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Brevetoxin-induced phosphorylation of Pyk2 and Src in murine neocortical neurons involves distinct signaling pathways

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Abstract

Brevetoxins (PbTx-1 to PbTx-10) are potent lipid soluble polyether neurotoxins produced by the marine dinoflagellate *Karenia brevis*. Brevetoxins bind to site 5 of the α -subunit of voltage-gated sodium channels (VGSCs) and augment Na^+ influx. In neocortical neurons brevetoxins elevate intracellular Ca^{2+} and augment NMDA receptor signaling. In this study, we explored the effects of PbTx-2 on Pyk2 and Src activation in neocortical neurons. We found that both Pyk2 and Src were activated following PbTx-2 exposure. PbTx-2-induced Pyk2 Tyr402 phosphorylation was dependent on elevation of Ca^{2+} influx through NMDA receptors. Moreover, Pyk2 Tyr402 phosphorylation was also found to require PKC activation inasmuch as RO-31-8425 and GF 109203x both attenuated the response. In contrast, PbTx-2-induced Src Tyr416 phosphorylation involved a Gq-coupled receptor inasmuch as U73122, a specific PLC inhibitor, abolished the response. This Gq-coupled receptor appears to be mGluR 5. The PKC δ inhibitor rottlerin abolished PbTx-2-induced Src activation demonstrating that this isoform of PKC is involved in the activation of Src by PbTx-2. Considered together these data suggest that although activation of neuronal Pyk2 and Src result from PbTx-2 stimulation of VGSC, engagement of these two non-receptor tyrosine kinases involves distinct signaling pathways.

Keywords

Brevetoxin; mGluR; Neocortical neuron; PKC; Pyk2; Src

1. Introduction

Voltage-gated sodium channels (VGSCs) are involved in the generation of action potentials in neurons. VGSCs represent the molecular target for several groups of neurotoxins that alter channel function by binding to specific sites on the alpha subunit of the channel (Cestele and Catterall, 2000). One such group of neurotoxins are the brevetoxins (PbTx-1 to PbTx-10) which are potent lipid-soluble, polyether neurotoxins produced by the marine dinoflagellate *Karenia brevis* (formerly known as *Gymnodinium breve* and *Ptychodiscus brevis*), an organism linked to periodic red tide blooms in the Gulf of Mexico along the western Florida coastline (Baden, 1989) and New Zealand (Ishida et al., 1994). Brevetoxins interact with site 5 of the α -subunit of the VGSC and augment Na^+ influx through VGSC by increasing the mean open time of the channel, inhibiting channel inactivation and shifting the activation potential to more negative values (Jeglitsch et al., 1998).

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K. brevis blooms have been implicated in massive fish kills, bird deaths, and marine mammal mortalities (O'Shea et al., 1991; Bossart et al., 1998). In humans, two distinct clinical entities, depending on the route of exposure, have been identified. Ingestion of bivalve molluscs contaminated with brevetoxins leads to neurotoxic shellfish poisoning (NSP), the symptoms of which include nausea, cramps, paresthesias, weakness and difficulty in movement, paralysis, seizures and coma (Baden and Mende, 1982; Ellis, 1985; McFarren et al., 1965). Inhalation of the aerosolized brevetoxins from sea spray results in respiratory irritation as well as dizziness, tunnel vision and skin rashes (Baden and Mende, 1982; Pierce, 1986). Brevetoxins are known to accumulate in the central nervous system (CNS) at concentrations sufficient to affect CNS function when administered systemically in animals (Cattet and Geraci, 1993; Templeton et al., 1989). These brain concentrations of brevetoxins range from 2.12 nM (Benson et al., 1999) to 6.8 nM (Cattet and Geraci, 1993) and are therefore sufficient to produce significant fractional occupancy (42–70%) of VGSCs ($[^3\text{H}]\text{PbTx-3 } K_D = 2.9 \text{ nM}$ for site 5 of VGSC (Poli et al., 1986). These findings underscore the importance of studying the cellular consequences of brevetoxin exposure on the CNS.

Src-family kinases (SFKs) are widely expressed in the mammalian CNS (Kalia et al., 2004) and are involved in a range of cellular functions. One of the major functions of SFKs is to regulate the activity of voltage-gated ion channels (Cataldi et al., 1996; Fadool et al., 1997) and ionotropic neuro-transmitter receptors (Moss et al., 1995; Wan et al., 1997; Wang et al., 2004). Src was also found to be involved in synaptic transmission and plasticity as well as excitotoxicity since Src regulated NMDA receptor activity (Wang and Salter, 1994; Yu et al., 1997). It has been reported that activation of the Gq-protein coupled receptors enhances the NMDA receptor function through Src kinase activity (Grishin et al., 2005; Heidinger et al., 2002; Lu et al., 1999). One of the upstream regulators of SFKs is proline-rich tyrosine kinase (Pyk2) that autophosphorylates on Tyr-402 resulting in a SH2 binding site that recruits SFKs, which results in autophosphorylation on Tyr-416 of Src (Dikic et al., 1996; Huang et al., 2001; Park et al., 2004). However, recent evidence suggest that Pyk2 Tyr-402 phosphorylation is dependent on SFKs activation in cardiomyocytes, leukocytes, fibroblasts cells and the hippocampus (Andreev et al., 2001; Bayer et al., 2003; Butler and Blystone, 2005; Heidkamp et al., 2005; Huo et al., 2006). These data suggest that in addition to Src autophosphorylation following association with Pyk2, other signaling pathways may exist in which activation of SFKs is upstream of Pyk2 activation.

Our laboratory has previously demonstrated that brevetoxin enhances glutamate release and produces acute neuro-toxicity in cerebellar granule cells (CGC) (Berman and Murray, 1999). Brevetoxin induces Ca^{2+} influx in CGC that is responsible for this neurotoxic action (Berman and Murray, 2000). In contrast, in neocortical neurons, brevetoxin primarily affects the ERK–CREB–BDNF cascade that is involved in neuronal growth and survival (Dravid et al., 2004). Brevetoxin augments NMDA receptor signaling in neocortical neurons, and this response may be mediated by Src kinase activity (Dravid et al., 2005). Additionally, recent reports also suggest that increasing intra-cellular sodium concentration ($[\text{Na}^+]_i$) may regulate NMDA receptor function through Src kinase activity (Yu and Salter, 1998; Yu, 2006). However, upregulation of Src and/or Pyk2 by activation of VGSC remains to be demonstrated. In the present study, using PbTx-2 as a probe, we have characterized the effects of VGSC stimulation on Pyk2 and Src activation. We found that the PbTx-2-induced Pyk2 phosphorylation required PKC activation, SFK(s) activity and intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) elevation, while PbTx-2-induced Src activation required mGluR 5 activation and engagement of a phospholipase C and PKC δ activity.

