Amiloride Inhibition of Human Cytomegalovirus Replication (43167)

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Abstract. **Amiloride, an inhibitor of Na^+/H^+ exchange, interfered with cytomegalovirus (CMV) DNA synthesis, blocked the formation of nuclear inclusions, and reduced CMV infectious yields. The reduction of CMV infectious yields was concentration dependent with an ED_{90} of 46 μM. Amiloride at a concentration of 150 μM reduced CMV yields by about 100-fold. Reduction of infectious yields appeared to be related to interference with the formation of nuclear inclusions and to inhibition of CMV DNA synthesis. Nuclear inclusions were much reduced in size and demonstrated poorly defined cellulae in the amiloride-treated cells. CMV DNA synthesis was inhibited by approximately 70% when cells were treated with 150 μM amiloride. The reduction in CMV yields could not be related to the reported inhibitory effect of amiloride on protein synthesis. In amiloride (150 μM)-treated, CMV-infected cells, late, yet not immediate-early or early, protein synthesis was markedly decreased relative to untreated, CMV-infected cells. Accordingly, CMV DNA synthesis and the replication of CMV may be related to Na^+ entry through an amiloride-sensitive pathway.** [P.S.E.B.M. 1991, Vol 196]

Human cytomegalovirus (CMV) is a member of the β-herpesviridae family. Viruses within this family are characterized by a relatively slow replication cycle, a propensity to establish persistent infections, and, for human CMV, a ubiquitous distribution among the human population. Although widely recognized as a prevalent and often devastating agent of virally induced congenital abnormalities (1), CMV has more recently been the focus of attention due to its association with morbidity and mortality among immunosuppressed individuals (2, 3), particularly those suffering from acquired immune deficiency syndrome (4).

Recent research from our laboratory has demonstrated that CMV infection results in a characteristic series of morphologic and possibly related physiologic responses (5). These physiologic responses include Ca^{2+} influx, the intracellular sequestering of Ca^{2+}, a substantial increase in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) (6), and changes in the cellular levels of cyclic nucleotides (5). Earlier studies have shown that inhibitors of the Ca^{2+} and/or cyclic nucleotide responses interfere with CMV replication and substantially reduce infectious yields (7).

We have also shown previously that CMV induces Na^+ entry, an increase in Na^+/K^+-ATPase activity and that the entry of Na^+ is related to the development of cytomegaly and the formation of nuclear inclusions (NI) (8). That study was based primarily on data derived from pharmacologic inhibition of Na^+ entry with amiloride, an inhibitor of Na^+/H^+ exchange, and on physiologic inhibition of Na^+ entry with medium containing 4 mM Na^+ and 139 mM N-methyl-D-glutamine. The present study was undertaken to determine whether there is a relationship between the amiloride-sensitive cellular responses to CMV infection and CMV replication.

Materials and Methods

Cells and Viruses. Human embryo skin muscle (SM) cells were used in all experiments. Details regarding their derivation and method of propagation have been published previously (9). Frozen stocks of the AD169 strain of CMV prepared as described previously (11) were employed in all studies. Infectivities of the CMV stocks ranged from 5×10^6 to 2×10^7 plaque-forming units (PFU)/ml. Uninfected cell lysates prepared in the same manner as virus stocks were used in experiments for mock infection (6).

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Drugs. Amiloride was purchased from Sigma (St. Louis, MO). 9-(1,3-Dihydroxy-2-propoxymethyl) guanine (DHPG) was supplied by the Syntex Corp. (Palo Alto, CA). Stock solutions were prepared just prior to each experiment at a concentration of 2 mg/ml in water. Working solutions were made by diluting stock solution in maintenance medium (Eagle's minimal essential medium with Earle's salts supplemented with 5% γ-irradiated fetal calf serum and 0.15% $NaHCO_3$). Papaverine was supplied by Eli Lilly (Indianapolis, IN). Details regarding preparation of working solutions of papaverine have been reported previously (7).

CMV Replication Assay. CMV infectious yield assays were done as described previously (7). Selected concentrations of amiloride and other drugs were added to infected cells at 0-hr postinfection (PI) and fresh medium containing drug was added at 48-hr PI. The infectivity of virus yields was measured at 96-hr PI.

