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Increased intracellular calcium is required for neurite outgrowth induced by a synthetic peptide ligand of NCAM

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Abstract We have recently identified a synthetic peptide, termed C3, capable of binding the first immunoglobulin-like module of neural cell adhesion molecule (NCAM) by means of combinatorial chemistry and shown that this NCAM ligand promotes neurite outgrowth. By means of single cell calcium imaging using the calcium-sensitive probe fura-2-acetomethyl ester, we here show that the C3-peptide induced an increase in intracellular calcium in primary hippocampal neurons and PC12-E2 cells, presumably requiring mobilization of calcium from both extracellular and intracellular stores. We further observed that C3-induced neurite outgrowth was inhibited by antagonists of voltage-dependent calcium channels as well as by an inhibitor of intracellular calcium mobilization, TMB-8. These findings demonstrate at the single cell level that a synthetic NCAM ligand directly can induce an increase in intracellular calcium and suggest that NCAM-dependent neurite outgrowth requires calcium mobilization from both extracellular and intracellular calcium stores. Thus, the C3-peptide may be regarded as a useful tool for the study of NCAM-dependent signal transduction. Furthermore, the peptide may be of considerable therapeutical interest for the treatment of neurodegenerative disorders.

Key words: Calcium; Combinatorial chemistry; Fura2; Imaging; Neural cell adhesion molecule; Neurite outgrowth

1. Introduction

The neural cell adhesion molecule (NCAM) plays a key role in the development of the nervous system by modulating axonal outgrowth and fasciculation. In addition, NCAM has been shown to be involved in synaptic plasticity associated with learning and memory [1–3]. Thus, spatial learning is impaired in NCAM-deficient mice [4] and intracranial injection of NCAM-antibodies in a time window of 5–7 h after a learning session inhibits learning in rats or chicks in models of passive avoidance learning [5,6]. In addition, long-term potentiation is impaired in hippocampal slice cultures from NCAM-deficient mice and in normal tissue slices after injection of NCAM antibodies [7–10].

NCAM binds homophilically (NCAM binding to NCAM) as well as heterophilically (NCAM binding to another ligand or counter-receptor), thereby mediating intercellular adhesion and adhesion between cells and the extracellular matrix. Homophilic NCAM binding probably depends on a double reciprocal interaction between the immunoglobulin-I (IgI) and IgII modules of two opposing NCAM molecules [11–15] although interactions between two opposing IgIII modules [16], or an antiparallel interaction between opposing IgI–IgV modules [17] also have been suggested to contribute to NCAM binding. Upon homophilic NCAM binding, neurite outgrowth is induced via activation of a signalling cascade that presumably includes activation of fibroblast growth factor (FGF) receptors, PKC and the Ras-MAPK pathway [18–20].

It has been shown that neurite outgrowth induced by homophilic NCAM binding in cocultures of neurons and NCAM-transfected fibroblasts is inhibited by antagonists of voltage-dependent calcium channels (VDCCs), suggesting that a calcium influx through VDCCs may be necessary for NCAM-induced neurite outgrowth [21]. Likewise, the homophilic binding of another cell adhesion molecule, L1, has been shown to induce neurite outgrowth [18] and it has been reported that soluble Fc-chimeras of L1 induce a calcium influx through VDCCs although without a concomitant rise in bulk intracellular calcium [22]. Moreover, by spectrofluorimetry NCAM antibodies have been shown to induce a long-lasting increase in intracellular calcium in PC12 cells [23] and primary cerebellar neurons [24].

By means of a combinatorial library of synthetic peptides, we have recently identified a ligand of the N-terminal NCAM Ig module (IgI), termed the C3-peptide, which promotes neurite outgrowth from primary hippocampal neurons and PC12-E2 cells. This effect can be inhibited by verapamil and ω-conotoxin GVIA, indicating a signalling pathway involving calcium influx through VDCCs [25]. We here studied the role of calcium channels in neurite outgrowth induced by the C3-peptide and further used single cell calcium imaging to investigate whether the C3-peptide affected intracellular calcium in primary hippocampal neurons and PC12-E2 cells.

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Abbreviations: AgaTxTK, ω-agatoxin TK; BSA, bovine serum albumin; CgTx MVIIA, ω-conotoxin MVIIA; FBS, fetal bovine serum; HS, horse serum; Ig, immunoglobulin; NCAM, neural cell adhesion molecule; VDCC, voltage-dependent calcium channel

