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17. The mouse c-*myc* probe plasmid contains an Xba I–Sac I fragment spanning the second intron junction. Linearization of this plasmid with Pst I produces a probe that protects a 220-nucleotide RNA fragment. Linearization with Pvu II produces a probe that protects a 457-nucleotide RNA fragment. HeLa, 143 tk^-, and MG63 cells were induced with poly(I)-poly(C) alone by incubation in serum-free medium containing poly(I)-poly(C) at 100 μg/ml until the time of RNA preparation. SP6 probe plasmids were provided by R. Kingston (*hsp70*), P. Mellon (MT-1), W. Kruijer (mouse c-*fos*), and R. Treisman (human c-*fos*).
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Amiloride Selectively Blocks the Low Threshold (T) Calcium Channel

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More than one type of voltage-gated calcium channel has been identified in muscle cells and neurons. Many specific organic and inorganic blockers of the conventional, slowly inactivating high threshold (L) calcium channel have been reported. No specific blockers of the low threshold (T) channel have been as yet identified. Amiloride, a potassium sparing diuretic, has now been shown to selectively block the low threshold calcium channel in mouse neuroblastoma and chick dorsal root ganglion neurons. The selective blockade of the T-type calcium channel will allow identification of this channel in different tissues and characterization of its specific physiological role.

IN ELECTRICALLY EXCITABLE CELLS THE influx of calcium through voltage-gated Ca^{2+} channels plays an important role in neurohormonal secretion, excitation-contraction coupling, and neuronal integration and signaling. More than one type of voltage-gated Ca^{2+} channel exists in neuronal and muscle cells (*1–4*). The physiological role of each channel type, however, remains speculative. One difficulty encountered in attempting to dissect the physiological function of the different types of channels is the lack of pharmacological agents that selectively block the low threshold (T) channel (*5–8*). For instance, dihydropyridines and ω-congotoxin VIA, compounds that block the (L) Ca^{2+} channels, fail to suppress the Ca^{2+} current through the T-type channels (*6–8*). On the other hand, verapamil (≥100 μ*M*) suppresses both high and low threshold Ca^{2+} channels almost equally (*9*). Ni^{2+}, which shows preferential block of the T-type channels at low concentrations [dissociation constant (K_D), 47 μ*M*] in neuroblastoma cells (*7, 10*), also blocks the L-type channels at higher concentrations (K_D = 300 μ*M*). Thus the separation of the two channel types based on their pharmacological specificities is difficult, especially in neuronal tissues. We have now shown that amiloride, a K^+ sparing diuretic, effectively blocks the low threshold Ca^{2+} channel in isolated chick dorsal root ganglion (DRG) and mouse neuroblastoma cells but has no significant effect on the high threshold Ca^{2+} channel.

Mouse neuroblastoma (N18) cells were voltage-clamped with the whole-cell clamp technique (*11*). Neuroblastoma cells were plated into petri dishes 1 to 3 days prior to their use. The internal dialyzing and bathing solutions were chosen to optimize the Ca^{2+} current and to minimize the outward K^+ current. The internal solution generally contained 80 m*M* CsCl, 20 m*M* NaCl, 20 m*M* tetraethylammonium (TEA) chloride, 20 m*M* Hepes, 10 m*M* EGTA, 2 m*M* magnesium adenosine triphosphate, and 0.2 m*M* adenosine 3′,5′-monophosphate (cAMP) and was titrated to *p*H 7.3 with CsOH. The external bathing solution contained 140 m*M* NaCl, 5 m*M* $CaCl_2$, 10 m*M* Hepes, and 5 μ*M* tetrodotoxin (TTX) and was buffered to *p*H 7.3. TTX was used to block the Na^+ current. In some experiments external Na^+ was replaced entirely with *N*-methylglucamine. All experiments were carried out at room temperature (23° to 25°C).