Development of Nuclear Inclusions. To study the development of NI, infected or mock-infected cells treated with or without amiloride were fixed in Bouin's fixative at selected times PI and stained with hematoxylin and alcoholic eosin as described previously (10).

DNA Analysis and Separation. The procedure for isolation and analysis of CMV DNA has been described in an earlier report (11). Briefly, infected and mock-infected cells were pulse labeled with [*methyl*-^{3}H]thymidine ([^{3}H]TdR, 10 μCi/ml; sp act 20 Ci/mmol; ICN Radiochemicals, Irvine, CA) for 12- or 24-hr periods, followed by disruption of the cells using a lysis buffer consisting of 0.1% Sarkosyl NL30 (Chemical Additives Co., Farmingville, NY), 0.02 *M* EDTA, and 0.1% Pronase. Cell and virus DNA were separated by isopycnic centrifugation in CsCl and the amount of acid-precipitable radioactivity was determined in a Beckman LS9000 liquid scintillation spectrophotometer.

Total Protein Synthesis Determinations. Total protein synthesis was estimated by determining the radioactivity in acid-precipitable material after pulse-labeling confluent SM cells in 35-mm tissue culture dishes for 24 hr with [^{3}H]leucine (10 μCi/ml, sp act 50 Ci/mmol), followed by disruption of the cells (15 min, 4°C) with a lysis buffer (0.01 *M* Tris-HCl [pH 7.4], 0.5% NP 40, 10% glycerol, and 0.5% sodium deoxycholate).

Polyacrylamide Gel Electrophoresis. Separation of CMV polypeptides was accomplished with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in a vertical slab gel according to the method of Laemmli (12). Confluent SM cells were pulse labeled in 35-mm tissue culture dishes for consecutive 6-hr periods with [^{35}S]methionine (15 μCi/ml, sp act 1000 Ci/mmol). At selected times PI, the cells were lysed, precleared with CMV-nonreactive antisera, and incubated (1 hr, 0°C) with convalescent sera reactive with CMV. Antigen-antibody complexes were precipitated with *Staphylococcus aureus* cells (Immunoprecipitin; BRL, Gaithersburg, MD), collected by sedimentation in a microfuge (9200 *g*, 2 min), and washed four times with washing buffer (0.5 *M* LiCl, 1% 2-mercaptoethanol, 0.1 *M* Tris-HCl, pH 9.0). Antigens were disassociated from antibody by boiling for 1 min in a sample mixing solution (1% SDS, 15% glycerol, 1% 2-mercaptoethanol, 0.004% bromophenol blue, 0.0625 *M* Tris-HCl, pH 6.7) before being applied to the gel. Each lane of the gel was loaded with the same amount of protein lysate after determination of the protein concentration by the Coomassie Blue G-250 method described for the Pierce protein assay (Pierce Chemical Co., Rockford, IL).

The separating gel contained 7% acrylamide, 0.25% bis-acrylamide, 0.025% TEMED, and 0.1% SDS in 0.375 *M* Tris-HCl (pH 8.9). The stacking gel was composed of 3% acrylamide, 0.08% bis-acrylamide, 0.025% TEMED, and 0.1% SDS in 0.125 *M* Tris-HCl (pH 6.7). A freshly made solution of 12% ammonium persulfate was added to each of the mixtures to a final concentration of 0.7% to initiate polymerization. The gel was subjected to electrophoresis in an electrode buffer containing 0.1% SDS, 0.05 *M* Tris, and 0.19 *M* glycine (pH 8.3) with a constant current of 25 mA until the bromophenol blue marker reached the bottom of the gel. The gel was then fixed and stained with a solution containing 2% Coomassie brilliant blue in 59% ethanol and 7% acetic acid. Autoradiography was performed following the method recommended by New England Nuclear (Boston, MA) using a fluorescence enhancer (EN^{3}HANCE) to treat the gel before vacuum drying and exposure to X-OMAT-R film (Eastman Kodak, Inc., Rochester, NY). Densitometry of exposed gels was performed on a Hoeffer model GS 300 scanning densitometer and analyzed with Hoeffer GS 360 densitometer software (Hoeffer Scientific Instruments, San Francisco, CA).

