

# Neural Cell Adhesion Molecule Induces Intracellular Signaling via Multiple Mechanisms of $\text{Ca}^{2+}$ Homeostasis

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The neural cell adhesion molecule (NCAM) plays a pivotal role in the development of the nervous system, promoting neuronal differentiation via homophilic (NCAM–NCAM) as well as heterophilic (NCAM–fibroblast growth factor receptor [FGFR]) interactions. NCAM-induced intracellular signaling has been shown to affect and be dependent on the cytoplasmic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ). However, the molecular basis of this remains unclear. In this study, we determined  $[\text{Ca}^{2+}]_i$  regulating mechanisms involved in intracellular signaling induced by NCAM. To mimic the effect of homophilic NCAM interaction on  $[\text{Ca}^{2+}]_i$  in vitro, we used a peptide derived from a homophilic binding site of NCAM, termed P2, which triggers signaling cascades similar to those activated by NCAM–NCAM interaction. We found that P2 increased  $[\text{Ca}^{2+}]_i$  in primary hippocampal neurons. This effect depended on two signaling pathways. The first pathway was associated with activation of FGFR, phospholipase  $\text{C}\gamma$ , and production of diacylglycerol, and the second pathway involved Src-family kinases. Moreover, NCAM-mediated  $\text{Ca}^{2+}$  entry required activation of nonselective cation and T-type voltage-gated  $\text{Ca}^{2+}$  channels. These channels, together with the Src-family kinases, were also involved in neuritogenesis induced by physiological, homophilic NCAM interactions. Thus, unanticipated mechanisms of  $\text{Ca}^{2+}$  homeostasis are shown to be activated by NCAM and to contribute to neuronal differentiation.

## INTRODUCTION

The neural cell adhesion molecule (NCAM) belongs to the Ig superfamily and has a variety of functions in the development and maintenance of the nervous system (reviewed in Povlsen *et al.*, 2003; Hinsby *et al.*, 2004). The extracellular domain of NCAM consists of five Ig modules (IgI–V) and two fibronectin type III homology modules (F3, I and II). Importantly, NCAM promotes neurite outgrowth via homophilic (NCAM–NCAM) as well as heterophilic (NCAM–fibroblast growth factor receptor [FGFR]) interactions (Kiselyov *et al.*, 2005), which activate a number of intracellular signaling cascades (reviewed in Walmod *et al.*, 2004). Recently, a novel mechanism of homophilic NCAM binding has been proposed, in which the IgI and IgII modules mediate dimerization of NCAM molecules situated on the same cell surface (*cis* interactions), whereas *trans* interactions between the IgI and IgIII, IgII and IgII, and the IgII and IgIII modules of NCAM determine cell–cell adhesion (Soroka *et al.*, 2003).

Although NCAM-induced intracellular signaling has been demonstrated to affect and be dependent on cytoplasmic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) (reviewed in Kiryushko *et al.*, 2004), data concerning the role of  $\text{Ca}^{2+}$  in NCAM-mediated signal transduction are limited. Neurite outgrowth induced

by homophilic binding of cell adhesion molecules (CAMs) in dorsal root ganglia neurons has been shown to depend on a  $\text{Ca}^{2+}$  influx via voltage-dependent  $\text{Ca}^{2+}$  channels (VDCCs) (Doherty *et al.*, 1991; Archer *et al.*, 1999). It has also been demonstrated by spectrofluorimetry that NCAM antibodies elicit a long-lasting increase of  $[\text{Ca}^{2+}]_i$  in PC12 cells (Schuch *et al.*, 1989) and primary cerebellar neurons (Von Bohlen Und Halback *et al.*, 1992). However, the molecular mechanisms of these events have not been elucidated.

The aim of this study was to investigate  $[\text{Ca}^{2+}]_i$ -regulating mechanisms involved in the intracellular signaling triggered by NCAM, and their importance for NCAM-induced neurite outgrowth. Previously, either cocultures of neurons grown on a monolayer of NCAM-expressing cells or stimulation with soluble NCAM have been used to mimic homophilic NCAM binding in vitro (Kiryushko *et al.*, 2004). However, the coculture approach has significant limitations because of difficulty in presenting NCAM to appropriate ligands in a quantitative manner. Also, stimulation with soluble NCAM is hampered due to NCAM homodimerization via homophilic interaction. These factors impede studies of NCAM effects on rapidly changing cellular parameters (e.g.,  $[\text{Ca}^{2+}]_i$ ).

To mimic the effect of homophilic NCAM binding on  $[\text{Ca}^{2+}]_i$  in vitro, we therefore used a synthetic peptide ligand of NCAM, termed P2. The peptide represents a part of the IgII module involved in homophilic NCAM interactions, and it binds to the IgI module of NCAM with a  $K_d$  similar to that of NCAM IgII (Soroka *et al.*, 2002). The tetrameric form of this peptide (P2d) in vitro induces neurite outgrowth in the absence of homophilic *trans* NCAM binding (Soroka *et al.*, 2002) and affects NCAM-mediated cell adhesion (Li *et al.*, 2005). Moreover, the peptide triggers intracellular signaling cascades similar to those activated by NCAM itself (Soroka *et al.*, 2002). Therefore, P2d was used as a tool to study which  $[\text{Ca}^{2+}]_i$ -regulating mechanisms are involved in intracellular signaling induced by NCAM.

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Abbreviations used: 2-AG, 2-arachidonoyl glycerol; AA, arachidonic acid; DAG, diacylglycerol; GAP, growth-associated protein; NSCC, nonselective cation channel; PLC, phospholipase C; RTK, receptor tyrosine kinase; TRP, transient receptor potential; VDCC, voltage-dependent  $\text{Ca}^{2+}$  channel.

## MATERIALS AND METHODS

### Materials

Tetrodotoxin, the inhibitor of voltage-gated sodium channels, and Hank's balanced salt solution (HBSS) were from Sigma-Aldrich (Copenhagen, Denmark). The receptor tyrosine kinase inhibitors lavendustin A and B; blockers of voltage-gated calcium channels of T-type (flunarizine), L-type (nifedipine), N-type ( $\omega$ -conotoxin MVIIA), and P/Q-type ( $\omega$ -agatoxin TK); and a blocker of Ca<sup>2+</sup> uptake into the endoplasmic reticulum (ER) stores, thapsigargin, were all from Calbiochem (Merck, Darmstadt, Germany). The phospholipase C (PLC) inhibitor U-73122 and the diacylglycerol (DAG)-lipase inhibitor RHC-80267 were from BIOMOL Research Laboratories (Plymouth Meeting, PA). A T-type Ca<sup>2+</sup> channels inhibitor mibefradil (dihydrochloride salt) was a gift from F. Hoffmann-La Roche (Basel, Switzerland). P2d (GRILARGEINFK) and scrambled P2d (NLFEKGRRAIGI) peptides were produced as described by Soroka *et al.* (2002).