Two distinct components of Ca^{2+} current may be separated in mouse neuroblastoma cells based on their voltage dependence and inactivation kinetics (Fig. 1) (*10*). The low threshold Ca^{2+} channel current is distinguished by its more negative activation threshold (Fig. 1A, upper traces) and its rapid inactivation kinetics (τ = 24 msec at −20 mV). The high threshold current, on the other hand, activated at more positive potentials (Fig. 1A, lower traces) and inactivated much more slowly (τ > 500 msec). Two populations of the Ca^{2+} channels were also indicated by comparison of the voltage dependence [current-voltage (*IV*) curves] of the transient (Fig. 1B) and the maintained (Fig. 1C) component of the Ca^{2+} current. Addition of amiloride [Figs. 1, A (middle traces) and B (open circles)] effectively suppressed the transient component of the Ca^{2+} current that activated at more negative potentials but had little or no effect on the maintained component of the Ca^{2+} current that activated at more positive potentials (Fig. 1C).

Amiloride blocked the low threshold Ca^{2+} current at relatively small concentrations. The effect of amiloride on the T-type channel is dose-dependent (Fig. 2). The dose-response relation was fitted with a theoretical curve with a K_D of 30 μ*M*. At much higher amiloride concentrations (>500 μ*M*), there was a variable but small (<20%) inhibitory effect of the drug on the high threshold Ca^{2+} current. Because at higher concentrations amiloride inhibits the Na^+-H^+ exchanger (*12*), the inhibition of the L-type channel may be related in part to such a mechanism. The rapid onset of the drug action ($t_{1/2}$ < 1 second), its quick washout, and the finding that its addition to the dialyzing internal solution had no effect on the calcium current (I_{Ca}) suggested that amiloride may block the low threshold channel from an external site.

In chick DRG neurons, where the low and high threshold Ca^{2+} channels have been well characterized (*1, 3*), amiloride also preferentially and strongly suppressed the low threshold T-type Ca^{2+} current activated in the range of potentials between −50 and −10 mV (*13*). Thus, irrespective of cell type or species, amiloride blocked specifically the low threshold (T) Ca^{2+} channels with little or no effect on the high threshold L-type channels. Consistent with the specificity of amiloride effect, the drug (up to 500 μ*M*) had no effect on the TTX-sensitive Na^+

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current in the neuroblastoma cells.

Because Ca^{2+} channels can carry Na^+ current in the absence of extracellular Ca^{2+} (*14, 15*), we also examined whether the amiloride block of T-type channel by amiloride was dependent on the permeating cation. Na^+ current through the Ca^{2+} channel could be activated in divalent-free 1 m*M* EGTA-containing solutions. Under such conditions, the current through the channel was about ten times larger, and the peak *IV* relation was shifted by about 20 mV to more negative potentials. In both DRG neurons (*13*) and neuroblastoma cells, amiloride blocked the low threshold Ca^{2+} channel in its Na^+-conducting mode in a dose-dependent manner (Fig. 3).