2. Results

2.1. PbTx-2 exposure increases Pyk2 and Src phosphorylation on Tyr-402 and Tyr-416, respectively

Our previous studies have shown that PbTx-2 exposure produces elevation of intracellular Ca^{2+} and also augments NMDA receptor signaling in neocortical neurons. The latter response may involve Src activation (Dravid et al., 2004). Src activation involves autophosphorylation on tyrosine 416 while Pyk2 activation is associated with autophosphorylation on tyrosine 402. We therefore used antibodies that specifically recognize p-Tyr₄₀₂-Pyk2 and p-Tyr₄₁₆-Src to evaluate PbTx-2-induced influence on Src and Pyk2. Src and Pyk2 phosphorylation on Tyr-416 and Tyr-402 were respectively increased by exposure of neocortical neurons to PbTx-2. The PbTx-2 concentration–response profile demonstrated that both Pyk2 and Src display peak responses at 100 nM PbTx-2 (Fig. 1a). Using the 100 nM concentration of PbTx-2, we then explored the time-course of Src and Pyk2 phosphorylation. We found that 100 nM PbTx-2 produced a rapid rise in both Src and Pyk2 phosphorylation (about 2 fold for Pyk2, $p<0.01$, 1.8 fold for Src, $p<0.01$) with a peak response at 2.5 min followed by a gradual decrease in their phosphorylation (Fig. 1b). No changes in total Pyk2 or Src levels were observed after PbTx-2 treatment.

2.2. PbTx-2-induced Pyk2 and Src activation is a consequence of activation of voltage-gated sodium channels

To ascertain the role of VGSC in the actions of PbTx-2 on Src and Pyk2, we assessed the effects of tetrodotoxin (TTX), a VGSC channel blocker, on PbTx-2-induced Src and Pyk2 phosphorylation. As expected, pretreatment with 1 μM TTX for 15 min markedly attenuated PbTx-2-induced Pyk2 Tyr-402 phosphorylation from $180\pm6\%$ (mean $\pm$ S.E.M.) to $88\pm12\%$ ($n=6$, $p<0.01$) (Fig. 2a). Similarly, TTX pretreatment reduced PbTx-2-induced Src Tyr-416 phosphorylation from $202\pm10\%$ to $109\pm12\%$ ($n=6$, $p<0.01$) (Fig. 2b). TTX alone did not show any significant effect on the Src or Pyk2 baseline phosphorylation. These results suggest that PbTx-2-induced Src and Pyk2 phosphorylation were triggered by VGSC activation with attendant Na^+ influx.

2.3. PbTx-2-induced Pyk2 activation requires Src-family kinase activity

To determine whether PbTx-2 induced Pyk2 Tyr-402 phosphorylation is dependent on SFK(s) activity, neocortical cells were pretreated with PP-2 (1 μM), a specific SFK(s) inhibitor, and the inactive analog PP-3 (1 μM) prior to exposure to 100 nM PbTx-2. We observed that 1 μM PP-2 abrogated PbTx-2-induced Pyk2 Tyr-402 phosphorylation from $192\pm26\%$ of control to $29\pm18\%$ ($n=6$, $p<0.01$). PP-2 pretreatment also decreased basal Pyk2 Tyr-402 phosphorylation to $27\pm11\%$ of control. PP-3 pre-treatment, however, did not influence either PbTx-2-induced or basal Pyk2 Tyr-402 phosphorylation (Fig. 3a). We also evaluated the effects of PP-2 and PP-3 on PbTx-2 induced Src Tyr-416 phosphorylation. As expected (Fig. 3b), PP-2 abrogated the PbTx-2 induced Src Tyr-416 phosphorylation ($182\pm13\%$ vs. $56\pm15\%$, $n=6$, $p<0.01$) and reduced the basal level of phosphorylated Src ($68\pm3\%$ of baseline) as well. In contrast, PP-3 treatment did not significantly affect PbTx-2-induced Src Tyr-416 phosphorylation ($182\pm13\%$ vs. $152\pm12\%$, $n=6$, $p>0.05$). These data suggest that PbTx-2-induced Pyk2 Tyr-402 phosphorylation requires SFK(s) activity.

2.4. PbTx-2-induced Pyk2 activation requires Ca^{2+} influx through NMDA receptors, whereas PbTx-2-induced Src activation requires elevation of intracellular Ca^{2+} from cytoplasmic compartments

We hypothesized that PbTx-2-induced Pyk2 activation would be dependent on intracellular Ca^{2+} elevation as found with other stimuli associated with Pyk2 activation. In order to address

this, neocortical cells were incubated with the membrane-permeant compound BAPTA/AM, which is hydrolyzed to the active Ca^{2+} chelator BAPTA upon entry into cells. We found that PbTx-2-induced Pyk2 Tyr-402 phosphorylation was dramatically decreased by 40 μM BAPTA/AM pretreatment, and also that BAPTA/AM pretreatment decreased the basal level of phosphorylated Pyk2 (Fig. 4a). To test whether Pyk2 activation was dependent on extracellular Ca^{2+} influx, we first examined the effects of low extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_e = 0.2 \text{ mM}$) on PbTx-2-induced Pyk2 Tyr-402 phosphorylation. We found that reducing the $[\text{Ca}^{2+}]_e$ did not alter the basal level of phosphorylated Pyk2, however, the PbTx-2-induced Tyr-402 phosphorylation of Pyk2 was completely inhibited (Fig. 4a). Previously, we reported that brevetoxin induced Ca^{2+} influx in cerebellar granule cells occurred through manifold routes (Berman and Murray, 2000). Accordingly, we tested the contribution of Ca^{2+} influx through NMDA receptors and L-type voltage-gated Ca^{2+} channels (VGCC) on Pyk2 activation. As shown in Fig. 4b, MK-801 (1 μM), a blocker of the NMDA receptor, modestly attenuated the PbTx-2-induced Pyk2 activation from $216 \pm 8\%$ to $155 \pm 19\%$ ($n=5$, $p<0.05$), whereas pretreatment with 1 μM of nifedipine, an antagonist of VGCC, had no effect on the response to 100 nM PbTx-2. Neither MK-801 nor nifedipine affected basal Pyk2 phosphorylation. These data, in agreement with our hypothesis, suggest that PbTx-2-induced Pyk2 activation depends partly on extracellular Ca^{2+} influx through NMDA receptors. We also evaluated the dependency of Src activation on intracellular Ca^{2+} elevation. Pretreatment with BAPTA/AM abrogated the PbTx-2-induced Src Tyr-416 phosphorylation, but was without effect on basal Src phosphorylation (Fig. 4c). These data indicate that PbTx-2-induced Src activation is also dependent on an elevation of intracellular Ca^{2+} . We therefore tested the effect of low $[\text{Ca}^{2+}]_e$ on Src Tyr-416 phosphorylation. Unlike the inhibition of PbTx-2-induced Pyk2 phosphorylation, low $[\text{Ca}^{2+}]_e$ did not affect basal or PbTx-2-induced Src Tyr-416 phosphorylation. This was further confirmed by pretreatment with either MK-801 or nifedipine. Neither MK-801 nor nifedipine exhibited any effect on PbTx-2-induced Src Tyr-416 phosphorylation (data not shown). Thus, although PbTx-2-induced phosphorylation of both Src Tyr416 and Pyk2 Tyr402 are dependent on elevation of intracellular Ca^{2+} , these results suggest the involvement of distinct Ca^{2+} signaling pathways in the regulation of the two tyrosine kinases.

