Cambridge, MA). We determined the specificity by testing the reactivity of purified recombinant murine IL-1β precursor in the ELISA. At concentrations of 20 pg/ml and 10 ng/ml, respectively, mature IL-1β and precursor IL-1β were recognized equally, indicating a cross-reactivity of 0.2%. The IL-1α antibody was from Genzyme (Cambridge, MA), and the TNF-α and IL-6 antibodies were from Biosource International (Camarillo, CA).
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Requirement for Phosphatidylinositol-3 Kinase in the Prevention of Apoptosis by Nerve Growth Factor

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Nerve growth factor (NGF) induces both differentiation and survival of neurons by binding to the Trk receptor protein tyrosine kinase. Although Ras is required for differentiation, it was not required for NGF-mediated survival of rat pheochromocytoma PC-12 cells in serum-free medium. However, the ability of NGF to prevent apoptosis (programmed cell death) was inhibited by wortmannin or LY294002, two specific inhibitors of phosphatidylinositol (PI)-3 kinase. Moreover, platelet-derived growth factor (PDGF) prevented apoptosis of PC-12 cells expressing the wild-type PDGF receptor, but not of cells expressing a mutant receptor that failed to activate PI-3 kinase. Cell survival thus appears to be mediated by a PI-3 kinase signaling pathway distinct from the pathway that mediates differentiation.

Apoptosis plays a key role in the normal development of the nervous system. Up to 50% of many types of developing neurons are eliminated by apoptosis (*1*). The survival of developing neurons is dependent on specific neurotrophic factors secreted by their target cells. NGF is the prototype of this family of neurotrophins, which also includes brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4 (*2*). NGF induces both cell differentiation and survival by binding to the Trk receptor protein tyrosine kinase (*3*). In rat pheochromocytoma PC-12 cells, neurite outgrowth is induced by activation of a signaling pathway that includes the Ras guanine nucleotide–binding protein and the protein kinases, Raf and mitogen-activated protein (MAP) kinase (*4*, *5*). NGF also prevents apoptosis of PC-12 cells in serum-free medium (*6*), but the signaling pathway that promotes cell survival has not been identified. Here, we report that prevention of apoptosis by NGF is independent of Ras but requires the activity of PI-3 kinase.

We first investigated whether prevention of apoptosis by NGF was dependent on Ras signaling. PC-12 cells in serum-free medium underwent apoptosis (*6*), as indicated by characteristic internucleosomal DNA fragmentation, starting at 3 hours and continuing for 24 hours after serum deprivation. Apoptosis was prevented by NGF, epidermal growth factor (EGF), or insulin, all of which activate receptors that are protein tyrosine kinases (Fig. 1). To determine whether Ras was required for the inhibition of apoptosis, we used a PC-12 cell line (M-M17-26) that expresses the dominant inhibitory mutant RasN17, which interferes with normal Ras function (*7*). RasN17 expression in this cell line effectively inhibits Raf activation and neuronal differentiation in response to NGF (*5*). Activation of MAP kinase was similarly inhibited by RasN17 expression (Fig. 1A). In contrast, the expression of RasN17 in these cells did not interfere with the ability of NGF, EGF, or insulin to prevent apoptosis (Fig. 1B). Similar results were obtained with two additional subclones of PC-12 cells that expressed RasN17. It therefore appears that cell survival, but not cell differentiation, is mediated by a Ras-independent signaling pathway. Ras is similarly not required for NGF-mediated survival of sympathetic neurons (*8*).

In addition to Ras, the signaling molecules activated by protein tyrosine kinases include phospholipase C-γ (PLC-γ) and PI-3 kinase (*9*). PLC-γ catalyzes the hydrolysis of PI-4,5-bisphosphate (PIP_2), which yields diacylglycerol and inositol triphosphate and results in the activation of protein kinase C. Because direct activation of protein kinase C by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) failed to inhibit apoptosis (*6*) (Fig. 1), participation of PLC-γ in a signaling pathway that promotes cell survival seems unlikely. On the other hand, phosphorylation of PIP_2 by PI-3 kinase yields PI-3,4,5-trisphosphate (PIP_3), the function of which has not been established (*10*). PI-3 kinase is activated by various receptor protein tyrosine kinases, including Trk (*11*). Although Ras may contribute to its activation, PI-3 kinase also appears to be activated by Ras-independent pathways (*12*). We therefore investigated the possible role of PI-3 kinase in signaling pathways that promote cell survival.

