3 μM, 2–3 min) still caused robust inhibition (Fig. 7*A* and *B*). Since the MAP kinase assay indicated that activity was capable of up- or downregulation, we tested whether this observation could be extended to the presumptive target – I_{sAHP}. When a MEK inhibitor was bath applied (U0126, 10 μM) after a stable control period, I_{sAHP} gradually increased in amplitude (Fig. 7*C* and *D*), while the inclusion of activated MAP kinase within the recording pipette caused a gradual loss of the current (Fig. 7*E* and *F*).

Discussion

These results add new facets to the signal transduction requirements of the postsynaptic metabotropic action of KARs on pyramidal cells. The data suggest a sequence

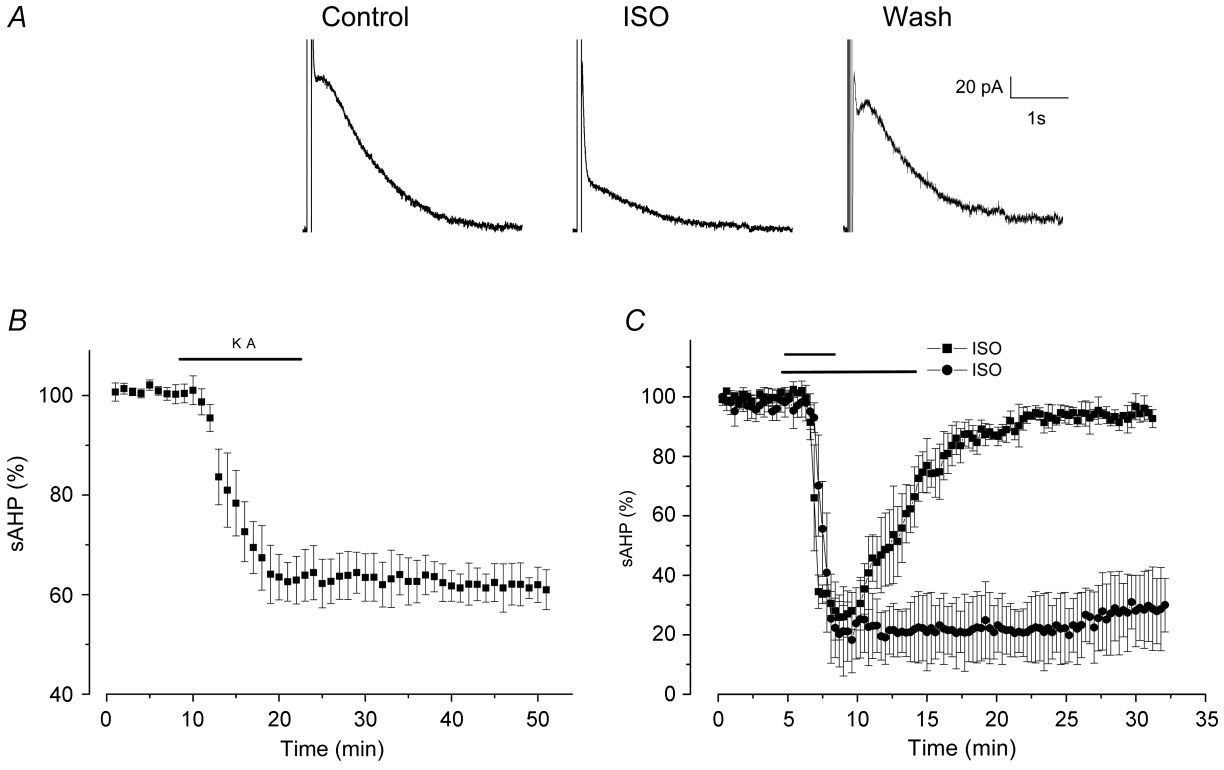

Figure 5. Effect of isoproterenol and kainate on the amplitude of I_{sAHP}
A, sample of traces of I_{sAHP} obtained in control conditions (left) after extracellular application of 5 μM isoproterenol (middle trace) and after 20 min washout (right). Scale bars indicate 20 pA and 1 s. *B*, the time course of I_{sAHP} amplitude after extracellular application of kainate (200 nM, $n = 5$). *C*, time course of I_{sAHP} amplitude for short ($n = 4$, ■) and prolonged (10 min, ●) application of 5 μM isoproterenol.