### Primary Cultures of Rat Hippocampal Neurons and Analysis of Neurite Outgrowth

Hippocampal neurons were prepared from embryonic day (E) 19 Wistar rat embryos as described by Maar *et al.* (1997). Neurons were seeded at a density of 10,000 cells/cm<sup>2</sup> in eight-well Lab-Tek tissue culture chambers with a growth surface of Permaxox plastic (NUNC, Roskilde, Denmark). After plating, cultures were maintained in Neurobasal medium as described previously (Soroka *et al.*, 2002). For long-term cultures, cytosine arabinofuranoside (final concentration 5  $\mu$ M; Sigma-Aldrich) was added after 2 d *in vitro* to inhibit glial cell proliferation. Analysis of neurite outgrowth was performed as described previously (Soroka *et al.*, 2002).

### Coculture of Hippocampal Neurons and L929 Fibroblasts without or with NCAM Expression

Hippocampal neurons were plated on top of a confluent monolayer of L929 fibroblasts without or with NCAM expression (Kolkova *et al.*, 2000). Inhibitors were added to the medium immediately after plating. Cocultures were maintained during 24 h at 37°C, 5% CO<sub>2</sub> in Neurobasal-A medium supplemented with 2% (vol/vol) B27, 5% (vol/vol) fetal calf serum, and 2 mM GlutaMAX (all reagents from Invitrogen, Carlsbad, CA).

### Fluorometric Ca<sup>2+</sup> Measurements

Dye loading and experiments were performed in modified HBSS at room temperature (22–24°C). A modified HBSS used in experiments with physiological extracellular Ca<sup>2+</sup> was produced by adjusting Ca<sup>2+</sup> concentration to 2 mM, pH 7.4. A low calcium HBSS was obtained by adjusting the Ca<sup>2+</sup> concentration to 200  $\mu$ M and adding CdCl<sub>2</sub> to the solution to a final concentration of 200  $\mu$ M. Primary neurons were seeded on eight-well poly-L-lysine-coated Lab-Tek chambered coverglass slides (NUNC) at a density of 50,000 cells/well and grown for 7–10 d. Attached cells were loaded with a Ca<sup>2+</sup>-sensitive dye fura-2 acetyloxymethyl ester (AM) (5  $\mu$ M; Invitrogen) or fluo-4 AM (2  $\mu$ M; Invitrogen). To eliminate the effect of synaptically induced Ca<sup>2+</sup> fluxes, all experiments were performed in the presence of 1  $\mu$ M tetrodotoxin. The regions of interest were positioned on somata of the neurons, which were identified by their morphological characteristics. Data were collected from at least three sets of cultures obtained from different animals; each experiment provided simultaneous measurements from up to eight neurons.

### Microscopy and Data Analysis

Immunostaining of primary neurons and image scanning were performed as described by Soroka *et al.* (2002). Rabbit anti-rat-growth-associated protein (GAP)-43 polyclonal antibodies (Cell Signaling Technology, Beverly, MA) were used.

Calcium imaging employing fura-2 AM was performed using a Sencam 12-bit cooled charge-coupled device camera (PCO, Keilheim, Germany) and a J&M monochromator (J&M, Aalen, Germany) connected to a Zeiss Axiovert 135 TV microscope (Zeiss Fluor oil immersion UV objective, 40 $\times$ , 1.3 numerical aperture). The cellular fluorescence signals were corrected for the background fluorescence, which was measured in a separate region in the vicinity of the cell. Ratio images (340/380 nm excitation; 510 LP emission) were collected after

background subtraction at 2- to 5-s intervals. The calibration constants and the estimated [Ca<sup>2+</sup>]<sub>i</sub> were calculated from a fluorescence ratio, R, as described by Rønn *et al.* (2002). Data acquisition and analysis were performed using the Imaging Workbench software (Molecular Devices, Sunnyvale, CA).

### Immunoblotting Assay

Hippocampal neurons were seeded in 60-mm tissue culture dishes (10<sup>6</sup> cells) and grown for 4 h before treatment. Immunoblotting was performed as described previously (Kolkova *et al.*, 2000). Rabbit anti-phospho-PLC $\gamma$ 1 antibodies (diluted 1:300; Cell Signaling Technology) or rabbit anti-phospho-Src antibodies (diluted 1:600; Cell Signaling Technology) were used. Protein bands were visualized using the enhanced chemiluminescence substrate Western Dura (Pierce Chemical, Rockford, IL) and processed with the GenTools software package (Syngene, Cambridge, United Kingdom). To estimate the total amount of PLC $\gamma$ 1 or actin, membranes were stripped and reprobed with an anti-PLC $\gamma$ 1 or anti-actin antibodies (both developed in rabbit; Cell Signaling Technology and Sigma-Aldrich, respectively).

### Statistics and Graphical Presentation

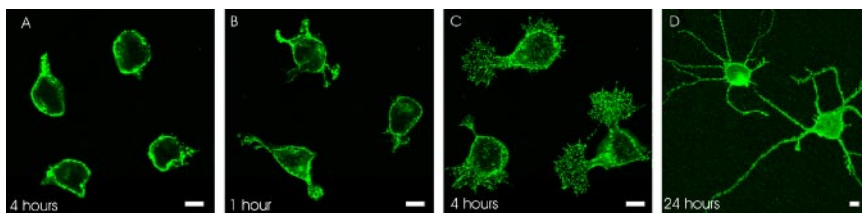
Statistics and graphical presentations were carried out using the Origin version 6.0 software package (OriginLab, Northampton, MA). Statistical evaluations were performed using a two-sided Student's *t* test. The results are given as mean  $\pm$  SEM. Unless otherwise stated asterisks indicate the statistical significance of \**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001 compared with control.

## RESULTS

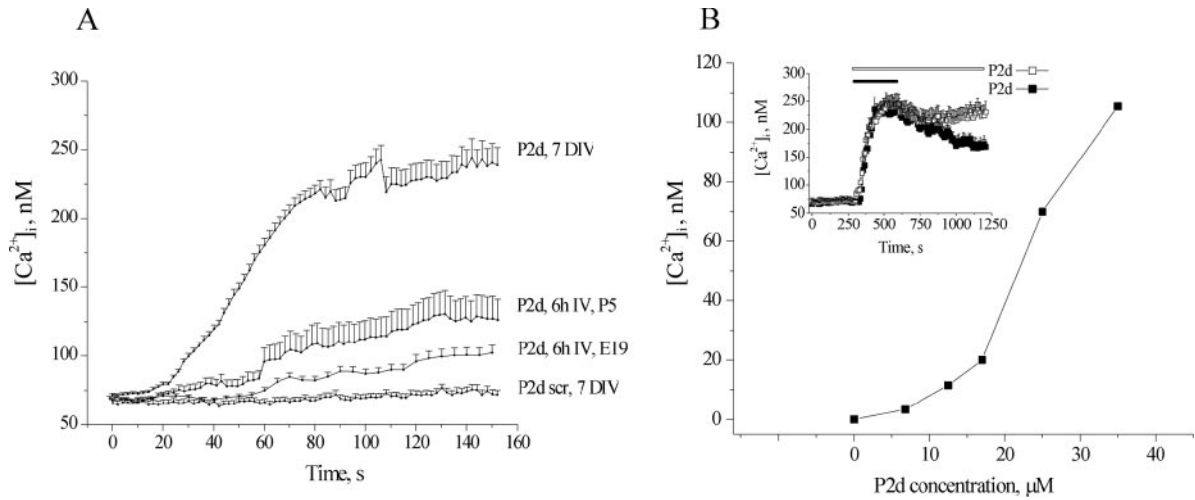
### P2d Induces Both Rapid Neurite Extension and a [Ca<sup>2+</sup>]<sub>i</sub> Elevation in Primary Hippocampal Neurons

It has previously been shown by Soroka *et al.*, (2002) that tetrameric P2 (P2d), but not a scrambled variant of P2d (scrP2d) induces differentiation of primary hippocampal neurons. We determined the time course of the P2d-triggered neurite extension. Neurons stimulated with scrP2d did not change morphology even after 4 h of treatment (Figure 1A). In contrast, P2d induced the formation of initial protrusions already 1 h after application (Figure 1B), and after 4 h, the formation of primary neurites was observed (Figure 1C). After 24 h, the P2d-treated neurons had an average neurite length of  $\sim$ 100  $\mu$ m per cell (Figure 1D).

