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Protease-activated receptor dependent and independent signaling by kallikreins 1 and 6 in CNS neuron and astroglial cell lines

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Abstract

While protease-activated receptors (PARs) are known to mediate signaling events in CNS, contributing both to normal function and pathogenesis, the endogenous activators of CNS PARs are poorly characterized. In this study, we test the hypothesis that kallikreins (KLKs) represent an important pool of endogenous activators of CNS PARs. Specifically, KLK1 and KLK6 were examined for their ability to evoke intracellular Ca^{2+} flux in a PAR-dependent fashion in NSC34 neurons and Neu7 astrocytes. Both KLKs were also examined for their ability to activate mitogen-activated protein kinases (extracellular signal-regulated kinases, C-Jun N-terminal kinases, and p38) and protein kinase B (AKT) intracellular signaling cascades. Cumulatively, these studies show that KLK6, but not KLK1, signals through PARs. KLK6 evoked intracellular Ca^{2+} flux was mediated by PAR1 in neurons and both PAR1 and PAR2 in astrocytes. Importantly, both KLK1 and KLK6 altered the activation state of mitogen-activated protein kinases and AKT, suggestive of important roles for each in CNS neuron and glial differentiation, and survival. The cellular specificity of CNS–KLK activity was underscored by observations that both proteases promoted AKT activation in astrocytes, but inhibited such signaling in neurons. PAR1 and bradykinin receptor inhibitors were used to demonstrate that KLK1-mediated activation of extracellular signal-regulated kinases in neurons occurred in a non-PAR, bradykinin 2 (B2) receptor-dependent fashion, while similar signaling by KLK6 was mediated by the combined activation of PAR1 and B2. Cumulatively results indicate KLK6, but not KLK1 is an activator of CNS PARs, and that both KLKs are poised to signal in a B2 receptor-dependent fashion to regulate multiple signal transduction pathways relevant to CNS physiologic function and dysfunction.

Keywords

astrocyte; bradykinin; mitogen-activated protein kinase; neuron; protease-activated receptor; protein kinase B

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The kallikreins (KLKs) comprise a family of 15 secreted serine proteases whose genes are localized in tandem on chromosome 19q13.4. While best characterized in terms of their utility as serum biomarkers in steroid hormone related cancers, emerging evidence links altered tissue, sera, or CSF levels to neurological disorders including Alzheimer's (Little *et al*. 1997; Zarghooni *et al.* 2002), multiple sclerosis (MS) (Scarisbrick *et al.* 2002; Blaber *et al.* 2004; Scarisbrick *et al.* 2008), stroke (Uchida *et al.* 2004), and trauma (Terayama *et al.* 2004; Scarisbrick *et al.* 2006b). The physiological actions of KLKs are only beginning to be understood, but roles for other serine proteases and/or their endogenous inhibitors in CNS are known to include neuron migration (Seeds *et al.* 1990), neurite outgrowth (Monard 1988; Siconolfi and Seeds 2001; Smirnova *et al.* 2001), synaptic plasticity (Liu *et al.* 1994), neurogenic pain (Vergnolle *et al.* 2001), and neurodegeneration (Tsirka *et al.* 1997). We have become particularly interested in determining the CNS specific roles of two KLKs, KLK1 and KLK6, as these are expressed at moderate to high levels in normal human brain, are elevated in CNS and serum of MS patients, and each has been shown to cause profound loss of neurites and degeneration of cortical neurons *in vitro* (Scarisbrick *et al.* 2002, 2008).

Kallikrein 6 is the most abundant KLK in CNS with highest levels of expression in brainstem and spinal cord (Scarisbrick *et al.* 1997, 2001, 2006a). In normal CNS, KLK6 is primarily associated with neurons and oligodendrocytes (Scarisbrick *et al.* 2000), however in response to injury or disease, KLK6 is additionally expressed by reactive astrocytes and microglia/macrophages (Scarisbrick *et al.* 2000, 2002, 2006b). Despite the fact that KLK6 is one of the most highly expressed serine proteases in adult CNS, with predicted broad substrate specificity (Scarisbrick *et al.* 1997), that includes hydrolysis of extracellular matrix and myelin proteins (Bernett *et al.* 2002; Blaber *et al.* 2002; Scarisbrick *et al.* 2002), relatively little is known regarding its physiologic activities.

Kallikrein 1 is moderately expressed in CNS (Raidoo *et al.* 1996; Scarisbrick *et al.* 2006a) and like other KLKs its CNS specific activities are poorly characterized. KLK1 cleaves an array of peptide precursors, including insulin (Ole-MoiYoi *et al.* 1979), and apolipoprotein B-100 (Cardin *et al.* 1984). KLK1 is perhaps best characterized by its ability to cleave low molecular weight kininogen to release Lysbradykinin which binds to G protein linked bradykinin receptor 2 (B2). KLK1 is implicated in regulation of blood pressure, sodium and water homeostasis, tumor growth, and inflammatory events (Bhoola *et al.* 2001). Interestingly, delivery of exogenous KLK1 in rodent models of ischemia improves neurologic function, limits inflammation and suppresses oxidative stress to enhance survival of neurons and glia in a B2 receptor-dependent fashion (Xia *et al.* 2004).

Recent evidence indicates certain KLKs, including KLK6, mediate their physiologic effects in part by activation of protease-activated receptors (PARs). Four G protein linked PARs have been described (PAR1–4) which are activated by N-terminal hydrolysis exposing a tethered ligand that binds intramolecularly triggering signal transduction. PARs are best characterized in terms of thrombin which activates PAR1, -3, and -4, and trypsin which can activate all four (Steinhoff *et al.* 2005). KLK5, KLK6, and KLK14 were recently shown to mobilize Ca^{2+} in rat v-K-ras transformed Normal Rat Kidney PAR2 over-expressing cells and to mediate aortic ring relaxation, in a PAR2-dependent fashion. KLK14 was additionally shown to activate PAR1, triggering Ca^{2+} flux in human embryonic kidney cells, and PAR4, to promote aggregation of rat platelets (Oikonomopoulou *et al.* 2006a,b).

While the activity of PAR is best characterized in the hemostatic and blood coagulation systems, these receptors are also widespread in CNS neurons and glia (Weinstein *et al.* 1995; Striggow *et al.* 2000; Wang *et al.* 2002a; Junge *et al.* 2004; Bushell *et al.* 2006). Proposed roles for CNS PARs include those related to normal brain function such as potentiation of NMDA receptors (Gingrich *et al.* 2000), as well as pathogenic mechanisms associated with ischemia

(Striggow *et al.* 2000; Junge *et al.* 2003), neurodegeneration (Smith-Swintosky *et al.* 1997), astrogliosis (Wang *et al.* 2002b; Sorensen *et al.* 2003; Nicole *et al.* 2005), hyperalgesia (Vergnolle *et al.* 2001), and neurogenic inflammation (Steinhoff *et al.* 2000). Despite these reports the endogenous activators of PARs in CNS have not been clearly identified. We hypothesize select tissue KLKs represent endogenous activators of CNS PARs. In this study, we determine the ability of two KLKs, KLK1 and KLK6, to mobilize intracellular Ca^{2+} in a PAR-dependent fashion in CNS derived neuron and astroglial cell lines and determine whether downstream signaling cascades related to cell differentiation and survival are activated.

Materials and methods

Cell culture

The NSC34 neuron and Neu7 astrocyte cell lines were used to determine the potential signaling activities elicited by KLK1 and KLK6 in CNS. NSC34 neurons are a murine spinal motor neuron hybridoma cell line which exhibits properties similar to motor neurons, including neurite extension, generation of action potentials, and acetylcholine production (Eggett *et al.* 2000; Scarisbrick *et al.* 2006b). Neu7 astrocytes are a murine cortical astrocyte cell line created by retroviral immortalization. Mirroring glial scar properties *in vivo*, Neu7 astrocytes produce an extracellular matrix which is inhibitory to neurite outgrowth (Fok-Seang *et al.* 1995).

NSC34 neurons were grown in high glucose Dulbecco's modified Eagle's medium, 10% fetal calf serum, 50 U/mL penicillin–streptomycin, and 2 mM Glutamax for expansion. NSC34 neurons were then differentiated for 48 h on surfaces coated with 10 μg/mL laminin under serum-free conditions. We have previously shown that growth in Neurobasal A media with 2% B27 supplement, 50 U/mL penicillin-streptomycin, 2 mM Glutamax, and 0.45% glucose (Sigma, St. Louis, MO, USA) promotes differentiation of NSC34 neurons and extensive neurite outgrowth (Scarisbrick *et al.* 2006b).

Neu7 astrocytes were adapted for growth under defined serum-free conditions (Neurobasal A with 2% B27 supplement, 1% N_2 supplement, 50 U/mL penicillin-streptomycin, 0.45% glucose, 2 mM Glutamax, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol; Sigma). Neu7 astrocytes were grown on surfaces coated with 10 μg/mL poly-D-lysine (Sigma). All cells were maintained at 37°C in 95% air and 5% CO_2. All cell culture reagents were obtained from Invitrogen (Carlsbad, CA, USA) unless otherwise indicated.

Primary cortical neurons and astrocytes were isolated from embryonic day 15 or postnatal day 1 C57/BL6J mice (Jackson Laboratories, Bar Harbor, ME, USA), essentially as described previously (McLaughlin *et al.* 1998). Primary neurons and astrocytes were differentiated for 96 h as described above for their cell line counterparts. All procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

RNA isolation and quantitative PCR

Total RNA was isolated from differentiated cells and from whole adult mouse brain or spinal cord using RNA STAT-60 (Tel-Test Inc., Friendswood, TX, USA). The level of PAR1–4 mRNA expression in each case was determined in 0.5 μg of RNA using Light Cycler RNA Amplification SYBR Green I (Roche, Basel, Switzerland) and either a Light Cycler (Roche) or iCycler iQ5 system (BioRad, Hercules, CA, USA). mRNA copy number in each case was determined using a standard curve prepared by parallel amplification of cDNA clones diluted to a known copy number as previously described in detail (Christophi *et al.* 2004). Primers used for amplification are provided in Table 1. Amplification of the housekeeping gene glyceraldehyde phosphate 3-dehydrogenase was used as a loading control. The mean and SE of PAR1–4 copy number was determined using three samples from independent cultures.