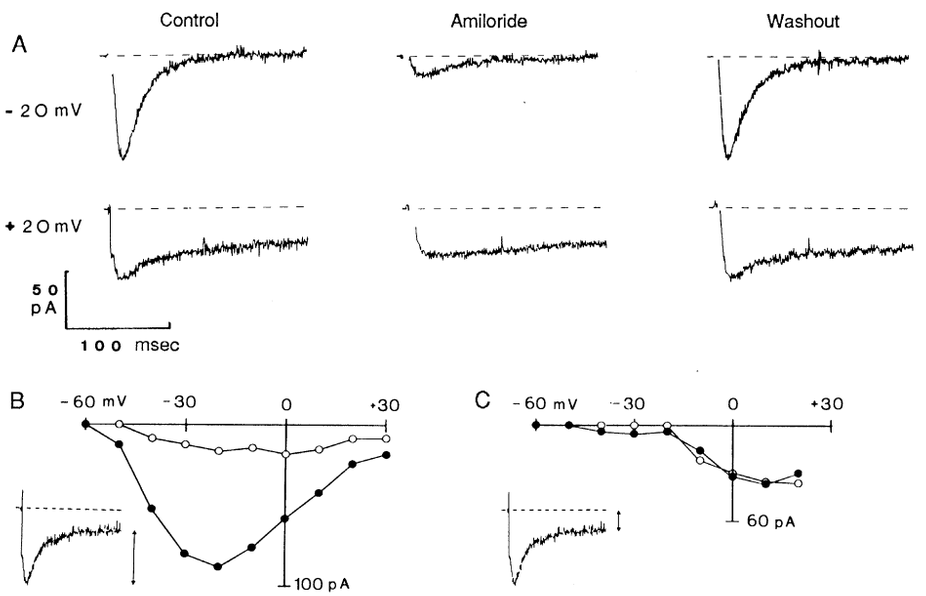

Fig. 1. Effect of amiloride on the two types of Ca^{2+} current in a neuroblastoma cell. Calcium currents were measured under voltage clamp conditions that minimized other ionic currents. The cell was held at −90 mV and depolarized to potentials between −60 mV and +30 mV. (**A**) Upper traces show amiloride (250 μ*M*) reversibly blocks the transient (T) Ca^{2+} current activated by depolarization to −20 mV. The lower traces show amiloride has no effect on the slowly inactivating (L) current activated by depolarization to +20 mV. (**B**) The transient current during a 200-msec pulse is plotted as a function of voltage (●). During application of amiloride this transient current is markedly suppressed (○). (**C**) The noninactivating component of the Ca^{2+} current is plotted as a function of voltage (●). Amiloride had little effect on this noninactivating component of the current.

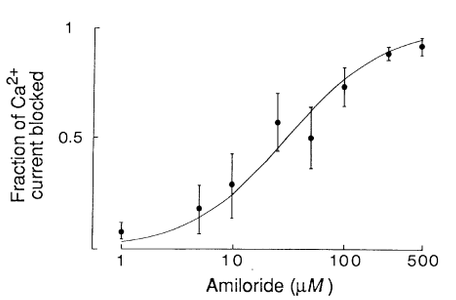

Fig. 2. Dose-response curve of amiloride on neuroblastoma cells. The fraction of Ca^{2+} current reversibly blocked by amiloride is plotted as a function of concentration. Each of 14 cells was tested at two or three different concentrations. Bars show one standard deviation from the mean. Smooth line is a plot of theoretical Michaelis-Menton–type curve with a K_D of 30 μ*M*.

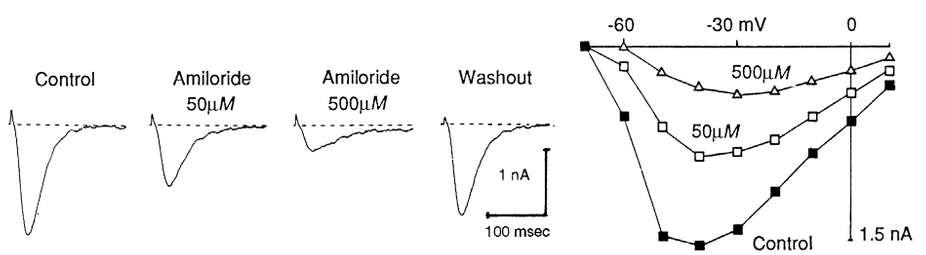

Fig. 3. Effect of amiloride on the Na^+ current through the low threshold Ca^{2+} channel. In the presence of external solution with no added Ca^{2+}, 1 m*M* EGTA, 140 m*M* NaCl, 10 m*M* Hepes, and 5 μ*M* TTX, a large inward Na^+ current can be measured under voltage clamp conditions in neuroblastoma cells. Amiloride reversibly blocks the Na^+ current through the Ca^{2+} channel. (**A**) Traces show currents evoked by voltage clamping to −40 mV from a holding potential of −90 mV. (**B**) *IV* curve as a function of voltage between −70 mV and +10 mV (■, control; □, 50 μ*M* amiloride; △, 500 μ*M* amiloride) (*26*). Amiloride also blocks this same Na^+ current in DRG neurons (*13*).