Homophilic binding of CAMs is known to increase the cytoplasmic Ca<sup>2+</sup> level (Archer *et al.*, 1999). We therefore tested whether P2d affected [Ca<sup>2+</sup>]<sub>i</sub> in cultured hippocampal neurons at various times after plating. Indeed, P2d induced Ca<sup>2+</sup> responses, although of differing amplitudes, in hippocampal neurons grown for 6 h and 7–10 d *in vitro*, whereas no change of [Ca<sup>2+</sup>]<sub>i</sub> was observed after application of scrambled P2d (Figure 2A). Thus, only the neuritogenically active peptide was capable of elevating the cytoplasmic Ca<sup>2+</sup> level, and the magnitude of the [Ca<sup>2+</sup>]<sub>i</sub> rise depended on the time neurons had been cultured. An amplitude of P2d-induced Ca<sup>2+</sup> responses also depended on the age of the animals, from which primary neurons were obtained, being at 6 h *in vitro* approximately twofold higher in the cells isolated from P5 than in the cells isolated from E19 animals (Figure 2A). In subsequent experiments, we used P2d at a concentration of 35  $\mu$ M (calculated per monomer), because this concentration both induced neurite outgrowth (Soroka *et al.*, 2002) and a prominent Ca<sup>2+</sup> response (Figure 2B). However, lower concentrations of P2d also elevated



**Figure 1.** P2d induces a fast neuritogenic response from primary hippocampal neurons. (A–D) Confocal images of hippocampal neurons treated for the indicated lengths of time with 5  $\mu$ M scrambled P2d (A) or 5  $\mu$ M P2d (B–D). The cells were immunostained for GAP-43. Representative neurons are shown. Bars, 5  $\mu$ m.



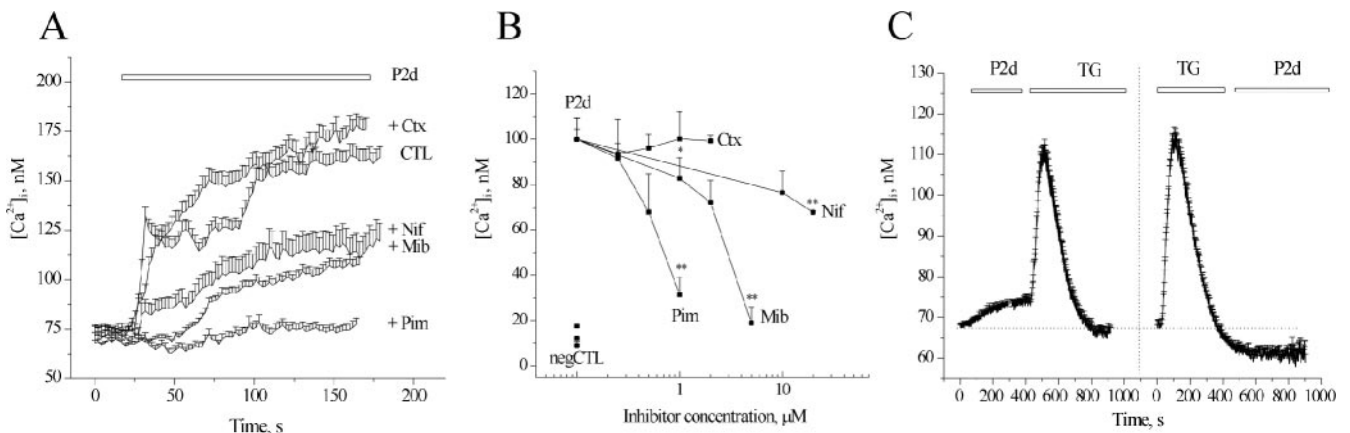
**Figure 2.** P2d increases  $[Ca^{2+}]_i$  in primary hippocampal neurons. (A)  $Ca^{2+}$  responses induced by P2d and scrP2d ( $30 \mu M$  both) in primary hippocampal neurons. (B) Dose-response relationship for P2d-triggered  $[Ca^{2+}]_i$  rise in primary hippocampal neurons cultured for 7–10 d. Inset,  $Ca^{2+}$  responses induced by P2d present either for 15 min (open squares) or for 3 min (filled squares). DIV, days in vitro. In all fluorometric experiments, number of cells  $n = 40-60$ .

$[Ca^{2+}]_i$  with a threshold of activation observed at  $\sim 10 \mu M$  P2d (Figure 2B). In the presence of the peptide, the P2d-induced  $Ca^{2+}$  responses sustained for at least 20 min (Figure 2B, inset). When P2d was washed out, a slow recovery of  $[Ca^{2+}]_i$  to the resting level was observed (Figure 2B, inset). Hippocampal cultures grown for 7 d in vitro were used for  $Ca^{2+}$  fluorometry because of their robust high-magnitude  $Ca^{2+}$  responses to P2d. Selected experiments were also reproduced in cultures grown for 6 h in vitro, yielding similar results (our unpublished data).