2.5. PbTx-2-induced Src activation involves the mGluR 5 receptor

In as much as intracellular Ca^{2+} release may be associated with Gq coupled receptor activation, we evaluated the role of phospholipase C (PLC) in the response to PbTx-2, using the inhibitor U73122. Pre-incubation of neocortical neurons with U73122 (3 μM) eliminated PbTx-2-induced Src Tyr-416 phosphorylation from $182 \pm 13\%$ to $31 \pm 8\%$ of control ($n=6$, $p<0.01$), and also reduced basal Src phosphorylation to $41 \pm 2\%$ (Fig. 5a). These data suggest that PbTx-2-induced Src phosphorylation may require Gq-coupled receptor(s) activation. Inasmuch as we have shown previously that brevetoxin produces glutamate release from intact neurons (Berman and Murray, 1999), we assessed the effects of mGluR 1 and mGluR 5 antagonists on the response to PbTx-2. As shown in Fig. 5b, S-4-CPG (500 μM) an antagonist of mGluR 1 receptors had no effect on PbTx-2-induced Src Tyr-416 phosphorylation. Pretreatment with the mGluR 5 antagonist, MTEP, however, did attenuate PbTx-2-induced Src Tyr-416 phosphorylation from $186 \pm 8\%$ to $123 \pm 14\%$ ($n=5$, $p<0.01$). MTEP pretreatment also slightly attenuated basal Src phosphorylation, but this was not statistically significant. We also ascertained the influence of inhibition of mGluR 1, mGluR 5 or PLC on PbTx-2-induced Pyk2 activation. No inhibitory effects of PbTx-2-induced Pyk2 activation were observed when cells were exposed to either S-4-CPG, MTEP or U73122 (data not shown). These data suggest that mGluR 5 is involved in PbTx-2-induced Src activation, but not PbTx-2-induced regulation of Pyk2.

2.6. PbTx-2-induced Pyk2 Tyr-402 and Src Tyr-416 phosphorylation are mediated by distinct PKC isozymes

Previous studies have indicated that Pyk2 is regulated by protein kinase C (PKC) (Alier and Morris, 2005). We therefore determined whether PbTx-2-induced Pyk2 Tyr-402 phosphorylation was dependent on PKC activity. We utilized two PKC inhibitors, RO-31-8425 and GF 109203x. Both RO-31-8425 and GF 109203x dramatically diminished the PbTx-2-induced Pyk2 Tyr-402 phosphorylation from $204 \pm 6\%$ to $129 \pm 11\%$ ($n=5$, $p<0.01$) and $126 \pm 16\%$ ($n=5$, $p<0.01$), respectively (Fig. 6a). We also determined whether PbTx-2-induced Src phosphorylation involves PKC activation. For that, we again utilized the two PKC inhibitors, RO-31-8425 and GF 109203x. Application of RO-31-8425 (1 μ M) did not affect PbTx-2-induced Src Tyr-416 phosphorylation ($173 \pm 7\%$ vs. $187 \pm 14\%$, $n=5$, $p<0.05$) (Fig. 6b); however, pretreatment with GF 109203x markedly attenuated PbTx-2-induced Src activation from $173 \pm 7\%$ to $99 \pm 18\%$ ($n=5$, $p<0.01$) (Fig. 6b). RO-31-8425 inhibits conventional PKC isozymes including PKC α , PKC β I, PKC β II, and PKC γ as well as the novel PKC, PKC ϵ (Wilkinson et al., 1993); whereas GF109203x inhibits PKC α -, β I-, β II-, γ -, δ -, and ϵ -isoforms. RO-31-8425 and GF 109203x therefore have partially overlapping selectivities for PKC isozymes. The ability of GF 109203x, but not RO-31-8425, to inhibit PKC δ suggests that the differential influence on PbTx-2-induced Src activation is related to their distinct PKC δ inhibition profiles. To confirm this hypothesis, we used the selective PKC δ inhibitor, rottlerin. As shown in Fig. 6c, 5 μ M rottlerin completely inhibited PbTx-2-induced Src Tyr416 phosphorylation. Considered together these results suggest that PbTx-2-induced Src Tyr416 and Pyk2 Tyr402 phosphorylation involves distinct PKC isozymes.

3. Discussion

We have previously reported that brevetoxin augments NMDA receptor signaling in neocortical neurons and that this effect may be mediated by Src kinase activity (Dravid et al., 2005). In the present study, we directly evaluated Src activation and its upstream signaling pathways in response to PbTx-2 exposure. We found that Src Tyr-416 was activated following PbTx-2 treatment with attendant $[Na^+]_i$ elevation (data not shown). These data support the suggestion that an increase in $[Na^+]_i$ may influence NMDA receptor function through Src kinase activity (Yu and Salter, 1998; Yu, 2006). Src phosphorylation is thought to be regulated by Pyk2 activity (Dikic et al., 1996; Huang et al., 2001; Park et al., 2004). Based on this notion, we assessed Pyk2 Tyr-402 phosphorylation level following PbTx-2 treatment. PbTx-2 treatment produced a rapid increase in Pyk2 Tyr-402 phosphorylation.

