the availability of putative human stem cell assays (*29*) provide an important framework to begin studies on gene transfer into human stem cells. Additional improvements in gene transfer may be realized through the use of higher titer amphotropic viruses or the development of techniques to enrich for transduced LAD stem cells such as those based on cell surface expression of CD11 and CD18.

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Cell Cycle Dependence of Chloride Permeability in Normal and Cystic Fibrosis Lymphocytes

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Cystic fibrosis (CF) is a genetic disease characterized by abnormal regulation of epithelial cell chloride channels. Nonepithelial cells, including lymphocytes and fibroblasts, may exhibit a similar defect. Two independent techniques were used to assess the macroscopic chloride permeability (P_{Cl}) of freshly isolated B lymphocytes and of B and T lymphocyte cell lines. Values for P_{Cl} increased specifically during the G_1 phase of the cell cycle and could be further enhanced by increasing intracellular adenosine 3′,5′-monophosphate (cAMP) or calcium. In lymphocytes from CF patients, regulation of P_{Cl} during the cell cycle and by second messengers was absent. Characterization of the cell cycle–dependent expression of the chloride permeability defect in lymphocytes from CF patients increases the utility of these cells in the analysis of the functional consequences of mutations in the CF gene.

MANY OF THE SALT TRANSPORT ABnormalities of cells from patients with CF are due to impaired second messenger activation of Cl^- permeability (P_{Cl}) (*1*). Patch-clamp studies of airway cells reveal a defect in the activation of an outwardly rectified Cl^- channel, both in response to agonists that act by means of cAMP as measured during cell-attached recording and in excised, inside-out membrane patches exposed to cAMP-dependent protein kinase. In single-channel studies, a similar Cl^- channel was identified in B and T lymphocyte cell lines that was also defective in its activation (*2*). However, these results were difficult to confirm, either because of low channel density (*2*) or failures in channel activation by agonists (*3*). To avoid the sampling problems inherent in single-channel measurements, we used two independent techniques to assess the macroscopic Cl^- permeabilities in normal lymphocytes and those from CF patients: fluorescence digital imaging microscopy (FDIM)

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and whole-cell patch-clamp. FDIM, performed with the halide-sensitive fluorophore 6-methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ), permitted assessment of P_{Cl} in single cells (*4*). Whole-cell patch-clamp was then used to define the contribution of Cl^- conductance to P_{Cl} and resolve detailed conductance properties.

The Cl^- permeabilities of individual cells from T (Jurkat) and B lymphocyte cell lines were heterogeneous (Fig. 1, A and B). The fluorescence intensity of some cells decreased rapidly when extracellular Cl^- was replaced by Br^-, indicating a high P_{Cl}, whereas the intensity in other cells decreased slowly, indicating a low P_{Cl}. The fluorescence quench rates calculated for individual cells fell into two subsets and indicated that the P_{Cl} of one subset was about four times as great as the other (Fig. 1C). An uncontrolled variable in these initial studies was cell cycle phase. Cytosolic Ca^{2+} (*5*) and cation channel activity (*6*) vary with the cell cycle in lymphocytes. We therefore examined P_{Cl} at specific cell cycle phases.

Resting B lymphocytes were isolated from peripheral venous blood samples of normal individuals (*7*) and mitogenically activated with mouse monoclonal antibodies to the F_c fragment of human immunoglobulin M (IgM) (25 μg/ml) (*8*) and with B cell growth factor (from T lymphocyte–conditioned medium) to produce cells in three different cell cycle phases. Cell cycle synchronization eliminated the heterogeneity in P_{Cl} characteristic of the unsynchronized populations (Fig. 2A). G_0 and S phase cells had uniformly low P_{Cl}, whereas G_1-S phase cells had uniformly high P_{Cl}. Similar results were obtained in Epstein-Barr virus (EBV)–transformed B cells from normal subjects that were synchronized in G_1-S or S by hydroxyurea treatment (*9*); these are the same cell lines used previously (*2, 3, 10*).