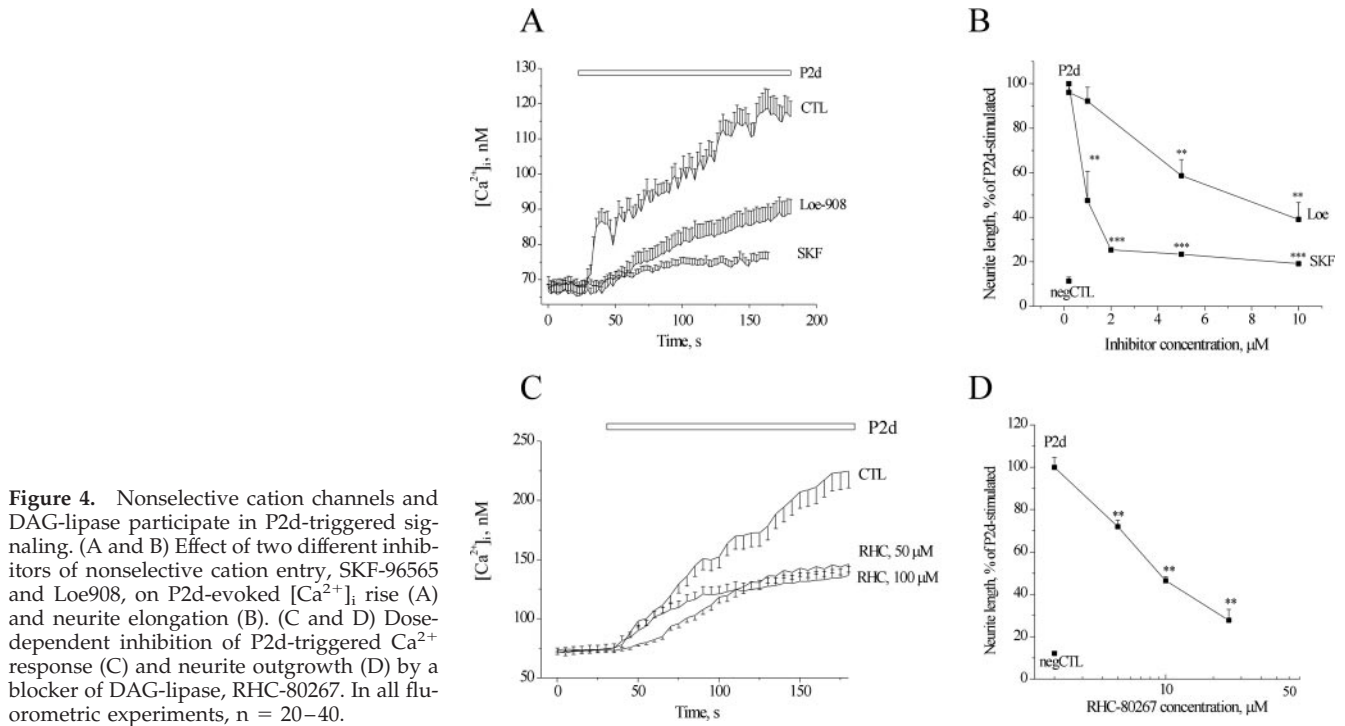
**The P2d-induced Rise of  $[Ca^{2+}]_i$  Involves VDCCs as Well as  $[Ca^{2+}]_i$  Stores**

Activity of VDCCs has been shown to be important for NCAM-induced neurite outgrowth (Doherty *et al.*, 1991). Using specific inhibitors, we checked whether this type of channels was involved in the P2d-induced  $Ca^{2+}$  response. Blocking

L-type VDCCs with nifedipine or T-type VDCC with mibefradil led to a partial ( $\sim 50\%$ ) decrease in the amplitude of the P2d-evoked  $[Ca^{2+}]_i$  elevation (Figure 3A). The partial inhibition of the  $Ca^{2+}$  response by mibefradil might be due to this drug also being a potent blocker of calcium-activated chloride channels (Nilius and Droogmans, 2003), which produce hyperpolarization of hippocampal neurons at this developmental stage (Stein *et al.*, 2004). However, a more selective T-type VDCCs inhibitor, pimozide, completely blocked the  $Ca^{2+}$  response to P2d (Figure 4A). In contrast, inhibition of N-type VDCC by  $\omega$ -conotoxin did not influence the magnitude of the P2d-induced  $[Ca^{2+}]_i$  elevation (Figure 3A). Neither did the P/Q-type  $Ca^{2+}$  channels inhibitor agatoxin have any effect (our unpublished data). In agreement with the fluorometric data, P2d-triggered neurite outgrowth was not affected by agatoxin (our unpublished data) or  $\omega$ -conotoxin (Figure 3B). Nifedipine caused a dose-dependent inhibition of the P2d-induced neuro-



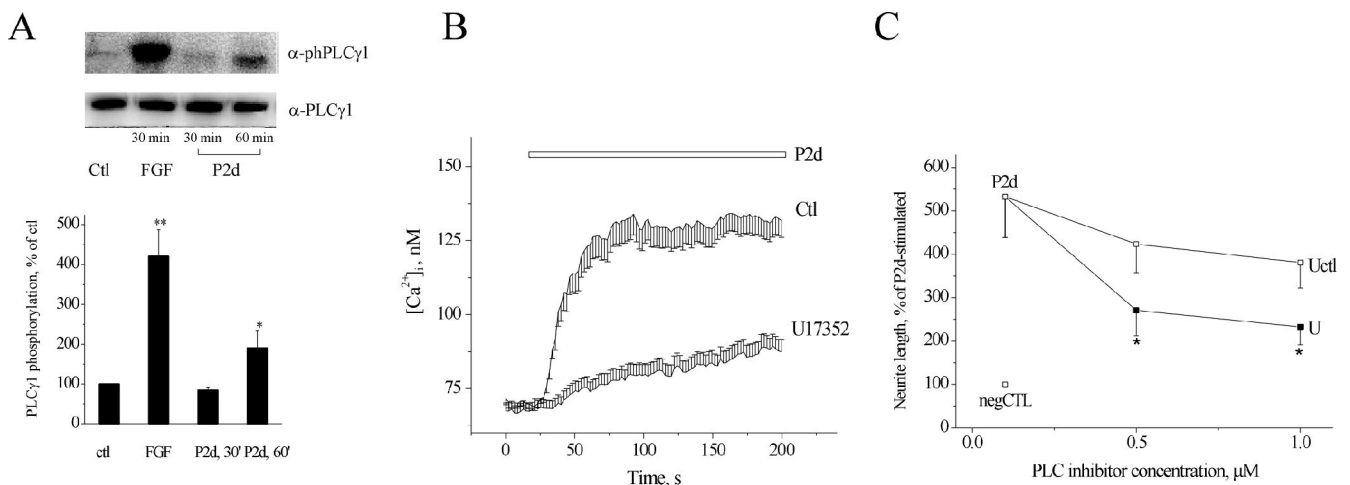
**Figure 3.** P2d induces  $Ca^{2+}$  entry via VDCCs and triggers  $Ca^{2+}$  release from intracellular stores. (A and B) Effects of different blockers of VDCCs on P2d-induced  $[Ca^{2+}]_i$  rise and neurite outgrowth, respectively. CTL, no inhibitor added; Nif, nifedipine ( $10 \mu M$ ), a blocker of L-type VDCCs; Mib, mibefradil and Pim, pimozide ( $5 \mu M$  both), blockers of T-type VDCCs; and Ctx, conotoxin ( $2 \mu M$ ), a blocker of N-type VDCCs. Here and henceforth, 100% indicates neurite outgrowth from cultures stimulated with P2d in the absence of inhibitors, and negCTL indicates neurite outgrowth from nonstimulated cultures. (C) PC12 cells were challenged with P2d either before (left;  $n = 22$ ) or after (right;  $n = 18$ ) application of an irreversible inhibitor of  $Ca^{2+}$  uptake into the ER stores, thapsigargin (TG,  $200 nM$ ), in a low  $Ca^{2+}$  medium. In all fluorometric experiments,  $n = 20-40$ .