Ratiometric calcium imaging

Differentiated NSC34 neurons or Neu7 astrocytes were grown on coated chambered cover glass (Lab-Tek II #1.5; Nalge Nunc International, Rochester, NY, USA) at a density of 25 or 50×10^3 cells per cm^2, respectively. The cells were rinsed with imaging media (0.137 M NaCl, 5 mM KCl, 5.6 mM glucose, 20 mM HEPES, 0.59 mM KH_2PO_4, 0.014 mM $CaCl_2$, 0.009 mM $MgSO_4$, and 10 mM $NaHCO_3$, pH 7.4) (Sigma), loaded with fura-2AM (0.05 mM) (Invitrogen) in imaging media for 1 h at 20°C then washed with the same media. Cells were imaged using a Zeiss Axiovert (Carl Zeiss, Inc., Thornwood. NY, USA) inverted microscope, a 40× oil immersion lens, and the Attofluor RatioVision System (Atto Bioscience, Rockville, MD, USA).

For ratiometric imaging, cells were alternately excited at 340 and 380 nm and emission collected between 470 and 550 nm. Changes in the $F_{340}/F_{380\ nm}$ fluorescence ratio were determined from selected regions encompassing the cytoplasm of a single cell; 10–20 cells were imaged per well, and 30–60 cells were quantified per condition. Each experiment was repeated at least three times and five representative traces are shown. Cells were imaged initially for 2 min to establish a baseline $F_{340}/F_{380\ nm}$ ratio. Agonists were then added and changes in the fluorescence ratio monitored. For cross desensitization experiments, 4 min after application of the first agonist, a second agonist was added and changes in the fluorescence ratio monitored. Images were taken every 10 s for a period of 10 min. When quantification of Ca^{2+} responses was performed the data were normalized by subtraction of the baseline $F_{340}/F_{380\ nm}$ reading. Agonists used: 1 μM bradykinin, 1 U thrombin, 100 nM trypsin (Sigma), 40 μM PAR1-activating peptide (AP) (TFLLR-amide), 200 μM PAR2-AP (SLIGRL-amide), 200 μM PAR4-AP (GYPGFK-amide) (Peptides International, Louisville, KY, USA), 400 nM KLK1, and 400 nM KLK6. Recombinant active KLK1 and KLK6 were expressed using a baculovirus/insect system and purified as described previously (Bernett *et al.* 2002; Blaber *et al.* 2002; Laxmikanthan *et al.* 2005).

Western blots

Western blots were performed using standard methodology Briefly, membranes were blocked for 1 h at 20°C using either 2% bovine serum albumin or 5% milk. Following block, primary antibodies were applied at concentrations of 1 : 1000 overnight at 4°C. Primary antibodies used were phospho-extracellular signal-regulated kinases (ERK), ERK, phospho-C-Jun N-terminal kinases (JNK), JNK, phospho-p38, p38, phospho-protein kinase B (AKT), and AKT all from Cell Signaling Technology (Danvers, MA, USA). Membranes were washed and horseradish peroxidase-conjugated secondary antibody (Santa Cruz, Santa Cruz, CA, USA) applied at 1 : 25 000 for 1 h at 20°C. Membranes were then washed and Supersignal Pico (Pierce, Rockford, IL, USA) added for detection. Westerns were repeated three times, scanned, quantified by densitometry using NIH ImageJ (Bethesda, MD, USA), and finally normalized to the non-phosphorylated form of each protein. All density measurements are expressed as a percent of the maximal response observed.

To determine the dependence of KLK1 and KLK6 signaling on PAR1 or B2 receptors, NSC34 neurons were pre-treated for 30 min with either the PAR1 antagonist SCH79797 (70 nM; Tocris, Ellisville, MO, USA), the B2 antagonist Icatibant (1 μM; Sigma), or vehicle alone. Cells were then exposed to KLK1 or KLK6 for 5 min, as described above, and ERK activation examined by western. The significance of changes in activation of signaling proteins was evaluated using unpaired Student's *t*-test.

Results

Functionally active PAR1 and PAR2 expression in NSC34 neurons and Neu7 astrocytes

An essential aspect of these studies was to determine the complement of PAR expressed by CNS neurons and astrocytes and whether these were functional. Importantly, PAR expression in NSC34 neurons and Neu7 astrocytes closely mirrored expression levels observed in primary cortical neurons and astrocytes isolated from the developing brain of C57/BL6J mice and allowed to mature in culture (Fig. 1). PAR1, also known as the thrombin receptor, was by far the most abundant PAR in both neurons and astrocytes. Significant levels of PAR2 were detected in astrocytes, but only very low levels were detectable in neurons. PAR3 and PAR4 were also detected in both cell types, but at low levels relative to PAR1. In parallel, the thrombin receptor was by far the most abundant PAR in adult murine brain and spinal cord while the other PARs were expressed at levels at least 10-fold lower. These results point to the potential for all 4 of the known PARs to participate in signaling in CNS but indicate the likely predominant role played by PAR1.

Ratiometric Ca^{2+} imaging was used to determine whether PARs observed in the neuron and astrocyte cell lines by quantitative PCR were present in sufficient quantity to produce a Ca^{2+} signal in response to stimulation with specific peptides that mimic the PARs tethered ligand. In NSC34 neurons, while PAR1-AP (40 μM) produced a Ca^{2+} signal (Fig. 2a), both PAR2-AP (200 μM) (Fig. 2c) and PAR4-AP (200 μM) (Fig. 2e) were unable to elicit a similar robust response even at concentrations up to 500 μM. In Neu7 astrocytes, both PAR1-AP (40 μM) (Fig. 2b) and PAR2-AP (200 μM) (Fig. 2d), but not PAR4-AP (200–500 μM) (Fig. 2f) elicited a robust Ca^{2+} response.

KLK6 evokes Ca^{2+} signaling in NSC34 neurons and Neu7 astrocytes

Ratiometric calcium imaging was used to monitor changes in intracellular Ca^{2+} in response to KLK treatment. The normal level of KLK6 expressed in human CSF is approximately 40 nM (Zarghooni *et al.* 2002) which is likely representative of levels in brain overall. Cells were treated with doses of KLK6 at physiologic concentration as well as a 10-fold above (400 nM) and below (4 nM). Forty-four percent of NSC34 neurons responded at the 400 nM concentration while no response was observed at 40 or 4 nM under the assay conditions used. When Neu7 astrocytes were treated with identical concentrations of KLK6, 92% of the cells responded at 400 nM, and 86% at 40 nM, but no response was observed at the lowest concentrations examined, 4 nM. As the 400 nM concentration of KLK6 produced the most robust response in both cell types (Fig. 3c and d), 400 nM KLK6 was used in all subsequent experiments. KLK1 did not evoke a Ca^{2+} signal in either cell type when applied at similar concentrations (Fig. 3a and b).

KLK6 activates PAR1 on both NSC34 neurons and Neu7 astrocytes

To determine the ability of KLK6 to activate PAR1 in neurons, ratiometric Ca^{2+} imaging and a series of cross desensitization experiments were performed using KLK6, PAR1-AP, and the NSC34 neuron cell line. Initial treatment of NSC34 neurons with KLK6 (400 nM) generated a robust but transient Ca^{2+} response and this reduced subsequent PAR1-AP (40 μM) induced Ca^{2+} responses by 81.29 ± 9.53% (Fig. 4a). Initial treatment with PAR1-AP (40 μM) also generated a robust Ca^{2+} response and this abolished subsequent KLK6 (400 nM) evoked Ca^{2+} responses (Fig. 4b). A second PAR1 agonist, thrombin, was used to verify findings with PAR1-AP. Initial treatment with KLK6 (400 nM) evoked a transient Ca^{2+} response which reduced subsequent thrombin (1 U) induced Ca^{2+} signaling by 68.7 ± 18.1% (Fig. 4c). Initial exposure of NSC34 neurons to thrombin generated the expected transient Ca^{2+} peak and this abolished subsequent KLK6 (400 nM) Ca^{2+} mobilization (90.6 ± 4.65% reduction) (Fig. 4d). To determine if KLK1 could disarm PAR1, KLK1 was applied to NSC34 neurons followed

by addition of either PAR1-AP or thrombin. In neither case did KLK1 treatment alter the subsequent Ca^{2+} response (data not shown).

To determine whether KLK6 activates PAR1 in astrocytes, additional cross desensitization experiments were performed using the Neu7 astrocyte cell line. Similar to results observed in NSC34 neurons, in Neu7 astrocytes initial exposure to KLK6 (400 nM) generated a robust and transient Ca^{2+} spike and KLK6 abolished subsequent Ca^{2+} flux in response to PAR1-AP (40 μM) (90.7 ± 3.66% reduction) (Fig. 5a). KLK6 pre-treatment only partially reduced subsequent thrombin (1 U) (Fig. 5c) induced Ca^{2+} signaling (66.9 ± 16.9% reduction). By contrast to NSC34 neurons, exposure to thrombin (1 U) induced a strong transient Ca^{2+} spike which reduced but did not abolish subsequent KLK6 (400 nM) Ca^{2+} signaling (82.9 ± 16.0% reduction) (Fig. 5d). Similarly, exposure of Neu7 astrocytes to PAR1-AP (40 μM) only partially reduced KLK6 (400 nM) mediated Ca^{2+} flux (56.0 ± 11.9% reduction) (Fig. 5b). The ability of thrombin to desensitize KLK6 signaling in astrocytes was shown to be dose dependent (Fig. S1a).