We then examined the Cl^- permeabilities of transformed and untransformed lymphocytes in G_1-S phase from patients with CF. Both freshly isolated B cells and B cell lines were synchronized to G_1-S with hydroxyurea before the P_{Cl} measurements. In contrast to the findings from normal lymphocytes (Fig. 2A), the G_1-S phase CF lymphocytes were essentially impermeable to Cl^- (Fig. 2B).

Results obtained from whole-cell patch-clamp studies indicate that the P_{Cl} differences between normal and CF lymphocytes in G_1-S are due to differences in their Cl^- conductances. Chloride currents were measured in CF and normal B cell lines under voltage-clamp with Cl^- as the principal permeant ion in the pipette and bath solutions. The normal cells in G_1-S show an outwardly rectified current-voltage relation (Fig. 3A); the currents in cells from CF patients were small (less than 100 pA at ±100 mV). Ion replacement studies showed that these currents were carried by Cl^-. In the physiologic voltage range (−40 to −60 mV) the Cl^-

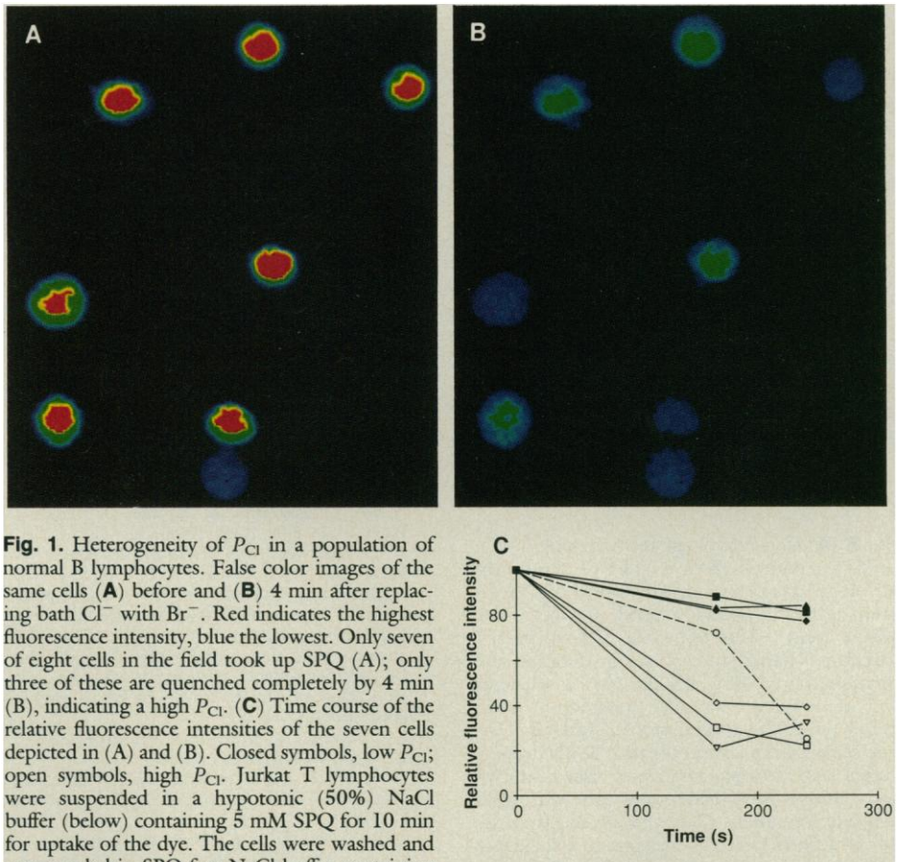

Fig. 1. Heterogeneity of P_{Cl} in a population of normal B lymphocytes. False color images of the same cells (**A**) before and (**B**) 4 min after replacing bath Cl^- with Br^-. Red indicates the highest fluorescence intensity, blue the lowest. Only seven of eight cells in the field took up SPQ (A); only three of these are quenched completely by 4 min (B), indicating a high P_{Cl}. (**C**) Time course of the relative fluorescence intensities of the seven cells depicted in (A) and (B). Closed symbols, low P_{Cl}; open symbols, high P_{Cl}. Jurkat T lymphocytes were suspended in a hypotonic (50%) NaCl buffer (below) containing 5 mM SPQ for 10 min for uptake of the dye. The cells were washed and resuspended in SPQ-free NaCl buffer containing 150 mM NaCl, 1.25 mM K_2SO_4, 1 mM $CaSO_4$, 1 mM $MgSO_4$, 1.25 mM Na_2HPO_4, 1.25 mM NaH_2PO_4, and 10 mM glucose, pH 7.4. Dye-loaded cells were plated on a cover glass in a perfusion chamber (volume 1 ml) on the stage of an inverted microscope fitted for