A previous report demonstrated that in response to KCl depolarization, the phosphorylation of Tyr-402 of Pyk2 was unaltered by PP-2, whereas total tyrosine phosphorylation of Pyk2 was dramatically reduced (Corvol et al., 2005). Unlike the present results in neocortical neurons, the KCl-induced depolarization of hippocampal slices in the earlier study evoked Ca^{2+} influx through voltage-gated calcium channels rather than NMDA receptors (Corvol et al., 2005). We found that Pyk2 Tyr-402 auto-phosphorylation in response to PbTx-2 was inhibited by the SFK inhibitor, PP-2 (1 μ M), but not the inactive analogue, PP-3 (1 μ M). This phenomenon has been similarly observed by several groups with different specific SFK inhibitors (Bayer et al., 2003; Butler and Blystone, 2005; Sorokin et al., 2001). Moreover, it has been reported that in SFK(s) deficient cells or mouse models, Pyk2 activation is reduced. For example, lysophosphatidic acid failed to activate Pyk2 in fibroblasts deficient in Src, Fyn and Yes (Andreev et al., 2001); and hippocampal slices derived from mice lacking the SFK Fyn exhibited a dramatic reduction in Pyk2 tyrosine phosphorylation including Tyr-402 (Corvol et al., 2005). Hence, it is reasonable to suggest that Src and/or other SFKs could act as an upstream positive regulators of Pyk2 activity. However, it remains unclear how SFKs induce auto-

phosphorylation at Pyk2 Tyr-402 in neocortical cells and which member of the SFKs is specifically involved in this response.

In agreement with previous studies in which the elevation of $[Ca^{2+}]_i$ resulted in Pyk2 Tyr-402 phosphorylation (Alier and Morris, 2005; Lev et al., 1995) [1, 33], we also found that PbTx-2-induced Pyk2 phosphorylation required an elevation of $[Ca^{2+}]_i$. PbTx-2-induced Pyk2 phosphorylation appeared to be dependent on Ca^{2+} influx rather than Ca^{2+} release from intracellular stores. This Ca^{2+} influx moreover may involve an NMDA receptor pathway since MK-801, but not nifedipine, attenuated the PbTx-2-induced Pyk2 Tyr-402 phosphorylation. These data suggest that the PbTx-2-induced Pyk2 Tyr-402 phosphorylation displays Ca^{2+} source-specificity. This mechanism is similar to that seen in neuronal excitotoxicity (Berman and Murray, 2000; Kato and Murota, 2005) as well as in activity dependent dendritic arborization (Wayman et al., 2006) in which Ca^{2+} entry is found to be primarily mediated through NMDA receptors rather than other pathways of Ca^{2+} influx. Conversely, PbTx-2-induced Src Tyr-416 phosphorylation involves Ca^{2+} release from intracellular Ca^{2+} stores rather than Ca^{2+} influx. Further dissection of this signaling pathway revealed that PbTx-2-induced Src activation involves PLC activity, a downstream effector of G_{aq} -coupled receptors inasmuch as U73122 (3 μ M) abolished the PbTx-2 induced Src Tyr-402 phosphorylation. Our data further suggest that mGluR 5, but not mGluR 1, mediates the PbTx-2-induced Src Tyr-416 phosphorylation. This response to PbTx-2 exposure is distinct from that produced by DHPG, a type I mGluR agonist, in which NMDA receptor upregulation by Pyk2/Src is mediated by mGluR1, but not mGluR5 (Heidinger et al., 2002). This difference may be related to the use of neocortical neurons plated on established glial monolayer in the earlier report, whereas the present data were obtained with an enriched neocortical neuron preparation in the absence of glial cells. In hippocampal neurons, it has been shown that mGluR 5 regulates NMDA receptor currents via Src activity (Lu et al., 1997; Mannaioni et al., 2001). Similarly, in mouse cortical wedges activation of mGluR 5 enhances NMDA responses (Attucci et al., 2001). It is noteworthy that the MTEP inhibition of PbTx-2-induced Src activation was not complete, whereas Src activation was completely abolished by a PLC inhibitor. It is therefore reasonable to infer that in addition to mGluR 5, there may be additional GPCRs that are activated in response to PbTx-2 exposure. In contrast to the regulation of Src activation, Pyk2 phosphorylation is not influenced by mGluR 5 activation. Similarly, and in stark contrast to Src, PbTx-2-induced phosphorylation of Pyk2 was unaltered by the PLC inhibitor U73122. These data further demonstrate that Src and Pyk2 activation involves distinct pathways.

It has been reported that PKC can directly bind to and activate Src (Moyers et al., 1993; Vondriska et al., 2001). Pyk2 activation was also found to be dependent on PKC activity. We propose that in addition to previous demonstrations of a $PKC \rightarrow Pyk2 \rightarrow Src$ pathway, there is an additional signaling pathway involving PKC activation of a Src-family kinase with downstream stimulation of Pyk2 Tyr-402 auto-phosphorylation in neocortical cells. This inference is based on our demonstration of a profound inhibition of Pyk2 Tyr-402 phosphorylation produced by the SFK inhibitor PP2.

We found that Src activation also involved $PKC\delta$ activity. Although $PKC\delta$ is not Ca^{2+} sensitive, we found PbTx-2-induced Src Tyr-416 phosphorylation required intracellular Ca^{2+} elevation. This elevation of $[Ca^{2+}]_i$ may be necessary to facilitate the vesicular release of glutamate. These data suggest a pathway in neocortical cells in which PbTx-2-induced glutamate release results in the activation of mGluR 5 with attendant coupling to G_{aq} and $PKC\delta$. This signaling pathway is similar to that observed in glioblastoma cells, where PMA-induced EGF receptor transactivation was mediated by a $PKC\delta/c-Src$ pathway (Amos et al., 2005). Taken together, we conclude that the observation that Pyk2 and Src phosphorylation require different PKC isozymes further demonstrates that Pyk2 and Src activation involve distinct pathways (Fig. 7).

VGSC are vital for normal CNS functioning and abnormal gating of these ion channels may lead to pathophysiological conditions such as epileptiform diseases (Kohling, 2002). VGSC are also the molecular targets for numerous naturally occurring neurotoxins (Cestele and Catterall, 2000). The results from the present study characterize the molecular signaling events triggered by exposure to a VGSC regulator. As mentioned previously, Pyk2 and Src have been shown to regulate important physiological function such as learning, memory, growth and survival of neurons (Girault et al., 1999; Kalia et al., 2004). The results of the present study therefore indicate that the cellular responses to brevetoxin exposure may not always manifest as neurotoxic sequelae (Berman and Murray, 1999), but by modulating key signaling pathways in the CNS may have a positive influence on neuronal plasticity and survival.