KLK6 activates PAR2 on Neu7 astrocytes

To determine whether KLK6 additionally activates PAR2 in Neu7 astrocytes, cross desensitization experiments using KLK6 and PAR2-AP were performed. Exposure of Neu7 astrocytes to KLK6 (400 nM) induced the expected robust yet transient Ca^{2+} spike and KLK6 reduced subsequent PAR2-AP (200 μM) induced Ca^{2+} signaling by 56.7 ± 13.7% (Fig. 6a). The desensitization of PAR2-AP by KLK6 was not as pronounced as that observed with subsequent PAR1-AP or thrombin exposure. Initial exposure of Neu7 astrocytes to PAR2-AP (200 μM) generated the expected strong Ca^{2+} peak, but this treatment did not significantly alter the Ca^{2+} peak observed upon subsequent exposure to KLK6 (400 nM) (27.5 ± 38.1% increase) (Fig. 6b). To determine whether KLK6 simultaneously signals through PAR1 and PAR2, cross desensitization experiments were performed with prior exposure to trypsin, a serine protease known to activate both PAR1 and PAR2 (Steinhoff *et al.* 2005). Initial treatment with KLK6 (400 nM) reduced subsequent trypsin (100 nM) induced signaling (Fig. 6c) (88.1 ± 4.24% reduction), while initial treatment with trypsin (100 nM) abolished subsequent KLK6 (400 nM) induced signaling (87.5 ± 5.42% reduction) (Fig. 6d). Notably, trypsin was shown to desensitize KLK6 signaling in astrocytes in a dose-dependent fashion (Fig. S1b).

KLK6 differentially regulates MAPK and AKT signaling in NSC34 neurons

Activation of mitogen-activated protein kinase (MAPK) family members (ERK, JNK, and p38) and AKT in response to KLK1 or KLK6 exposure was examined over a period of 1 h. NSC34 neurons were treated with either KLK1 (200 nM), KLK6 (200 nM), or the PAR1- (40 μM) or PAR2-APs (200 μM) for 5, 10, 15, 30, or 60 min. In NSC34 neurons, ERK phosphorylation was strongly induced by KLK1 (Fig. 7a) at 5 min after exposure. Subsequently, activated ERK remained elevated gradually decreasing over time. Exposure of NSC34 neurons to KLK6 by contrast induced significant ERK activation at 5 min, followed by return to baseline at 10 min, and a second peak of ERK activation at the 15 min time point (Fig. 7b). PAR1-AP induced strong ERK phosphorylation at 5 min while the phosphorylated form of ERK at subsequent time points was detected at far below basal levels (Fig. 7c). Both the elevation above and the drop below basal phosphorylation of ERK in response to PAR1-AP treatment were statistically significant. KLK1 treatment induced a significant increase in JNK activation at both 5 and 30 min after exposure (Fig. 7d). By contrast, KLK6 and the PAR1-AP did not induce statistically significant changes in JNK phosphorylation in NSC34 neurons (Fig. 7e and f). Neither p38, nor its phosphorylated form, were detected at significant levels in NSC34 neurons (not shown).

Both KLK1 and KLK6 exposure resulted in significant decreases in AKT phosphorylation 10 min following exposure, with the effect of KLK6 being most pronounced and visible at both

the 5 and 10 min time points (Fig. 7g and h). A transient increase in AKT phosphorylation 30 min after KLK6 exposure was observed in two of four replicates, but did not achieve statistical significance. PAR1-AP produced a noticeable but not statistically significant decrease in the active form of AKT (Fig. 7i). PAR2-AP did not alter any of the intracellular signaling pathways examined (data not shown). Quantification of KLK and PAR–AP induced intracellular signaling in neurons is shown in Fig. S2.

KLK1 and KLK6 activate MAPK and AKT signaling in Neu7 astrocytes

Parallel to studies in neurons, the ability of KLK1 (200 nM), KLK6 (200 nM), PAR1- (40 μM), and PAR2-APs (200 μM) to activate MAPK family members or AKT was also examined in Neu7 astrocytes. KLK1, KLK6, PAR1- and PAR2-AP all promoted rapid phosphorylation of ERK by 5 min after exposure (Fig. 8a–d). ERK activation in each case was sustained through the 60 min time point examined except in the case of PAR1-AP in which basal levels of phosphorylation were reached by 30 min post-exposure. Peak levels of ERK activation were reached at 15 min in the case of KLK1 and across the 10 and 15 min time points in the case of KLK6. While robust ERK activation was observed with both PAR1- and PAR2-APs, across the three replicates examined the magnitude of ERK activation was not sufficient to reach the level of statistical significance. The activated form of p38 was not detected in Neu7 astrocytes following treatment with either KLK1 or KLK6 (Fig. 8e and f). However, both PAR1- and PAR2-APs generated a robust and transient increase in p38 phosphorylation at 5 min after exposure (Fig. 8g and h). Phosphorylated JNK was not detected in Neu7 astrocytes following treatment with KLK1 or KLK6 and the PAR1- and PAR2-APs did not generate a consistent pattern of JNK phosphorylation across the three replicates examined (data not shown).

Exposure of Neu7 astrocytes to KLK1 resulted in robust activation of AKT by 5 and at 10 min following exposure and this response was sustained over the 60 min period examined (Fig. 8i). KLK6 exposure promoted AKT phos-phorylation at the 5 and 10 min time points examined, but then decreased to levels below baseline (Fig. 8j). While the trend was consistent across the three replicates examined, neither KLK1 nor KLK6-induced AKT phosphorylation was sufficiently robust to reach the level of statistical significance. In response to PAR1-AP a small but statistically significant decrease in AKT phosphorylation was observed (Fig. 8k). PAR2-AP treatment produced a robust and sustained decrease in AKT phosphorylation beginning at 10 min through the 60 min time point examined (Fig. 8l). Quantification of KLK and PAR–AP induced intracellular signaling in astrocytes is shown in Fig. S3.

Differential effects of KLK1 and KLK6 on PAR and bradykinin receptors

Exposure of neurons to the PAR1-specific inhibitor, SCH79797 (70 nM), resulted in a significant decrease in KLK6-induced ERK phosphorylation (Fig. 9b). By contrast, this inhibitor had no effect on KLK1-induced ERK activation (Fig. 9a). Pre-treatment of cells with the B2-specific inhibitor, Icatibant (1 μM), significantly reduced activation of ERK in response to both KLK1 and KLK6 (Fig. 9c and d).

To further investigate KLK6-B2-dependent signaling, the ability of a B2 agonist to induce Ca^{2+} signaling was investigated. Exposure of neurons to the B2 agonist brady-kinin (1 μM) resulted in a robust Ca^{2+} signal (Fig. 10a), while similar exposure of astrocytes did not generate a signal even at concentrations up to 100 μM (Fig. 10b). To determine the relative contributions of KLK6-PAR1 and KLK6-B2 activation to the Ca^{2+} spike observed NSC34 neurons were exposed to either the PAR1 antagonist SCH79797 or the B2 antagonist Icatibant for at least 5 min prior to KLK6 application. Prior exposure of neurons to the PAR1 antagonist significantly reduced but did not eliminate KLK6-induced calcium signaling. Prior exposure of neurons to the B2 antagonist also significantly reduced the ability of KLK6 to generate a Ca^{2+} spike and

this reduction was significantly greater than that generated by blocking PAR1 (Student's *t*-test, $p < 0.05$) (Fig. 10c–e).

Discussion

Given the abundant expression of KLKs in CNS, and their deregulation with CNS injury and disease (Scarisbrick *et al.* 2006b), it has become essential to further clarify their potential scope of physiologic action. In this study we demonstrate KLK1 and KLK6 use both PAR and non-PAR-dependent mechanisms to activate multiple signal transduc-tion pathways in neurons and astrocytes. Critical to understanding the role of KLK6 in CNS, we demonstrate this novel protease functionally activates PAR2 and the classic thrombin receptor, PAR1. The fundamental importance of KLK6 as a signaling molecule in CNS is further underscored by data which indicate KLK6 is also capable of activating the B2 receptor. As expected KLK1 had similar B2 activating effects, but notably was unable to activate PARs. Taken with the demonstrated ability of both KLK1 and KLK6 to activate components of the MAPK and AKT signaling cascades in CNS derived cells, these studies point to novel KLK functions in regulation of signal transduction pathways likely critical to CNS physiologic function, including roles in cellular differentiation and survival.

Our understanding of PAR function comes in large part from studies regarding their roles in hemostasis and thrombosis. PAR1 activates cellular responses to thrombin, as well as coagulation factor Xa, plasmin, and protein C. PAR2 is activated by trypsin-like serine proteases, including trypsin, mast cell tryptase, and factors VIIa and Xa. PAR3 and PAR4 signal primarily in response to thrombin, with PAR4 also activated by cathepsin G (Steinhoff *et al.* 2005). In cases of CNS injury or disease several of these proteases are up regulated, or extravasate across a compromised blood–brain barrier, resulting in ectopic PAR activation (Citron *et al.* 2000; Riek-Burchardt *et al.* 2002). In relation to this, a wealth of experimental work points to the activity of thrombolytic proteases in CNS pathogenesis. *In vivo*, thrombin promotes disruption of the brain-endothelial barrier, brain edema, seizures, and ischemia (Lee *et al.* 1997; Striggow *et al.* 2000; Kitaoka *et al.* 2002). Also, high doses of thrombin are neurotoxic *in vivo* (Festoff 1992; Nishino *et al.* 1993; Xue and Del Bigio 2001; Hamill *et al.* 2007), and *in vitro* (Jalink and Moolenaar 1992; Jiang *et al.* 2002). Thrombin additionally promotes astrogliosis (Nishino *et al.* 1993; Nicole *et al.* 2005), and microglial activation (Suo *et al.* 2002; Choi *et al.* 2003).