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2. Materials and methods

2.1. Cell culture

The PC12-E2 cell line [26], a gift from Dr. Klaus Seedorf, Hagedorn Research Institute, Gentofte, Denmark, was grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Paisley, Scotland, UK) supplemented with 10% horse serum (HS; Sigma, St. Louis, MO, USA), 5% fetal bovine serum (FBS; Sigma) penicillin (100 U/ml, Sigma) and streptomycin (100 μg/ml, Sigma). Dissociated hippocampal cells were obtained from embryonic day 18 Wistar rats as previously described [27]. Briefly, hippocampi were dissected in a cold modified Krebs–Ringer solution (Gibco BRL). After removal of meninges, the tissue was roughly homogenized by chopping with a scalpel blade and thereafter trypsinizing for 5 min at room temperature. Cells were then washed in the presence of DNase I (Sigma) and soybean trypsin inhibitor (Sigma) and thereafter triturated. All animals were handled in accordance with the national guidelines for animal welfare. For analysis of neurite outgrowth, 5000 cells/well were seeded in 8-well LabTek tissue culture chamber slides with a growth surface of Permanox plastic coated with fibronectin (10 μg/ml, Sigma) and grown in Neurobasal medium supplemented with B27 (Gibco, BRL), 20 mM HEPES (Gibco BRL), 0.4% w/v bovine serum albumin (BSA; Sigma), penicillin (100 U/ml) and streptomycin (100 μg/ml). After 21 h, cultures were fixed using 4% w/v paraformaldehyde, washed in phosphate-buffered saline and stained with Coomassie blue (4 g/l Coomassie brilliant blue, 45% v/v ethanol, 10% v/v acetic acid). Reagents to be tested were added immediately after seeding of cells and included ω-conotoxin MVIIA (CgTx MVIIA), nifedipine and ω-agatoxin TK (AgaTxTK; Alomone Labs, Jerusalem, Israel), TMB-8 (8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride; Calbiochem, La Jolla, CA, USA) and Igs recognizing NCAM purified from a rabbit polyclonal NCAM antiserum using a protein-A Sepharose column as previously described [7].

2.2. Analysis of neurite outgrowth

After 21 h in culture, images of cultures were grabbed and analyzed by means of computer-assisted microscopy. The total neurite length per cell was analyzed by means of a stereological method using the software Processlength [28]. Briefly, the total length of neurites per cell was estimated by counting the number of intersections between neurites and the test lines of an unbiased counting frame. The absolute neurite length (L) was calculated using the equation $L = (\pi \cdot d/2) \cdot I$ in which I represents the number of neurite intersections per cell and d represents the vertical distance between the test lines of the counting frame.

2.3. Calcium imaging

Hippocampal cells were seeded on 4- or 8-well fibronectin-coated LabTek chambered coverglass slides (NUNC, Roskilde, Denmark) at a density of 10–50 000 cells/well and grown for 1–21 days in Neurobasal medium supplemented with B27, 20 mM HEPES, 0,4% w/v BSA, penicillin (100 U/ml) and streptomycin (100 μg/ml). Approximately two thirds of the medium were exchanged every third day. PC12-E2 cells were dislodged mechanically by tapping and seeded at a density of 5000-30 000 cells/cm^2 in 4- or 8-well LabTek chambered coverglass slides (NUNC) with a growth surface of plastic coated with fibronectin (10 μg/ml, Sigma) and grown for 1–5 days. In some cases, neuronal differentiation was induced by changing the medium to DMEM supplemented with 1% FBS, 1% HS and nerve growth factor (NGF; 50 ng/ml, Alomone) or FGF-2 (10 ng/ml, Alomone). Cells were washed with Krebs–Ringer solution comprising KCl (5 mM), NaCl (116 mM), $NaHCO_3$ (20 mM), $MgCl_2$ (1 mM), Na_2HPO_4 (3 mM), $CaCl_2$ (1 mM), HEPES (5 mM) and loaded with Fura-2 acetomethyl ester (Fura-2-AM; 2 μM; Molecular Probes, Eugene, OR, USA) dissolved in dimethyl sulfoxide for 35 min at 20°C in the dark. In some cases Hanks' balanced salt solution (Sigma) comprising KCl (5.4 mM), NaCl (137 mM), $NaHCO_3$ (2 mM), $MgSO_4$ (0.8 mM), Na_2HPO_4 (0.27 mM), glucose (5.6 mM), $CaCl_2$ (1.25 mM), KH_2PO_4 (0.44 mM) was used instead of Krebs–Ringer solution. Subsequently, cells were washed four times and placed on the stage of an inverted Axiovert 135 TV microscope (Zeiss, Göttingen, Germany) equipped with an oil immersion UV objective (Zeiss Fluar 40×, 1.3 numerical aperture). Imaging was performed using a sensicam 12 bit cooled CCD camera (PCO, Keilheim, Germany) and a J&M monochromator (J&M, Aalen, Germany). In some cases, an Axiovert 135 inverted microscope (Zeiss) equipped with an oil immersion UV objective (Zeiss Achrostigmat 40×, 1.3 numerical aperture), a filter wheel, a CCD-72 video camera and a Gen II sys image intensifier (DAGE-MIJ, Michigan City, MI, USA) were used for calcium imaging. As software for data acquisition and analysis either Imaging Workbench (Axon, Foster City, CA, USA) or Metafluor (Universal Imaging, West Chester, PA, USA) were used. Ratio images were obtained after background subtraction from images collected at wavelengths over 510 nm after excitation at 340 and 380 nm respectively at sampling rates between 0.1 and 1 Hz. Calibration was performed using CaEGTA/K_2EGTA buffers with known concentrations of free calcium (Molecular Probes) and Fura-2 pentapotassium salt (5 μM, Molecular Probes). Concentration of free calcium was estimated as, $[Ca^{2+}]_i = K_d \times (R - R_{min})/(R_{max} - R) \times (F380_{max}/F380_{min})$, where R is the ratio of background subtracted fluorescence intensities obtained at excitation at 340 and 380 nm respectively, R_{max} is the ratio at saturating calcium, R_{min} is the ratio at zero free calcium, $F380_{min}$ is the intensity at saturating free calcium exciting at 380 nm, while $F380_{max}$ is the intensity at zero free calcium. R_{max} was determined in situ using Fura-2-AM-loaded cells in the presence of 5 μM ionomycin (Molecular Probes) and 10 mM extracellular calcium. Values determined were: R_{max} 9.0; R_{min} 0.68; $F380_{max}/F380_{min}$ 7.0. The K_d used was 236 nM [29]. Buffer with nominally zero calcium was prepared as described above except that the NaCl molarity was increased correspondingly to compensate for the decreased ionic strength. For experiments using zero calcium buffer, cells were carefully washed five times in the buffer before being used for imaging. Peptides and other compounds to be tested were applied directly to the cell culture chambers in a volume corresponding to half of the volume present in the chamber prior to application to ensure an even distribution of the compound.