Wortmannin inhibits PI-3 kinase both in vitro and in vivo (*13*). Addition of wortmannin to cells maintained in NGF induced a pattern of DNA fragmentation characteristic of apoptosis within 3 hours (Fig. 2A); this time course is similar to that of apoptosis induced by serum deprivation (*6*). The intensity of DNA fragmentation

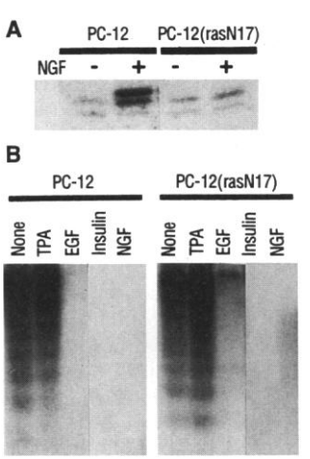

Fig. 1. Inhibition of apoptosis by growth factors is independent of Ras function. (**A**) Inhibition of MAP kinase activation by RasN17 expression. Normal PC-12 cells or M-M17-26 cells expressing the dominant inhibitory RasN17 mutant [PC-12 (rasN17)] (*5*) were incubated without (−) or with (+) NGF (100 ng/ml) for 5 min. Total cell lysates (20 μg of protein) were separated by SDS–polyacrylamide gel electrophoresis, and MAP kinase activity was determined by an in-gel assay using myelin basic protein as the substrate (*27*). (**B**) Inhibition of apoptosis by growth factors. Cells were plated on 100-mm culture dishes (2×10^6 cells per dish) in DMEM supplemented with fetal bovine serum (5%) and horse serum (10%). After 3 days, cells were washed five times with serum-free DMEM and were cultured in the presence of no additives (None), TPA (200 nM), EGF (100 ng/ml), insulin (5 μM), or NGF (100 ng/ml). After 24 hours, the cells attached to the culture dish and those suspended in the medium were collected by centrifugation. Soluble cytoplasmic DNAs were extracted (*28*), separated by electrophoresis in 1.8% agarose gels, blotted onto GeneScreen Plus membrane (NEN-DuPont, Boston, Massachusetts), and hybridized with ^{32}P-labeled, Eco RI–digested rat genomic DNA as a probe.

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induced by wortmannin declined with time, presumably because of the instability of wortmannin in culture medium (*14*).

To confirm the induction of apoptosis by wortmannin, we used an in situ terminal deoxynucleotidyltransferase assay to detect DNA fragmentation in individual cells (*15*). This analysis indicated that about 30% of wortmannin-treated cells underwent DNA fragmentation. Consistent with this finding, trypan blue staining indicated a 30 to 40% reduction in the number of viable cells 6 hours after wortmannin treatment. In contrast, the viability of control cells remained constant for at least 3 days in the presence of NGF. The induction of apoptosis by wortmannin was further confirmed by the observation of chromatin condensation in cells stained with propidium iodide.

The concentration of wortmannin required to induce apoptosis was then compared with that needed to inhibit PI-3 kinase, both in vitro and in vivo. At a wortmannin concentration of 10 nM (10^{-8} M), the activity of PI-3 kinase immunoprecipitated with antibody to its 85-kD subunit (p85) was inhibited by ~60%, and it was almost completely inhibited (~95%) at a wortmannin concentration of 100 nM (Fig. 2B) (*13*, *14*). The in vivo activity of NGF-stimulated PI-3 kinase, as assayed by intracellular production of PIP_3, was similarly inhibited at wortmannin concentrations of 10 to 100 nM (Fig. 2B). DNA fragmentation of cells maintained in NGF was induced by wortmannin at concentrations as low as 10 nM, and this fragmentation increased at a concentration of 100 nM (Fig. 2C). Similar concentrations of wortmannin also induced apoptosis of cells maintained in EGF or insulin (Fig. 2C). The concentration of wortmannin required to induce apoptosis of cells maintained in NGF or other growth factors thus correlated closely with that required for inhibition of PI-3 kinase.

