Inhibition of Neutrophil Chemokinesis on Vitronectin by Inhibitors of Calcineurin

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Migration of human polymorphonuclear neutrophils on vitronectin is dependent on repeated transient increases in the concentration of intracellular free calcium ($[Ca^{2+}]_i$). A specific peptide inhibitor of the Ca^{2+}-calmodulin–dependent phosphatase calcineurin was introduced into the cytoplasm of neutrophils. The peptide inhibited neutrophil migration on vitronectin by interfering with the release of the cells from sites of attachment. A similar reduction in motility on vitronectin occurred when cells were treated with the immunosuppressant FK506, which also inhibits calcineurin when bound to its binding protein, FKBP. These results indicate that a rise in $[Ca^{2+}]_i$ reduces integrin-mediated adhesion to vitronectin by a mechanism that requires calcineurin activity.

Repeated transient increases in $[Ca^{2+}]_i$ occur in motile neutrophils after stimulation with chemoattractants (*1*, *2*). These multiple transient increases in $[Ca^{2+}]_i$ function as a signal to regulate the cycles of detachment necessary for continued migration across adhesive surfaces. Blocking the $[Ca^{2+}]_i$ transients specifically inhibits cellular detachment from certain adhesive substrates (*3*) but does not prevent cell polarization, ruffling, or other processes associated with motility. The inhibition of $[Ca^{2+}]_i$ transients impairs neutrophil motility on fibronectin and vitronectin but not on albumin (*1*, *3*). Neutrophils encounter fibronectin and vitronectin in the connective tissue stroma (*4*) and may also encounter vitronectin at sites of tissue injury (*5*). Neutrophils express several types of integrins on their surfaces (*6*), and some of these may be responsible for the calcium-regulated adhesion to fibronectin and vitronectin (*7*).

Activation of members of the protein kinase C family increases the integrin-mediated adhesion of neutrophils (*8*–*10*), although the molecular basis for this increase in adhesion is unclear. Because kinases can increase adhesion, activation of a phosphatase might provide a mechanism for Ca^{2+}-activated detachment from sites of adhesion. Calcineurins (protein phosphatase 2B) (*11*) are a class of serine and threonine phosphatases found in a variety of cells and tissues that can be membrane-associated and are distinguished from other phosphatases by calcium-calmodulin regulation (*12*–*14*). The binding of calcium-calmodulin to calcineurin apparently activates the enzyme by displacing an autoinhibitory domain (*15*). The loss of either the calmodulin-binding domain or the autoinhibitory domain as a result of in vitro proteolytic cleavage results in an enzyme that is active independently of calcium-calmodulin (*15*). To test the hypothesis that Ca^{2+}-activated phosphatases are important for the release of adherent neutrophils, we have used a peptide inhibitor that resembles the autoinhibitory domain.

A peptide (Cn-412) containing a 25-residue sequence from the autoinhibitory domain of calcineurin is a noncompetitive inhibitor of calcineurin in vitro (*16*, *17*). This peptide inhibitor does not inhibit protein phosphatases 1 (PP1), 2A (PP2A), or calcium-calmodulin–dependent protein kinase II, nor does it interfere with calmodulin binding (*16*). An overlapping peptide, Cn-413, which contains some residues from the NH_2-terminal to Cn-412 but lacks regions of the autoinhibitory domain contained in Cn-412 (*17*), was less potent than Cn-412 in inhibiting calcineurin (Fig. 1B).

An edocytosis–osmotic shock procedure was used to load the peptide into the cytoplasm of neutrophils (*18*, *19*). The cells were expected to tolerate this treatment because hypoosmotic shock is used to lyse contaminating erythrocytes during the neutrophil isolation procedure. To verify that hydrophilic molecules may be introduced into the cytoplasm by this procedure, we determined cellular uptake of membrane-impermeant fluorescent dyes (*19*). After endocytosis, punctate fluorescence was observed, indicating endosomal-lysosomal localization of the dyes. After osmotic shock, punctate fluorescence was reduced, and a diffuse cytoplasmic fluorescence was seen in the cells (Fig. 2A). Overall, about 1 to 5% of the external concentration of a small hydrophilic molecule was delivered to the cytoplasm after endocytosis and osmotic shock (*19*).