2.4. Peptide synthesis

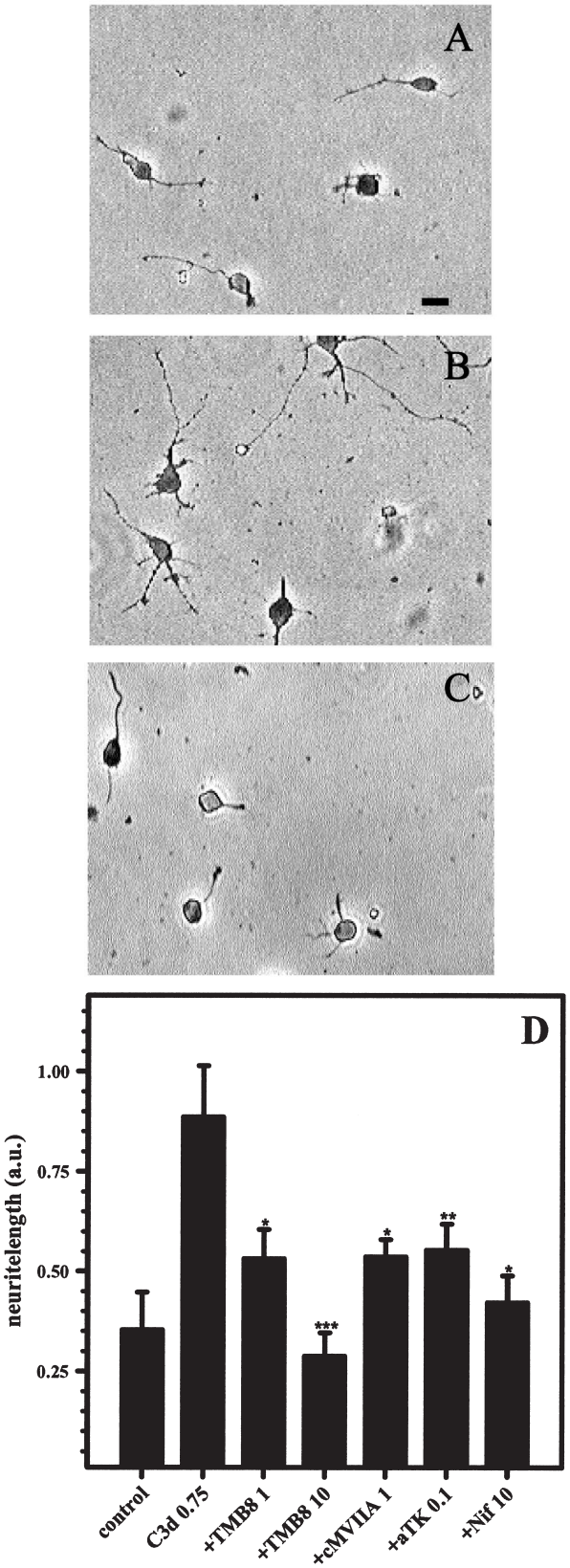
Peptide dendrimers consisting of four peptide monomers coupled to a lysine backbone were synthesized on a TentaGel resin (Rapp Polymere, Tübingen, Germany) with the Rink amide linker (Novabiochem, Läufelfingen, Switzerland) using 9-fluorenylmethoxycarbonyl-protected amino acids (Novabiochem) as previously described [25]. The peptides were at least 95% pure as estimated by HPLC. Concentrations of peptide dendrimers were calculated according to the amount of peptide monomers. The sequence of the C3-peptide is ASKKPKRNIKA. Two peptides comprising the C3-sequence with two or four alanine substitutions, ASKKPAANIKA (C3 2ala) and ASAAPAANIKA (C3 4ala), were used as controls for the C3-peptide. The dendrimer of the C3-peptide is termed C3d.

3. Results

3.1. Neurite outgrowth induced by C3d is inhibited by calcium channel antagonists

In order to investigate the role of intracellular calcium in the signalling pathway activated by the C3-peptide ligand of NCAM, primary hippocampal neurons were grown for 24 h in the presence of antagonists of VDCCs or in the presence of TMB-8, which has been reported to be an inhibitor of intracellular calcium mobilization [30]. As previously observed, the dendrimeric form of C3, C3d, stimulated neurite outgrowth from primary hippocampal neurons at a concentration of 0.75 μM (Fig. 1). Thus, in the presence of C3d, the neurite length per cell was approximately twice as large as the length of neurites from cells grown in the absence of peptide. This increase in neurite length was inhibited partially by the presence of either CgTx MVIIA (1 μM), nifedipine (10 μM) or AgaTxTK (0.1 μM), which are antagonists of N-type, L-type and P/Q-calcium channels, respectively. The possible involvement of N- and L-type calcium channels in NCAM-dependent neurite outgrowth induced by either the C3-peptide [25] or homophilic NCAM binding in neuron–fibroblast co-cultures [21] has previously been demonstrated using the compounds verapamil, diltiazem and ω-conotoxin GVIA. The

present findings support the involvement of N- and L-type calcium channels and furthermore imply that activation of P/Q-type calcium channels upon NCAM binding also may be necessary for neurite outgrowth stimulated by C3d.



