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13. Developing bean seeds were harvested 13 to 19 days after flowering and mRNA was isolated by the procedure described by T. C. Hall *et al.* [*Proc. Natl. Acad. Sci. U.S.A.* **75**, 3196 (1978)] except that the sucrose gradient centrifugations were omitted.
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2-Aminopurine Selectively Inhibits the Induction of β-Interferon, c-*fos*, and c-*myc* Gene Expression

KAI ZINN,* ANDREW KELLER, LISA-ANNE WHITTEMORE, TOM MANIATIS

The protein kinase inhibitor 2-aminopurine (2AP) blocks the induction of the human β-interferon gene by virus or poly(I)-poly(C) at the level of transcription. This inhibition is specific, since 2AP does not inhibit induction of either the *hsp*70 heat-shock gene by high temperature or the metallothionein gene by cadmium or dexamethasone. However, 2AP does block the induction of the c-*fos* and c-*myc* proto-oncogenes by serum growth factors or virus, suggesting that a protein kinase may be involved in the regulation of these genes, as well as of the β-interferon gene. However, different factors must be required for the induction of these three genes, since they are not coordinately regulated by the same inducers in most of the cell lines examined.

THE HUMAN β-INTERFERON (β-IFN) gene is induced by viral infection or by treatment of cells with synthetic double-stranded RNAs (dsRNAs) such as poly(I)-poly(C). The viral induction signal is thought to be dsRNA generated during infection (see *1* for review). Although the mechanism by which the presence of dsRNA leads to β-IFN gene activation is not understood, the DNA sequences required for induction have been extensively characterized (*2, 3*). A 40-bp region known as the IRE (interferon gene regulatory element) is sufficient for induction in mouse C127 cells. The IRE is a transcription enhancer composed of two distinct positive regulatory elements and an adjacent negative regulatory sequence (*4*). A second negative regulatory sequence is located 5′ to the IRE (*2*). Genomic deoxyribonuclease protection (footprinting) experiments show that proteins are bound to both negative regulatory sequences before induction. After induction, these proteins dissociate and other proteins bind to the positive regulatory sequences (*5*). In vitro DNA binding experiments have identified three different factors that specifically bind to the positive regulatory domains of the IRE (*6*). Two of these factors are detected in nuclear extracts from uninduced cells, whereas the third is detected exclusively in extracts from cells induced with virus or poly(I)-poly(C) (*6*). Since protein synthesis is not required for induction, the inactivation of the repressor and the inducible DNA binding activity may be the consequence of post-translational modifications of preexisting proteins. One possible mechanism by which transcription factors or repressors could be modified is phosphorylation. To examine the possibility that a protein kinase might be involved in the pathway of β-IFN induction, we studied the effect of the kinase inhibitor 2-aminopurine (2AP) (*7*) on β-IFN messenger RNA (mRNA) transcription in vivo.

Human MG63 osteosarcoma cells were induced with poly(I)-poly(C) in the presence or absence of 10 m*M* 2AP. RNA was prepared 4 to 6 hours after induction and analyzed for β-IFN and γ-actin mRNA by quantitative ribonuclease (RNase) mapping (*2*). As shown in Fig. 1, 2AP caused a dramatic (>100-fold) inhibition of the induction of β-IFN mRNA, but had no effect on the level of γ-actin mRNA. A similar inhibitory effect of 2AP was observed with Sendai virus induction. No cytotoxicity due to 2AP was apparent during the induction period. However, after incubation for 12