Immunofluorescence Staining. Indirect immunofluorescence staining of cells fixed on glass coverslips was accomplished as described previously (11). Either convalescent sera containing antibodies reactive to CMV or monoclonal antibody reactive against CMV immediate early (IE) (NEN product 9221) or late (NEN product 9220) antigen was used as the primary antibody. Cells were examined following addition of an appropriate fluorescein-conjugated antiserum (Cappel Labs. Inc., Cochranville, PA) with a Zeiss photomicroscope III equipped for fluorescence detection.

Determination of Cell Viability. The effect of amiloride on cell viability was determined by the ability of cells to exclude trypan blue. CMV-infected and mock-infected SM cells confluent in 25-cm^2 flasks were treated with amiloride (150 μM) at 0-hr PI. At selected times after addition of amiloride, the cells were washed, dissociated with trypsin, sedimented by centrifugation,

resuspended with a small volume of Eagle's minimal essential medium, and diluted 1/10 with a trypan blue solution (4%; Gibco, Grand Island, NY). Trypan blue-excluding (viable) cells were counted using a hemocytometer. LD_{50}'s were calculated as the drug concentration resulting in a 50% reduction in cell viability as determined by trypan blue exclusion.

Results

Amiloride Inhibition of CMV Replication. We initially examined the effect of the Na^+/H^+ exchange inhibitor, amiloride, on CMV replication, and compared the effect of this compound with two potent antiviral agents, DHPG (13) and papaverine (7). Concentrations of amiloride from 0 to 150 μM were chosen for these studies because concentrations within this range gave optimal inhibition of Na^+/K^+-ATPase increases as determined by ouabain-sensitive $^{86}Rb^+$ uptake with minimum cytotoxicity (8). Amiloride, at a concentration of 30 μM or less, had essentially no effect on CMV infectious yields (Fig. 1). There was a near-linear decrease in infectious yields as the concentration of amiloride was increased from 30 to 150 μM. The ED_{90} for inhibition of CMV replication in this experiment was 52 μM (similar results were obtained when this experiment was repeated with the ED_{90} for amiloride having a mean of 46 ± 9.2 μM). The effect of papaverine and DHPG on CMV yields was determined concurrently. The ED_{90}'s for papaverine and DHPG in this experiment were 1.4 and 1.6 μM, respectively.

Effect of Amiloride on the Development of CMV-Induced NI. The effect of amiloride on the development and progression of cytopathic effects within infected cells was examined from 0- to 96-hr PI. Consistent with our findings in an earlier study, amiloride (150 μM)-treated, CMV-infected cells failed to enlarge (8). Furthermore, the development of NI appeared to be incomplete (Fig. 2). Amiloride-treated, CMV-infected cells contained very small NI without the numerous characteristic cellulae seen in untreated, infected control cells (14, 15). Margination of the cellular chromatin, however, was evident in the amiloride-treated cells. Since the NI is thought to be the site of CMV DNA synthesis (16) and of assembly of nucleocapsids (14, 15), the decreased size of the NI suggested the possibility that amiloride interfered with CMV DNA synthesis (11).

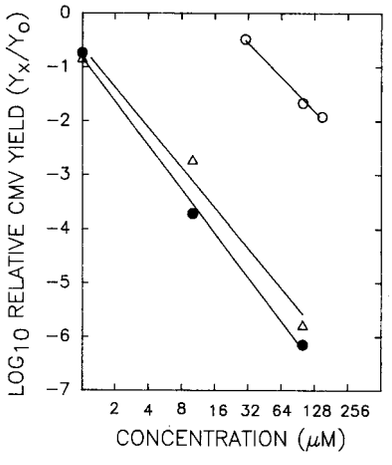

Figure 1. The effect of amiloride (○——○), DHPG (●——●), and papaverine (△——△) on the replication of CMV. SM cells were infected with CMV (5 PFU/cell) and treated at 0-hr PI with selected concentrations of the drugs. Fresh medium containing drug was added at 48-hr PI and virus yields were measured at 96-hr PI by plaque assay.