While it is increasingly apparent that thrombolytic proteases contribute to aberrant PAR activation in CNS disease, the endogenous activators of CNS PARs, which likely contribute to ongoing neuromodulatory activities (Gingrich *et al.* 2000; Almonte *et al.* 2007), are far from clear. Data presented herein, taken with that in the literature, support the hypothesis that select KLKs are endogenous CNS PAR activators. First, 12 of 15 KLKs are known to be expressed in uninjured human brain (KLK1, 2, and 5–14) (Clements *et al.* 2001; Scarisbrick *et al.* 2006a). Of these, KLK1, 2, 5, 6, 8, and 10–14 have known or predicted trypsin-like specificity hydrolyzing substrates C-terminal to either Arg or Lys (Borgono *et al.* 2007; Yoon *et al.* 2007). Activation of PAR1, -2, and -4 occurs with cleavage C-terminal to Arg. Activation of PAR3 requires cleavage C-terminal to Lys (Steinhoff *et al.* 2005). Thus, 10 of the 12 KLKs present in CNS are in a position to activate PAR. By contrast, KLK3, 7, and 9 are predicted to have chymotrypsin-like specificity (Borgono *et al.* 2007), and therefore unlikely to cleave PARs.

Further supporting the concept that KLKs activate CNS PARs are recent studies by Oikonomopoulou *et al.* (2006a,b) demonstrating physiologic activation of PAR2 by KLK5, KLK6 and KLK14 in non-neuronal cells. Mass spectral analysis of KLK6 generated cleavage products indicated this enzyme was also able to activate PAR1 (Oikonomopoulou *et al.*

2006a), while a fluorescence resonance energy transfer based cleavage assay suggested KLK6 could activate PAR2, but not PAR1 (Angelo *et al.* 2006). Results herein are the first, in any cell type, to clearly demonstrate KLK6 functionally activates PAR1. The present findings taken with the demonstrated abundance of both KLK6 (Scarisbrick *et al.* 1997,2006a) and PAR1 in CNS (Junge *et al.* 2004), underscores the likely importance of the KLK6-PAR1 axis in CNS function and perhaps dysfunction. KLK6 was additionally shown to mobilize Ca^{2+} in a PAR2-dependent fashion in astrocytes pointing to the pleiotropic nature of this enzyme and that PAR activation is an important mechanism of action in the KLK6 repertoire. Demonstrating the specificity of KLK–PAR interactions, functional activation of PAR4 by KLK6, or any PAR by KLK1, was absent or below the detection limit of our assay.

Functional KLK6 activation of both PAR1 and PAR2 in astrocytes, but only PAR1 in neurons, likely relates to the high levels of PAR2 in the former. These findings may also underlie the fact that 10-fold lower levels of KLK6 were needed to trigger Ca^{2+} responses in astrocytes compared with neurons. Both the level of available protease, as well as the full complement of cognate receptors is therefore an important feature shaping KLK–PAR physiologic responses. The ability of KLK6 to activate both PAR1- and PAR2-dependent mechanisms would allow for significant expansion of its signaling capacity and provides a clear mechanism by which this novel protease likely mediates its CNS physiological and pathophysiological effects. Even small changes in KLK6 activity therefore would be significantly amplified by coordinate changes in PAR1 and/or PAR2, which are known to occur with CNS injury (Citron *et al.* 2000; Striggow *et al.* 2000; Rohatgi *et al.* 2004) and disease (Choi *et al.* 1995; Boven *et al.* 2003; Noorbakhsh *et al.* 2006; Afkhami-Goli *et al.* 2007). Notably, robust elevations in KLK6 levels have been demonstrated in MS, stroke, and spinal cord injury (Scarisbrick *et al.* 2002; Uchida *et al.* 2004; Scarisbrick *et al.* 2006b, 2008). Moreover, like thrombolytic enzymes, KLK6 is found at high levels in human serum, and may therefore enter the CNS with blood–brain barrier breakdown to participate in aberrant, likely pathogenic, PAR activation. Given that both KLK5 and KLK14 are also expressed in CNS (Scarisbrick *et al.* 2006b), and serum (Borgono and Diamandis 2004), and activate PARs (Oikonomopoulou *et al.* 2006a,b), findings herein point to PARs as an important site of integration of multiple KLK signaling cascades.

Cumulatively, the inability of the PAR1 inhibitor SCH79797 to block KLK1-induced ERK activation, taken with the inability of KLK1 to promote a Ca^{2+} response, indicate it is very unlikely that KLK1 activates PAR1. Rather than being PAR dependent, KLK1 signaling in CNS is likely to occur in a partially B2-dependent fashion, as the B2 inhibitor Icatibant strongly inhibited KLK1-induced ERK activation. This later result is not unexpected as KLK1 is best characterized by its kininogenase activity resulting in activation of B2. Like PARs, B2 receptors are constitutively expressed in a wide variety of tissues including CNS and are G protein-linked evoking Ca^{2+} responses and signaling cascades in numerous cell types (Bhoola *et al.* 2001). The ability of a B2 agonist but the inability of KLK1 to evoke Ca^{2+} signaling herein likely reflects the lack of kininogens in the Ca^{2+} imaging buffer. These results also suggest there is insufficient kininogen for KLK1 cleavage secreted into the media or on the surface of cells under the Ca^{2+} imaging conditions of this study. Prior studies link B2 receptor activation to brain edema in cases of CNS injury (Hellal *et al.* 2003; Lehmberg *et al.* 2003) or disease (Lorenzl *et al.* 1996). Given the up regulation of KLK1 and B2 receptors in stroke (Groger *et al.* 2005), results herein underscore the likely importance of the bradykinin receptor, rather than the PAR pathway, in mediating the CNS-specific effects of KLK1.

Of particular interest were observations in this study that KLK6-induced ERK activation in neurons was like that of KLK1, shutdown by the B2 inhibitor Icatibant. Furthermore, studies following up on this observation showed KLK6 mediated Ca^{2+} signaling in neurons to be dependent not only on PAR1 but also B2. Indeed KLK6-mediated activation of ERK and

Ca^{2+} signaling appear to depend on the co-activation of PAR1 and B2. Thus in addition to activation of PAR1, observations herein point to a novel means of B2 activation in neurons mediated by KLK6. While understanding the precise mechanism of KLK6 activation of B2 will require substantial additional studies, several possible mechanisms of action can be considered. One possibility is that KLK6 directly or indirectly promotes generation of bradykinin receptor activating kinins. This could explain B2 activation in the cell culture systems examined, but is more difficult to reconcile with the Ca^{2+} imaging assays in which KLK1, a known potent activator of kininogen, did not generate kinins in sufficient quantities to trigger a Ca^{2+} signal. Although direct KLK6-mediated kinin release is not ruled out by the current studies, it is not the most likely explanation as cleavage of kininogen to release Lys-bradykinin requires cleavage C-terminal to Lys, and this is not the preferred substrate specificity of KLK6 (Bernett *et al.* 2002; Blaber *et al.* 2002). An alternative mechanism may be that KLK6 activates another enzyme which in turn mediates kinin liberation. A further possible mechanism is direct activation of the B2 receptor by KLK6. Direct receptor activation is supported by prior studies showing that a variety of proteases including KLK1, trypsin, cathepsin G, and endoarginase C are able to activate the B2 receptor in Chinese hamster ovary, Madin–Darby canine kidney, and human embryonic kidney cells stably expressing B2 (Biyashev *et al.* 2006; Hecquet *et al.* 2006). Despite these intriguing results, independent studies suggest that local production of bradykinin by these cells, rather than direct B2 receptor activation by proteases, was responsible for the observed signaling (Houle *et al.* 2003). The results of the present study identify likely activation of B2 by KLK6, the mechanism of which will need to be addressed in future studies.

While the mechanism by which KLK6 activates B2 receptors remains to be determined, these findings demonstrate a previously unknown functional link between the KLK-related peptidase KLK6, and the well characterized tissue KLK–kininogen activation pathway. Given the widespread function of kinins in health and disease, including CNS disease (Chao and Chao 2006), these novel observations have important implications for understanding KLK6 function and B2 receptor physiology. These results additionally highlight the fact that KLK6 utilizes multiple signaling mechanisms, the net effect of which will be to amplify small changes in its concentration which may occur in normal and pathological conditions. For example in neurons, data presented suggest that KLK6-mediated Ca^{2+} flux and ERK activation require the co-activation of both the B2 and PAR1 receptors. Similar co-operativity between PAR1 and B2 has been demonstrated in Chinese hamster ovary and human endothelial cells. In these cell types arachidonic acid release in response to B2 receptor activation is significantly elevated when PAR1 is also activated (Hecquet *et al.* 2006).

Evidence presented indicates both KLK1 and KLK6 activate intracellular signaling pathways known to be involved in cellular survival and differentiation. KLK6 signaling occurred via PAR1 and B2 receptor dependent mechanisms while KLK1 signaling occurred by a non-PAR, B2-receptor dependent mechanism. In neurons both proteases promoted an early and pronounced decrease in activated AKT. In relation to this, we have previously demonstrated KLK1 and KLK6 promote rapid neurite retraction and degeneration of cortical neurons *in vitro* (Scarisbrick *et al.* 2008). Taken with strong evidence that AKT activation is linked to promotion of cell survival in a wide range of cell types (Brunet *et al.* 2001), we suggest KLK1 and KLK6 when present in excess, suppress phosphorylation of neuronal AKT thereby compromising cell survival. Both KLKs also promoted activation of ERK, which has been linked to diverse downstream effects in neurons, including neuron cell death, nociception, and neuronal plasticity (Selcher *et al.* 2003). The very robust neurotoxic properties of KLK1 (Scarisbrick *et al.* 2008) may relate to findings that this protease additionally promoted activation JNK, a pro-apoptotic protein (Arthur *et al.* 2007). Cumulatively these studies support the concept that both KLK1 and KLK6 represent important targets for CNS neuroprotective therapies.

