# Voltage-Sensitive Calcium Channels in Normal and Transformed 3T3 Fibroblasts

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**Patch clamp recordings of whole-cell and single channel currents revealed the presence of two voltage-sensitive calcium channel types in the membrane of 3T3 fibroblasts. The two calcium channel types were identified by their unitary properties and pharmacological sensitivities. Both calcium channel types were present in all control 3T3 cells, but one type was selectively suppressed in 3T3 cells that had been transformed by activated c-H-*ras*, EJ-*ras*, v-*fms*, or polyoma middle T oncogenes. The presence of voltage-sensitive calcium channels in these nonexcitable cells and the control of their functional expression by transforming oncogenes raises questions about their role in the control of calcium-sensitive processes such as cell motility, cytoskeletal organization, and cell growth.**

FIBROBLAST CELL LINES ARE COMMONLY used for the study of cell growth, and there is evidence for an important role of intracellular $Ca^{2+}$ in the control of cell growth (*1*). However, little is known about the mechanisms regulating cytoplasmic $Ca^{2+}$ in these cells, particularly because no specific $Ca^{2+}$-transporting pathways have been identified in the membrane of a fibroblast cell.

We used the patch clamp technique (*2*) to obtain direct evidence for the presence of $Ca^{2+}$ channels in fibroblasts. Two types of $Ca^{2+}$ channel currents could be recorded from a 3T3 fibroblast (Fig. 1A). A test depolarization to +20 mV from a holding potential of −30 mV elicited an inward current that activated within 5 to 10 msec and remained well maintained during the depolarization. An additional transient component of inward current was activated at the same test potential when the fibroblast was held at −70 mV before depolarization. This current activated rapidly to a peak and then decayed to zero within about 50 msec (Fig. 1A, bottom trace). The two inward currents differ not only in their kinetics and dependence on the holding potential but also in their sensitivity to drugs. For example, the dihydropyridine (DHP) $Ca^{2+}$ channel agonist (+)202-791 (*3*) selectively increased the maintained current component but failed to affect the transient current (Fig. 1B).

Single channel currents underlying the maintained whole-cell $Ca^{2+}$ channel component are shown in Fig. 2, A and B: at +20 mV, brief openings of ~1.2 pA amplitude occurred in bursts throughout the depolarizing pulse, giving rise to a well-maintained average current. As expected from the whole-cell recordings, (+)202-791 greatly increased the averaged current. The single channel currents in the presence of (+)202-791 demonstrated the mechanism of drug action: the appearance of long-lasting channel openings, which occurred rarely in the absence of drug (Fig. 2A), was greatly enhanced by the DHP $Ca^{2+}$ channel agonist (*4*), thus leading to an overall increase in the probability of channel opening.

The unitary properties of the second $Ca^{2+}$ channel type are shown in Fig. 2C. Here, depolarizations to a more negative test potential (−20 mV) elicited inward currents of ~0.4 pA amplitude which tended to be grouped toward the beginning of the depolarization. The time course of the averaged current corresponded nicely to that of the transient whole-cell component, and, like the whole-cell current, the elementary activity was greatly reduced if the clamp potential was held positive to −60 mV and was insensitive to DHP drugs.

The macroscopic and microscopic kinetics, elementary conductances, and DHP sensitivities of the two $Ca^{2+}$ channel types in 3T3 cells are very similar to the L-type (long-lasting, large conductance) and T-type (transient) $Ca^{2+}$ channels that have been described in greater detail in excitable cells such as sensory neurons (*5*), cardiac muscle (*6*), smooth muscle (*7*), and anterior pituitary cell lines (*8*). We also found several classes of $K^+$ channels, $Cl^-$ channels, and nonselective cation channels in 3T3 fibroblasts. One of these showed measurable permeability to divalent ions but little selectivity for divalent over monovalent ions (*9*).