To investigate the involvement of intracellular calcium stores in NCAM-dependent neurite outgrowth, we tested the effect of TMB-8, an inhibitor of intracellular calcium mobilization [30], on neurite outgrowth induced by the C3-peptide. TMB-8 blocked the neurite outgrowth response to the C3-peptide completely in a concentration of 10 μM, and it was partially inhibiting at a concentration of 1 μM. This indicates that mobilization of calcium from intracellular stores may be required for neurite outgrowth induced by NCAM binding, and this mobilization probably acts in concert with a calcium influx through VDCCs in the plasma membrane.

3.2. The C3-peptide induces an increase in intracellular calcium in PC12-E2 cells

By means of spectrofluorometry, it has previously been shown that polyclonal antibodies against NCAM induce an increase in the average intracellular concentration of calcium in quin-2-loaded PC12 cells when applied in relatively high concentrations (0.4–1 mg/ml) [23]. Using a similar method, recombinant NCAM fragments or purified NCAM from mouse brain were shown to increase intracellular calcium in cerebellar neurons [24,31].

Here, we have used imaging of single Fura-2-loaded PC12-E2 cells to investigate whether the C3-peptide was capable of modulating intracellular calcium. When applied at a concentration of 50 μM, C3 induced a sustained increase in intracellular calcium in undifferentiated PC12-E2 cells as shown in Figs. 2 and 3A. The cells responded heterogenously, with some cells displaying slow oscillations in intracellular calcium. A similar response was observed after a neuronal phenotype had been induced in the PC12-E2 cells by pre-treatment with NGF or FGF-2 (not shown). When C3d was applied in nominally Ca^{2+}-free conditions, a significant reduction in the response was observed (Fig. 3B), indicating that the C3-induced increase in the intracellular calcium concentration is dependent on influx of calcium from extracellular sources. However, the finding that in the absence of extracellular calcium C3d still induced a small, transient increase in intracellular calcium suggests that mobilization of calcium from internal stores also contributes to the response. As the increase in the intracellular calcium concentration under these conditions was only transient, this indicates that VDCCs in the plasma membrane are necessary for the sustained increase in intracellular calcium observed after C3 application. However, the diminished Ca^{2+} signalling from internal stores in Ca^{2+}-free medium could also be due to a partial depletion of intracellular Ca^{2+} stores under these conditions. In the presence of TMB-8 the response to C3d was smaller and the onset was slower than in the absence of TMB-8, supporting the notion that mobilization of calcium from intracellular stores is induced by C3d (Fig. 3C). A C3-control peptide with no neu-

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Fig. 1. Effect of calcium channel antagonists on neurite outgrowth induced by C3d. A: Micrograph of primary hippocampal neurons, bar = 20 μm. B: Primary hippocampal neurons grown in the presence of C3d (0.75 μM). C: Primary hippocampal neurons grown in the presence of C3d (0.75 μM) and TMB-8 (10 μM). D: Total length of neurites in primary hippocampal cell cultures grown in the absence or presence of C3d and the calcium channel antagonists TMB-8, CgTx MVIIA (cMVIIA), AgaTxTK (aTK) and nifedipine (Nif) (concentrations in μM are indicated). The results are shown as means ± S.E.M. from six to nine independent experiments, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (paired *t*-test).