of events in which KAR activation is followed by a requirement for $G\alpha_{i/o}$ protein. Inhibition of I_{sAHP} by KARs shows a requirement for the adenylyl cyclase–PKA pathway. However, while KAR action is sensitive to PKA inhibitors, solitary activation of PKC is sufficient to mimic the actions of KAR. The downstream effector is the MAP kinase cascade which provides for a bidirectional modulation of I_{sAHP}.

The requirement of $G\alpha_{i/o}$ proteins for metabotropic KAR action is a consistent finding (Rodriguez-Moreno *et al.* 1997; Frerking *et al.* 2001; this paper). What is not yet understood is whether KARs couple to G proteins directly, or if there is some involvement of ancillary proteins. In this regard, the GluR6-containing receptor may be similar to AMPA, NMDA and nicotinic acetylcholine receptors, which also appear capable of activating second messenger systems (Sorenson & Gallagher, 1996; Wang *et al.* 1997; Mao *et al.* 2004). Given that the predicted sequence topology of GluR6-containing receptors is different from G protein-coupled receptors, the link between G proteins and GluR6 is not understood. Conceivably, conformational changes caused by glutamate binding to GluR6 may allow interaction with adaptor proteins that result in G protein activation. Nevertheless, the work presented here and elsewhere highlights $G\alpha_{i/o}$ protein activity as the leading candidate to mediate kainate receptor activation and the second messenger cascade.

Intracellular messengers and inhibition of I_{sAHP}

PKA is required for KAR modulation of excitatory (Rodriguez-Moreno & Sihra, 2004; Negrete-Diaz *et al.* 2006) but not inhibitory (Rodriguez-Moreno & Lerma, 1998) synaptic activity. Functionally, members of the $G\alpha_{i/o}$ family are most likely to inhibit adenylyl cyclase activity (Taussig *et al.* 1994). Although the various adenylyl cyclase isoenzymes exhibit different susceptibilities to $G\alpha_{i/o}$-mediated inhibition, it is unclear how KARs could increase the activity of PKA directly. Consequently, the second possibility is that constitutive PKA activity is required to maintain the viability of the KAR transduction mechanism. This scheme would match the resting PKA/phosphatase activity that is known to exist in pyramidal cells (Pedarzani *et al.* 1998). Since PKA inhibition does not prevent the action of PKC activators, the PKA requirement cannot be downstream of PKC, and modulation is unlikely to be at PKC itself because the phosphorylation sites are not targets for PKA (Newton, 2003). A co-dependence on PKA and PKC has been reported in neurones of the enteric system. It was observed that inhibition of a slow AHP in response to 5-HT_1 receptor stimulation was linked to G_o and cooperatively mediated by PKA and PKC (Pan *et al.* 1997). Here it was suggested that 5-HT receptors acted through G_o, then phospholipase C and PKC, followed by adenylyl cyclase and PKA. Although our data have a similar pharmacological profile, our observations in the hippocampus indicate that PKC can act either downstream of or independently from PKA.