4. Experimental procedures

4.1. Materials

Trypsin, penicillin, streptomycin, heat-inactivated fetal bovine serum, horse serum and soybean trypsin inhibitor were obtained from Atlanta Biologicals (Norcross, GA, USA). Minimum essential medium, deoxyribonuclease (DNase), poly-L-lysine, cytosine arabinoside, anti-rabbit, anti-mouse IgG were from Sigma (St. Louis, MO, USA). Anti-phospho-Tyr416-Src was purchased from cell signaling technology (Beverly, MA, USA). Neurobasal and B-27 Supplement were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Tetrodotoxin (TTX), Brevetoxin-2 (PbTx-2) and (*S*)-4-Carboxyphenylglycine (*S*-CPG) were purchased from Tocris Cookson, Inc. (Ellisville, MO, USA). Anti-Src and Anti-Pyk2 antibodies were purchased from Upstate (Charlottesville, VA, USA). Rottlerin, 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), 4-Amino-7-phenylpyrazolo[3,4-*d*]pyrimidine (PP3), 3-((2-Methyl-1,3-thiazol-4-yl)ethynyl) pyridine (MTEP), 1,2-*bis*(*o*-Aminophenoxy) ethane-*N,N,N,N*-tetraacetic Acid Tetra (acetoxymethyl) Ester (BAPTA/AM), 2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (GF 109203x), 2-[8-(Aminomethyl)-6,7,8,9-tetrahydropyrido [1,2-*a*]indol-3-yl]-3-(1-methyl-1H-indol-3-yl) maleimide (RO-31-8425) and anti-phospho-Tyr402-Pyk2 were purchased from Calbiochem (La Jolla, CA, USA). ECL kit was purchased from Amersham Biosciences (Piscataway, NJ, USA). 1-(6-(17-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl-1H-pyrrole-2,5-dione (U73122) was from Biosource (Camarillo, CA, USA).

4.2. Neocortical neuron culture

Primary cell cultures of neocortical neurons were obtained from Swiss–Webster mice on embryonic day 16. Embryos were extracted following euthanasia via CO₂ asphyxiation and their neocortices were collected. Isolated neocortices were then removed of their meninges, minced by trituration using a Pasteur pipette, and treated with trypsin for 20 min at 37 °C. The cells were further dissociated *via* two successive trituration and sedimentation steps in isolation buffer containing soybean trypsin inhibitor and DNase. The cells underwent another centrifugation step and were resuspended in a neuron-plating medium containing Eagles's minimal essential medium with Earle's salt (MEM), along with 2 mM L-glutamine, 10% fetal bovine serum, 10% horse serum, 100 I.U./mL penicillin and 0.10 mg/mL streptomycin, pH=7.4. Cells were plated onto poly-L-lysine treated, 12-wells culture plates at a density of 1.8×10⁶ cells/well. Plates were incubated at 37 °C with 5% CO₂ and 95% humidity. On day 2, post-plating, cells were treated with Cytosine arabinoside (10 μM) to prevent proliferation of non-neuronal cells. The culture media was changed both on days 5, 7, 9 using a serum-free growth medium containing Neurobasal Medium supplemented with B-27, 100 I.U./mL penicillin, 0.10 mg/mL streptomycin, and 0.2 mM L-glutamine. Cultures were used in experiments between 9 and 12 days *in vitro* (DIV). All animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC).

4.3. Drug treatment

Cells were washed three times with Locke's buffer and then allowed to equilibrate in Locke's for 15–30 min. Any inhibitors were added during the equilibration period as well. After this period, cultures were treated with the indicated drugs diluted in Locke's buffer and incubated at 37 °C for specified times. Cultures were then transferred to an ice slurry to terminate treatment. After washing with ice cold PBS, cells were harvested in ice cold lysis buffer containing 50 mM Tris, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 µg/mL leupeptin, 1 µg/mL aprotinin, 1 µg/mL pepstatin and 1 mM phenylmethylsulfonyl fluoride just prior to use and incubated for 20 min at 4 °C. Cell lysates then underwent sonification and were centrifuged at 16,000×g for 10 min at 4 °C.

4.4. Western blotting

The supernatant obtained after centrifugation of lysates was assayed by the Bradford method to determine protein content. Equal amounts of protein were mixed with the Laemmli sample buffer and boiled 5 min. The samples were loaded onto a 9% SDS-PAGE gel and transferred to a nitrocellulose membrane by electroblotting. The membranes were blocked in TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween 20) with 5% skimmed milk for 1 h at room temperature. After blocking, membranes were incubated overnight at 4 °C in primary antibody diluted in TBST containing 5% skimmed milk. The blots were washed and incubated with the secondary antibody conjugated with horseradish peroxidase for 1 h, washed four times in TBST and exposed with ECL plus for 3 min. Blots were exposed to Kodak hyperfilm and developed. Membranes could be stripped with stripping buffer (63 mM Tris base, 70 mM SDS, 0.0007% 2-mercaptoethanol, pH=6.8) and reblotted for further use.

4.5. Data analysis

Western blot densitometry data was obtained using AIS software® (Imaging Research, Inc.). ANOVA and graphing were completed using GraphPad Prism® (GraphPad Software, Inc., San Diego, CA).

Abbreviations

CGC	cerebellar granule cells
mGluR	metabotropic glutamate receptor
PbTx-2	Brevetoxin-2
PKC	protein kinase C
PLC	phospholipase C
SFK	Src-family kinase
VGCC	voltage-gated calcium channel
VGSC	