DNA Synthesis in Amiloride-Treated, CMV-Infected Cells. To determine the effect of amiloride on DNA synthesis, CMV-infected cells were treated with amiloride (150 μM) and pulse labeled for 12- or 24-hr intervals at selected times PI with [^{3}H]TdR. Figure 3 shows the data for sedimentation to equilibrium in cesium chloride of DNA extracted from CMV-infected cells in the presence and absence of amiloride. Amiloride inhibited viral DNA synthesis at all times after infection. CMV DNA synthesis before 36 hr was inhibited by approximately 30%. Inhibition of viral DNA synthesis reached 72% and 67% between 36- to 48 and 48- to 72-hr PI, respectively. The substantial increase in the degree of inhibition of CMV DNA synthesis at later times may indicate that late phase cellular responses to CMV infection may be more sensitive to amiloride inhibition than early phase responses. Amiloride (150 μM) also inhibited cellular DNA synthesis in mock-infected control cells as has been reported previously (19).

Effect of Amiloride on CMV-Induced Antigen Synthesis. In order to examine the effect of amiloride on CMV-induced antigen synthesis at a time consistent with the expression of IE and early CMV proteins, infected cells were examined at 6-hr PI by indirect immunofluorescence with human convalescent sera reactive to CMV IE gene products. In addition, a monoclonal antibody reactive against the major CMV IE gene product was used as a primary reagent. Using

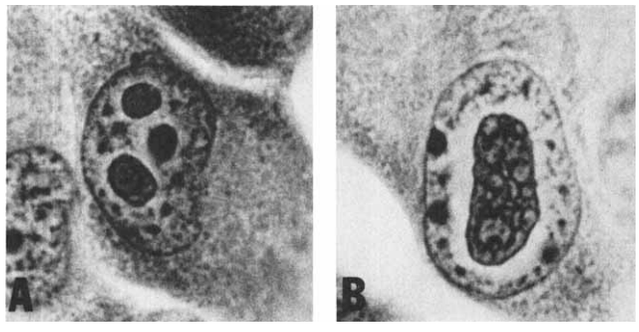

Figure 2. The effect of amiloride (150 μM) on the morphology of CMV-induced NI in SM cells. (A) Amiloride-treated, CMV-infected cells 72-hr PI. (B) CMV-infected cells in the absence of amiloride, 72-hr PI (original magnification ×915).