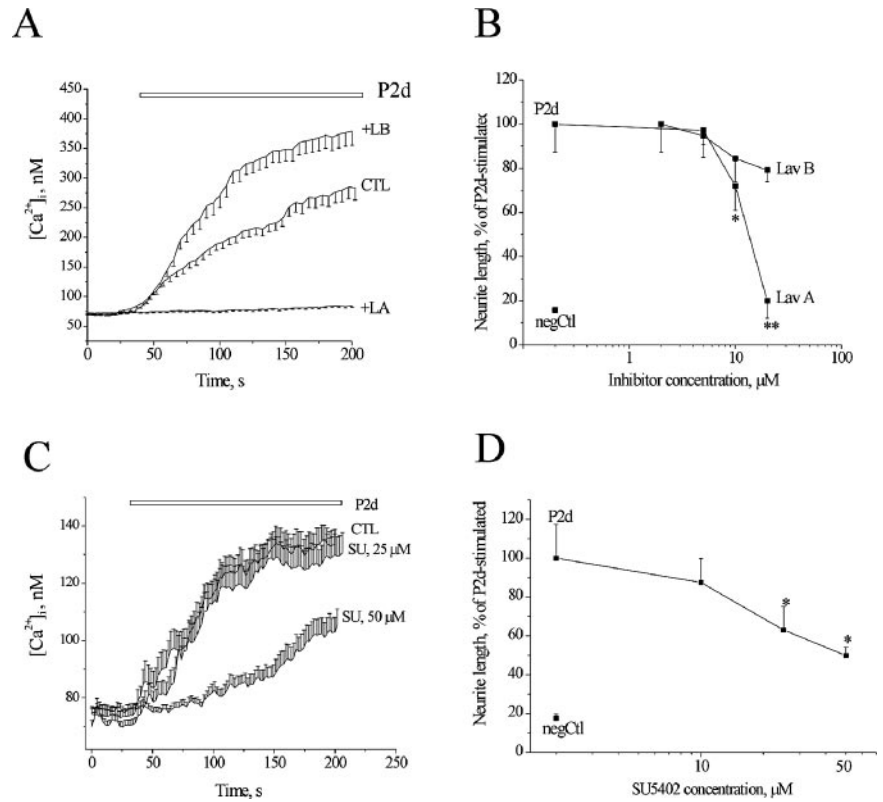
togenic response with a 40% reduction in average neurite length at a concentration of  $20 \mu M$  (Figure 3B), whereas mibefradil and pimozide almost completely blocked P2d-induced neurite extension at concentrations close to their  $2 \times K_{1/2}$  values ( $2.5$  and  $2.2 \mu M$ , respectively). This suggested that the activity of T-type, but not L-type,  $Ca^{2+}$  channels was crucial for neurite induction by P2d.

Because  $Ca^{2+}$  retained within the ER also might contribute to the P2d-triggered  $[Ca^{2+}]_i$  rise, we checked, whether application of the peptide led to a  $Ca^{2+}$  release from the intracellular ER stores. Cultured hippocampal neurons have been reported to possess overlapping  $Ca^{2+}$ - (ryanodine) and inositol trisphosphate-sensitive stores; however, no detectable  $Ca^{2+}$

response could be induced in the neurons by application of drugs activating the respective receptors on the surface of the stores (Irving and Collingridge, 1998). Therefore, we used the NCAM-expressing PC12E2 cell line known to possess a high amount of releasable  $Ca^{2+}$  in the stores. To estimate the P2d-induced  $Ca^{2+}$  release from the ER, the stores were challenged with P2d in a low  $Ca^{2+}$  medium (see *Materials and Methods* for composition). A long-lasting low-amplitude  $[Ca^{2+}]_i$  transient was observed (Figure 3C, left). After the P2d-induced response, thapsigargin (TG), a high-affinity blocker of  $Ca^{2+}$  pumps of the ER, still was able to release  $Ca^{2+}$  from the lumen of ER, indicating that P2d did not deplete the stores (Figure 3C, left). When TG was applied before P2d, thereby emptying the stores,



**Figure 5.** P2d-induced cellular responses depend on PLC $\gamma$ . (A) Top, increase in Tyr783 phosphorylation of PLC $\gamma$ 1 induced by FGF2 (FGF;  $10 \text{ nM}$ ) and P2d (P2d;  $5 \mu M$ ), representative immunoblot of four individual experiments is shown. Bottom, results from four independent experiments. (B and C) Effects of the inhibitor of phosphoinositide-specific PLCs (U17352) on P2d-induced  $Ca^{2+}$  responses (B, inhibitor concentration  $1 \mu M$ ) and neurite extension (C). U, U17352; Uctl, U17350 (a control compound for U17352).



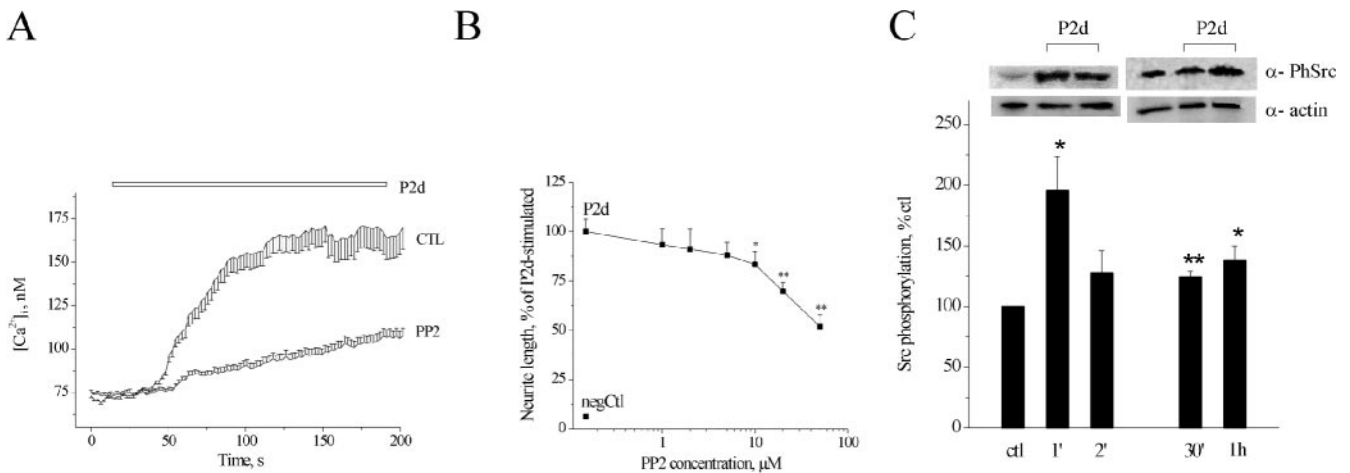
**Figure 6.** P2d-triggered signaling is mediated by RTKs and/or PTKs but only partially depends on FGFR. Effect of the inhibitors of RTK (lavendustin A) and FGFR1 (SU5402) on P2d-induced  $[Ca^{2+}]_i$  rise (A and C, respectively) and neurite outgrowth (B and D, respectively). LA, lavendustin A; LB, lavendustin B (a control compound to LA); SU, SU5402; n = 20–40.

the cells were no longer able to mount a  $Ca^{2+}$  response to P2d (Figure 3C, right), indicating that P2d indeed induced a  $Ca^{2+}$  release from the ER.