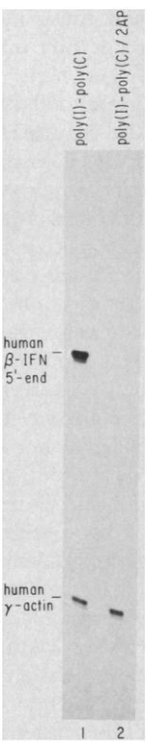

Fig. 1. Effects of 2AP on induction of the β-IFN gene in human MG63 cells. Total cellular RNA (10 μg per lane) was hybridized to a mixture of labeled hybridization probes specific for the 5′ end of human β-IFN mRNA (*2*) and the coding region of a human γ-actin gene (*12*) and analyzed by RNase mapping (*12*). Correctly initiated β-IFN mRNA protects a 277-nucleotide RNA fragment, and γ-actin mRNA protects a 145-nucleotide RNA fragment. Cells were induced with poly(I)-poly(C) as described elsewhere (*12*) in the absence (lane 1) or presence (lane 2) of 10 m*M* 2AP. Longer exposures of this and other autoradiograms indicate that 2AP inhibits induction of β-IFN mRNA by greater than 100-fold.

Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138.

*Present address: Department of Biochemistry, University of California, Berkeley, CA 94720.

hours in 10 m*M* 2AP, cell death was observed.

Inhibition of β-IFN induction by 2AP could act at the level of transcription initiation or at a subsequent step in the accumulation of β-IFN mRNA, such as cytoplasmic transport or mRNA stability. To distinguish between these possibilities, we carried out nuclear transcription experiments with human MG63 cells. Treatment with poly(I)-poly(C) results in a significant increase in the rate of transcription (Fig. 2). However, this increase is blocked when the cells are treated with 2AP. A low level of induction of antisense transcription was also observed, and this induction was also blocked by 2AP.

To determine whether inhibition by 2AP was specific to β-IFN induction, we examined the effect of the compound on the induction of the heat-shock gene *hsp*70 and the metallothionein MT-1 gene in mouse NIH 3T3 cells. Induction of the *hsp*70 gene by heat shock is unaffected by 2AP (Fig. 3A). In the case of the MT-1 gene, 2AP augmented induction by cadmium (Cd) or dexamethasone (Dex) and was also capable of inducing MT-1 mRNA when used alone (Fig. 3B). Both Cd and Dex induce MT-1 gene expression by a factor of 3 to 4 in these cells. We also detected unspliced MT-1 precursor RNA, and this was highly inducible (>20-fold) by Cd or 2AP but not by Dex (Fig. 3B). The high level of precursor relative to mature mRNA suggests that Cd and 2AP may inhibit splicing of newly synthesized MT-1 precursor RNA.

We also examined the effects of 2AP on induction of the c-*fos* and c-*myc* genes in NIH 3T3 cells. These genes, the cellular homologs of the viral oncogenes from FBJ murine sarcoma virus and avian MC29 virus, respectively, are induced at the level of transcription by serum growth factors, phorbol ester tumor promoters, and many other agents (*8*). The c-*fos* and c-*myc* genes are also inducible by poly(I)-poly(C) and virus in Balb/c-3T3 cells (*9*). C-*fos* mRNA is induced within 15 minutes of the addition of serum or purified growth factors to quiescent cells and accumulates to maximal levels by about 30 minutes. C-*myc* mRNA is induced more slowly, reaching a peak at 1 to 2 hours after serum addition, and its induction ratio (<10-fold) is usually smaller than that of c-*fos* mRNA (up to 50-fold). Induction of these genes does not require new protein synthesis.

When quiescent NIH 3T3 cells were incubated with medium containing 20% fetal calf serum, induction of c-*myc* and c-*fos* mRNA was observed, and this induction was suppressed by 2AP. However, 2AP had no effect on the basal level of c-*myc*, c-*fos*, or γ-actin mRNA in quiescent cells (Figs. 4, A and B). Since both c-*myc* and c-*fos* mRNAs are known to be very unstable (*10*), it is possible that 2AP does not inhibit constitutive transcription of these genes. C-*fos* precursor RNA can also be detected, and its splicing, like that of the MT-1 precursor, appears to be inhibited by 2AP (Fig. 4B). We find that c-*fos* mRNA is inducible by poly(I)-poly(C) or virus in HeLa cells, and this induction is also inhibited by 2AP (*11*).