The KAR itself is an obvious candidate site for phosphorylation. The most likely candidate subunit is GluR6. Pharmacological evidence suggests that GluR5 subunits are not involved in this response since the GluR5-selective agonist ATPA did not produce the same effect as kainate or domoate (Melyan *et al.* 2002). Subunit deletion studies have shown that GluR6−/− or KA2−/−, but not GluR5−/− mice, lack kainate-induced inhibition of the slow AHP (Fisahn *et al.* 2004; Ruiz

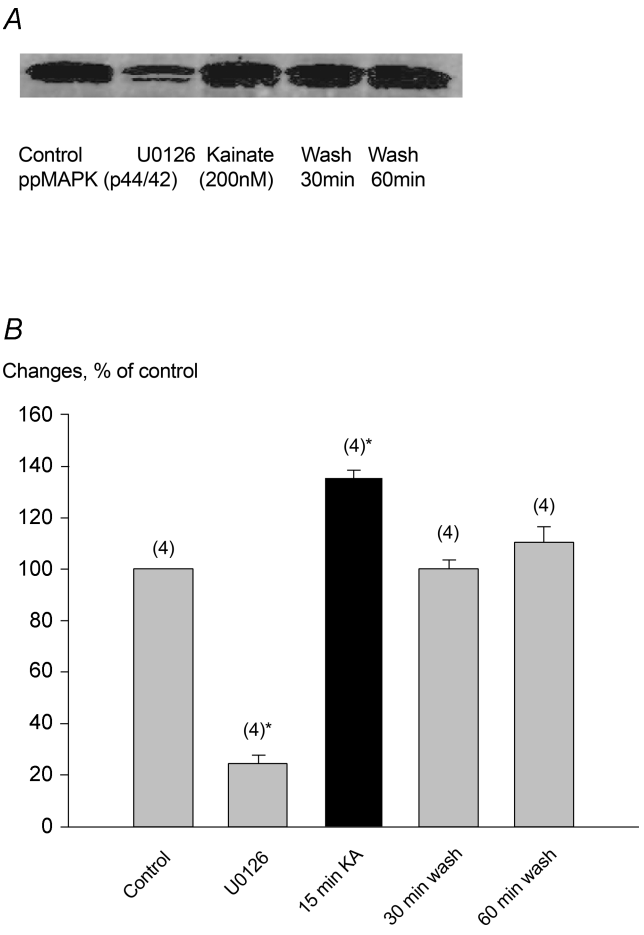

Figure 6. KARs lead to activation of MAP kinase
A, representative Western blots, following 15 min incubation with 200 nm kainate or 200 nm kainate and 10 μM U0126. Further slices were treated as above but subjected to a 30 or 60 min wash. Slices were extracted and 25 μg of hippocampal protein subjected to Western blotting with ppMAPK antisera. Relative fluroscence across treatments was measured using Licor Odyssey system and changes p-MAPK activity represented as changes now *versus* control. *B*, summarized data of *in vitro* ppMAPK assay shows that kainate elevated levels of ppMAPK and the effect was suppressed in the presence of MAPK kinase inhibitor U0126. The data represent the means ± S.E.M. from four independent experiments. $^*P < 0.01$