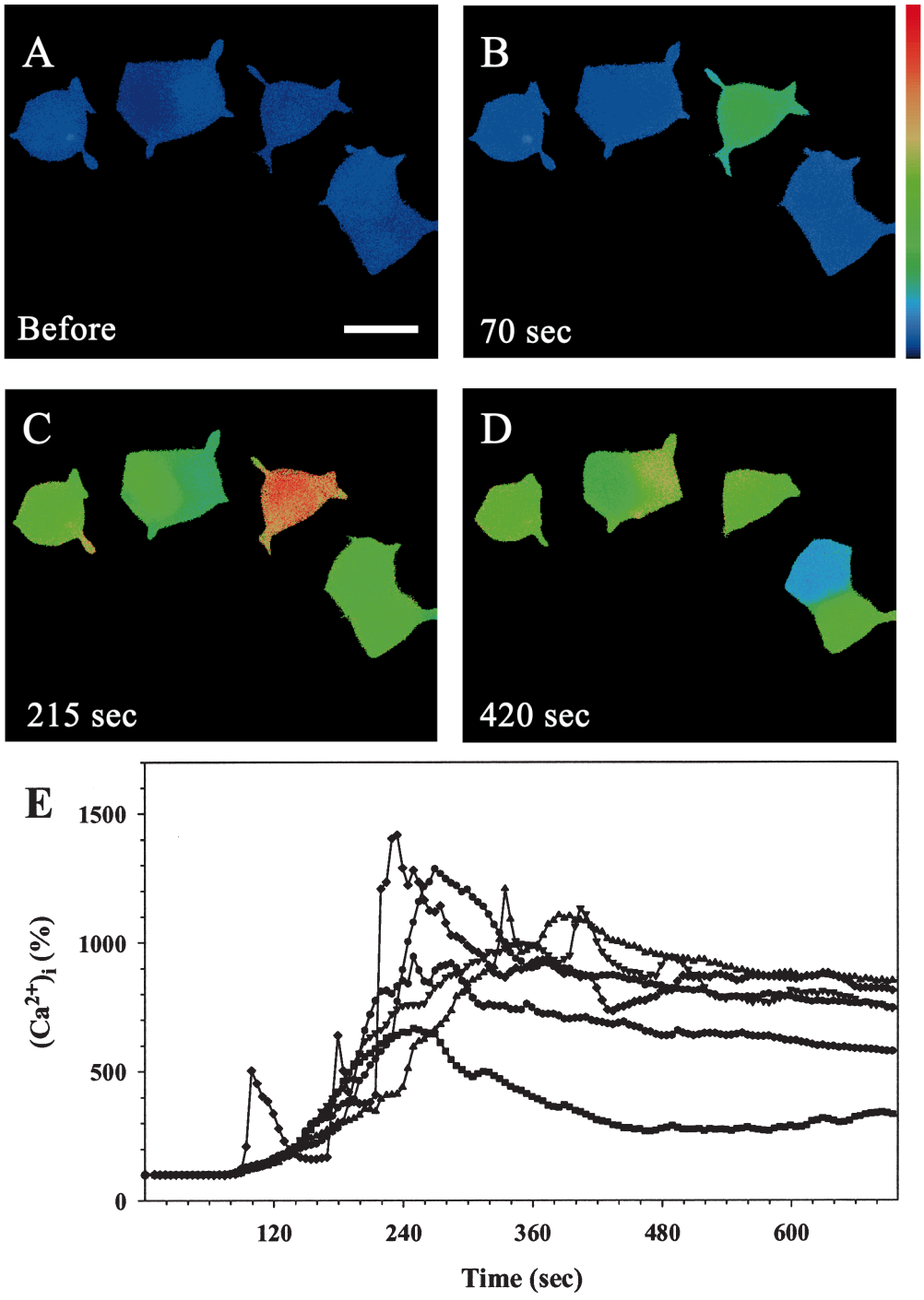

Fig. 2. C3d induces an increase in intracellular calcium in PC12-E2 cells. A: Micrograph of a Fura-2-AM-loaded PC12-E2 cell before C3d application, bar: 20 μm. B–D: Micrographs of PC12-E2 cells 70 s, 215 s and 420 s after application of C3d (50 μM). E: Time course of the change in intracellular calcium concentration ($[Ca^{2+}]_i$) calculated from the Fura-2 fluorescence ratio following application of C3d (50 μM). Each trace represents an individual cell.

ritogenic activity [25], C3d 2ala, had no measurable effect on intracellular calcium (Fig. 3D).

3.3. *The effect of the C3-peptide on intracellular calcium in primary hippocampal neurons*

We subsequently studied the effect of the C3-peptide on Fura-2-loaded primary hippocampal neurons in order to relate the apparent dependence of C3-induced neurite outgrowth to the observed changes in intracellular calcium (Fig. 4). As in PC12-E2 cells, a sustained increase in intracellular calcium was observed in primary hippocampal neurons upon application of 54 μM C3d (Fig. 4A). In contrast, a C3-control peptide with no neuritogenic activity, C3d 4ala [25], had no measurable effect on intracellular calcium. In addition, polyclonal antibodies against NCAM elicited an increase in intracellular calcium, while Igs from non-immune serum had no effect (Fig. 4B). When lower concentrations of C3d were applied, the responses were heterogeneous. Gen-