Recently another conductance state of the Ca^{2+} channel has been shown to exist in neurons, which is activated by rapid increases in extracellular proton concentrations and causes a transient loss of voltage-gating and a shift from divalent to monovalent selectivity of the channel (*16, 17*). Amiloride suppressed the proton-activated Na^+ current [$I_{Na(H)}$] through the Ca^{2+} channels. Amiloride suppresses $I_{Na(H)}$ more strongly at −80 mV than at 0 mV (Fig. 4). At amiloride concentrations of 10 μ*M* the percent inhibition of $I_{Na(H)}$ was about 20% at +20 mV, compared to 80% at −120 mV (n = 5). These findings suggest that amiloride may block $I_{Na(H)}$ in a voltage-dependent manner, consistent with the effect of the drug on epithelial Na^+ channels (*18*). The positive charge on amiloride at physiological *p*Hs, resulting from acylguanidium side group (pK_a = 8.7), most likely contributes to the voltage-dependent effect of the drug. Because the T-type Ca^{2+} channel is activated at more negative potentials, the voltage-dependent blocking property of amiloride may contribute to its selectivity for the T-type Ca^{2+} channel. This possibility, however, could not be directly tested because activation of the two types of Ca^{2+} channels is highly voltage dependent.

Amiloride and its derivatives block the epithelial TTX-insensitive Na^+ channel in submicromolar concentrations (*18, 19*). The diuretic effect of amiloride, in fact, occurs secondary to the blockade of the Na^+ channels in renal tubules (*18*). There are no reports, however, as to the presence of this type of Na^+ channel in neuronal or cardiac tissues.

Amiloride also inhibits the Na^+-H^+ and Na^+-Ca^{2+} exchange systems (*12, 20*). The latter effects, however, require much higher concentrations (>1 m*M*) than the blockade of the low threshold Ca^{2+} current in our experiments. One possible interpretation of multiple blocking effects of amiloride is that these transport processes share common structural homologies. Such homologies appear to be present, for example, between the dihydropyridine-sensitive moiety of the Ca^{2+} channel (*21*) and the Na^+ channel. Thus the low threshold T-type Ca^{2+} channel may have structural homologies with the TTX-insensitive Na^+ channels of epithelial cells.

Consistent with the regulatory effects of amiloride on Ca^{2+} influx, amiloride slows