FDIM and maintained at 37°C. Excitation light passed through a 370-nm short-pass filter (SPQ excitation maximum, 350 nm), and emitted light, through a 410-nm long-pass filter. Images were collected with a microchannel plate image intensifier (KS-1380, Videoscope International, Washington, D.C.) coupled to a Newvicon video camera (Dage-MTI, Inc., Michigan City, Indiana). Each image was digitized; the signal was averaged over 32 successive frames, and then it was recorded on videotape. Background-subtracted fluorescence intensity was determined for each cell in the field as the difference between the average intensity of a 5 by 5 pixel array in the center of the cell and the intensity of a similar array off of the cell. We determined P_{Cl} from the initial rate of SPQ fluorescence quench during cellular Cl^- and Br^- exchange induced by replacing perfusate NaCl with NaBr.

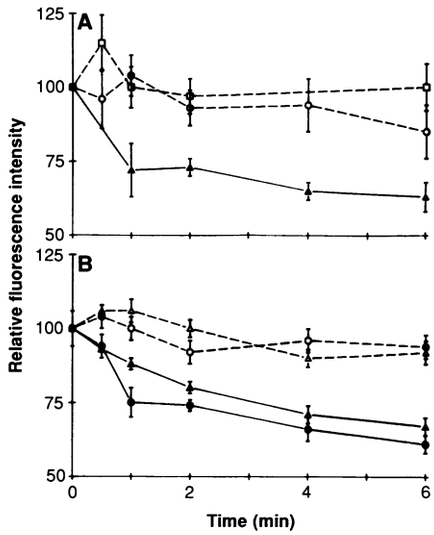

Fig. 2. (**A**) Chloride ion permeability determinations in three cell cycle–synchronized populations of normal B lymphocytes. Resting cells [G_0 (○), n = 7 cells] were isolated from a tonsil (*7*). G_1-S phase cells [(▲) n = 5 cells] were produced by activating resting cells with mouse monoclonal antibodies specific for human IgM (25 μg/ml) and with B cell growth factor in the presence of hydroxyurea (1 mM) for 48 hours. S-phase cells [(□) n = 6 cells] were produced by activating resting cells in the absence of hydroxyurea for 72 hours (the time of peak DNA synthesis). (**B**) P_{Cl} determinations in G_1-S phase transformed and freshly isolated B lymphocytes. Transformed cells were synchronized with 1 mM hydroxyurea. Untransformed lymphocytes were isolated from the peripheral blood of three [(○) CF, n = 5; (△) CF transformed, n = 42] patients with CF and five normal individuals [(●) control, n = 15; (▲) control transformed, n = 23].

currents in normal cells were approximately four times that of their CF counterparts, indicating that the differences in P_{Cl} detected by SPQ fluorescence are due to differences in cellular Cl^- conductances.