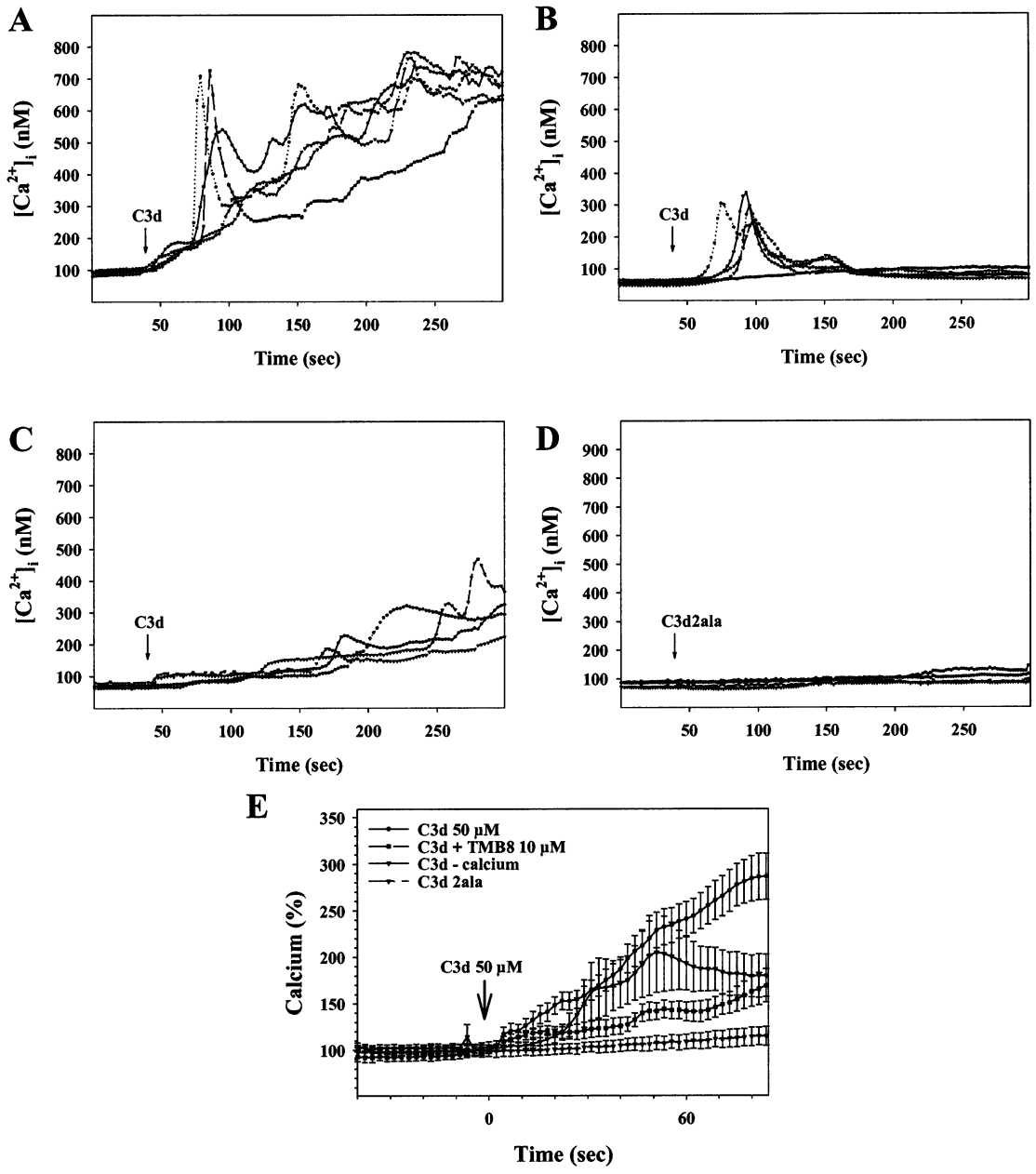

Fig. 3. C3d induces mobilization from intracellular calcium stores in PC12-E2 cells. A: Effect of C3d application (50 μM) on the intracellular calcium concentration in Fura-2-AM-loaded PC12-E2 cells. B: Effect of C3d on intracellular calcium under nominally calcium-free conditions. C: Effect of C3d on intracellular calcium of PC12-E2 cells pre-treated with TMB-8 (10 μM). D: Effect of C3-control peptide, C3d 2ala, on intracellular calcium in PC12-E2 cells. Data in (A–D) are representative of 4–12 independent experiments, each trace representing an individual cell. E: Effect of C3d or C3-control peptide (C3d 2ala) on intracellular calcium in PC12-E2 cells in the presence or absence of TMB-8 (10 μM) or in nominally calcium-free conditions (−calcium). Data points are means ± S.E.M. of 4–12 independent experiments.

erally, only few cells responded measurably to 0.54 μM, while a higher fraction of cells responded with an increase in intracellular calcium, when C3d was applied in a concentration of 5.4 μM (Fig. 4C). The C3-induced change in intracellular calcium also appeared to depend on the in vitro differentiation state of the hippocampal cells. When C3d was applied in concentrations of 0.54 or 5.4 μM to cells after 24 h in culture only very few cells ($< 5\%$) responded measurably. After 72 h in culture a higher but variable fraction of the cells showed a change in intracellular calcium after application of C3d while most cells grown for a period of 10–21 days responded to the C3-peptide. Furthermore, the increase in intracellular calcium in the responding cells was variable, some cells exhibiting oscillations in intracellular calcium (Fig. 4D) while others did not.

4. Discussion

We here show that C3, a synthetic peptide ligand of the NCAM IgI domain induces an increase in intracellular calcium in primary hippocampal neurons and PC12-E2 cells. The findings demonstrate at the level of individual cells that NCAM ligands directly can induce an increase in the average intracellular concentration of calcium in accordance with pre-