Delivery of calcineurin inhibitor Cn-412 to the cytoplasm inhibited neutrophil chemokinesis on vitronectin in response to formylmethionyl-leucyl-phenylalanine (fMLP). The inhibition was dependent on the concentration of the inhibitory peptide present during the endocytic uptake of the peptide (Fig. 2B). Use of the endocytosis–osmotic shock procedure (n = 17 experiments) reduced the percentage of motile cells from 55 ± 11% for untreated control cells to 24 ± 8% for cells exposed to a 100 μM external concentration of Cn-412. The effect of endocytosis–osmotic shock without peptide was assessed in five experiments; 47 ± 10% of the shocked control cells were motile. Peptide Cn-413, which is less potent as a calcineurin inhibitor than Cn-412 (Fig. 1B), is also less potent in blocking neutrophil motility on vitronectin. A higher concentration of Cn-413 was required during the endocytosis–osmotic shock to reduce the number of motile cells (Fig. 2B). The reduction in the percentage of motile cells by calcineurin inhibitors is similar to that seen when $[Ca^{2+}]_i$ transients are buffered by introducing (2-{[2-bis-(carboxymethyl)amino-5-methylphenoxy]methyl}-6-methoxy-8-bis{carboxymethyl}-aminoquinoline (quin 2) into the cytoplasm (*3*). Both treatments also resulted in similar morphology. The cells ruffled, polarized, and extended their leading edge and appeared to be unable to retract their tail (Fig. 3, A to C). The quin 2–buffered cells and the Cn-412 cells were frequently observed to revert to their starting positions and to make repeated ineffective attempts to move.

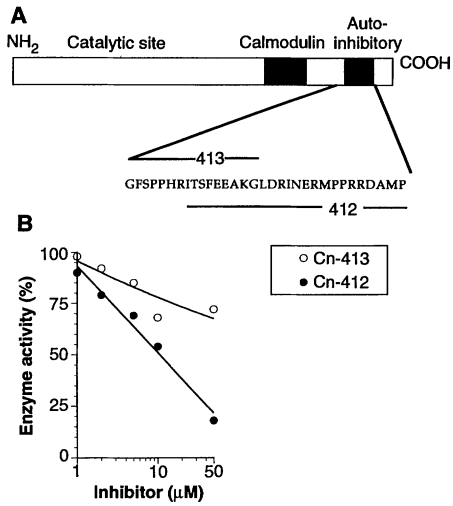

Fig. 1. Schematic diagram of calcineurin A and inhibition of calcineurin by peptide inhibitors. (**A**) Diagram of catalytic, calmodulin-binding (*27*), and inhibitory domains of calcineurin subunit A. Inhibitory peptides Cn-412 and Cn-413 are derived from the peptide sequences in the indicated areas of calcineurin A. (**B**) Effect of Cn-412 and Cn-413 on the in vitro enzyme activity of calcineurin A. Calcineurin was isolated from bovine brain, and enzyme activity was measured with 30 nM calcineurin, 100 μM Ca^{2+}, 30 nM calmodulin, and 1 μM substrate peptide (*28*). Data were normalized to the initial rates of the uninhibited enzyme.