By contrast to effects observed in neurons, in astrocytes there was a trend towards KLK1 and KLK6 promoting activation of AKT. Along with parallel elevations in activated ERK, we postulate KLK1 and KLK6 likely promote astrocyte survival and astrogliosis. Supporting this, prior studies indicate AKT activation promotes formation of reactive astrocytes (Choi *et al.* 2005; Neary *et al.* 2005), and PAR1 mediated ERK activation stimulates astrocyte proliferation (Wang *et al.* 2002a; Sorensen *et al.* 2003; Nicole *et al.* 2005; Luo *et al.* 2006; Wang *et al.* 2007). Notably, thrombin causes reversal of astrocyte stellation (Park *et al.* 2006) and KLK1 promotes astrocyte migration (Xia *et al.* 2004), each a hallmark of astrogliosis. The precise role of KLK1 and KLK6 in astrogliosis warrants further attention and is currently the subject of ongoing studies in our laboratory. This is a particularly important question in CNS injury as KLK6 is virtually absent from all astrocytes of normal CNS white matter, but is induced in astrocytes in cases of CNS injury or disease (Scarisbrick *et al.* 2002, 2006b). We propose autocrine or paracrine activity of astrocytic KLK6 activates PAR and bradykinin receptors to drive astrogliosis and neuron injury. Modulation of the KLK–PAR–bradykinin receptor axis therefore may offer an opportunity to modulate astrocytic glial scar formation and create an environment favorable to repair in the injured CNS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used

AKT, protein kinase B; AP, activating peptide; B2, bradykinin receptor 2; ERK, extracellular signal-regulated kinases; JNK, C-Jun N-terminal kinases; KLK, kallikrein; MAPK, mitogen-activated protein kinase; MS, multiple sclerosis; PAR, protease-activated receptor; RT, room temperature.

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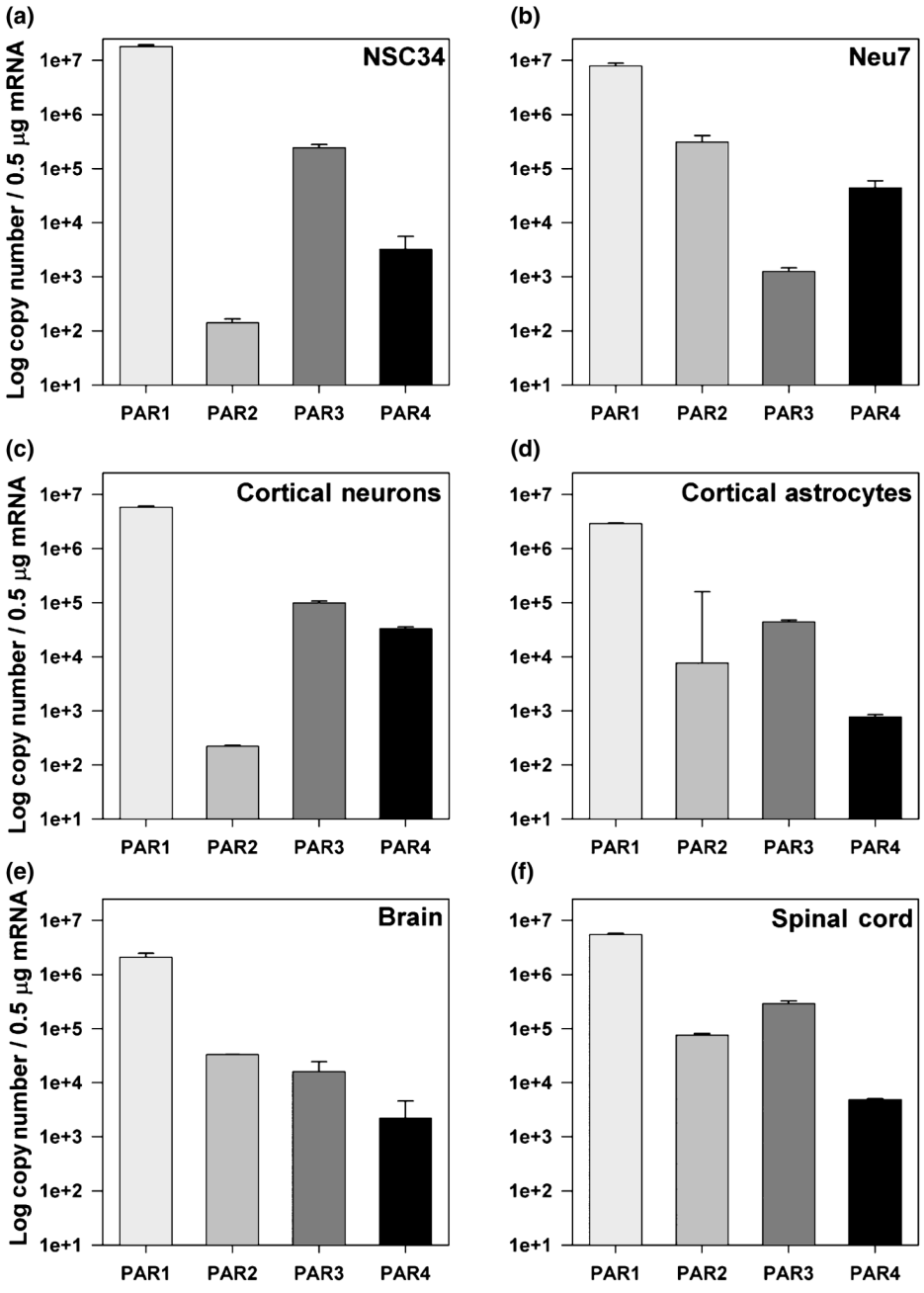

Fig. 1.

PAR expression in the murine CNS and CNS derived cells. Quantitative PCR showing relative mRNA expression of PAR1–4 in NSC34 neurons (a), Neu7 as-trocytes (b), primary cortical neurons (c), primary cortical astrocytes (d), adult mouse brain (e), and adult mouse spinal cord (f).

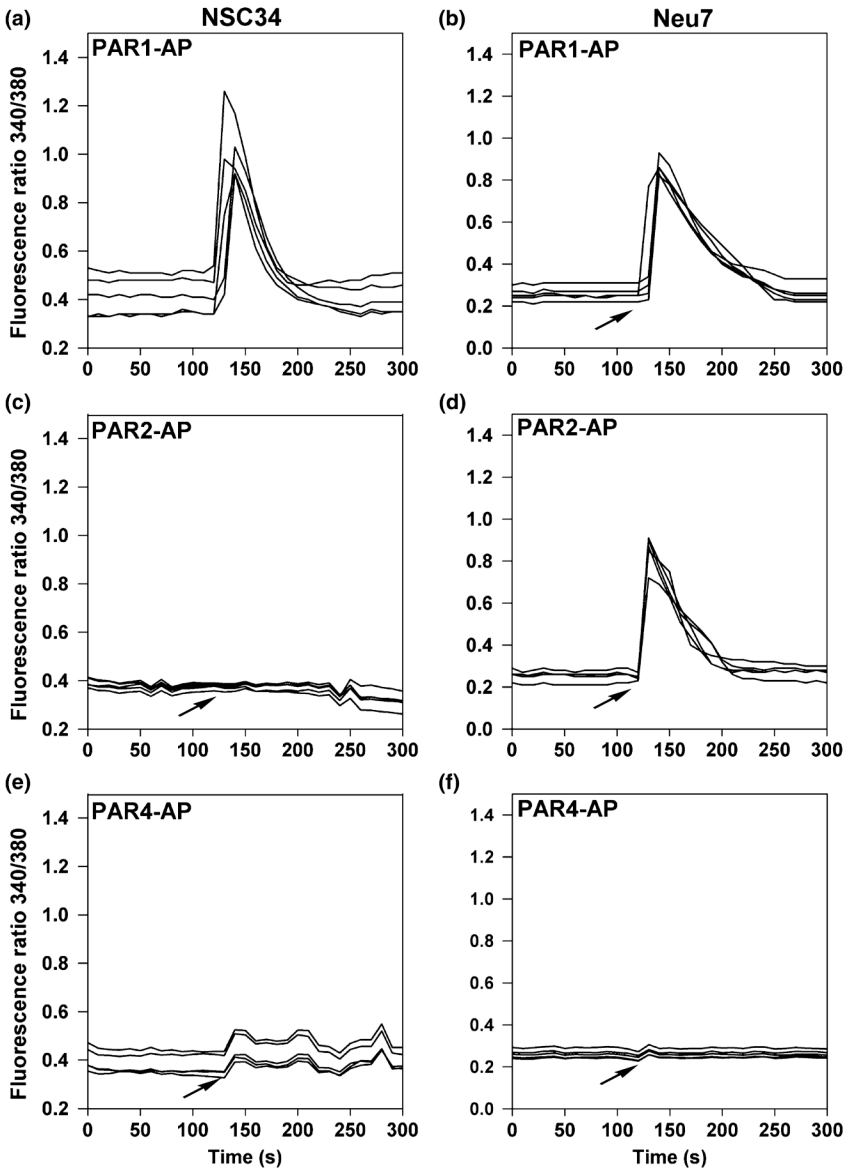

Fig. 2.

Presence of functional PARs on NSC34 neurons and Neu7 astrocytes. Representative traces from five cells show Ca^{2+} responses measured by changes in the fluorescence ratio (F_{340}/$F_{380\ nm}$) of fura-2 loaded NSC34 neurons (a, c, and e) or Neu7 astrocytes (b, d, and f). Arrows indicate the time of agonist application. (a) NSC34 neurons treated with PAR1-AP (40 μM) (n = 30); (b) Neu7 astrocytes treated with PAR1-AP (40 μM) (n = 30); (c) NSC34 neurons treated with PAR2-AP (200 μM) (n = 30); (d) Neu7 astrocytes treated with PAR2-AP (200 μM) (n = 30); (e) NSC34 neurons treated with PAR4-AP (200 μM) (n = 30); (f) Neu7 astrocytes treated with PAR4-AP (200 μM) (n = 30); n indicates total number of cells quantified.