voltage-gated sodium channel

References

- Alier KA, Morris BJ. Divergent regulation of Pyk2/CAKbeta phosphorylation by Ca²⁺ and cAMP in the hippocampus. *Biochim Biophys Acta* 2005;1745:342–349. [PubMed: 16120467]
- Amos S, Martin PM, Polar GA, Parsons SJ, Hussaini IM. Phorbol 12-myristate 13-acetate induces epidermal growth factor receptor transactivation via protein kinase Cdelta/c-Src pathways in glioblastoma cells. *J Biol Chem* 2005;280:7729–7738. [PubMed: 15618223]
- Andreev J, Galisteo ML, Kranenburg O, Logan SK, Chiu ES, Okigaki M, Cary LA, Moolenaar WH, Schlessinger J. Src and Pyk2 mediate G-protein-coupled receptor activation of epidermal growth factor receptor (EGFR) but are not required for coupling to the mitogen-activated protein (MAP) kinase signaling cascade. *J Biol Chem* 2001;276:20130–20135. [PubMed: 11274221]
- Attucci S, Carla V, Mannaioni G, Moroni F. Activation of type 5 metabotropic glutamate receptors enhances NMDA responses in mice cortical wedges. *Br J Pharmacol* 2001;132:799–806. [PubMed: 11181420]
- Baden DG. Brevetoxins: unique polyether dinoflagellate toxins. *FASEB J* 1989;3:1807–1817. [PubMed: 2565840]
- Baden DG, Mende TJ. Toxicity of two toxins from the Florida red tide marine dinoflagellate, *Ptychodiscus brevis*. *Toxicon* 1982;20:457–461. [PubMed: 6896247]
- Bayer AL, Heidkamp MC, Howes AL, Heller Brown J, Byron KL, Samarel AM. Protein kinase C epsilon-dependent activation of proline-rich tyrosine kinase 2 in neonatal rat ventricular myocytes. *J Mol Cell Cardiol* 2003;35:1121–1133. [PubMed: 12967635]
- Benson JM, Tischler DL, Baden DG. Uptake, tissue distribution, and excretion of brevetoxin 3 administered to rats by intratracheal instillation. *J Toxicol Environ Health, Part A* 1999;57:345–355. [PubMed: 10405188]
- Berman FW, Murray TF. Brevetoxins cause acute excitotoxicity in primary cultures of rat cerebellar granule neurons. *J Pharmacol Exp Ther* 1999;290:439–444. [PubMed: 10381810]
- Berman FW, Murray TF. Brevetoxin-induced autocrine excitotoxicity is associated with manifold routes of Ca²⁺ influx. *J Neurochem* 2000;74:1443–1451. [PubMed: 10737600]
- Bossart GD, Baden DG, Ewing RY, Roberts B, Wright SD. Brevetoxicosis in manatees (*Trichechus manatus latirostris*) from the 1996 epizootic: gross, histologic, and immunohistochemical features. *Toxicol Pathol* 1998;26:276–282. [PubMed: 9547868]
- Butler B, Blystone SD. Tyrosine phosphorylation of beta3 integrin provides a binding site for Pyk2. *J Biol Chem* 2005;280:14556–14562. [PubMed: 15695828]
- Cataldi M, Tagliabata M, Guerriero S, Amoroso S, Lombardi G, di Renzo G, Annunziato L. Protein-tyrosine kinases activate while protein-tyrosine phosphatases inhibit L-type calcium channel activity in pituitary GH3 cells. *J Biol Chem* 1996;271:9441–9446. [PubMed: 8621613]
- Cattet M, Geraci JR. Distribution and elimination of ingested brevetoxin (PbTx-3) in rats. *Toxicon* 1993;31:1483–1486. [PubMed: 8310449]
- Cestele S, Catterall WA. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie* 2000;82:883–892. [PubMed: 11086218]
- Corvol JC, Valjent E, Toutant M, Enslen H, Irinopoulou T, Lev S, Herve D, Girault JA. Depolarization activates ERK and proline-rich tyrosine kinase 2 (PYK2) independently in different cellular compartments in hippocampal slices. *J Biol Chem* 2005;280:660–668. [PubMed: 15537634]
- Dikic I, Tokiwa G, Lev S, Courtneidge SA, Schlessinger J. A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature* 1996;383:547–550. [PubMed: 8849729]
- Dravid SM, Baden DG, Murray TF. Brevetoxin activation of voltage-gated sodium channels regulates Ca dynamics and ERK1/2 phosphorylation in murine neocortical neurons. *J Neurochem* 2004;89:739–749. [PubMed: 15086530]
- Dravid SM, Baden DG, Murray TF. Brevetoxin augments NMDA receptor signaling in murine neocortical neurons. *Brain Res* 2005;1031:30–38. [PubMed: 15621010]
- Ellis S. Brevetoxins: chemistry and pharmacology of 'red tide' toxins from *Ptychodiscus brevis* (formerly *Gymnodinium breve*). *Toxicon* 1985;23:469–472. [PubMed: 4040668]