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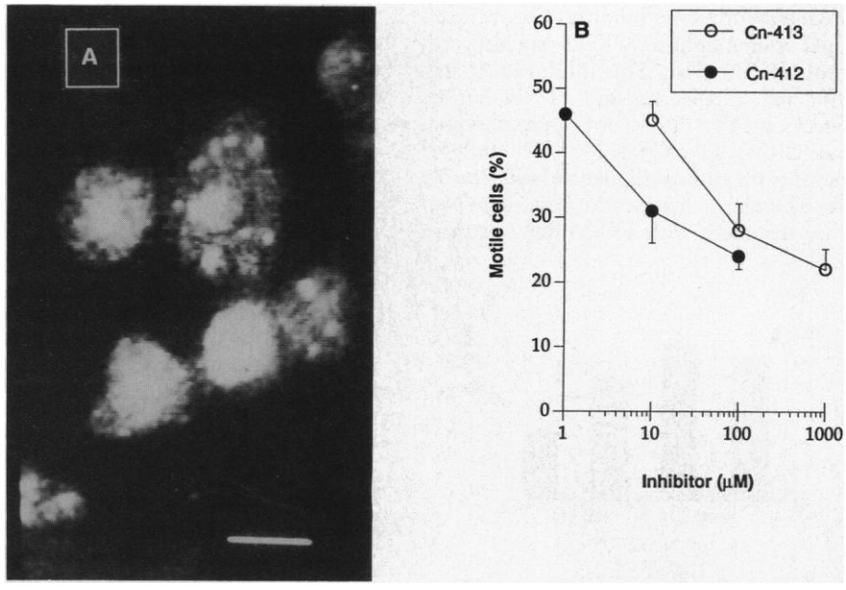

Fig. 2. Cytoplasmic loading of cells with FITC-dextran and inhibition of fMLP-induced neutrophil chemokinesis by calcineurin peptide inhibitors. (**A**) Neutrophils were loaded with FITC-dextran (70 kD) and osmotically shocked (*19*). Note large areas of diffuse cytoplasmic fluorescence and punctate vesicular fluorescence. Scale bar, 10 μm. (**B**) Effect of cytoplasmic Cn-412 and Cn-413 on neutrophil motility. The concentration shown is that of the peptide in the external media before the osmotic shock. The percentage of cells that were motile for each treatment is shown (mean ± SEM from 3 to 17 separate experiments). Polymorphonuclear leukocytes (neutrophils) were isolated from whole human blood donated by healthy volunteers by a single-step separation over a Ficoll-hypaque solution. Contaminating erythrocytes were lysed by a 30- to 40-s hypotonic shock (*3*). All experiments were done in incubation buffer [150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 20 mM Hepes (pH 7.4)]. The final cell preparation consisted of more than 97% polymorphonuclear leukocytes (*3*). Peptide inhibitors (Cn-412 and Cn-413) or FITC-dextran were loaded into neutrophils by an osmotic shock procedure (*19*). Neutrophils were maintained in incubation buffer at 20°C to prevent clumping ing. For motility assays, the neutrophils (10^3 to 10^4 cells) were plated onto the cover slip area of the experimental chamber that had been coated with vitronectin (10 μg/ml) (Telios Pharmaceuticals, San Diego, California) (*3*) and maintained at 37°C. The neutrophils were allowed to attach for 5 min and were then rinsed in incubation buffer. After 5 min, the chemoattractant fMLP (10 nM) was added. Cell motility was monitored by the use of a Lietz Divert microscope equipped with Nomarski differential interference contrast (DIC) optics (*3*). A video camera (CCD-72; Dage-MTI Inc., Michigan City, Indiana) and an optical memory disk recorder (Panasonic; Matsushita Electronics Corp., Osaka, Japan) were used to record single frames every 10 s for a period of 200 s for later analysis. Migrating cells were defined as those in which both the leading edge and tail of the cell were observed to move at least 7 μm from their initial position in 200 s. Separate dishes were used for each treatment, and three sequential fields were recorded from each dish. The cell motility (number of motile cells divided by the number of cells observed) was determined for each treatment group.

Treatment with Cn-412 did not reduce the percentage of motile cells on a purified albumin substrate (Fig. 4A), confirming that the effect of calcineurin peptide inhibitors on vitronectin motility was not due to a more general inhibition of motility nor was it due to a toxic effect on the cells. The inhibition of motility caused by Cn-412 was similar to the inhibition of motility caused by buffering $[Ca^{2+}]_i$ transients: both treatments reduced motility on vitronectin, whereas motility on albumin was unaffected (*3*).