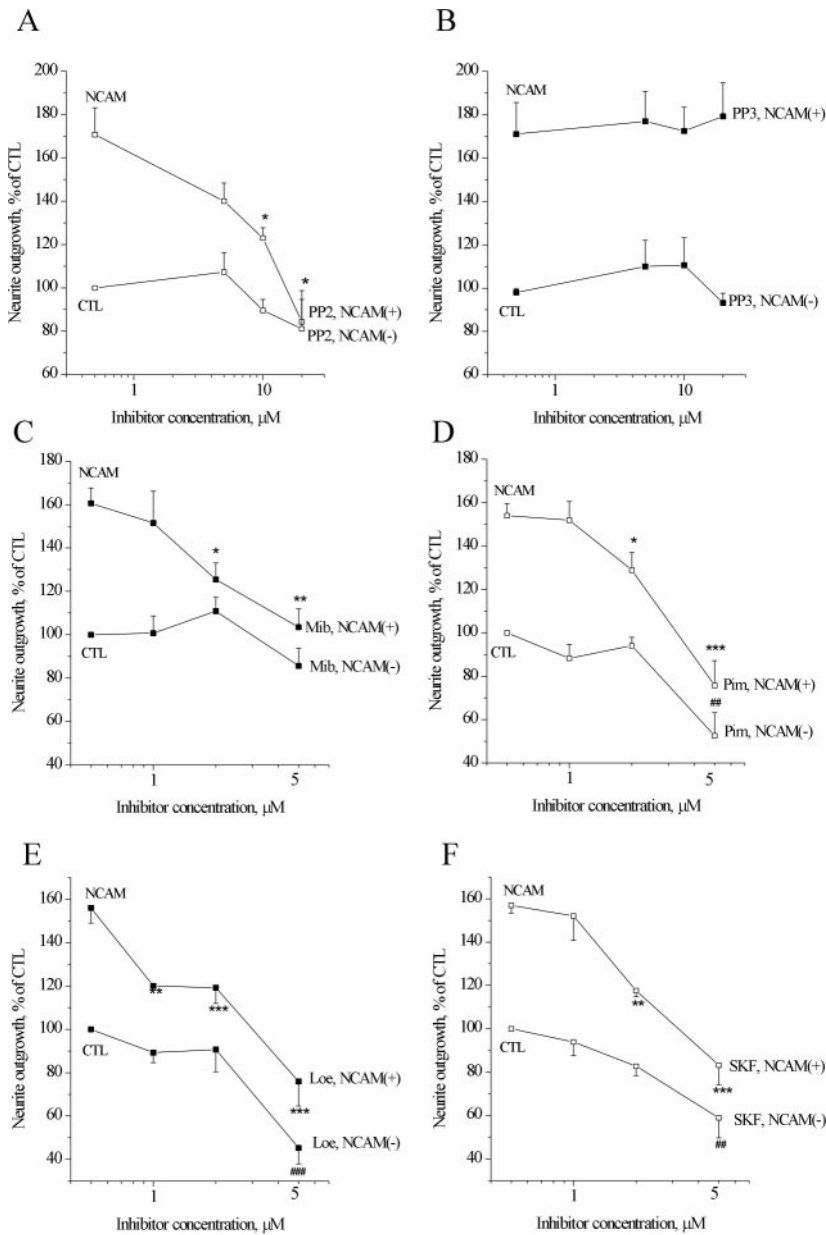
**The P2d-induced  $[Ca^{2+}]_i$  Rise Involves Nonselective Cation Channels and Requires Activation of the FGFR–PLC $\gamma$ –DAG Signaling Pathway**

We next tested, whether any other types of ion channels were involved in the P2d-induced  $Ca^{2+}$  response. NCAM homophilic binding results in the phosphorylation of FGFR, lead-

ing to an activation of the phosphoinositide-specific PLC $\gamma$  and to a formation of DAG, which can further be hydrolyzed by DAG-lipase to 2-arachidonoylglycerol (2-AG), and subsequently to arachidonic acid (AA) (reviewed in Kiryushko *et al.*, 2004). All these messengers activate nonselective cation channels (NSCCs) (reviewed in Hardie 2003). We found that two different inhibitors of NSCCs, Loe-908 and SKF-96562, decreased the P2d-induced  $Ca^{2+}$  response (Figure 4A) as well as neurite elongation (Figure 4B) in a dose-dependent manner. This indicated that the nonselective  $Ca^{2+}$  entry, possibly along-



**Figure 7.** Src-family kinases are involved in P2d-induced cellular responses. (A and B) Effect of the inhibitor of Src-family kinases (PP2) on P2d-induced  $Ca^{2+}$  responses (A) and neurite outgrowth (B). (C) Top, increase in Tyr416 phosphorylation of Src-family kinases induced by P2d (5  $\mu$ M), representative immunoblots of three or four individual experiments are shown. Bottom, results from three or four independent experiments.



**Figure 8.** Src-family kinases, VDCCs, and NSCCs are involved in the NCAM-induced neurite outgrowth. (A and B) Effect of the inhibitors of Src-family kinases PP2 (A) and its inactive analog PP3 (B) on NCAM-induced neurite outgrowth. (C and D) Effect of the inhibitors of T-type VDCCs mibefradil (Mib) (C) and pimoizide (Pim) (D) on NCAM-induced neurite outgrowth. (E and F) Effect of the inhibitors of NSCCs Loe908 (Loe) (E) and SKF-96365 (SKF) (F) on NCAM-induced neurite outgrowth. NCAM and control (CTL), neurite outgrowth from neurons grown on monolayers of fibroblasts with (NCAM+) and without (NCAM-) NCAM expression, respectively, in the absence of inhibitors. Asterisk (\*) indicates significance compared with NCAM; pound (#) indicates significances compared with CTL.

side with Ca<sup>2+</sup> entry via T-type VDCCs, was responsible for initiation of the Ca<sup>2+</sup> response to P2d. To test, whether DAG and/or its downstream products were involved in P2d-induced NSCC activation, DAG-lipase was blocked by the specific inhibitor RHC-80267, resulting in a dose-dependent decrease of P2d-triggered Ca<sup>2+</sup> transients (Figure 4C) and neurite outgrowth (Figure 4D). An NCAM-induced signaling cascade leading to DAG generation was most probably associated with PLC $\gamma$  (Hinsby *et al.*, 2004, and references therein). Indeed, in hippocampal neurons, P2d phosphorylated PLC $\gamma$ 1 on the Tyr783 residue (Figure 5A), an event known to be both necessary and sufficient for PLC $\gamma$ 1 activation (Kim *et al.*, 1991). PLC $\gamma$ 2 was not expressed in detectable amounts in hippocampal neurons (Kiryushko *et al.*, 2006). Thus, P2d launched intracellular signaling via PLC $\gamma$ 1, and the hydrolysis of DAG to 2-AG and/or AA contributed to the induction of the cellular response to the peptide.

Interestingly, blocking of DAG-lipase did not inhibit the P2d-induced Ca<sup>2+</sup> response completely (Figure 4, C and D),

suggesting either that DAG itself also plays a role or that other signaling cascade(s) also may contribute to the P2d-induced Ca<sup>2+</sup> response. In that case, inhibition of PLC $\gamma$  should not result in a complete blocking of the P2d-induced Ca<sup>2+</sup> rise. Indeed, U17352, an inhibitor of phosphoinositide-specific PLCs, did not abolish P2d-induced Ca<sup>2+</sup> elevation or neurite outgrowth, although decreasing both by ~70% (Figure 5, B and C). This indicated that P2d might activate other relevant signaling cascade(s) besides that associated with PLC.