Wortmannin also induced apoptosis of PC-12 cells maintained in serum, although this effect required concentrations of wortmannin that were about 10 times those required for the growth factors (Fig. 2C). This difference might reflect serum stimulation of a distinct species of PI-3 kinase, activated by heterotrimeric guanine nucleotide binding protein (G protein)–coupled receptors, that is less sensitive to wortmannin than is the p85-containing PI-3 kinase activated by tyrosine kinases (*16*). Consistent with the possibility that the survival of PC-12 cells in serum requires PI-3 kinase, we observed that the activity of PI-3 kinase immunoprecipitated from cell extracts with antibody to phosphotyrosine decreased within 5 min of serum withdrawal to one-third to one-fourth that of cells maintained in serum. In contrast, addition of NGF after serum withdrawal resulted in an initial increase in PI-3 kinase activity, which then returned to that observed in cells maintained in serum. The comparable activities of PI-3 kinase in cells maintained in serum and in NGF imply that PI-3 kinase may participate in cell survival signaling pathways that are mediated by serum as well as by NGF. However, because serum is a complex mixture of factors, it may also mediate cell survival through signaling pathways that are independent of PI-3 kinase.

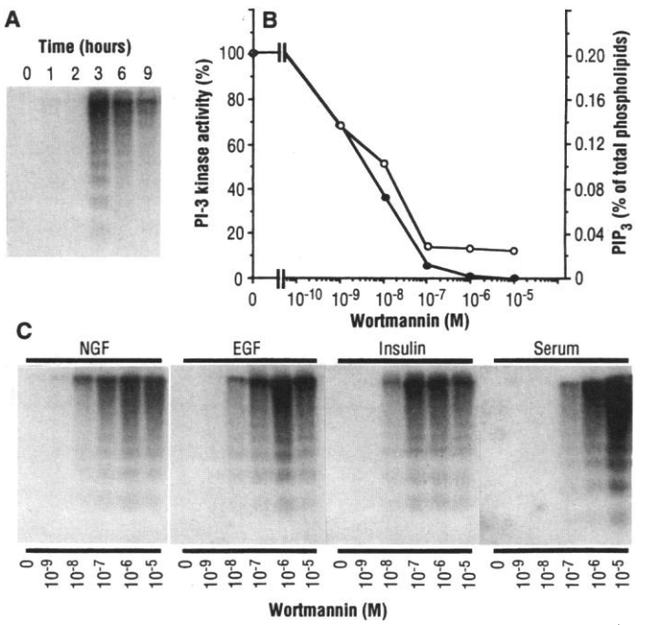

Fig. 2. Induction of apoptosis of PC-12 cells by wortmannin. (**A**) Kinetics of DNA fragmentation. PC-12 cells were cultured as described in Fig. 1 and were maintained in serum-free DMEM supplemented with NGF for 24 hours. Wortmannin (Sigma) was then added to a final concentration of 200 nM, and cells were incubated for the indicated times before analysis of DNA fragmentation. (**B**) Inhibition of PI-3 kinase. For in vitro assays, PI-3 kinase was immunoprecipitated from PC-12 cells with antibody to its p85 subunit (UBI, Lake Placid, New York) and was assayed for activity (*13*) in the presence of the indicated concentrations of wortmannin. Results are presented as percent activity (●) compared with a control sample without wortmannin. To assay intracellular production of PIP_3, 60-mm dishes of PC-12 cells were labeled with [^{32}P]orthophosphate (100 μCi/ml) for 2 hours and were then incubated with the indicated concentrations of wortmannin for 30 min. Cells were stimulated by NGF (100 ng/ml) for 5 min; lipids were extracted and analyzed by thin-layer chromatography (TLC) (*14*) on oxalated silica gel TLC plates (mean pore diameter 60 Å) with a solvent consisting of 40 parts $CHCl_3$, 13 parts methanol, 15 parts acetone, 12 parts acetic acid, and 8 parts water. Spots were identified with standards synthesized by PI-3 kinase in vitro, and radioactivity was quantitated on a Phosphorimager. Results are presented as the percentage of lipid corresponding to PIP_3 (○), which was 0.06% in unstimulated cells. (**C**) Induction of apoptosis by various concentrations of wortmannin. PC-12 cells were cultured as described in Fig. 1 and were incubated in DMEM containing NGF, EGF, insulin, or serum (5% fetal bovine serum and 10% horse serum) for 24 hours. Wortmannin was then added to the indicated concentrations, and DNA fragmentation was assayed after 3 hours of incubation.