Although the inhibition of motility on vitronectin caused by blocking $[Ca^{2+}]_i$ transients was similar to the inhibition of motility caused by inhibiting calcineurin, the treatments produced different results when the substrate was fibronectin. Treatment with Cn-412 did not reduce motility on fibronectin substrates (Fig. 4A), whereas treatments that reduce $[Ca^{2+}]_i$ transients caused an inhibition of motility on both fibronectin and vitronectin (*3*). Thus, inhibition of motility associated with calcineurin inhibitors was more selective than the inhibition caused by the reduction of calcium transients. This suggests that activation of calcineurin by calcium regulates motility on vitronectin substrates, but that motility on fibronectin substrates requires other calcium-dependent processes.

The immunosuppressant FK506 inhib-

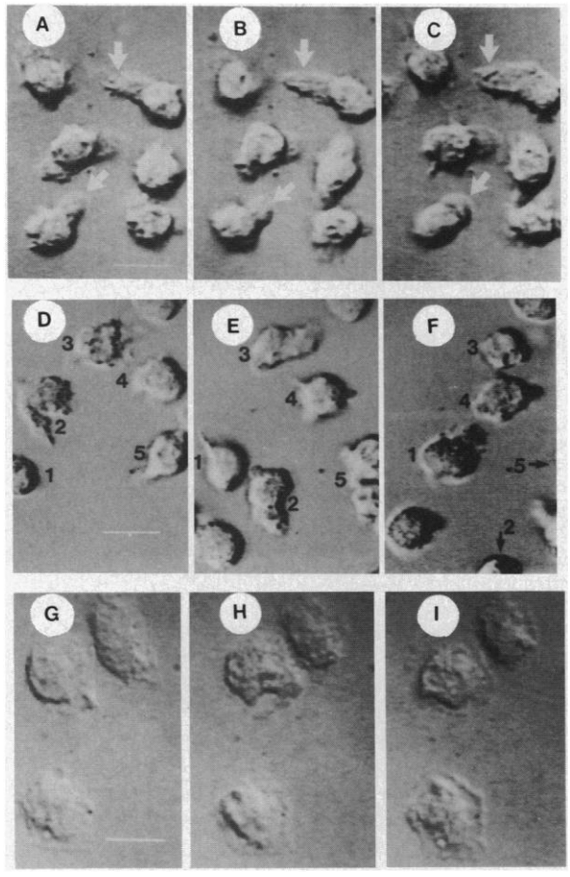

Fig. 3. Effect of phosphatase inhibitors on the morphology and motility of neutrophils on vitronectin. (**A** to **C**) Reduced chemokinetic motility of neutrophils loaded with 100 μM Cn-412. (A) Neutrophils are polarized after stimulation with fMLP (10 nM); arrows indicate attachment sites for two of the cells. After 100 s (B) and 200 s (C), cells remain attached at the same sites. In (**D**) to (**F**), an RGD peptide [GRGDSP (100 μg/ml), Telios Pharmaceuticals, San Diego, California] was added to the medium of Cn-412–loaded cells for 5 min before stimulation with fMLP. Numbers refer to individual cells and allow tracking of the cells from their starting positions (D) to those after 100 s (E) and 200 s (F). Effect of okadaic acid (1 μM) added to the media for 5 min before stimulation with fMLP (**G** to **I**). The polarized morphology is lost and replaced by a flattened symmetrical morphology; no motility is noted after 100 (H) or 200 (I) s. All bars, 10 μm.