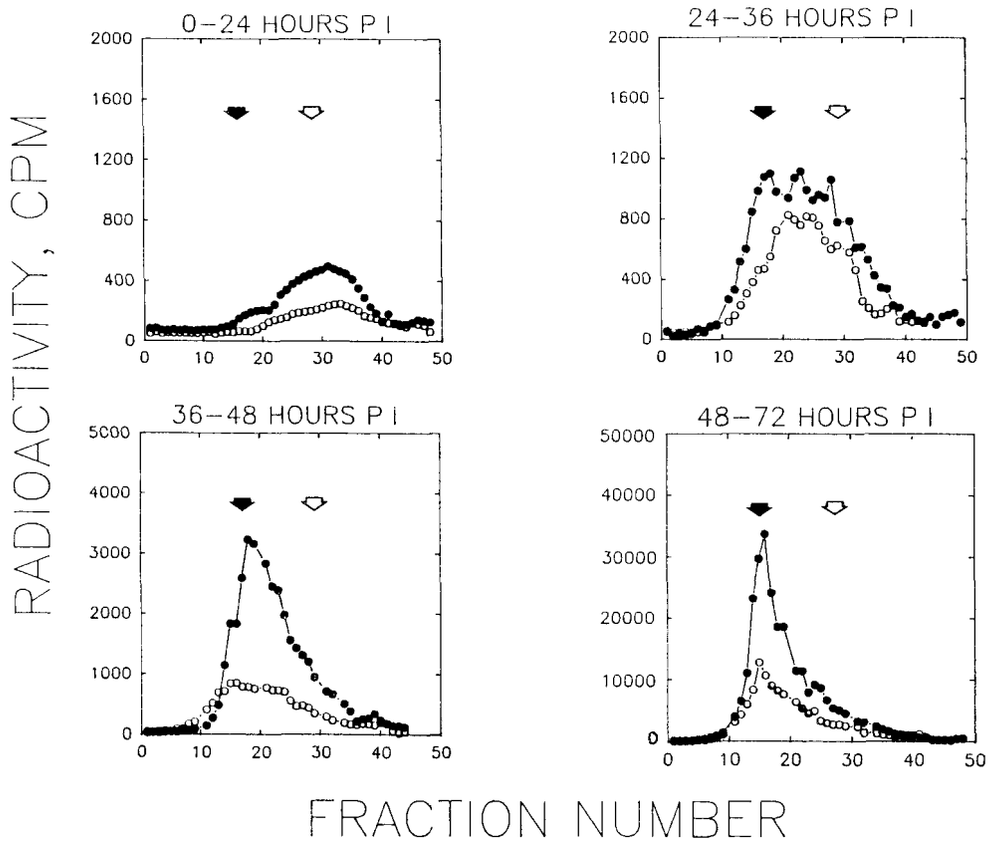

Figure 3. The effect of amiloride on the synthesis of CMV DNA. Amiloride (150 μM) was added to CMV-infected cells (5 PFU/cell) at 0-hr PI. Cells were labeled with [^{3}H]TdR at the indicated times and DNA was extracted as described in Materials and Methods. CMV and cellular DNA were separated by isopycnic density centrifugation through CsCl. ○——○, amiloride; ●——●, no amiloride. Maximum radioactivity for CMV DNA (closed arrow) or cellular DNA (open arrow) was usually found in Fraction 16 or 29, respectively.

either antibody preparation, bright nuclear fluorescence of similar intensity and location was observed in about the same proportion of amiloride-treated and the untreated, CMV-infected cells (Fig. 4, A and B). In contrast, at a later time (96-hr PI), when CMV-infected cells were examined using convalescent sera reactive with late structural CMV polypeptides of the virus, a substantial difference was evident in the fluorescence staining of infected cells in the presence and absence of amiloride. Bright fluorescence was observed in the nucleus and cytoplasm of the majority of the untreated, CMV-infected cells, yet fewer of the amiloride-treated, CMV-infected cells showed fluorescence with a similar intensity. A similar disparity was observed in cells imaged after reaction with a monoclonal antibody reactive against a CMV late polypeptide. In these experiments uninfected control cells in the presence or absence of amiloride demonstrated no detectable fluorescence

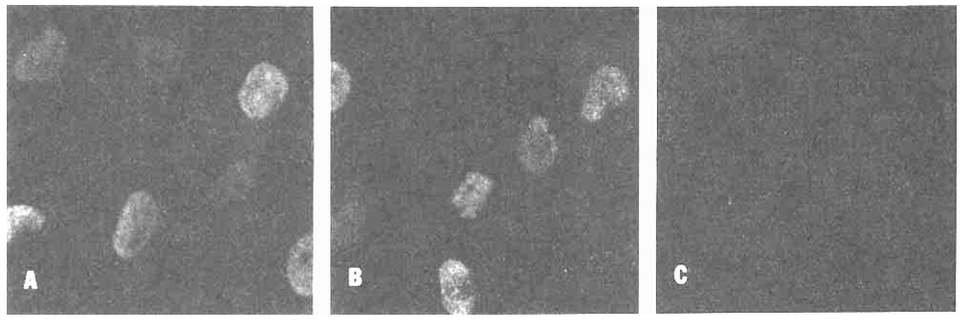

Figure 4. The influence of amiloride on detection of CMV-specific immediate early proteins with monoclonal antibody NEN 9221 at 6-hr PI. (A) CMV-infected cells in the absence of amiloride. (B) CMV-infected cells treated with amiloride (150 μM) from 0- to 6-hr PI. (C) Mock-infected cells without amiloride.

after treatment with either the IE or late monoclonal antibodies (Fig. 4C).

Since the immunofluorescence experiments suggested a possible effect of amiloride on CMV-induced protein synthesis, particularly at late times PI, a more detailed examination of the effect of amiloride on CMV-induced protein synthesis was undertaken. Peptides from amiloride-treated and untreated, CMV-infected cells were analyzed after immunoprecipitation and separation by polyacrylamide gel electrophoresis. No substantial differences were detected in the abundance of CMV polypeptides immunoprecipitated with convalescent sera from cells labeled at IE and early times PI (Fig. 5, A and B). Consistent with the observed inhibition of CMV DNA synthesis and the decreased fluorescence at late times (48- to 96-hr PI) in amiloride-treated cells, substantial reduction in the abundance of all detectable CMV-induced late polypeptides was observed in the presence of amiloride (Fig. 5, C and D).