- Fadool DA, Holmes TC, Berman K, Dagan D, Levitan IB. Tyrosine phosphorylation modulates current amplitude and kinetics of a neuronal voltage-gated potassium channel. *J Neurophysiol* 1997;78:1563–1573. [PubMed: 9310443]
- Girault JA, Costa A, Derkinderen P, Studler JM, Toutant M. FAK and PYK2/CAKbeta in the nervous system: a link between neuronal activity, plasticity and survival? *Trends Neurosci* 1999;22:257–263. [PubMed: 10354603]
- Grishin AA, Benquet P, Gerber U. Muscarinic receptor stimulation reduces NMDA responses in CA3 hippocampal pyramidal cells via Ca²⁺-dependent activation of tyrosine phosphatase. *Neuropharmacology* 2005;49:328–337. [PubMed: 15993905]
- Heidinger V, Manzerra P, Wang XQ, Strasser U, Yu SP, Choi DW, Behrens MM. Metabotropic glutamate receptor 1-induced upregulation of NMDA receptor current: mediation through the Pyk2/Src-family kinase pathway in cortical neurons. *J Neurosci* 2002;22:5452–5461. [PubMed: 12097497]
- Heidkamp MC, Scully BT, Vijayan K, Engman SJ, Szotek EL, Samarel AM. PYK2 regulates SERCA2 gene expression in neonatal rat ventricular myocytes. *Am J Physiol, Cell Physiol* 2005;289:C471–C482. [PubMed: 15829561]
- Huang Y, Lu W, Ali DW, Pelkey KA, Pitcher GM, Lu YM, Aoto H, Roder JC, Sasaki T, Salter MW, MacDonald JF. CAKbeta/Pyk2 kinase is a signaling link for induction of long-term potentiation in CA1 hippocampus. *Neuron* 2001;29:485–496. [PubMed: 11239437]
- Huo JZ, Dykstra CM, Gurd JW. Increase in tyrosine phosphorylation of the NMDA receptor following the induction of status epilepticus. *Neurosci Lett* 2006;401:266–270. [PubMed: 16600505]
- Ishida H, Nozawa A, Nukaya H, Tsuji K, Kaspar H, Berkett N, Kosuge T. Isolation and structure determination of a new marine neurotoxin from the New Zealand shellfish, *Austrovenus stutchburyi*. *Toxicon* 1994;32:1672–1674. [PubMed: 7725335]
- Jeglitsch G, Rein K, Baden DG, Adams DJ. Brevetoxin-3 (PbTx-3) and its derivatives modulate single tetrodotoxin-sensitive sodium channels in rat sensory neurons. *J Pharmacol Exp Ther* 1998;284:516–525. [PubMed: 9454792]
- Kalia LV, Gingrich JR, Salter MW. Src in synaptic transmission and plasticity. *Oncogene* 2004;23:8007–8016. [PubMed: 15489918]
- Kato K, Murota SI. NMDA receptor stimulation in the absence of extracellular Ca²⁺ potentiates Ca²⁺ influx-dependent cell death system. *Brain Res* 2005;1035:177–187. [PubMed: 15722057]
- Kohling R. Voltage-gated sodium channels in epilepsy. *Epilepsia* 2002;43:1278–1295. [PubMed: 12423377]
- Lev S, Moreno H, Martinez R, Canoll P, Peles E, Musacchio JM, Plowman GD, Rudy B, Schlessinger J. Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions. *Nature* 1995;376:737–745. [PubMed: 7544443]
- Lu YM, Jia Z, Janus C, Henderson JT, Gerlai R, Wojtowicz JM, Roder JC. Mice lacking metabotropic glutamate receptor 5 show impaired learning and reduced CA1 long-term potentiation (LTP) but normal CA3 LTP. *J Neurosci* 1997;17:5196–5205. [PubMed: 9185557]
- Lu WY, Xiong ZG, Lei S, Orser BA, Dudek E, Browning MD, MacDonald JF. G-protein-coupled receptors act via protein kinase C and Src to regulate NMDA receptors. *Nat Neurosci* 1999;2:331–338. [PubMed: 10204539]
- Mannaioni G, Marino MJ, Valenti O, Traynelis SF, Conn PJ. Metabotropic glutamate receptors 1 and 5 differentially regulate CA1 pyramidal cell function. *J Neurosci* 2001;21:5925–5934. [PubMed: 11487615]
- McFarren EF, Silva FJ, Tanabe H, Wilson WB, Campbell JE, Lewis KH. The occurrence of a ciguatera-like poison in oysters, clams, and *Gymnodinium breve* cultures. *Toxicon* 1965;3:111–123. [PubMed: 5867066]
- Moss SJ, Gorrie GH, Amato A, Smart TG. Modulation of GABAA receptors by tyrosine phosphorylation. *Nature* 1995;377:344–348. [PubMed: 7566089]
- Moyers JS, Bouton AH, Parsons SJ. The sites of phosphorylation by protein kinase C and an intact SH2 domain are required for the enhanced response to beta-adrenergic agonists in cells overexpressing c-src. *Mol Cell Biol* 1993;13:2391–2400. [PubMed: 7681147]
- O'Shea TJ, Rathbun GB, Buergelt CD, Odell DK. An epizootic of Florida manatees associated with a dinoflagellate bloom. *Mar Mamm Sci* 1991;7:165–179.

- Park SY, Avraham HK, Avraham S. RAFTK/Pyk2 activation is mediated by trans-acting autophosphorylation in a Src-independent manner. *J Biol Chem* 2004;279:33315–33322. [PubMed: 15166227]
- Pierce RH. Red tide (*Ptychodiscus brevis*) toxin aerosols: a review. *Toxicon* 1986;24:955–965. [PubMed: 3824403]
- Poli MA, Mende TJ, Baden DG. Brevetoxins, unique activators of voltage-sensitive sodium channels, bind to specific sites in rat brain synaptosomes. *Mol Pharmacol* 1986;30:129–135. [PubMed: 2426567]
- Sorokin A, Kozlowski P, Graves L, Philip A. Protein–tyrosine kinase Pyk2 mediates endothelin-induced p38 MAPK activation in glomerular mesangial cells. *J Biol Chem* 2001;276:21521–21528. [PubMed: 11278444]
- Templeton CB, Poli MA, Solow R. Prophylactic and therapeutic use of an anti-brevetoxin (PbTx-2) antibody in conscious rats. *Toxicon* 1989;27:1389–1395. [PubMed: 2629179]
- Vondriska TM, Zhang J, Song C, Tang XL, Cao X, Baines CP, Pass JM, Wang S, Bolli R, Ping P. Protein kinase C epsilon-Src modules direct signal transduction in nitric oxide-induced cardioprotection: complex formation as a means for cardioprotective signaling. *Circ Res* 2001;88:1306–1313. [PubMed: 11420308]
- Wan Q, Man HY, Braunton J, Wang W, Salter MW, Becker L, Wang YT. Modulation of GABAA receptor function by tyrosine phosphorylation of beta subunits. *J Neurosci* 1997;17:5062–5069. [PubMed: 9185543]
- Wang YT, Salter MW. Regulation of NMDA receptors by tyrosine kinases and phosphatases. *Nature* 1994;369:233–235. [PubMed: 7514272]
- Wang K, Hackett JT, Cox ME, Van Hoek M, Lindstrom JM, Parsons SJ. Regulation of the neuronal nicotinic acetylcholine receptor by SRC family tyrosine kinases. *J Biol Chem* 2004;279:8779–8786. [PubMed: 14679211]
- Wayman GA, Impey S, Marks D, Saneyoshi T, Grant WF, Derkach V, Soderling TR. Activity-dependent dendritic arborization mediated by CaM-kinase I activation and enhanced CREB-dependent transcription of Wnt-2. *Neuron* 2006;50:897–909. [PubMed: 16772171]
- Wilkinson SE, Parker PJ, Nixon JS. Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. *Biochem J* 1993;294 (Pt 2):335–337. [PubMed: 8373348]
- Yu XM. The role of intracellular sodium in the regulation of NMDA-receptor-mediated channel activity and toxicity. *Mol Neurobiol* 2006;33:63–80. [PubMed: 16388111]
- Yu XM, Salter MW. Gain control of NMDA-receptor currents by intracellular sodium. *Nature* 1998;396:469–474. [PubMed: 9853755]
- Yu XM, Askalan R, Keil GJ, Salter MW. NMDA channel regulation by channel-associated protein tyrosine kinase Src. *Science* 1997;275:674–678. [PubMed: 9005855]

Fig. 1.