The c-*fos* and c-*myc* genes are induced by poly(I)-poly(C) and virus as well as by platelet-derived growth factor (PDGF) in Balb/c-3T3 cells (*9*). These observations, together with our data on inhibition of induction of these genes by 2AP, suggested that the three genes could be coordinately regulated. To examine this question, we analyzed induction of the c-*fos* and c-*myc* genes in several cell lines that differ in their capacity for β-IFN gene induction. Human MG63 and mouse C127 cells are highly inducible by virus or poly(I)-poly(C), whereas HeLa and human 143 tk^- cells are inducible only after pretreatment with IFN (priming). Unprimed HeLa and 143 tk^- cells were shown to lack an IFN-induced trans-acting factor or factors required for β-IFN gene induction (*12*).

To determine whether the IFN-induced factor or factors might also be involved in c-*fos* and c-*myc* induction, we induced the four cell lines with poly(I)-poly(C), Sendai virus, or serum, and analyzed expression of all three genes. Both primed and unprimed HeLa and 143 tk^- cells were examined. RNA was prepared from cells before treatment with inducer, and at 30, 60, 120, and 180 minutes after induction. In HeLa cells, β-IFN mRNA is induced efficiently by poly(I)-poly(C) or virus only if the cells have been primed by IFN (*12*). However,

Table 1. Summary of c-*fos*, c-*myc*, and β-IFN gene induction in various cell lines. ++, High-level (>500 transcripts per cell) induction of the β-IFN gene is observed; +, normal induction (5- to 10-fold for c-*myc*, 20- to 50-fold for c-*fos*, <500 transcripts per cell of β-IFN mRNA) is observed; +/−, low-level (2- to 4-fold) induction of c-*fos* or c-*myc* is observed; −, no induction; ND, not determined.

Inducer	Cell line	Gene		β-IFN
		fos	*myc*	
Virus	HeLa	+	−	−
	Primed HeLa	+	−	+
	143 tk^-	−	+/−	−
	Primed 143 tk^-	−	+/−	+
	MG63	−	−	++
	C127	−	−	++
	NIH 3T3	ND	ND	−
Poly(I)-poly(C)	HeLa	+	−	−
	Primed HeLa	+	−	+
	143 tk^-	−	+/−	−
	Primed 143 tk^-	−	+/−	+
	MG63	−	−	++
	C127	−	−	++
Serum	Hela	+	−	−
	Primed HeLa	+	−	−
	143 tk^-	+/−	+	−
	Primed 143 tk^-	+/−	+	−
	MG63	+	−	−
	C127	+	−	−
	NIH 3T3	+	+	ND

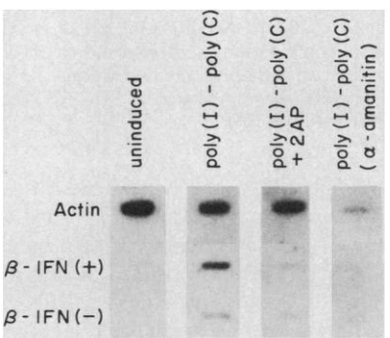

Fig. 2. 2AP inhibition of β-IFN gene expression acts at the level of transcription. Human MG63 cells were induced with poly(I)-poly(C) for 2 minutes in the presence or absence of 10 m*M* 2AP. Nuclei were isolated and nascent transcripts elongated in the presence of ^{32}P-labeled uridine triphosphate according to published procedures (*15*). Where indicated 2 μg of α-amanitin per milliliter was included in the transcription reaction. Labeled transcripts were hybridized to excess DNA immobilized on a nitrocellulose filter, and the filters were exposed to x-ray film. DNA probes were as follows: actin, dsDNA containing the human γ-actin gene (*12*); IFN (+) and IFN (−), phage M13 single-stranded DNA containing the human β-IFN gene in either orientation (*16*). The notations (+) and (−) refer to sense and antisense transcription, respectively. No hybridization was observed with the M13 vector DNA.