Evaluation of Possible Toxicity of Amiloride. To assess the possibility that the observed reductions in CMV replication and macromolecular synthesis are a result of general toxic effects of amiloride, we evaluated the effect of amiloride on cell viability and overall ability to incorporate protein and DNA precursors. The reduction of infectious yields by amiloride was apparently not the result of cellular toxicity since the number of viable cells was reduced by less than 20% at 96-hr PI in the absence or presence of amiloride (150 μM) (data not shown). Under similar conditions, however, the uninfected, amiloride-treated (150 μM) cells did not incorporate comparable amounts of [^{3}H]TdR to those cells which were not treated with amiloride. This observation is consistent with those reported previously by Stiernberg *et al.* (19) for inhibition of cellular DNA synthesis and suggests further that inhibition of DNA synthesis is the primary mode of action for amiloride.

The extent to which amiloride affects the ability of CMV-infected cells to synthesize proteins in general was measured by the incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable material from 0- to 96-hr PI (Fig. 6). CMV- and mock-infected cells were pulsed labeled with [^{3}H]leucine for 24-hr intervals in the presence and absence of amiloride. CMV infection enhanced the level of protein synthesis in untreated cells by 135 and 283% during the labeling from 24- to 48- and 72- to 96-hr PI, respectively. Amiloride (150 μM) had about the same effect on protein synthesis in mock- and CMV-infected cells, reducing the radioactivity by 38% and 30% in mock- and CMV-infected cells, respectively, during the period from 24- to 48-hr PI. Greater reductions of 67% and 51% were observed for mock- and CMV-infected cells, respectively, at 72- to 96-hr PI. Thus, even in the presence of amiloride, CMV enhanced protein synthesis (6634 cpm) to a level greater than that for the mock-infected controls (3542 cpm) in the absence of amiloride.

Discussion

In earlier work it was demonstrated that CMV infection results in Na^+ entry that is amiloride-sensitive and related to the development of cytomegaly (8). In this study amiloride treatment of CMV-infected cells substantially reduced yields of infectious virus progeny. The present findings suggest that there may be a relationship between Na^+ entry and CMV replication. Apparently, the intracellular environment resulting from Na^+ entry (i.e., in the cytomegalic cells) is related to the success of CMV replication, and in particular to CMV DNA synthesis which was reduced by about 70% at late times in the presence of amiloride.

Amiloride inhibited CMV yields in a concentration-dependent manner between 30 and 150 μM. Concentrations less than 30 μM appeared to have very little, if any, effect on CMV yields or cytopathology. Concentrations of 200 μM or greater demonstrated some cellular toxicity and therefore were not used. Amiloride has been reported to affect Na^+ channels in fibroblasts with a K_i between 0.01 and 1 μM (17). Our findings suggest that amiloride concentrations affecting primarily Na^+ channels are not sufficient to inhibit CMV replication, since amiloride at concentrations of 30 μM or less had essentially no inhibitory effect on CMV yields. Amiloride has a reported K_i for inhibition of Na^+/H^+ exchange in fibroblasts between 3 and 300 μM (17). Thus, the present findings of reduction of CMV yields at concentrations greater than 30 μM are consistent with the hypothesis that activation of the Na^+/H^+ exchanger may be a part of the cellular responses induced by CMV (8) and may be involved in the replication of CMV, as well as in the development of cytomegaly.

How Na^+ entry is involved in CMV replication is not known at this time, although there may be some parallels between the cellular response to CMV infection and induction of cellular DNA synthesis. Initiation of cell DNA synthesis by growth factors is a complex process involving both monovalent (e.g., Na^+, H^+) and divalent (e.g., Ca^{2+}) cations. Two major components of this process appear to be alterations in $[Ca^{2+}]_i$ (18) and amiloride-sensitive Na^+ entry and H^+ efflux through the Na^+/H^+ antiport (19, 20). The resulting increase in cellular pH is postulated to be part of the signal initiating cellular DNA synthesis (18). Platelet-derived growth factor, for example, is reported to stimulate the Na^+/H^+ antiport and accordingly Na^+ entry and intracellular alkalinization (18, 20). Apparently, the physiologic responses associated with Na^+ entry and increased cellular pH that are characteristic of cell activation, provoke development of an intracellular environment consistent with the replication of cellular