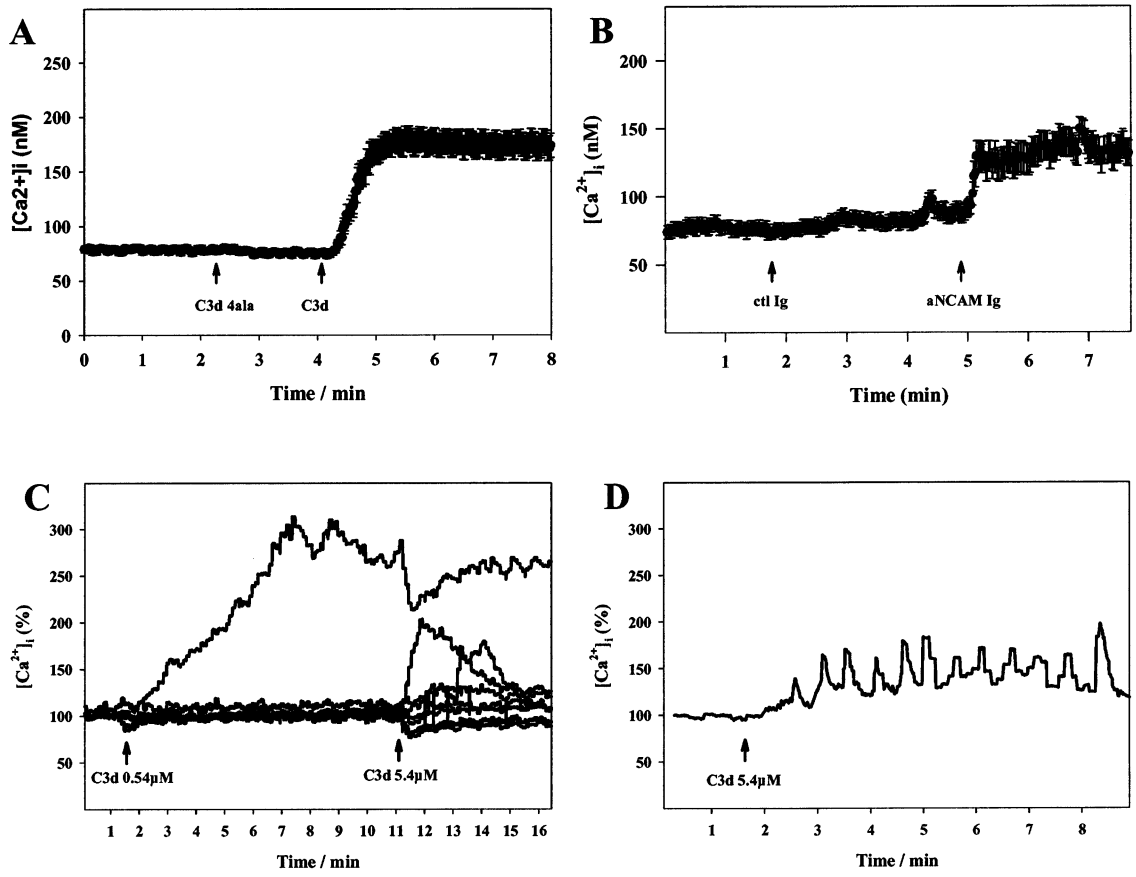

Fig. 4. Effect of C3d on intracellular calcium in primary hippocampal neurons. A: Effect of C3d (54 μM) and a C3-control peptide, C3d 4ala, on intracellular calcium in Fura-2-loaded hippocampal cells after 11 days in culture. B: Effect of Igs directed against NCAM (aNCAM Ig, 1 mg/ml) and Igs from non-immune serum (ctl Ig) on intracellular calcium in hippocampal cells after 15 days in culture. Datapoints represent means ± S.E.M. of 40 (A) or 24 (B) cells. C: Effect of C3d (0.54 or 5.4 μM) on intracellular calcium in hippocampal cells after 3 days in culture. Each trace represents one individual cell. D: Oscillations in intracellular calcium induced by C3d (5.4 μM) in hippocampal cell after 3 days in culture.

vious reports of increases in the average intracellular calcium concentration in populations of neuronal cells measured by spectrofluorimetry [23,24,31]. In addition, our data suggest that mobilization of calcium from intracellular stores also contributes to the increase in intracellular calcium induced by NCAM binding, although extracellular calcium is necessary for a pronounced and sustained increase to occur (Fig. 4).

Both in PC12-E2 cells and primary hippocampal neurons the calcium response to C3d appears to be heterogeneous. Interestingly, in some cells the application of C3d induced oscillations in intracellular calcium, but it remains to be determined whether calcium oscillations induced by NCAM-activation are of functional significance. In the hippocampal cell cultures the heterogeneous calcium response may be explained by the heterogeneity in differentiation of the cells. Alternatively, differences in the 'binding status' of the cells, in particular with respect to NCAM may be speculated to be of importance to the C3-induced increase in intracellular calcium. In this respect it is of interest that intracellular calcium has been shown to increase in PC12 cells adhering to other PC12 cells when compared to non-adherent cells [23].

The finding that neurite outgrowth induced by C3d is inhibited by VDCC antagonists and by a presumed inhibitor of mobilization of intracellular calcium, TMB-8, indicates that C3d upon NCAM-binding activates a signalling pathway that leads to an increase in intracellular calcium dependent on both intracellular and extracellular sources. The stimulatory effect of C3d on neurite outgrowth was readily detected when primary hippocampal cells were grown for 24 h. However, when C3d was applied to Fura-2-loaded hippocampal cells grown for 24 h only, few cells responded to low concentrations of C3d with a detectable increase in intracellular calcium. Therefore, it cannot be ruled out that although C3d and other NCAM ligands can induce an increase in intracellular calcium, other mechanisms may be responsible for neurite outgrowth induced by NCAM stimulation. An alternative explanation may be that only small localized calcium fluxes are necessary to promote neurite outgrowth, and that such calcium fluxes are not readily detected by means of the here-employed calcium imaging method. This is in accordance with the hypothesis that relatively low changes in intracellular calcium induce neurite outgrowth whereas more pronounced increases in intracellular calcium do not.

In summary, the present study shows that the synthetic peptide ligand C3 induces an increase in intracellular calcium, which appears to be necessary for NCAM-dependent neurite outgrowth, presumably requiring mobilization of calcium from both intracellular and extracellular calcium stores. Furthermore, the study demonstrates the feasibility of studying

signal transduction induced by NCAM in real time at the single cell level by means of imaging techniques.