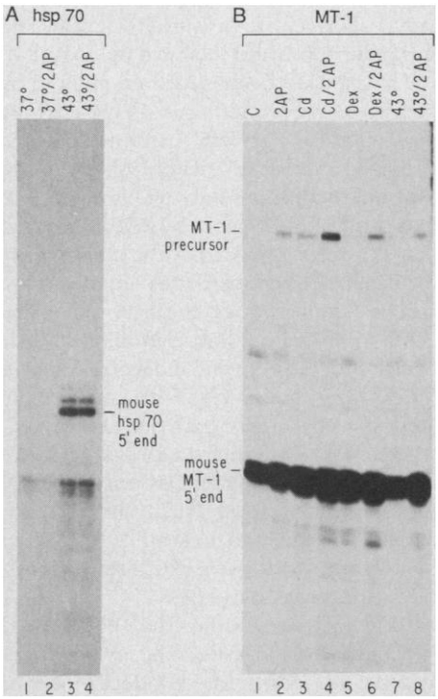

Fig. 3. Effects of 2AP on induction of the *hsp*70 and MT-1 genes. (**A**) Induction of *hsp*70. Total cellular RNA (20 μg per lane) was hybridized to a mouse *hsp*70 5′ SP6 RNA probe (*17*). Correctly initiated *hsp*70 mRNA protects a 250-nucleotide RNA fragment. NIH 3T3 cells were incubated at 37°C or 43°C with or without 10 m*M* 2AP, and RNA was prepared after 2 hours. (**B**) MT-1 induction. Total cellular RNA (20 μg per lane) was hybridized to a mouse MT-1 5′ SP6 RNA probe (*17*). Correctly initiated MT-1 mRNA protects a 101-nucleotide RNA fragment; MT-1 precursor RNA protects a 218-nucleotide RNA fragment. NIH 3T3 cells were treated with 10 m*M* 2AP, 20 μ*M* $CdSO_4$, 100 n*M* dexamethasone, or combinations of these as indicated. RNA was prepared at 6 hours after induction. The lanes labeled 43°C were incubated at 43°C for 2 hours with or without 2AP.

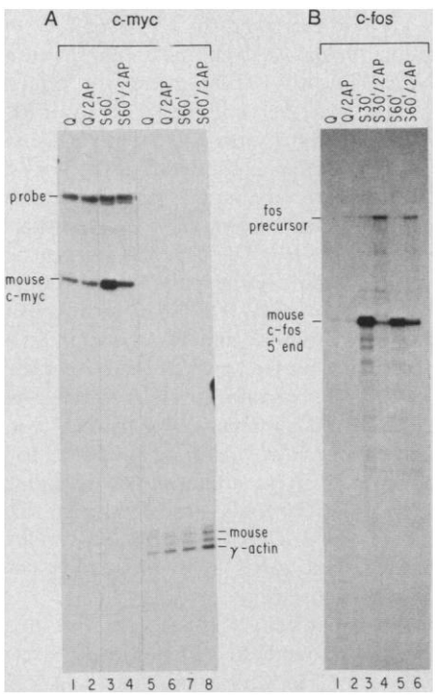

Fig. 4. Effects of 2AP on induction of the c-*myc* and c-*fos* genes. (**A**) C-*myc* induction. Total cellular RNA (20 μg per lane) was hybridized to a mouse c-*myc* Sp6 RNA probe that spans the 3′ border of the second exon (*17*). C-*myc* mRNA protects a 220-nucleotide RNA fragment. The upper band is an artifact that is always observed with this probe. The same RNA samples were hybridized separately to the human γ-actin probe, which protects a 65-nucleotide fragment when hybridized to mouse γ-actin mRNA (*12*). NIH 3T3 cells were starved by incubation in 0.5% serum for 2 days, then induced with 20% serum in the presence of absence of 10 m*M* 2AP. RNA was prepared at 60 minutes and 120 minutes after induction. The Q (quiescent) lanes contain RNA from starved cells that were incubated with 0.5% serum in the presence or absence of 10 m*M* 2AP for 120 minutes. (**B**) C-*fos* induction. Total cellular RNA (20 μg per lane) was hybridized to a 5′ mouse c-*fos* probe (*17*). Correctly initiated c-*fos* mRNA protects 289-nucleotide RNA fragment. Unspliced c-*fos* precursor RNA protects a 637-nucleotide fragment. NIH 3T3 cells were starved and induced in the presence or absence of 2AP as described above, and RNA prepared at 30 minutes and 60 minutes postinduction.