its calcineurin after binding to its intracellular immunophilin, FK506-binding protein (FKBP) (*20*). This inhibition is specific for calcineurin, and FK506 has no effect on PP1, PP2A, or protein phosphatase C (*21*). Like Cn-412, FK506 inhibited motility on vitronectin but allowed motility on albumin or fibronectin (Fig. 4B). Neither treatment with FK506 nor treatment with Cn-412 blocked the multiple $[Ca^{2+}]_i$ transients that occurred in fMLP-stimulated neutrophils. The morphology of the FK506-treated cells was similar to that of the Cn-412– or quin 2–treated cells. The dependence of FK506 inhibition on the substrate was most evident during the first 200-s observation period beginning 60 s after stimulation with fMLP. In the next two observation periods, some loss of motility was observed on fibronectin and albumin (*22*). The reduced motility on albumin and variable motility on fibronectin evident in Fig. 4B are the result of averaging the motility observed during the three observation periods. Both the substrate-specific and time-dependent effects of FK506 on motility could be reversed by removing the inhibitor from the incubation media. At higher concentrations, nonspecific effects were more evident, and FK506 inhibited motility independently of substrate. The concentration of FK506 needed to inhibit motility is higher than that required to inhibit T cell activation (*21*). However, the concentration of FK506 required to inhibit calcineurin-dependent cell functions should be dependent on the relative amounts of FKBP and calcineurin in the cells (*21*). Concentrations of FKBP and calcineurin are not known for either neutrophils or lymphocytes, and this makes comparison difficult.

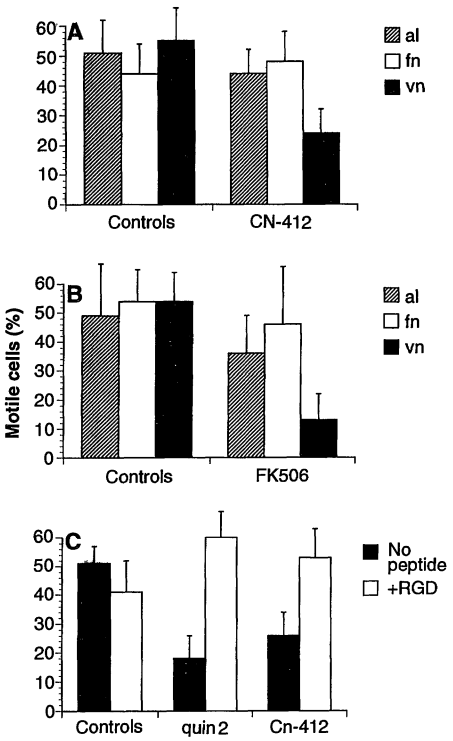

Fig. 4. Inhibition of neutrophil motility on vitronectin by calcineurin inhibitors and restoration of motility by RGD peptides. (**A**) The Cn-412 cells were loaded with Cn-412 (100 μM) in the external media (*19*) and fMLP-stimulated motility was measured as in Fig. 2A. Motility of cells loaded with Cn-412 and of control (no inhibitor) cells was determined in 17 experiments on vitronectin (vn), 7 experiments on fibronectin (fn), and in 6 experiments on albumin (al). (**B**) Motility of cells treated with FK506 and of control cells was compared on vitronectin and fibronectin in eight experiments and on albumin in six experiments. Cells were incubated with FK506 (100 ng/ml) at 20°C for a minimum of 2 hours before the experiment (*29*). (**C**) Reversal of the effect of quin 2 and Cn-412 by an RGD peptide. GRGDSP (100 μg/ml) was added to the medium as in Fig. 3, and motility on vitronectin was measured in 9 experiments with control cells, 4 experiments with quin 2–buffered cells, and 11 experiments with cell-loaded Cn-412. Cells were loaded with quin 2–AM (acetoxymethyl ester of quin 2) (50 μM) for 40 min and allowed to recover for a minimum of 20 min before the start of the experiment (*3*). We made the substrates by coating the experimental chamber with either fibronectin (100 μg/ml), vitronectin (10 μg/ml), or purified albumin (1 mg/ml) (*3*). Data shown are the mean ± SD.

Because the inhibition of neutrophil motility induced by the calcineurin peptide inhibitors is substrate-specific, we attempted to restore that motility by blocking specific attachment sites. Vitronectin receptors are members of the integrin family and recognize a specific region of vitronectin containing an RGD peptide sequence. RGD peptides have been shown to block cellular attachment to vitronectin (*23*). The addition of peptide GRGDSP to the incubation media restored motility to cells loaded with Cn-412 and to cells in which the calcium was buffered with quin 2 (Fig. 4C). Addition of a control peptide, GRADSP (100 μg/ml), did not reduce the quin 2 inhibition; only 20 ± 6% of the quin 2–treated cells were motile in the presence of GRADSP and 18 ± 8% were motile with no peptide present (n = 4 experiments). The peptide GRADSP also failed to reverse Cn-412 inhibition in two control experiments. Thus, by blocking RGD-mediated attachment to the substrate, motility was restored. This indicates that inhibition of calcineurin reduces cell motility by maintaining adhesion to vitronectin by an integrin.