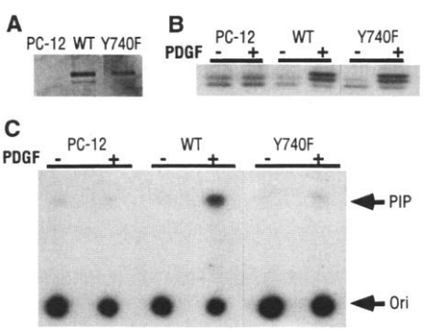

Fig. 3. Characterization of PC-12 cells expressing PDGF receptors. Normal PC-12 cells (PC-12) and PC-12 cells expressing the wild-type PDGF receptor (WT) or the Y740F mutant PDGF receptor (Y740F) were cultured in DMEM supplemented with plasma-derived horse serum (5%) and plasma-derived newborn calf serum (5%). (**A**) Immunoblot analysis. Total cell proteins (20 μg) were immunoblotted with antibody to the PDGF receptor (UBI). All lanes shown are from the same immunoblot. (**B**) Induction of MAP kinase. Cells were incubated without (−) or with (+) PDGF (50 ng/ml) for 7 min. MAP kinase activity was determined as described in Fig. 1. (**C**) Induction of PI-3 kinase. Cells were treated as above, and PI-3 kinase was immunoprecipitated with antibody to phosphotyrosine (UBI) and assayed as described in Fig. 2B. An autoradiogram of the TLC plate is shown; arrows indicate the positions of radioactivity corresponding to PI-3-monophosphate (PIP) and the origin (Ori).

The ability of wortmannin to induce apoptosis was confirmed by experiments with another PI-3 kinase inhibitor, LY294002 (*17*). LY294002 induced apoptosis of PC-12 cells maintained in NGF within 3 hours at concentrations of 10 to 20 μM; this result is consistent with its potency as a PI-3 kinase inhibitor (*17*).

We further evaluated the role of PI-3 kinase by studying PC-12 cell lines into which genes encoding either the wild-type platelet-derived growth factor (PDGF) receptor or the Y740F mutant PDGF receptor

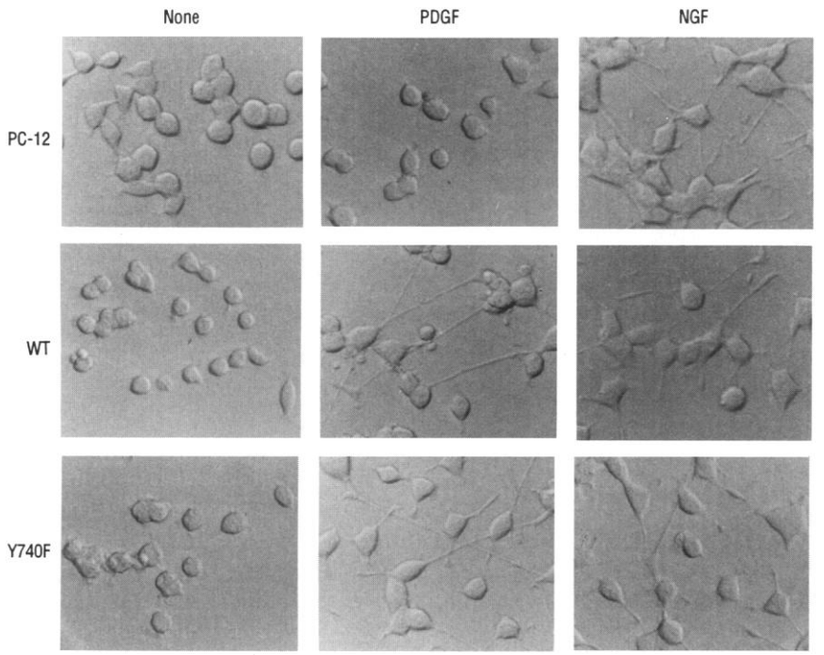

Fig. 4. Differentiation of normal PC-12 cells and PC-12 cells expressing wild-type or Y740F mutant PDGF receptors. Cells were plated on 24-well dishes coated with poly-L-lysine (1000 cells per well) in serum-containing medium and were incubated for 24 hours. The medium was then replaced by DMEM supplemented with fetal bovine serum (0.5%) and containing no additives (None), PDGF (50 ng/ml), or NGF (100 ng/ml). Cells were photographed 3 days after addition of growth factors.