NCAM-induced intracellular signaling has previously been shown to be mediated by FGFR, a member of the receptor tyrosine kinase (RTK) family (Saffell *et al.*, 1997; Kiselyov *et al.*, 2005). An antagonist of RTKs and protein tyrosine kinases, lavendustin A, but not its inactive analog lavendustin B, abolished P2d-induced Ca<sup>2+</sup> entry and neurite outgrowth (Figure 6, A and B, respectively). The observed effect of lavendustin A could be partially attributed to the inhibition of FGFR1, a ubiquitous FGFR type in primary neurons, because a specific blocker of FGFR1 (SU5402)

decreased both  $[Ca^{2+}]_i$  rise (Figure 6C) and neurite extension induced by P2d (Figure 6D). However, even at the maximal antagonist concentration the inhibition was only ~50%, suggesting that another signaling mechanism might be involved in the induction of cellular responses by P2d.

#### *Src-Family Kinases Are Involved in the P2d-induced Intracellular Signaling*

Because lavendustin A inhibits both RTKs and the Src-family kinases known to be involved in NCAM-induced signaling (reviewed in Walmod *et al.*, 2004), we tested, whether Src-family kinases participated in the P2d-induced  $Ca^{2+}$  signaling using a specific inhibitor, PP2. Indeed, PP2 caused a significant reduction in both  $[Ca^{2+}]_i$  rise (Figure 7A) and neurite outgrowth (Figure 7B) in response to P2d. We therefore checked whether P2d increased the phosphorylation of Src-family kinases on the Tyr416 residue shown to be involved in the activation of these enzymes. As seen in Figure 7C, the peptide induced phosphorylation of the Src-family kinases with a maximum activation observed already 1 min after application. The increase in the amount of the phosphorylated enzyme form was also observed 30 min and 1 h after peptide application. Thus, P2d as well as NCAM itself (Beggs *et al.*, 1997) triggered Src-mediated signaling.

#### *Src-Family Kinases, T-type VDCC, and NSCC Are Involved in the NCAM-induced Neurite Outgrowth*

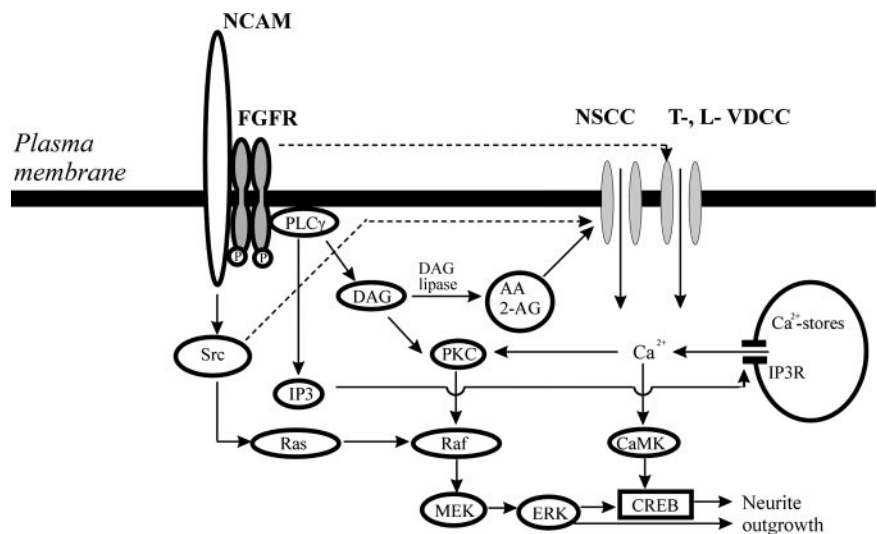
We next checked, whether the mechanisms of  $Ca^{2+}$  homeostasis involved in the signaling triggered by P2d were of importance for physiological NCAM-induced differentiation. In this assay, primary hippocampal neurons are grown on monolayers of fibroblasts expressing NCAM, a condition known to mimic physiological NCAM homophilic *trans* interactions. Under these circumstances, NCAM is known to induce neurite extension from a variety of neuronal cell types (reviewed in Hinsby *et al.*, 2004). As seen in Figure 8, hippocampal neurons grown on monolayers of NCAM-expressing fibroblasts extended ~60% longer neurites (NCAM control) than neurons grown in coculture with fibroblasts without NCAM expression (negative control). NCAM-specific neurite outgrowth could be abolished by the blocker of Src-family kinases PP2 (Figure 8A), whereas the inactive PP2 analog PP3 had no effect (Figure 8B).

Blockers of both T-type VDCCs (mibefradil, Figure 8C; and pimozone, Figure 8D) and NSCC (Loe908, Figure 8E; and SKF-96365, Figure 8F) all significantly reduced NCAM-specific neurite extension when applied in low ( $\sim K_{1/2}$ ) concentrations. At concentrations close to their  $2 \times K_{1/2}$ , the inhibitors also affected neurite extension from the neurons grown on fibroblasts without NCAM expression. Thus, the activity of T-type VDCCs and NSCCs was important for NCAM-dependent neurite extension, but it probably also played a role in the neurite outgrowth response induced by other cell surface-associated neuritogenic cues.

## DISCUSSION

In this study, we have investigated  $[Ca^{2+}]_i$  regulating mechanisms involved in NCAM signaling using a synthetic NCAM mimetic, the P2 peptide. Like NCAM itself, the peptide both triggered neurite extension and a  $[Ca^{2+}]_i$  rise in primary neurons. The amplitude of the observed  $[Ca^{2+}]_i$  responses to P2d was dependent on how long neurons had been grown in culture, being about threefold higher in cultures grown for 7 d than in cultures grown for only 6 h. Moreover, at identical times *in vitro*, an amplitude of P2d-induced  $[Ca^{2+}]_i$  rise was significantly higher in the neurons isolated from the P5 animals than in the neurons isolated from the E19 animals, suggesting a developmental change of  $Ca^{2+}$  responses to the peptide. This difference might be caused by changing levels of NCAM/FGFR expression, or more likely, by an age-dependent increase in the density of L-type VDCCs (Porter *et al.*, 1997), which together with T-type channels, are the main factors responsible for depolarization-induced  $Ca^{2+}$  influx into the somata of hippocampal neurons. In accordance with this, the P2d-induced  $[Ca^{2+}]_i$  could be significantly inhibited by blockers of both L- and T-type but not N-type VDCCs (summarized in Figure 9). Because N- and P/Q-type VDCCs are sparsely distributed on the neuronal somata but abundant in growth cones (Catterall, 2000), it cannot be ruled out that these channels may mediate P2d-induced  $Ca^{2+}$  entry into the nerve terminals. However, in P2d-triggered neurite outgrowth they seemed to play a minor role.