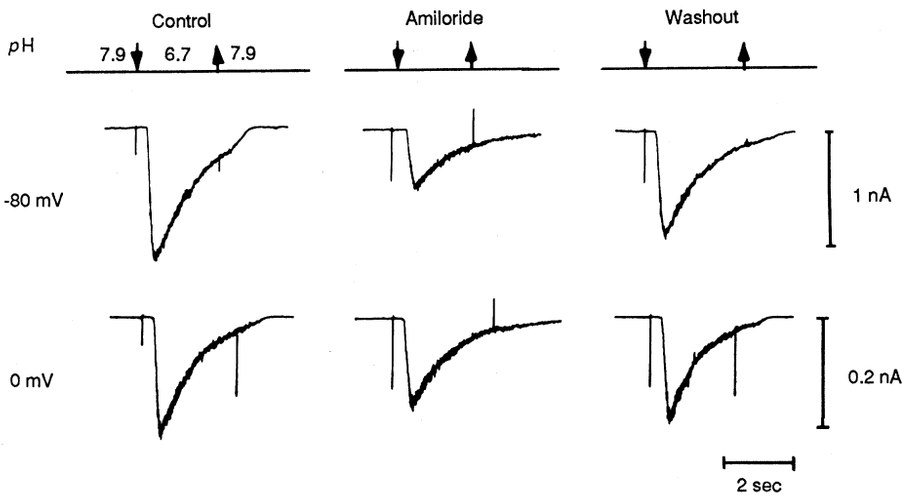

Fig. 4. Voltage-dependent block of $I_{Na(H)}$ by amiloride. In a voltage-clamped neuroblastoma cell, rapid increase of the extracellular proton concentration from *p*H 7.9 to 6.7 activates a transient inward Na^+ current which can be deactivated when the proton concentrations are step reduced to *p*H 7.9 (upward arrow). Amiloride blocks this current in a voltage-dependent manner (middle panels). At a holding potential of −80 mV, 10 μ*M* of amiloride suppressed approximately half of the current, while there is only minimal suppression at 0 mV. The external solution contained 140 m*M* Na^+, 1 m*M* Ca^{2+}, 10 m*M* Pipes and 2 μ*M* TTX. The internal solution contained 80 m*M* Cs^+, 20 m*M* TEA, 20 m*M* Na^+, 20 m*M* Hepes, and 10 m*M* EGTA and was buffered to *p*H 7.3.

impulse conduction through the atrioventricular node and suppressed the pacing rate in the sinoatrial node (*22, 23*). Because the T-type Ca^{2+} channel appears to mediate pacing in heart cells (*24*), we tested the effect of amiloride on I_{Ca} and found that it selectively blocked the T-type current in guinea pig atrial myocytes (*25*).

The suppressive effect of amiloride on the Ca^{2+} current is shared by a number of its chemical derivatives. In particular, the addition of substituted benzyl moieties to the guanidine side group greatly enhanced the blocking properties of amiloride derivatives ($K_D < 10^{-6}M$). Thus amiloride and its derivatives can serve as specific inhibitors of the T-type Ca^{2+} channel, making it possible to dissect the physiological role of this channel in different neuronal and muscle tissues.

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26. Return of the external solution to the normal external solution containing 5 m*M* $CaCl_2$, 140 m*M* NaCl, 10 m*M* Hepes, and 5 μ*M* TTX brought back the usual amiloride-sensitive Ca^{2+} current (not shown in figure).

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Maternally Inherited Transposon Excision in *Drosophila simulans*

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A mutation in the *white* gene of *Drosophila mauritiana* resulting from insertion of the transposable element *mariner* exhibits genetic instability in germline and somatic cells. The instability is greatly enhanced in the presence of the trans-acting autosomal factor *Mos*, giving eye-color mosaics with pigmented sectors of tissue on an otherwise peach-colored background. The *Mos* factor, when introduced into the genome of the sibling species *Drosophila simulans*, exhibited a dramatic maternal effect on expression of the mosaic phenotype. When *D. simulans* mosaic females (heterozygous for *Mos*) were crossed with non-mosaic males, two distinct classes of mosaic offspring occurred, one resulting from a maternal effect in which the non-*Mos* offspring were nevertheless mosaic. The maternal effect was mediated by a product acting after fertilization, and was expressed to varying extents in different backcross strains.

CYTOPLASMIC TRANSMISSION OF SUBstances regulating the mobilization of transposable elements is an important feature of hybrid dysgenesis in *Drosophila melanogaster* (*1, 2*). In hybrid dysgenesis, unidentified factors transmitted through the maternal cytoplasm can suppress mobilization of the transposable P element (*1*). We have found a maternal effect, involving the dominant autosomal factor *Mos*, that is clearly distinct from hybrid dysgenesis in that maternally transmitted cytoplasmic factors enhance excision of the *mariner* element. The two effects are perhaps related in that the excision depends on factors transmitted through the egg.

The *white-peach* (w^{pch}) allele contains the 1.3-kb *mariner* transposable element inserted immediately upstream of the first exon of the X-linked *white* gene (*3*). The genome of *D. mauritiana* contains about 20 copies of *mariner*, one of which is the *Mos* factor (*4*). In combination with *Mos*, excision of the *mariner* element from the w^{pch} allele occurs at high frequency in somatic and germinal cells in *D. mauritiana*. Somatic excision results in mosaic eye-color with pigmented patches on an otherwise peach-colored back-

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