The density of $Ca^{2+}$ channels in 3T3 fibroblasts is considerably lower than that in heart or neurons but roughly comparable to that in certain vascular smooth muscle cells (*7*). With 20 m*M* $Ba^{2+}$ as the charge carrier, the maximal current (*I*) carried by either channel type in fibroblasts was about equal and averaged 0.5 to 0.6 pA per picofarad of cell capacitance (see Fig. 3). With values of the elementary current (*i*) and the opening probability (*p*) obtained from our single channel recordings and a specific capacitance of 1 $\mu F/cm^2$, we can estimate the density of functional channels ($N = I/ip$) as approximately one per 10 to 20 $\mu m^2$ of cell membrane for each type of $Ca^{2+}$ channel.

The role of voltage-dependent $Ca^{2+}$ channels in fibroblasts is not obvious (*10*). Under physiological ionic conditions the membrane potential can be as negative as −70 mV and can oscillate between −70 and about −10 mV (*11*), thus covering the physiological activation range for both channel types. Activation of $Ca^{2+}$ channels in fibroblasts may contribute to such $Ca^{2+}$-sensitive processes as control of secretion, shape change, motility, and phagocytosis (*12*).

Because of the important role of $Ca^{2+}$ in the control of cell growth (*1*), we investigat-

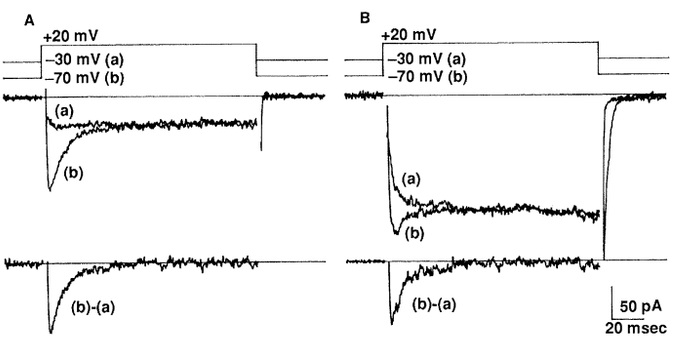


**Fig. 1.** Two types of $Ca^{2+}$ channels in Swiss 3T3 fibroblasts (*18*). Whole-cell currents carried by $Ba^{2+}$ (20 m*M*) in response to voltage clamp steps to +20 mV before (**A**) and after (**B**) addition of the DHP $Ca^{2+}$ channel agonist (+)202-791 (1 $\mu M$). Two currents elicited from holding potentials of −30 and −70 mV are superimposed. The extra component of current activated from the more negative holding potential is shown as the difference between the two current traces (bottom). Standard procedures were used for the patch clamp recordings and isolation of $Ca^{2+}$ channel currents (*2, 6*). The internal solution contained (in m*M*): 135 CsCl, 10 EGTA, 10 Hepes, 5 $MgCl_2$, 4 adenosine triphosphate, *p*H 7.5. The external solution contained: 20 $BaCl_2$, 135 tetraethylammonium (TEA)–chloride, 10 Hepes, *p*H 7.5. Depolarizing pulses were delivered every 3 to 4 seconds. Linear leak and capacity currents were subtracted digitally. Cell capacitance, 141 pF; temperature, 22°C.

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ed $Ca^{2+}$ channels in oncogenically transformed (*13*) cells. We found that fibroblasts transformed by activated c-H-*ras*, EJ-*ras*, v-*fms*, or polyoma middle T oncogenes (*14*) had densities of L-type currents similar to those of nontransformed cells but specifically lacked the T-type currents found in all control 3T3 cells (Fig. 3).

The observed suppression of $Ca^{2+}$ channel activity in transformed fibroblasts runs contrary to the generally accepted positive correlation between $Ca^{2+}$ and cell growth (*1*) and may point to a negative feedback mechanism for the control of cell growth by $Ca^{2+}$. T-type $Ca^{2+}$ channel activity may be suppressed because the morphological transformation interferes with channel function or through oncogene-induced inhibition of channel gene expression. A direct effect of an oncogene product on the channel seems unlikely because the products of the three oncogenes tested differ in their direct cellular actions: the *ras* product ($p21^{ras}$) is a guanine nucleotide–binding protein (*15*), whereas the other two oncogene products directly (*fms*) or indirectly (middle T, through binding to $pp60^{src}$) lead to increased tyrosine kinase activity (*16*). However, some messenger molecule formed later in a cascade of transforming biochemical events presumably common to all three oncogene products could inhibit T-type $Ca^{2+}$ channels. No second messengers with actions on T-type channels have yet been reported in these or other cell types.