poly(I)-poly(C) and virus induce c-*fos* mRNA in both primed and unprimed cells (Table 1). C-*fos* mRNA is also induced efficiently by serum in primed or unprimed HeLa cells, but induction of the β-IFN gene by serum is not observed (Table 1). Human 143 tk^- cells behave similarly to HeLa cells with respect to β-IFN mRNA induction in that they require priming with IFN for efficient induction (*12*). They behave differently with respect to induction of the c-*fos* and c-*myc* genes, however; c-*fos* mRNA is only slightly inducible by serum, and not inducible at all by poly(I)-poly(C) or virus in primed or unprimed 143 tk^- cells. C-*myc* mRNA is expressed at a very low level and is induced two- to threefold by all three agents. As in HeLa cells, the β-IFN gene is not inducible by serum (Table 1). MG63 and C127 cells are highly inducible for β-IFN mRNA by poly(I)-poly(C) or virus but not by serum, and this induction is unaffected by priming (*12*) (Table 1). C-*fos* mRNA is not inducible by virus in either of these lines, although it is highly inducible by serum. C-*myc* mRNA is not inducible by any of the three agents (Table 1).

The data indicate that the c-*fos* and c-*myc* and β-IFN genes are not coordinately regulated. In the three human cell lines examined, poly(I)-poly(C) or virus induces β-IFN but not c-*fos* or c-*myc* in MG63 cells, induces c-*fos* but not β-IFN or c-*myc* in unprimed HeLa cells, and induces c-*myc* but not β-IFN or c-*fos* in unprimed 143 tk^- cells. In addition, each does not always respond to all three inducers in a particular cell type. For example, c-*fos* is induced by poly(I)-poly(C), virus, or serum in HeLa or Balb/c-3T3 cells but by serum only in MG63 and C127 cells. The only correlations that can be made in the cell lines we have examined are that poly(I)-poly(C) and virus induction are always coordinate, and that in every case where the c-*fos* or c-*myc* genes can be induced by poly(I)-poly(C) or virus, they are also inducible by serum.

In summary, our data show that the kinase inhibitor 2AP inhibits induction of the β-IFN, c-*fos*, and c-*myc* genes by virus or poly(I)-poly(C). In addition, 2AP inhibits c-*fos* induction by serum growth factors. We have shown that 2AP inhibition of β-IFN induction is at the level of transcription initiation, and it appears that this compound may also inhibit splicing of the MT-1 and c-*fos* mRNA precursors. We do not know whether 2AP inhibits the induction of c-*fos* and c-*myc* at the level of transcription initiation.

At present, we have no evidence that the inhibitory effect of 2AP on induction of the β-IFN, c-*myc*, and c-*fos* genes results from the inactivation of a protein kinase. The two kinases that are known to be inhibited by 2AP in vitro are the heme-regulated and dsRNA-dependent eukaryotic initiation factors (eIF)–2α kinases (*7*). Although the specificity of the inhibitory effects of 2AP has not been systematically studied in vivo, it does not significantly alter the overall pattern of protein phosphorylation in HeLa cells (*13*). Thus, 2AP does not appear to be a general inhibitor of cellular kinases. We note that the dsRNA-dependent kinase is induced by IFN treatment (*14*, for review), and a factor required for β-IFN gene activation by poly(I)-poly(C) or virus in HeLa or 143 tk^- cells is also induced by IFN (*12*). These observations, together with the 2AP inhibition data, suggest the possibility that the dsRNA-dependent kinase could be involved in induction of the β-IFN gene. A direct test of this interesting possibility will require cloning of the kinase gene and its expression in HeLa or 143 tk^- cells.

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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

CHA-MIN TANG, FERNANDO PRESSER, MARTIN MORAD

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^+

University of Pennsylvania, Department of Physiology, Philadelphia, PA 19104–6085.