Cyclic AMP increases apical membrane Cl^- conductance and elicits Cl^- secretion across normal but not CF epithelial cells (*1*). Calcium stimulates Cl^- secretion in airway and sweat gland of both CF patients and normal subjects (*11*) and in intestine of normal subjects, but not CF patients (*12*). We examined the effects of cAMP and Ca^{2+} on lymphocyte Cl^- conductances with whole-cell patch-clamping. Basal Cl^- currents, elicited by test pulses to ±50 mV, were outwardly rectified (Fig. 3B). Perfusion with chlorophenylthio (cpt)-cAMP (400 μM) doubled the Cl^- currents in normal lymphocytes within 1 min. This effect was transient, reaching a peak between 1 and 2 min, and decreasing to control levels, even in the continued presence of the cAMP analog. Subsequent addition of ionomycin (2 μM) to raise intracellular Ca^{2+} also increased the Cl^- currents in normal lymphocytes, but unlike cpt-cAMP, its effect was sustained. Time-dependent increases in outward Cl^- currents during depolarizing voltage pulses were induced by both second messengers (Fig. 3B, expanded time course). In contrast to the findings in normal lymphocytes, neither cpt-cAMP nor ionomycin increased the whole-cell Cl^- currents of lymphocytes from CF patients (Fig. 3C).

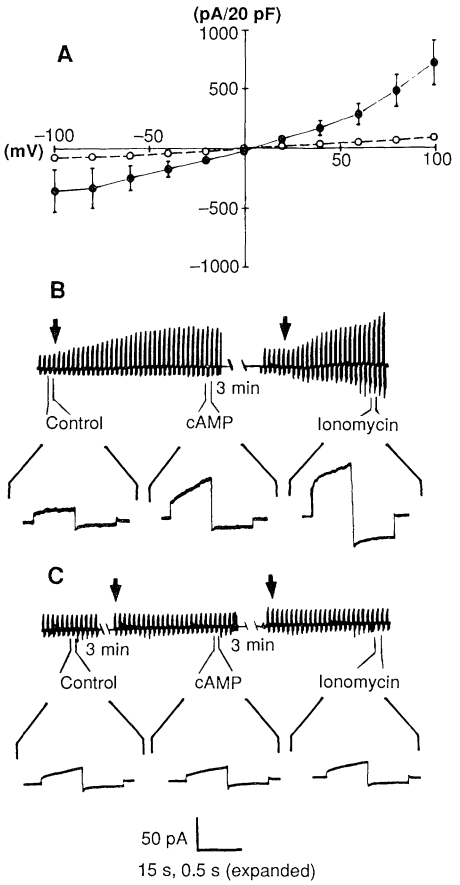

Fig. 3. (**A**) Current-voltage relations of CF [(○) $n = 6$] and normal [(●) $n = 5$] EBV-transformed B lymphocytes determined by whole-cell patch-clamp. Pulse protocols stepped the membrane voltage from −100 to +100 mV in 20-mV increments. Pulses were 50 ms in duration and interrupted by holding at 0 mV for 1 s. Currents were recorded 40 ms after initiation of each voltage pulse. Values are means ± SEM. (**B**) Current record from a transformed B cell from a normal subject; representative of data from five cells. Controls had no additions; cpt-cAMP (400 μM) and ionomycin (2 μM) were added at the indicated times (arrows). Membrane voltage was held at 0 mV and pulsed to ±50 mV for 0.5 s (**C**). Current record from a transformed B cell from a CF patient; representative of data from six cells. Whole-cell currents were recorded with standard techniques (*16*). Pipette solutions: 120 mM *N*-methyl-D-glutamine-chloride, 1 mM EGTA (10 nM free Ca^{2+}), 4 mM adenosine triphosphate, and 5 mM Hepes, pH 7.2; bath: 150 mM *N*-methyl-D-glutamine-chloride, 2 mM $CaCl_2$, 1 mM EGTA (1 mM free Ca^{2+}), and 5 mM Hepes, pH 7.2. The hypotonic pipette solution is required to prevent cell swelling under whole-cell patch-clamp (*17*).