Our results point out new differences between the two $Ca^{2+}$ channel types and further strengthen the view that even though the two channel types could share structural elements, each channel type is a separate molecular entity.

*Note added proof*: After completion of this work, we received a manuscript describing a transient type of $Ca^{2+}$ current in 3T3 fibroblasts (*17*).

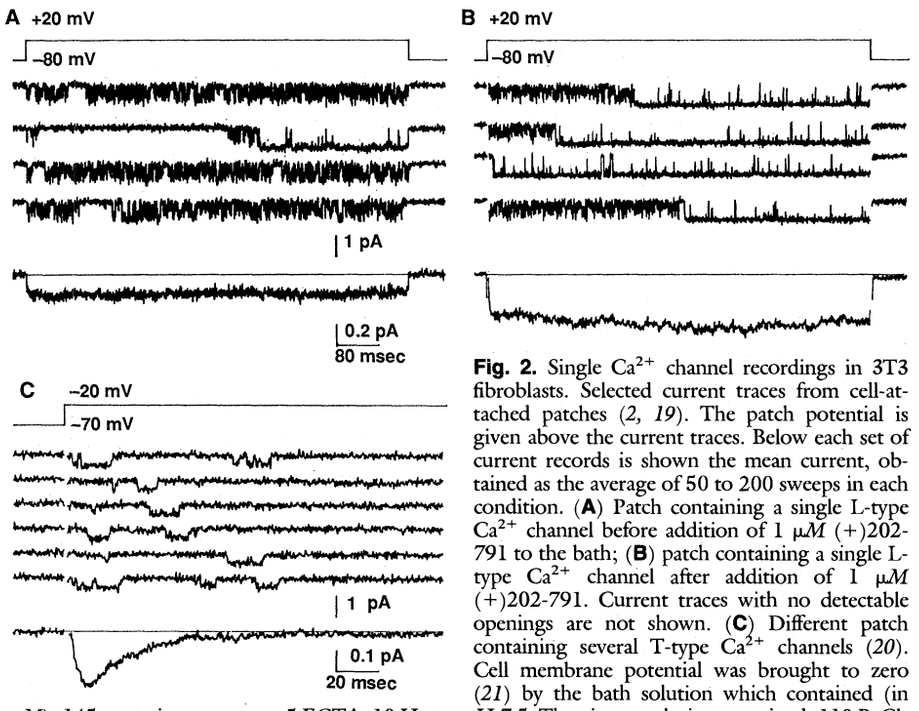


**Fig. 2.** Single $Ca^{2+}$ channel recordings in 3T3 fibroblasts. Selected current traces from cell-attached patches (*2, 19*). The patch potential is given above the current traces. Below each set of current records is shown the mean current, obtained as the average of 50 to 200 sweeps in each condition. (**A**) Patch containing a single L-type $Ca^{2+}$ channel before addition of 1 $\mu M$ (+)202-791 to the bath; (**B**) patch containing a single L-type $Ca^{2+}$ channel after addition of 1 $\mu M$ (+)202-791. Current traces with no detectable openings are not shown. (**C**) Different patch containing several T-type $Ca^{2+}$ channels (*20*). Cell membrane potential was brought to zero (*21*) by the bath solution which contained (in m$M$): 145 potassium aspartate, 5 EGTA, 10 Hepes, $p$H 7.5. The pipette solution contained: 110 $BaCl_2$ (A and B) or 110 $CaCl_2$ (C), 10 Hepes, $p$H 7.5. Linear leak and capacity currents were subtracted digitally. Sampling rate, 5 kHz; filter, 1 kHz (−3 dB, eight-pole Bessel filter); temperature, 22°C.