Upstream of the VDCCs, the P2d-mediated signaling involved RTKs; PLC $\gamma$ 1, an enzyme predominantly associated with RTKs; and DAG-lipase (Figure 9). This is in agreement with observations by Saffell *et al.* (1997) and Archer *et al.* (1999)



**Figure 9.** Schematic representation of signaling pathways activated by P2d interaction with NCAM. CaMK, calcium-calmodulin kinase; CREB, cAMP response element-binding protein; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; MEK, mitogen-activated protein kinase kinase; Src, Src-family kinases. See *Discussion* for details.

that homophilic CAM binding sequentially induces phosphorylation of FGFR, activation of PLC $\gamma$ , and generation of DAG. The major Ca<sup>2+</sup> channels type affected by DAG and/or its products has previously been suggested to be the L-type (Archer *et al.*, 1999), presumably activated either by a direct interaction with FGFR2 (Rosenthal *et al.*, 2001) or indirectly by the DAG metabolite AA (Archer *et al.*, 1999) and/or by the AA-activated cannabinoid receptor 1 (Williams *et al.*, 2003). However, to our knowledge, no evidence of direct activation by AA of voltage-gated Ca<sup>2+</sup> channels at near-basal membrane potentials exists. Moreover, AA has been shown to reduce L-type Ca<sup>2+</sup> current in cardiac cells (Petit-Jacques and Hartzell, 1996), rat sympathetic neurons (Liu and Rittenhouse, 2000), and rat ventricular myocytes (Xiao *et al.*, 1997). No effect of AA on the basal L-current density has been observed in cardiac myocytes (Petit-Jacques and Hartzell, 1996), and basic fibroblast growth factor (FGF), the high-affinity ligand of FGFR, does not affect activation or inactivation kinetics of L-current at the near-basal potentials (Rosenthal, *et al.*, 2001). Thus, given the significant depolarization required for opening of either L- or N-type Ca<sup>2+</sup> channels, it seems unlikely that AA activates them directly. Instead, 2-AG or AA might first activate their well-known effectors, the NSCCs. In agreement with this hypothesis, the P2d-induced [Ca<sup>2+</sup>]<sub>i</sub> rise was only partially inhibited by blockers of L-type VDCCs. However, both T-type VDCCs and NSCCs were important for induction of Ca<sup>2+</sup> responses by the NCAM mimetic. Thus, NSCCs might be the channels, which open first in response to P2d and mediate an "initial" Ca<sup>2+</sup> influx and depolarization of the plasma membrane. This then leads to the opening of low-voltage-activated T-type Ca<sup>2+</sup> channels, which mediate further depolarization, resulting in an opening of high-voltage-activated L-type VDCCs and in an increase of Ca<sup>2+</sup> entry. In addition, a direct activation of L-type of VDCCs by FGFR2, which also is expressed in cultured hippocampal neurons (Neiendam *et al.*, 2004), might contribute to the induced Ca<sup>2+</sup> influx (Rosenthal *et al.*, 2001; Figure 9, stippled arrow).

The NSCCs types mediating the P2d-induced Ca<sup>2+</sup> entry might be arachidonate-regulated Ca<sup>2+</sup> channels and/or the transient receptor potential (TRP) family Ca<sup>2+</sup>-permeable channels, both activated by 2-AG, AA, and its metabolites (reviewed in Hardie, 2003; Shuttleworth and Mignen, 2003). Recently, it has been shown that the TRPC1 channels of the TRP family colocalize with FGFR1 and contribute to the FGFR1-mediated Ca<sup>2+</sup> influx in embryonic rat neural stem cells by an unknown mechanism (Pla *et al.*, 2005). Because TRPC1 can heterotetramerize with other TRPCs expressed during brain developments (e.g., with TRPC3/TRPC6, TRPC4/TRPC5; Strubing *et al.*, 2003), it is possible that FGFR1 coexists in complexes with TRPCs in primary neurons. In this case, activation of FGFR1 would result in a local increase of DAG and its products, leading to an efficient activation of the adjacent TRP channels.

In our experiments, the P2d-elicited [Ca<sup>2+</sup>]<sub>i</sub> rise was only partially inhibited by blockage of the FGFR1-PLC $\gamma$ -DAG signaling pathway but abolished by an RTK/protein tyrosine kinase blocker lavendustin A. This can be explained by an "additional" activation of L-type VDCCs by FGFR2 (see *Discussion* above) and/or by Src-family kinases. Indeed, both P2d-induced neurite outgrowth and Ca<sup>2+</sup> responses were significantly inhibited by blocking of Src. Moreover, treatment with P2d resulted in the phosphorylation of the Src-family kinases on Tyr416, an event known to mediate Src activation, and the time course of the phosphorylation correlated to that of P2d-induced Ca<sup>2+</sup> responses. This is in accordance with previous reports demonstrating that Src kinases are phosphorylated by homophilic NCAM binding

(Beggs *et al.*, 1997) or by another synthetic peptide ligand of NCAM, C3d (our unpublished observations). Tyrosine phosphorylation of Src kinases can positively regulate NSCCs (Hisatsune *et al.*, 2004; Vazquez *et al.*, 2004) and L-type VDCC (Hou *et al.*, 2003, and references therein), thus explaining the dependence of P2d-elicited Ca<sup>2+</sup> responses on the activity of Src kinases (Figure 9, stippled arrow).

Activation of the Src-associated signaling cascade may not be universally required for the cellular effects exerted by NCAM. However, this cascade might be of importance under circumstances where the FGFR-associated signaling is not "sufficient" to induce a given cellular response. In particular, in cultures of embryonic hippocampal neurons, neurite extension cannot be elicited by an activation of FGFR with its high-affinity ligand FGF2 (Amoureux *et al.*, 2000). However, FGF2 has been demonstrated to induce neurite outgrowth from postnatal dorsal root ganglion and cerebellar neurons (Archer *et al.*, 1999; Neiendam *et al.*, 2004), thus exemplifying principal differences in the outputs of FGFR activation in the above-mentioned experimental systems.

Importantly, the Ca<sup>2+</sup>-regulating mechanisms shown to be involved in the P2d-induced intracellular signaling (Src-family kinases, T-types VDCCs, and NSCCs) are also crucial for NCAM-induced differentiation of primary neurons. However, our data suggest that T-type VDCCs and NSCCs are not only involved in the NCAM-induced neurite outgrowth but also may participate in the induction of neurite extension induced by other factors.

In our experiments, we found that the relative contribution of FGFR- and Src-mediated signaling pathways in P2d-versus NCAM-induced responses differed. The reason might be that homophilic NCAM binding is mediated by interactions between several NCAM modules, namely, the IgI and IgII modules mediate dimerization of NCAM molecules situated on the same cell surface, whereas the IgI and IgIII, IgII and IgII, and the IgII and IgIII modules of NCAM mediate *trans* homophilic binding (Soroka *et al.*, 2003). The P2d-peptide selectively mimicks only the IgI to IgII binding. Therefore, the P2d- and NCAM-induced responses probably result in a different dynamics of NCAM clustering and thus in different contribution of Src (Fyn) phosphorylation known to be induced by this clustering (Beggs *et al.*, 1997).

In conclusion, we have identified two signaling pathways associated with FGFR and Src-family kinases, respectively, mediating the intracellular Ca<sup>2+</sup> signaling induced by the NCAM mimetic P2d. Nonselective cation conductances and T-type VDCCs, but not L-type VDCCs, were crucial for the induction of P2d-mediated cellular responses. Moreover, Src-family kinases, T-type VDCCs, and NSCCs all were important for NCAM-induced neurite extension. This suggests that multiple signaling pathways and mechanisms of Ca<sup>2+</sup> homeostasis contribute to NCAM-triggered neuronal differentiation depending on cell type and developmental stage.

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