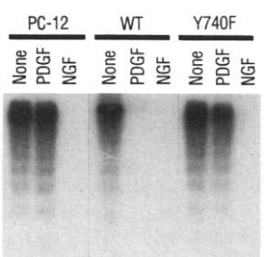

Fig. 5. Prevention of apoptosis by PDGF. Normal PC-12 cells and PC-12 cells expressing wild-type or Y740F mutant PDGF receptors were incubated in serum-free DMEM containing no additives (None), PDGF (50 ng/ml), or NGF (100 ng/ml) for 16 hours. DNA fragmentation was analyzed as described in Fig. 1.

(which lacks a PI-3 kinase binding site) had been introduced (*18*). PI-3 kinase binds to phosphotyrosine residues 740 and 751 of the PDGF receptor (*19*). Because Tyr^{751} also serves as the binding site of the Nck adapter protein (*20*), we selected the Y740F mutant in which Tyr^{740} is substituted by Phe; this substitution substantially reduces the receptor association of PI-3 kinase after PDGF stimulation (*19*). The amount of PDGF receptor expression in PC-12 cells expressing the Y740F mutant receptor was about half that in cells expressing the wild-type receptor (Fig. 3A), but PDGF treatment of cells expressing either the wild-type receptor or the Y740F mutant receptor stimulated the activity of MAP kinase to similar extents—that is, about five times that observed in untreated cells (Fig. 3B). In contrast, PI-3 kinase activity was stimulated about 10 times in PDGF-treated cells expressing the wild-type receptor, but only about 2 times in PDGF-treated cells expressing the Y740F mutant receptor (Fig. 3C). The Y740F mutation thus substantially inhibited receptor association of PI-3 kinase without affecting activation of the Ras-Raf-MAP kinase pathway.

PDGF was found to induce neurite outgrowth in PC-12 cells expressing either the wild-type receptor (*21*) or the Y740F mutant receptor (Fig. 4). This observation is consistent with the activation of the MAP kinase pathway in these cells. Thus, PI-3 kinase appears not to be required for induction of differentiation by NGF; this result is in agreement with previous studies that used PDGF receptor–NGF receptor chimeras (*22*). Transient inhibition of PI-3 kinase with wortmannin at the time of NGF stimulation similarly does not affect differentiation of PC-12 cells, but chronic treatment with wortmannin inhibits the late phase of neurite extension (*14*). The difference between this observation and the results obtained with receptor mutants (*22*) (Fig. 4) may be because the mutants reduce but do not abolish PI-3 kinase activity.

In contrast to its lack of effect on differentiation, the Y740F mutation completely suppressed the prevention of apoptosis by PDGF (Fig. 5). In PC-12 cells expressing the wild-type receptor, PDGF was as effective as NGF in preventing apoptosis induced by serum deprivation. However, PDGF did not prevent apoptosis of PC-12 cells expressing the Y740F mutant receptor. Activation of PI-3 kinase was thus required for the prevention of apoptosis by PDGF; this finding supports the conclusion that induction of apoptosis by wortmannin and LY294002 results from the inhibition of PI-3 kinase.

These results indicate that distinct signaling pathways activated downstream of protein tyrosine kinases are responsible for the induction of differentiation and the prevention of apoptosis by NGF and other growth factors. Differentiation is induced by the Ras-Raf-MAP kinase pathway (*4, 5*) and does not require PI-3 kinase (*22*) (Fig. 4). In contrast, cell survival is dependent on the activation of PI-3 kinase and does not require Ras.

The activation of PI-3 kinase results in the production of PIP_3, which has been reported to activate the ζ isotype of protein kinase C (*23*) and to lead to stimulation of the 70-kD S6 kinase (*24*). PI-3 kinase has been implicated in a variety of cellular responses to growth factors, including mitogenesis, insulin stimulation of glucose transport, and membrane trafficking (*13, 25*). Our results indicate that prevention of apoptosis is at least one important function of the PI-3 kinase pathway in PC-12 cells. Because wortmannin also induces apoptosis of Rat-1 fibroblasts (*26*), the role of PI-3 kinase in signaling pathways that promote cell survival may not be restricted to neuronal cells and may extend to other cell types.