We examined whether other phosphatases could have the same effect on motility and adhesion as calcineurin. Although it is unlikely that the peptide inhibitors would affect other phosphatases, it has been proposed that calcineurin can act by initiating a cascade of phosphatase activity by the activation of PP1 (*24*). Okadaic acid was used because it enters cells and inhibits PP2A at 1 nM and PP1 at 10 nM (*25*). Of neutrophils treated with okadaic acid (100 nM), 44 ± 8% were motile on vitronectin and 53 ± 8% were motile on albumin, whereas 49 ± 8% of untreated control cells were motile on vitronectin and 51 ± 14% were motile on albumin in four matched experiments. These results suggest that PP1 and PP2A do not inhibit motility. We also tested the effect of 1 μM okadaic acid because high concentrations may be needed in some cells to overcome high intracellular concentrations of PP1 and PP2A (*25*). After treatment with 1 μM okadaic acid, the cells became more spread, lost their polarized appearance, and motility was inhibited (Fig. 3, G to I). Unlike the calcineurin peptide inhibition, this inhibition occurred on either albumin or vitronectin. The mechanism by which high concentrations of okadaic acid inhibit motility are not clear. However, this inhibition apparently represents a general interference with the motile apparatus of the cell rather than a specific regulation of substrate detachment.

In preliminary studies, we have found that treatment with Cn-412 does not cause gross changes in protein phosphorylation in intact neutrophils. However, a limited number of proteins are more phosphorylated when Cn-412–treated cells are compared to control cells on two-dimensional gels (not shown). It is not clear what role these proteins may play in regulating detachment from vitronectin or whether the phosphorylation of these proteins is directly affected by calcineurin or by a cascade of calcineurin-regulated phosphoserine (*24*) or phosphotyrosine phosphatases (*26*). A further characterization of the vitronectin receptor in neutrophils is necessary to determine whether $[Ca^{2+}]_i$ and calcineurin directly affect the avidity of the receptor or whether these effects are mediated by receptor-associated processes, such as the disassembly of a receptor cytoskeletal complex or the localization of cell substrate attachment sites.

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17. The sequence of Cn-412 is ITSFEEAKGLDRINER-MPPRRDAMP, encompassing residues 476 to 501 of subunit A-β of calcineurin and is identical to "peptide 3" in (*16*). Cn-413 has the sequence GSFPPHRITSFEEAKG (residues 468 to 484). Abbreviations for the amino acid residues are: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; R, Arg; S, Ser; T, Thr.
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19. After isolation, neutrophils were allowed to take up by endocytosis a cell-impermeable dye [Lucifer yellow (500 μg/ml) or fluorescein isothiocyanate (FITC)–dextran (50 mg/ml, 70 kD)] or calcineurin inhibitor peptides in incubation buffer for 30 to 40 min at 37°C. The neutrophils were then subjected to a 30-s hypoosmotic shock in water, rinsed in phosphate-buffered saline, and resuspended in incubation buffer. After a minimum of 15 min, motility assays were performed on the cells (Fig. 2). For fluorescence microscopy, the cells were washed three times after exposure to the fluorescent dyes, plated on a glass surface, allowed to spread, and examined on a microscope both before and after an osmotic shock. The cytoplasmic localization of the dye was confirmed by adding trypan blue (200 μg/ml) to quench any residual cell surface fluorescence. In addition, cytoplasmic fluorescence was sensitive to a low concentration of digitonin, a treatment that preferentially permeabilizes the plasma membrane [D. J. Yamashiro, S. R. Fluss, F. R. Maxfield, *J. Cell Biol.* **97**, 929 (1983)]. An assessment of cellular loading was also performed with the use of a homogenization procedure [R. R. Ratan, M. L. Shelanski, F. R. Maxfield, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5136 (1986)]. The homogenization studies indicated that the cells had taken up 5 ± 4.1% (n = 4) of the external concentration of the dye. In the cytoplasm, the concentration was equal to 4 ± 3.6% of the external concentration of the dye. The amount of dye found in the vesicular fraction ranged from a negligible amount to one-half the amount found in the cytoplasmic fraction. The variability in the compartmentalization of the dye may reflect differences in the effectiveness of osmotic shock or indicate that the homogenization procedure resulted in the artifactual destruction of endosomes and subsequent overestimation of cytoplasmic concentration. A conservative estimate is that the cytoplasmic concentration of the peptide after endocytosis–osmotic shock was 1% of the extracellular concentration of the peptide.
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Diverse Migratory Pathways in the Developing Cerebral Cortex