Complementary experiments were performed with FDIM to determine the effects of cpt-cAMP and ionomycin on the Cl^- permeability of G_1-S phase normal lymphocytes (*13*). Ionomycin increased the SPQ quench rate threefold above that in unstimulated cells, confirming the effect of ionomycin on whole-cell Cl^- currents. We did not detect a cpt-cAMP–induced increase in P_{Cl} with FDIM, probably because of the transient nature of the response. In the SPQ protocols, the time required for replacement of Cl^- by Br exceeded the duration of the response to cpt-cAMP detected by whole-cell patch-clamp (*4*).

Our findings offer several explanations for the variations in the properties of lymphocytes from normal controls and CF patients observed in prior studies (*2, 3*). First, a significant Cl^- conductance is spontaneously present only during the G_1 phase of the cell cycle. We do not know if Cl^- channels are present only during G_1-S, or in all cell cycle phases (which would imply that second messengers regulate P_{Cl} during the cell cycle). Second, the increase in P_{Cl} induced by cpt-cAMP is transient. Thus, it is not surprising that investigators using single-channel techniques have had variable success in detecting active Cl^- channels and have been unable to consistently demonstrate cAMP-induced activation of Cl^- channels in cell-attached patches. The macroscopic measures of Cl^- conductance that we have used can more readily detect these transient phenomena.

Like epithelial cells, lymphocytes exhibit a regulated Cl^- conductance that can be augmented by increased intracellular cAMP or Ca^{2+}. The primary physiological regulator of Cl^- conductance during lymphocyte cell cycling is probably intracellular Ca^{2+}. Cytosolic Ca^{2+} is low in G_0 and S and increases threefold in G_1 (*5*). The role of Cl^- conductance changes in lymphocyte activation and cell cycle progression is currently unknown, and it will be interesting to determine whether mitogen-induced lymphocyte activation progresses more slowly in CF cells.

Recently, the CF gene was identified (*14*). The most common mutation, a three-base deletion that removes phenylalanine from position 508 of the amino acid sequence, occurs in about 70% of CF chromosomes; additional mutations account for the remainder of CF cases. Lymphocytes should serve as a valuable system to further define the role of the CF gene product in regulating plasma membrane Cl^- conductance. Standard techniques for transformation and transfection are available (*15*), so that continuous lymphocyte cell lines can be established from individuals of known genotype. Thus, lymphocytes offer the opportunity to examine the functional consequences of various CF mutations, an approach that is impractical with epithelial cells.

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Flunarizine Protects Neurons from Death After Axotomy or NGF Deprivation

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Systemically administered flunarizine enhanced neuronal survival in lumbar sensory ganglia in newborn rats after axotomy. Flunarizine-treated rats lost 71 percent fewer neurons than the untreated control rats at the end of 1 week. In cell culture, flunarizine at 30 to 40 μM also prevented neuronal death in nerve growth factor–dependent embryonic sensory and sympathetic neurons after the abrupt withdrawal of neurotrophic support. The drug may cause this effect by acting at an intracellular site, one distinct from its blockade of voltage-dependent calcium channels.

SENSORY AND SYMPATHETIC NEURONS depend on nerve growth factor (NGF) for maintenance or survival (or both) in vivo and in vitro. Characterization of the relation between these neural crest–derived cells and NGF provides much of the basis for the neurotrophic theory (*1*). Neuronal death is a highly controlled and intricate process that occurs naturally during development in multiple neural groups in the nervous system. Neurotrophic factors are important determinants in this process (*2*). NGF is critical during development for the survival of dorsal root ganglion (DRG) and superior cervical ganglion (SCG) neurons (*3*). Postnatally, sympathetic SCG neurons remain dependent on NGF for survival, whereas sensory DRG neurons remain dependent on NGF only for maintenance of their morphologic features and biochemical properties, but not for survival (*4*). Interruption of the physiologic trophic support between end-organ and neuron can result in neuronal death. After axotomy, death in DRG neurons occurs to varying degrees contingent on several factors, such as the age of the animal, location and type of lesion, and the success of subsequent regeneration (*5*). Exogenous NGF can prevent the neuronal death that occurs in the DRG after axotomy in newborn (*6*) or adult rats (*7*). The mechanism whereby NGF promotes survival in these neurons is not known. The ability of the Ca^{2+} channel blocking agent flunarizine [(*E*)-1-bis-(4-fluorophenyl)methyl-4-(3-phenyl-2-propenyl)piperazine dihydrochloride] to protect neurons from death after withdrawal of trophic support was examined both in vitro and in vivo. Two other Ca^{2+} channel–blocking agents, nimodipine and lidoflazine, were tested in vitro.