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References

[1] Fields, R.D. and Itoh, K. (1996) Trends Neurosci. 19, 473–480.
[2] Rønn, L.C.B., Hartz, B.P. and Bock, E. (1999) Exp. Gerontol. 33, 853–864.
[3] Crossin, K.L. and Krushel, L.A. (2000) Dev. Dyn. 218, 260–279.
[4] Cremer, H., Lange, R., Christoph, A., Plomann, M., Vopper, G., Roes, J., Brown, R., Baldwin, S., Kraemer, P., Scheff, S., Baerhels, D., Rajewsky, K. and Wille, W. (1994) Nature 367, 455–459.
[5] Doyle, E., Nolan, P.M., Bell, R. and Regan, C.M. (1992) J. Neurochem. 59, 1570–1573.
[6] Scholey, A.B., Rose, S.P.R., Zamani, M.R., Bock, E. and Schachner, M. (1993) Neuroscience 55, 499–509.
[7] Rønn, L.C.B., Bock, E., Linnemann, D. and Jahnsen, H. (1995) Brain Res. 677, 145–151.
[8] Luthi, A., Laurent, J.P., Figurov, A., Muller, D. and Schachner, M. (1994) Nature 372, 777–779.
[9] Muller, D., Wang, C., Skibo, G., Toni, N., Cremer, H., Calaora, V., Rougon, G. and Kiss, J.Z. (1996) Neuron 17, 413–422.
[10] Cremer, H., Chazal, G., Carleton, A., Goridis, C., Vincent, J.D. and Lledo, P.M. (1998) Proc. Natl. Acad. Sci. USA 95, 13242–13247.
[11] Kiselyov, V.V., Berezin, V., Maar, T.E., Soroka, V., Edvardsen, K., Schousboe, A. and Bock, E. (1997) J. Biol. Chem. 272, 10125–10134.
[12] Thomsen, N.K., Soroka, V., Jensen, P.H., Berezin, V., Kiselyov, V.V., Bock, E. and Poulsen, F.M. (1996) Nat. Struct. Biol. 3, 581–585.
[13] Jensen, P.H., Soroka, V., Thomsen, N.K., Ralets, I., Berezin, V., Bock, E. and Poulsen, F.M. (1999) Nat. Struct. Biol. 6, 486–493.
[14] Atkins, A.R., Osborne, M.J., Lashuel, H.A., Edelman, G.M., Wright, P.E., Cunningham, B.A. and Dyson, J.H. (1999) FEBS Lett. 451, 162–168.
[15] Kasper, C., Rasmussen, H., Kastrup, J.S., Ikemizu, S., Jones, E.Y., Berezin, V., Bock, E. and Larsen, I.K. (2000) Nat. Struct. Biol. 7, 389–393.
[16] Rao, Y., Zhao, X. and Siu, C.H. (1994) J. Biol. Chem. 269, 27540–27548.
[17] Ranheim, T.S., Edelman, G.M. and Cunningham, B.A. (1996) Proc. Natl. Acad. Sci. USA 93, 4071–4075.
[18] Doherty, P. and Walsh, F.S. (1996) Mol. Cell. Neurosci. 8, 99–111.
[19] Schmid, R.S., Graff, R.D., Schaller, M.D., Chen, S., Schachner, M., Hemperly, J.J. and Maness, P.F. (1999) J. Neurobiol. 38, 542–558.
[20] Kolkova, K., Novitskaya, V., Pedersen, N., Berezin, V. and Bock, E. (2000) J. Neurosci. 20, 2238–2246.
[21] Doherty, P., Ashton, S.V., Moore, S.E. and Walsh, F.S. (1991) Cell 67, 21–33.
[22] Archer, F.R., Doherty, P., Collins, D. and Bolsover, S.R. (1999) Eur. J. Neurosci. 11, 3565–3573.
[23] Schuch, U., Lohse, M.J. and Schachner, M. (1989) Neuron 3, 13–20.
[24] von Bohlen und Halbach, F., Taylor, J. and Schachner, M. (1992) Eur.J. Neurosci. 4, 896–909.
[25] Rønn, L.C.B., Olsen, M., Østergaard, S., Kiselyov, V.V., Berezin, V., Mortensen, M.T., Lerche, M.H., Jensen, P.H., Soroka, V., Saffell, J.L., Doherty, P., Poulsen, F.M., Bock, E. and Holm, A. (1999) Nat. Biot. 17, 1000–1005.
[26] Wu, Y.Y. and Bradshaw, R.A. (1995) J. Cell. Physiol. 164, 522–532.
[27] Maar, T.E., Rønn, L.C.B., Bock, E., Berezin, V., Moran, J., Pasantes-Morales, H. and Schousboe, A. (1997) J. Neurosci. Res. 47, 163–172.
[28] Rønn, L.C.B., Ralets, I., Hartz, B., Berezin, V., Beck, M., Berezin, A., Møller, A. and Bock, E. (2000) J. Neurosci. Methods 100, 25–32.
[29] Groden, D.L., Guan, Z. and Stokes, B.T. (1991) Cell. Calcium 12, 279–287.
[30] Malagodi, M.H. and Chiou, C.Y. (1974) Pharmacology 12, 20–31.
[31] Frei, T., von Bohlen und Halbach, F., Wille, W. and Schachner, M. (1992) J. Cell Biol. 118, 177–194.