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During early development of the mammalian cerebral cortex, young neurons migrate outward from the site of their final mitosis in the ventricular zone into the cortical plate, where they form the adult cortex. Time-lapse confocal microscopy was used to observe directly the dynamic behaviors of migrating cells in living slices of developing cortex. The majority of cells migrated along a radial pathway, consistent with the view that cortical neurons migrate along radial glial fibers. A fraction of cells, however, turned within the intermediate zone and migrated orthogonal to the radial fibers. This orthogonal migration may contribute to the tangential dispersion of clonally related cortical neurons.

Newly generated neurons in developing cerebral cortex migrate outward from the ventricular zone, through a cell-sparse intermediate zone, and into the cortical plate below the pial surface of the brain (*1*). Glial fibers that extend radially from the ventricular to pial surfaces offer a direct pathway for this migration (*2*, *3*). Rakic (*4*) has proposed that cortical neuronal migration is strictly radial, preserving a point-to-point mapping between the ventricular zone and cortical plate. Lineage studies with retroviral markers, however, indicate that clones derived from single progenitors can have a wide tangential dispersion across the cortex (*5*, *6*). One possible explanation for this result is that the pathways for neuronal migration are more complex than the simple routes offered by radial glia.

To examine directly the migratory behavior and pathways of individual cortical cells, we fluorescently labeled cells in living brain slices and observed their movement using time-lapse confocal microscopy (*7*). Brain slices allow such imaging studies yet maintain the complex three-dimensional environment in which neurons normally migrate (*8*, *9*). Coronal slices were prepared from neonatal ferret cortex during upper-layer neurogenesis (*10*) and were maintained in roller tube cultures (*11*). The following day cells were labeled by focal injection of DiI into the ventricular zone (*12*), and fluorescent cells were imaged in the intermediate zone 12 to 48 hours later (*13*). Images were obtained 30 to 60 μm below the slice surface to avoid following cells that had migrated beyond the slice onto the cover slip or into the collagen gel. Single optical sections were collected at 1- to 2-min intervals for up to 45 hours, and the pathways and rates of migration were determined.

Visualization of DiI-labeled cells in the intermediate zone revealed cells with bipolar morphologies, elongated cell bodies, thick leading processes, and thinner trailing processes (Fig. 1A). Leading processes extended complex lamellate and filopodial expansions and frequently branched (Figs. 1, 2, and 3). Trailing processes were simpler but also extended filopodia. These morphological features are similar to those of cells in fixed brains presumed to be migrating neurons (*2*).

Time-lapse sequences revealed the dynamics of migration. Four images of a single cell that exhibited characteristic migratory behavior (Fig. 1) show that both leading and trailing processes rapidly extended and retracted filopodia, behaviors typical of cells exploring their environment (*14*). The trajectory of the leading process anticipated the direction of movement of the cell soma, although the soma often moved at an independent rate. Here, the leading process advanced rapidly with little cell body movement (Fig. 1, B and C); subsequently, the soma lurched forward (Fig. 1D). The cell body changed shape during this movement, as if squeezing past an obstruction in its environment. The behavior of these cells is reminiscent of that observed in co-cultures of dissociated cerebellar neurons migrating on glia (*3*).

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