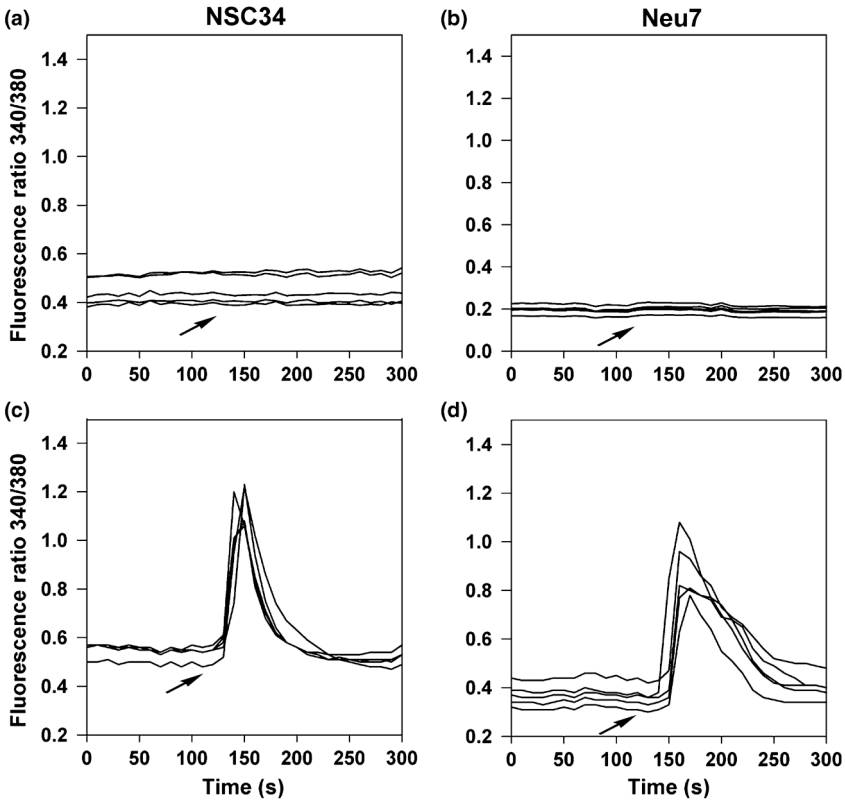

Fig. 3.

KLK6, but not KLK1, induces Ca^{2+} signaling in NSC34 neurons and Neu7 as-trocytes. Traces show Ca^{2+} responses measured by changes in the fluorescence ratio ($F_{340}/F_{380\ nm}$) of fura-2 loaded NSC34 neurons (a and c) or Neu7 astrocytes (b and d). Representative traces from five cells are shown. Arrows indicate the time of addition of agonist. (a) NSC34 neurons (n = 30) or (b) Neu7 astrocytes exposed to KLK1 (400 nM) (n = 30); (c) NSC34 neurons (n = 30) or (d) Neu7 astrocytes exposed to KLK6 (400 nM) (n = 30); n indicates total number of cells quantified.

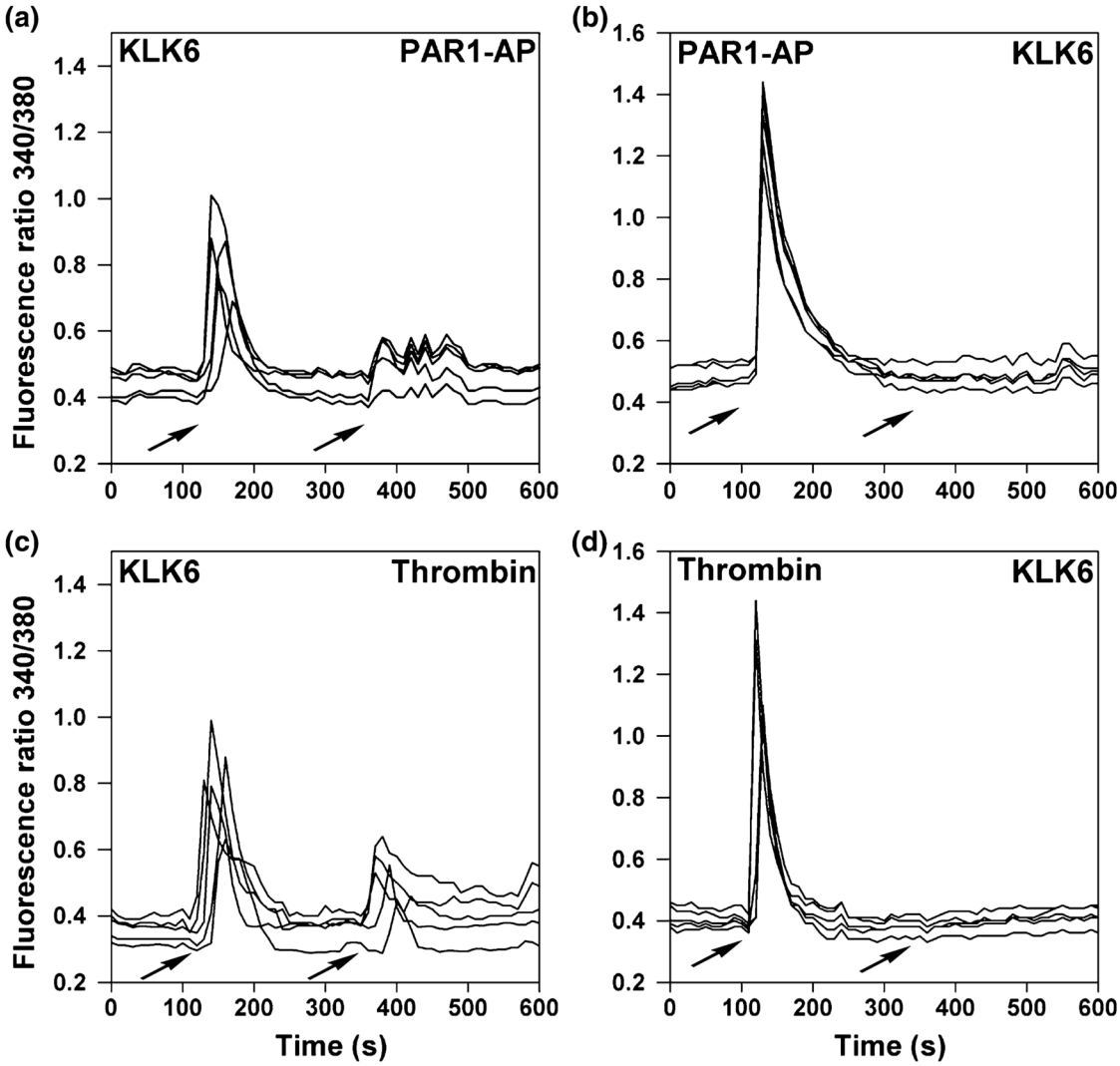

Fig. 4.

KLK6 Ca^{2+} signaling is mediated by PAR1 in NSC34 neurons. Traces show Ca^{2+} responses in fura-2 loaded NSC34 neurons in response to PAR cross desen-sitization. Changes in the fluorescence ratio ($F_{340}/F_{380\ nm}$) are shown from five representative cells. Arrows indicate the time of agonist application. (a) NSC34 neurons treated initially with KLK6 (400 nM) were desensitized to subsequent PAR1-AP (40 μM) exposure ($n = 40$); (b) NSC34 neurons initially exposed to PAR1-AP (40 μM) were desensitized to subsequent exposure to KLK6 (400 nM) ($n = 40$); (c) NSC34 neurons exposed initially to KLK6 (400 nM) showed a reduced signal to subsequent thrombin exposure (1 U) ($n = 40$); (d) NSC34 neurons exposed initially to thrombin (1 U) were desensitized to subsequent exposure to KLK6 (400 nM) ($n = 40$); *n* indicates total number of cells quantified.

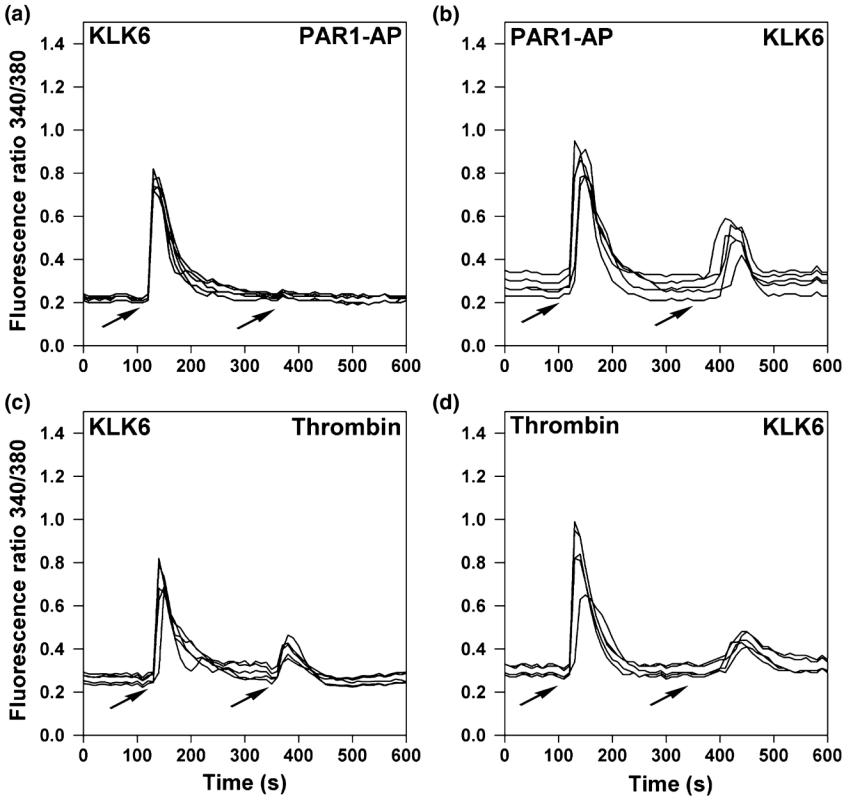

Fig. 5.