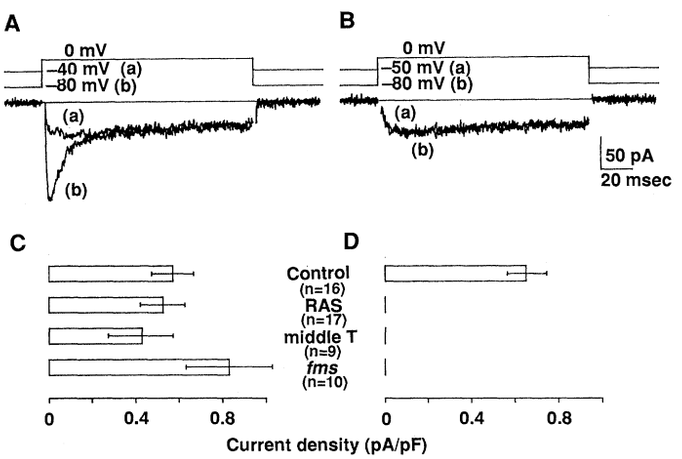


**Fig. 3.** 3T3 fibroblasts transformed by activated c-H-*ras*, EJ-*ras*, v-*fms*, or polyoma middle T oncogenes (*22–27*) specifically lack T-type current. Superposition of whole-cell $Ba^{2+}$ currents elicited from two holding potentials. (**A**) Control virus-infected NIH 3T3 cell (*28*) has both components of $Ca^{2+}$ channel current. Cell capacitance, 95 pF. (**B**) NIH 3T3 cell transformed by the activated c-H-*ras* oncogene lacks T-type current. Capacitance, 76 pF. (**C** and **D**) Mean values ± SEM of the current densities of the two $Ca^{2+}$ channel types in control and transformed cells. The number of cells ($n$) studied is indicated for each group. The mean densities of the L-type current did not differ significantly between control and transformed cells. Solutions and patch clamp methods are the same as in Fig. 1, except that aspartate (cesium aspartate, TEA-aspartate) and acetate (barium acetate) were used in place of $Cl^-$. Cell capacitance was measured from the capacity current elicited by a small depolarization. Currents were measured after leak subtraction at the peak of the current-voltage relation (−20 mV and +20 mV for T- and L-currents, respectively) with 20 m$M$ $Ba^{2+}$ as the charge carrier.

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18. Swiss 3T3 fibroblasts were obtained from H. Green (Department of Physiology and Biophysics, Harvard Medical School) and cultured in Dulbecco's modified Eagle's medium and 10% fetal calf serum. For the whole-cell recordings, individual cells were obtained by brief (5 to 10 minutes) trypsinization (0.05% trypsin + 0.5 m*M* EDTA). The cell suspension was allowed to settle in the recording chamber, and currents were measured from individual cells not connected to their neighbors.
19. Cell-attached recordings were obtained from cells in cultures without trypsinization as well as from the dissociated cells used for whole-cell recordings. We noticed no difference in the unitary channel properties between the two groups.
20. The conductance of the L-type $Ca^{2+}$ channel with 110 m*M* $BaCl_2$ was 25 pS. The conductance of the T-type $Ca^{2+}$ channel with 110 m*M* $CaCl_2$ was 8 pS. Unlike the L-type channel (*6, 21*), the T-type channel had similar conductance and kinetics with either $Ca^{2+}$ or $Ba^{2+}$ as the charge carrier (*6, 18*).
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22. The cell lines were grown from G418-resistant colonies selected after infection with a recombinant retrovirus (*23*) carrying the neomycin/G418 resistance gene and the following inserts: (i) no second gene (control virus-infected cells); (ii) activated c-H-*ras*-1 complementary DNA–encoding leucine in place of glutamine at codon 61 [M. J. Corbley, unpublished data; (*24*)]; (iii) v-*fms*, the transforming gene of the McDonough strain of feline sarcoma virus (*25*); and (iv) polyoma virus middle tumor antigen (middle T) (*26*). NIH 3T3 cells were also grown from a transformed focus after transfection with EJ-*ras* DNA (*27*). The results from cells infected with activated c-H-*ras* ($n = 10$) or transfected by EJ-*ras* ($n = 7$) were similar and were therefore pooled and are shown as "RAS" in Fig. 3.
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28. We found no difference in the whole-cell $Ca^{2+}$ currents of virus-infected NIH 3T3, noninfected NIH 3T3, and noninfected Swiss 3T3 cells.
29. We thank D. K. Morrison and V. Cherington for providing cell lines and H. Green and O. Kehinde for cells, advice, and use of their culture facilities. Supported by grants from the U.S. PHS (HL37124 to P.H.; CA21082 to T.M.R.) and the American Cancer Society (CD342 to P.H.).