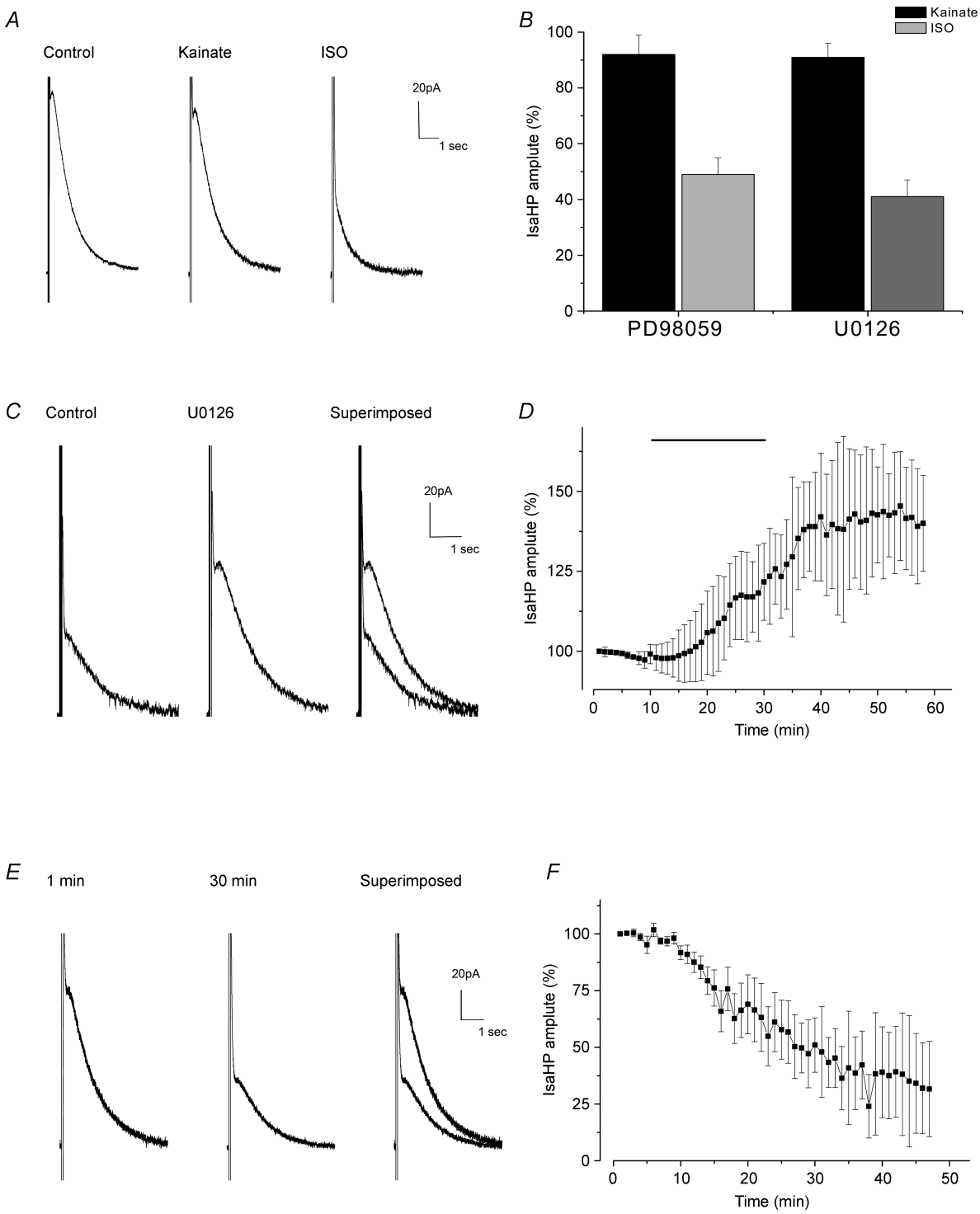

Figure 7. The MAPK signalling pathway is involved in kainate suppression of I_{sAHP}
A, whole-cell voltage-clamp recordings from a CA1 pyramidal neurone dialysed with 10 μM U0126 through the recording pipette. The MEK inhibitor blocked the inhibitory effect of kainate (200 nM, 10 min) but not of brief application of isoproterenol (ISO, 3 μM, 2–3 min). Scale bars indicate 20 pA and 1 s. *B*, summary data demonstrating that inclusion of distinct MEK inhibitors PD98059 (10 μM, $n = 5$) and U0126 (10 μM, $n = 5$) in recording pipettes blocked the metabotropic actions of kainate (200 nM) but not isoproterenol (ISO, 3 μM). *C*, effect of extracellular application of U0126 (10 μM) on the amplitude of I_{sAHP}. *D*, pooled data of the effect of U0126 on the I_{sAHP}. Bar indicates time of U0126 application. The data represent the means ± S.D. of four independent experiments. *E*, intracellular dialysis of a cell with activated MAPK (100 μM) resulted in suppression of the amplitude of I_{sAHP}. *F*, pooled data show the effect of activated MAPK on the I_{sAHP}. The data represent the means ± S.D. of three independent experiments.

et al. 2005). It has been noted that in both GluR5−/− and GluR6−/− mice, downregulation of KA2 also occurs (Christensen *et al.* 2004). While this implies that changes in KA2 do not account for the actions on the slow AHP, the observation also suggests that caution is required in interpreting kainate subunit deletions.