PbTx-2 increases Pyk2 Tyr-402 and Src Tyr-416 phosphorylation. (a) Concentration–response profile for PbTx-2 effects on Pyk2 Tyr-402 and Src Tyr-416 phosphorylation. Neocortical neurons were exposed to 1–1000 nM PbTx-2 for 10 min. The phosphorylation levels of Pyk2 and Src were detected by western blot using phosphospecific antibodies against the active forms of the kinases (pPyk2(pY402), and pSrc(pY416)). The relative densities of phosphotyrosine were normalized to the total protein densities of both Pyk2 and Src for each treatment.

Individual bars represent the mean±S.E.M. from four independent experiments (*, $p < 0.05$; **, $p < 0.01$, PbTx-2 vs. control by ANOVA). (b) Time course for PbTx-2 (100 nM)-induced stimulation of Pyk2 Tyr-402 and Src Tyr-416 phosphorylation. Cells were exposed to PbTx-2 for 1–30 min. Bars indicate mean values of five independent experiments (*, $p < 0.05$; **, $p < 0.01$, PbTx-2 vs. control by ANOVA).

Fig. 2.

Effect of tetrodotoxin (TTX) on PbTx-2-induced Pyk2 and Src activation. Representative western blotting and quantification of Pyk2 Tyr-402 phosphorylation (a) and Src Tyr-416 phosphorylation (b) after exposure of neocortical cells to PbTx-2 for 5 min in the presence or absence of TTX (1 μ M). Bars indicate mean values from six independent experiments (**, $p < 0.01$, PbTx-2 vs. control; aa, $p < 0.01$, PbTx-2 vs. PbTx-2+TTX by ANOVA).

Fig. 3.

Effects of PP-2 and PP-3 on PbTx-2-induced Pyk2 and Src phosphorylation. Representative western blotting and quantification of Pyk2 Tyr-402 phosphorylation (a) and Src Tyr-416 phosphorylation (b) after exposure of neocortical cells to PbTx-2 for 5 min in the presence or absence of the Src-family kinase inhibitor PP-2 (1 μ M) or the inactive analog PP-3 (1 μ M). Bars depict mean values derived from six independent experiments (**, $p < 0.01$, PbTx-2 vs. control; aa, $p < 0.01$, PbTx-2 vs. PbTx-2+ PP-2 by ANOVA).

Fig. 4.

Influence of Ca²⁺ dynamics on PbTx-2-induced Pyk2 Tyr-402 and Src Tyr-416 phosphorylation. (a) Representative western blotting and quantification of Pyk2 Tyr-402 phosphorylation after exposure of neocortical cells to PbTx-2 for 5 min in the presence or absence of the Ca²⁺ chelator BAPTA/AM (40 μ M) or low Ca²⁺ (0.2 mM). Bars depict mean values derived from four independent experiments (**, $p < 0.01$, PbTx-2 vs. control; aa, $p < 0.01$, PbTx-2 vs. PbTx-2/0.2 mM Ca²⁺; bb, $p < 0.01$, PbTx-2 vs. PbTx-2+BAPTA by ANOVA). (b) Representative western blotting and quantification of Pyk2 Tyr-402 phosphorylation after exposure of neocortical cells to PbTx-2 for 5 min in the presence or absence of MK-801 (1 μ M) or nifedipine (1 μ M). Bars depict mean values derived from five independent experiments (**, $p < 0.01$, PbTx-2 vs. control; *, $p < 0.05$, PbTx-2 vs. PbTx-2+MK-801 by ANOVA). (c) Representative western blotting and quantification of Src Tyr-416 phosphorylation after exposure of neocortical cells to PbTx-2 for 5 min in the presence or absence of the Ca²⁺ chelator

BAPTA/AM (40 μ M) or low Ca^{2+} (0.2 mM). Bars depict mean values derived from four independent experiments (**, $p < 0.01$, PbTx-2 vs. control; aa, $p < 0.01$, PbTx-2 vs. PbTx-2 + BAPTA by ANOVA).

Fig. 5.

Effect of a PLC inhibitor U73122, a mGluR 1 antagonist S-4-CPG, and the mGluR 5 antagonist MTEP on PbTx-2-induced Src Tyr-416 phosphorylation. (a) Representative western blotting and quantification of Src Tyr-416 phosphorylation after exposure of neocortical cells to PbTx-2 for 5 min in the presence or absence of U73122 (3 μ M). Bars depict mean values derived from six independent experiments (**, $p < 0.01$, PbTx-2 vs. control; aa, $p < 0.01$, PbTx-2 vs. PbTx-2 +U73122 by ANOVA). (b) Representative western blotting and quantification of Src Tyr-416 phosphorylation after exposure of neocortical cells to PbTx-2 for 5 min in the presence or absence of S-4-CPG (500 μ M) or MTEP (1 μ M). Bars depict mean values derived from five independent experiments (**, $p < 0.01$, PbTx-2 vs. control; aa, $p < 0.01$, PbTx-2 vs. PbTx-2 +MTEP by ANOVA).

Fig. 6.

Effects of the PKC inhibitors on PbTx-2-induced Pyk2 Tyr-402 and Src Tyr-416 phosphorylation. (a) Representative western blotting and quantification of Pyk2 Tyr-402 phosphorylation after exposure of neocortical cells to PbTx-2 for 5 min in the presence or absence of RO-31-8425 (1 μ M) or GF 109203x (1 μ M). Bars depict mean values derived from five independent experiments (**, $p < 0.01$, PbTx-2 vs. control; aa, $p < 0.01$, PbTx-2 vs. PbTx-2 + RO-318425; bb, $p < 0.01$, PbTx-2 vs. PbTx-2 + GF 109203x by ANOVA). (b) Representative western blotting and quantification of Src-416 phosphorylation after exposure of neocortical cells to PbTx-2 for 5 min in the presence or absence of RO-31-8425 (1 μ M) or GF 109203x (1 μ M). Bars depict mean values derived from five independent experiments (**, $p < 0.01$, PbTx-2 vs. control; aa, $p < 0.01$, PbTx-2 vs. PbTx-2 + GF 109203x by ANOVA). (c) Effects of the PKC δ inhibitor rottlerin on PbTx-2-induced Src Tyr-416 phosphorylation. Representative western blotting and quantification of Src-416 phosphorylation after exposure of neocortical cells to PbTx-2 for 5 min in the presence or absence of rottlerin (5 μ M). Bars depict mean values derived from five independent experiments (**, $p < 0.01$, PbTx-2 vs. control; aa, $p < 0.01$, PbTx-2 vs. PbTx-2 + rottlerin by ANOVA).

Fig. 7. Schematic diagram showing proposed pathways underlying PbTx-2-induced Src Tyr416 and Pyk2 Tyr402 phosphorylation in mouse neocortical neurons.