KLK6 Ca^{2+} signaling in Neu7 astrocytes can be mediated by PAR1. Traces show Ca^{2+} responses in fura-2 loaded Neu7 astrocytes in response to PAR cross desensitization. Changes in the fluorescence ratio ($F_{340}/F_{380\ nm}$) are shown from five representative cells. Arrows indicate the time of agonist application. (a) Neu7 astrocytes initially exposed to KLK6 (400 nM) were desensitized to subsequent PAR1-AP (40 μM) exposure ($n = 60$); (b) Neu7 astrocytes initially exposed to PAR1-AP (40 μM) were partially desensitized to subsequent treatment with KLK6 (400 nM) ($n = 60$); (c) Neu7 astrocytes initially exposed to KLK6 (400 nM) were partially desensitized to subsequent thrombin (1 U) exposure ($n = 40$); (d) Neu7 astrocytes exposed to thrombin (1 U) were partially desensitized to subsequent KLK6 (400 nM) exposure ($n = 40$); *n* indicates total number of cells quantified.

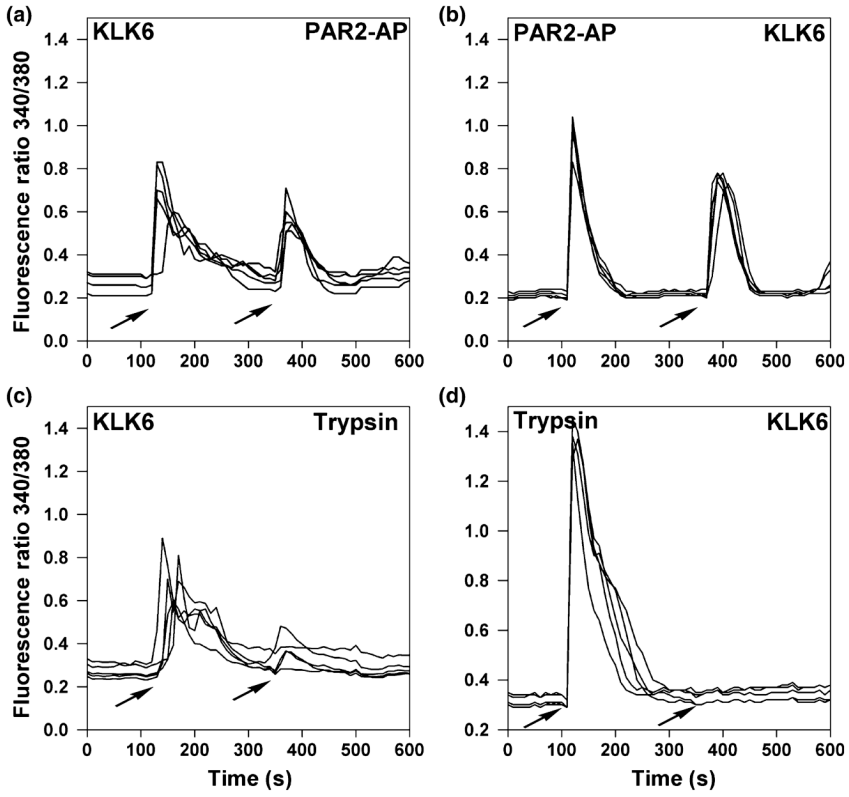

Fig. 6.

KLK6 Ca^{2+} signaling in Neu7 astrocytes can be mediated by PAR2. Calcium responses observed in fura-2 loaded Neu7 astrocytes in response to receptor cross desensitization. Changes in the fluorescence ratio ($F_{340}/F_{380\ nm}$) are shown for five representative cells in each case. Arrows indicate the time of agonist application. (a) Neu7 astrocytes initially exposed to KLK6 (400 nM) were partially desensitized to subsequent PAR2-AP (200 μM) exposure (n = 60); (b) Neu7 astrocytes initially exposed to PAR2-AP (200 μM) were not desensitized to subsequent KLK6 (400 nM) exposure (n = 60); (c) Neu7 astrocytes initially exposed to KLK6 (400 nM) were desensitized to subsequent trypsin (100 nM) exposure (n = 40); (d) Neu7 astrocytes exposed to trypsin (100 nM) were desensitized to subsequent exposure to KLK6 (400 nM) (n = 40); n indicates total number of cells quantified.

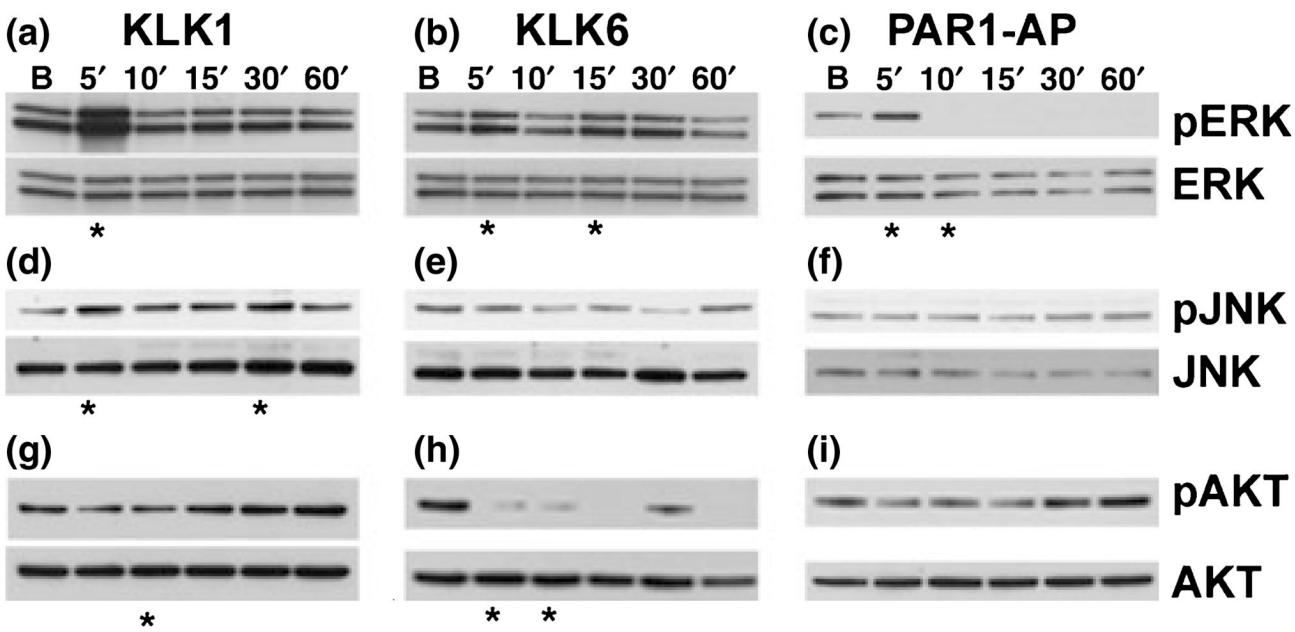

Fig. 7.

KLK1, KLK6, and PAR1-AP induced signaling in NSC34 neurons. Western blots for MAPK family members and AKT following exposure of NSC34 neurons to KLK1, KLK6, or PAR1-AP. ERK phosphorylation following exposure to (a) KLK1 (200 nM), (b) KLK6 (200 nM), or (c) PAR1-AP (40 μM). JNK phosphorylation following exposure to (d) KLK1 (200 nM), (e) KLK6 (200 nM), or (f) PAR1-AP (40 μM). AKT phosphorylation following exposure to (g) KLK1 (200 nM), (h) KLK6 (200 nM), or (i) PAR1-AP (40 μM); *$p < 0.05$.

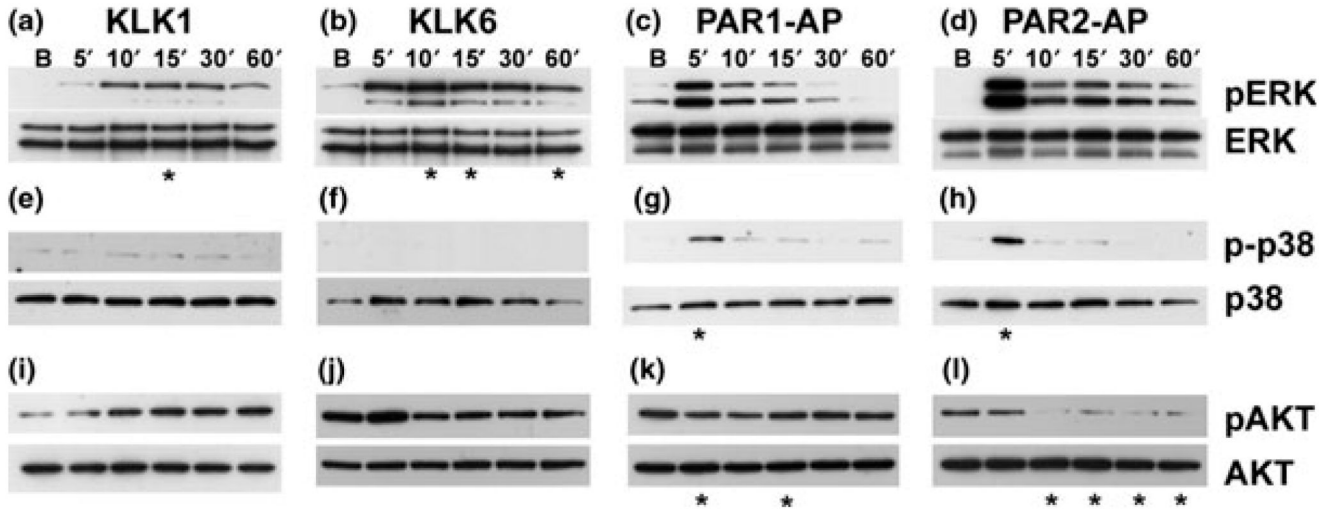

Fig. 8.