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# A Newly Characterized HLA $DQ_\beta$ Allele Associated with Pemphigus Vulgaris

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**The inheritance of particular alleles of major histocompatibility complex class II genes increases the risk for various human autoimmune diseases; however, only a small percentage of individuals having an allele associated with susceptibility develop disease. The identification of allelic variants more precisely correlated with disease susceptibility would greatly facilitate clinical screening and diagnosis. Oligonucleotide-primed gene amplification in vitro was used to determine the nucleotide sequence of a class II variant found almost exclusively in patients with the autoimmune skin disease pemphigus vulgaris. In addition to clinical implications, the disease-restricted distribution of this variant should provide insight into the molecular mechanisms underlying associations between diseases and HLA–class II genes.**

THERE IS A STRIKING GENETIC ASSOCIATION between susceptibility to human autoimmune disease and particular alleles of the major histocompatibility complex (HLA) class II (D) region (*1*). This multigene complex on chromosome 6 encodes the α and β polypeptides of the highly polymorphic DP, DQ, and DR heterodimers that regulate immune responsiveness (*2*). Given the significant effects of class II protein sequence variation on the specificity of the immune response in mice (*3*), there have been intensive efforts to identify autoimmune disease–unique or restricted class II allelic variants by serologic, cellular, and restriction fragment length polymorphism (RFLP) typing methods (*4*). Two $DQ_\beta$ RFLP variants that very clearly distinguish between patients with the autoimmune skin disease pemphigus vulgaris (PV) and class II–matched healthy controls were recently identified (*5*). We now report the first domain nucleotide sequence of both of these RFLP variants. One of these sequences represents a previously unidentified $DQ_\beta$ allele present in 13 of 13 DRw6,DQw1 Israeli PV patients but in only 1 of 13 DR- and DQ-matched Israeli controls.

Pemphigus vulgaris, a severe autoimmune disease of the skin characterized by intraepidermal blistering, is mediated by autoantibodies to an epidermal cell-surface protein (*6*). The disease is relatively frequent among Jews (*7*); it is strongly associated with the DR4 serologic specificity among Ashkenazi Jews and with the DR4 and DRw6 serologic specificities in non-Ashkenazi Jews (*5*). However, the association of PV, by RFLP analysis, with $DQ_\beta$ rather than $DQ_\alpha$ or $DR_\beta$ (*5*) indicates that $DQ_\beta$ may be the susceptibility locus. The previously reported DR associations might then reflect the known strong linkage disequilibrium between the DR and DQ loci. There are four serologically identified DQ alleles: DQw1, DQw2, DQw3, and DQWa (or DQwBLANK) (*8*). DR4 haplotypes are nearly always associated with DQw3, whereas most DRw6 haplotypes have the DQw1 specificity (*9*). The DQw1 serologic determinant is also found in DR1 and DR2 haplotypes, but DR1-, DR2-, and DRw6-associated DQw1 molecules have been differentiated at the protein and RFLP levels (*10*). Furthermore, there is DQ heterogeneity within the DRw6 haplotype (*11*) (Table 1).

When Bam HI–digested genomic DNA from several $DQw1^+$ PV patients and controls was examined by Southern blot and hybridized with a $DQ_\beta$ complementary DNA (cDNA) probe, the PV-associated 2.5-kb fragment (*5*) was found in three of three DRw6,DQw1 PV patients (Fig. 1, lanes 4, 6, and 10), three of three DR1,DQw1 homozygous typing cells (HTC) (lanes 1 to 3), one of three DR2,DQw1 HTC (lanes 11 to 13), and none of three DRw6,DQw1 HTC (lanes 7 to 9). The presence of this fragment in the DR1,DQw1 HTC and in the AZH HTC, an example of the non-Dw2, non-Dw12 DR2 subtype (lane 11), likely reflects the near identity of these $DQ_\beta$ alleles at the

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