Embryonic day 15 (E15) DRG neurons were dissociated and maintained for 10 days in the presence of NGF. The neurons were then acutely deprived of NGF by removal of the trophic factor from the medium and treatment of the cultures with 2% guinea pig antiserum to mouse NGF (*8*). The E21 SCG sympathetic neurons were dissociated and maintained in culture for 7 days, at which time the neurons were deprived of NGF in the same manner as described for the sensory neurons. A dose-response relation for the protective effects of flunarizine after withdrawal of NGF support was determined by adding various concentrations (1 to 80 μM) of the drug to the cell cultures. On each culture plate, both positive (NGF-maintained) and negative (NGF-deprived) control groups of neurons were grown without flunarizine. Neuronal death was determined by histologic examination with phase-contrast microscopy and scanning electron microscopy. Neurons were examined at regular intervals by phase-contrast microscopy for evidence of neuronal death, that is, neuronal atrophy, loss of perikaryal morphology, and presence of pyknotic cells. To further assess neuronal protection, we measured protein synthesis with an [^{35}S]methionine incorporation assay (*8*). In other groups of dissociated neurons after NGF deprivation, the cultures were fixed with glutaraldehyde, and prepared for examination by scanning electron microscopy.

The E15 DRG neurons, maintained in culture in the presence of NGF for 10 days, consistently died 48 to 72 hours after NGF deprivation. However, neurons similarly NGF deprived but treated with flunarizine at concentrations ≥30 μM were protected from death, both morphologically and biochemically. The protection of neurons and neurites is most clearly shown by scanning electron microscopy (Fig. 1). Dose-response studies showed incomplete neuronal protection at lower concentrations of flunarizine

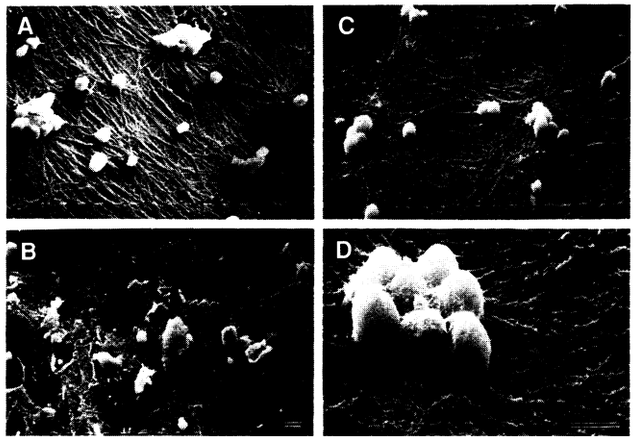

Fig. 1. Representative scanning electron micrographs from cell cultures of DRG neurons. (**A**) DRG neurons maintained in an NGF-containing medium. (**B**) DRG neurons deprived of NGF for 8 hours show loss of neurons and extensive disruption of neuritic network. (**C**) DRG neurons deprived of NGF for 48 hours in the presence of 30 μM flunarizine demonstrate protection of the neurons and their neurites. (**D**) Higher magnification of DRG neurons protected after NGF deprivation with 30 μM flunarizine. Scale bar in (B) and (D), 20 μm.

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