KLK1, KLK6, and PAR1- and PAR2-AP induced signaling in Neu7 astrocytes. Western blots for MAPK family members and AKT following exposure of Neu7 astrocytes to KLK1, KLK6, PAR1-, or PAR2-APs. ERK phosphorylation following exposure to (a) KLK1 (200 nM), (b) KLK6 (200 nM), (c) PAR1-AP (40 μM), or (d) PAR2-AP (200 μM). p38 phosphorylation following exposure to (e) KLK1 (200 nM), (f) KLK6 (200 nM), (g) PAR1-AP (40 μM), or (h) PAR2-AP (200 μM). AKT phosphorylation following exposure to (i) KLK1 (200 nM), (j) KLK6 (200 nM), (k) PAR1-AP (40 μM), or (l) PAR2-AP (200 μM); *$p < 0.05$.

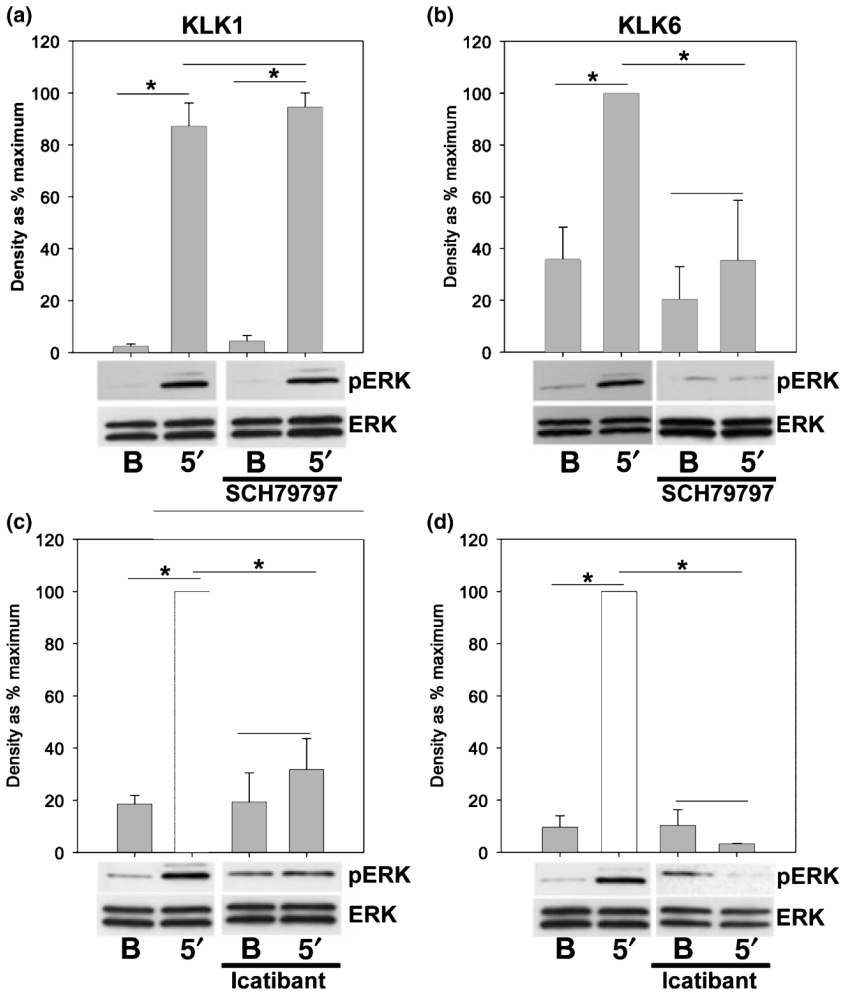

Fig. 9.

Differential inhibition of KLK1- and KLK6-mediated ERK activation by PAR1 and B2 receptor inhibitors. Western blots show the level of ERK phosphorylation in NSC34 neurons. Neurons were exposed to (a) KLK1 (200 nM), or (b) KLK6 (200 nM), in the presence or absence of the PAR1 antagonist SCH79797 (70 nM). In (c and d), neurons were exposed to KLK1 or KLK6, respectively, in the presence or absence of B2 antagonist Icatibant (1 μM); $*p < 0.05$.

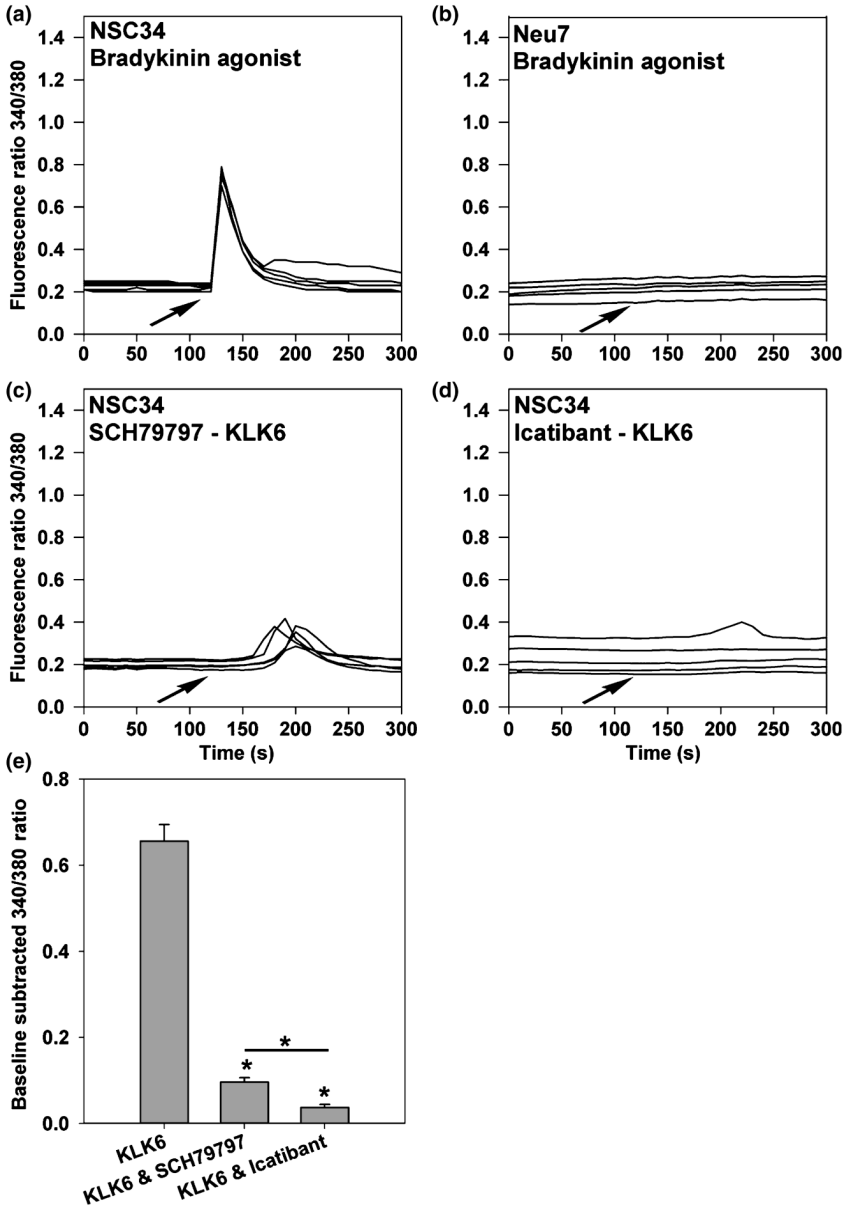

Fig. 10.

Inhibition of KLK6-mediated Ca^{2+} signaling by PAR1 and B2 receptor inhibitors. Traces show Ca^{2+} responses in fura-2 loaded cells. Changes in the fluorescence ratio ($F_{340}/F_{380\ nm}$) are shown from five representative cells. Traces show the Ca^{2} response of NSC34 neurons (n = 30) (a) or Neu7 astrocytes (n = 30) (b) to bradykinin (1 μM). Traces show the Ca^{2} response of NSC34 cells to KLK6 in the presence of the PAR1 antagonist SCH79797 (n = 60) (c) or the B2 antagonist Icatibant (n = 60) (d). Panel (e) shows a graph of the mean and SE of the baseline subtracted fluorescence ratio ($F_{340}/F_{380\ nm}$) of KLK6 response in NSC34 neurons; n indicates total number of cells quantified and $*p < 0.05$.

Table 1

Primers used for quantitative PCR (5′–3′)

Gene	Accession number	Sequence
PAR1 (f)	NM_010169.3	CTTGCTGATCGTCGCCC
PAR1 (r)		TTCACCGTAGCATCTGTCCT
PAR2 (f)	NM_007974.3	CCGGACCGAGAACCTTG
PAR2 (r)		CGGAAGAAAGACAGTGGTCAG
PAR3 (f)	NM_010170.3	TCCACACACCTTCACACCGA
PAR3 (r)		GAACGGGCCTTTAACGACTT
PAR4 (f)	NM_007975.3	CCCAGCATCTAGGATGATGTAGAG
PAR4 (r)		CAGCGTGTCACTGTCGTT
GAPDH (f)	NM_008084.2	ACCACCATGGAGAAGGC
GAPDH (r)		GGCATGGACTGTGGTCATGA

GAPDH, glyceraldehyde phosphate 3-dehydrogenase; PAR, protease-activated receptor. Forward (f) and reverse (r) primers were used to amplify murine PAR isoforms and GAPDH.