The metabotropic KARs which cause the postsynaptic action on the slow AHP seem likely to represent a distinct population from those that modulate GABA release. Notably, the GluR5 subunits that mediate KAR action on inhibitory terminals (Rodriguez-Moreno & Lerma, 1998; Cossart *et al.* 1998; Fisahn *et al.* 2004) show no functional changes as a result of PKA phosphorylation (Cho *et al.* 2003), although PKA dependence has been reported for kainate actions on glutamate release (Rodriguez-Moreno & Sihra, 2004). Nonetheless, GluR6-containing receptors remain the most likely explanation for the actions of kainate on the slow AHP.

We used two different drugs (indolactam V and OAG) to activate PKC directly; in both cases the result was an inhibition of I_{sAHP}. These observations support the results reported by Malenka *et al.* (1986) indicating that phorbol esters inhibit I_{sAHP}. The similar action suggests that this was a genuine effect of PKC activation, although there were quantitative differences. Concentrations of indolactam V as low as 300 nM were fully effective in blocking I_{sAHP}, and the effect was irreversible; the effect of 50 μM OAG resulted in partial blockade of I_{sAHP} and was reversible. A possible explanation for the differences is that since OAG is a synthetic fatty acid analogue of the natural ligand (DAG), it too is metabolized (Ebeling *et al.* 1985). Additionally, direct activators of PKC such as indolactam and phorbol esters might provide much stronger stimulation which overrides physiological controls. Note that both of these compounds caused much more complete inhibition of I_{sAHP} than OAG or KAR activation. Interestingly, Pan *et al.* (1997) similarly reported that OAG was less potent than phorbol esters at inhibiting slow afterhyperpolarizations in enteric neurones.

A mechanism for the persistent action of kainate

We attempted to uncover a putative mechanism to account for the long-lasting action of KAR stimulation. Pathways such as MAP kinases which are involved in late LTP (English & Sweatt, 1996; Coogan *et al.* 1999; Winder *et al.* 1999) are obvious candidates to test. It is also suggestive that kainate action requires PKC – one of the stimuli for this enzyme cascade (Roberson *et al.* 1999). Our results showing MAP kinase activation after kainate application are consistent with recently published data (Mao *et al.* 2004). The reduction in basal MAP kinase activity by MEK inhibitors that we observed was also reported recently (Lubin *et al.* 2005). The physiological correlates are that reduction of MAP kinase activity allows for enhanced I_{sAHP}, whereas stimulation of MAP kinase suppresses I_{sAHP}. Thus modulation of MAP kinase activity is reported as changes in neuronal excitability via regulation of I_{sAHP}.

Does MAP kinase represent a single common pathway by which many transmitters cause I_{sAHP} inhibition? Since both PKA and PKC have been shown to couple to MAP kinase (Roberson *et al.* 1999), our findings that MAP kinases are involved in the action of kainate receptors raises the unifying possibility that KARs and prolonged β-receptor stimulation both act via MAP kinase. In contrast to previous observations with brief β-receptor stimulation, we observed that prolonged β-receptor stimulation can lead to a persistent inhibition of I_{sAHP}. This mirrors the actions of KAR activation. Indeed, a differential coupling of β-receptors to MAP kinase signalling is known to depend on the duration of receptor activation (Shenoy *et al.* 2006). Accordingly, brief activity of the noradrenergic system may be insufficient to recruit the MAP kinase pathway required for more persistent modifications of excitability.

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