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High-Frequency Motility of Outer Hair Cells and the Cochlear Amplifier

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Outer hair cells undergo somatic elongation-contraction cycles in vitro when electrically stimulated. This "electromotile" response is assumed to underlie the high sensitivity and frequency selectivity of amplification in the mammalian cochlea. This process, presumably operating on a cycle-by-cycle basis at the frequency of the stimulus, is believed to provide mechanical feedback in vivo. However, if driven by the receptor potential of the cell, the mechanical feedback is expected to be severely attenuated at high frequencies because of electrical low-pass filtering by the outer hair cell basolateral membrane. It is proposed that electromotility at high frequencies is driven instead by extracellular potential gradients across the hair cell, and it is shown that this driving voltage is not subject to low-pass filtering and is sufficiently large. It is further shown that if the filtering properties of the cell membrane are canceled, taking advantage of the electrical characteristics of isolated outer hair cells in a partitioning glass microchamber, then the lower bound of the motor's bandwidth is approximately 22 kilohertz, a number determined only by the limitations of our instrumentation.

It is a common assumption that mammalian hearing owes its remarkable sensitivity and frequency-resolving capabilities to a local mechanical feedback process within the cochlea [for review, see (*1*)]. This process has been termed the "cochlear amplifier" (*2*) and it is assumed that its motor arm is a somatic shape change of outer hair cells (OHCs) (*3*). Such shape changes can be elicited by a natural stimulus to the cell [that is, stereociliary deflection (*4*)] and therefore can be driven in vivo. In order to be an effective form of mechanical feedback, somatic length changes are thought to occur on a cycle-by-cycle basis, counteracting the mechanical damping of the cochlear partition. Inasmuch as the high-frequency boundary of mammalian hearing exceeds 20 kHz in most species and 100 kHz in some, somatic length changes need to be made at remarkable speeds. It is known that distortion product otoacoustic emissions, assumed to be related to OHC motility in mammals, occur up to at least 100 kHz (*5*).

Because the receptor potential is severely attenuated at high frequencies due to the resistance-capacitance (RC) filtering of the cell membrane (*6*), it is difficult to understand how it may drive the cell's own motility except at relatively low frequencies in the apex of the cochlea, where OHC receptor potentials are sufficient to produce large electromotile responses. It is estimated that OHC motility in the 16- to 18-kHz range, where measurements are available, is about 20% of basilar membrane motion (*7*). It thus appears that the generally accepted model of cochlear amplification becomes questionable as the intracellular response becomes too small to drive motility at high frequencies where OHC motility presumably occurs in many small mammals. We propose that the effective electrical stimulus that powers the OHC motile response at high frequencies is not the cell's own receptor potential, but an extracellular voltage gradient, established across the hair cell, between the scala media and intra–organ of Corti fluid spaces. These gross potentials reflect voltage drops on the bulk impedance of the organ of Corti that are due to the weighted vector sum of receptor currents produced by all sound-stimulated OHCs.

For consideration of such a proposition, three questions need to be examined. First, why would not these extracellular voltages be subject to filtering limitations similar to those on the intracellular receptor potential? Second, is the extracellular potential larger than the intracellular one at high frequencies? Third, are the OHC motors intrinsically capable of responding in the high kilohertz range? The last issue has its own biological significance, because in order to understand the nature of the aggregate of molecular motors (*3*, *8*) that powers motility, it is of interest to determine its inherent speed. At this time, due to experimental limitations, quantitative data on the speed of motility have been reported only up to approximately 1 kHz (*6*).

Outer hair cells are partitioned in vivo between two fluid compartments: endolymph of the scala media and perilymph within the organ of Corti (Fig. 1A). OHCs dominate the production of extracellular stimulus-related potentials, which are voltage drops due to the receptor current on the various bulk impedances of cochlear tissues. Although the electrical circuit of the cochlea is complex, one can estimate (*9*) that, owing to the small shunt resistance, the total impedance between scala media and organ of Corti fluid space has a flat magnitude between ~6 and ~300 kHz. It is also known that extracellular ac electrical responses can be measured up to at least 100 kHz (*10*). Thus, extracellular voltage gradients are not significantly filtered.

The microchamber technique (*11*) simulates the electrical partitioning that exists in epithelia, such as the organ of Corti (Fig.

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