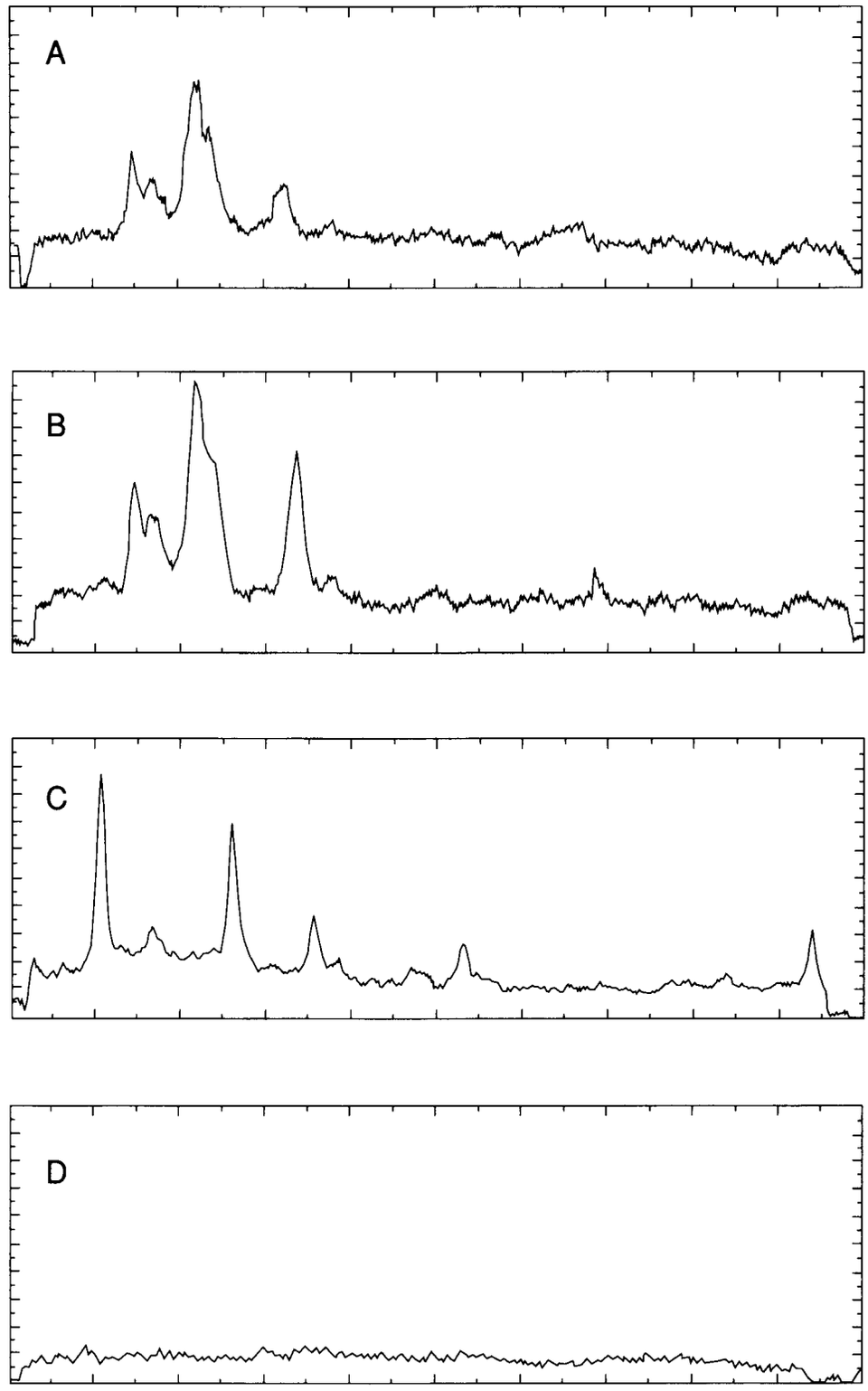

Figure 5. Densitometer tracings of the autoradiograms of electrophoretically separated, immunoprecipitated [^{35}S]methionine-labeled polypeptides from CMV-infected cells. (A) No drug, isotopically labeled 0- to 6-hr PI. (B) Amiloride (150 μM), isotopically labeled 0- to 6-hr PI. (C) No drug, isotopically labeled 90- to 96-hr PI. (D) Amiloride (150 μM), isotopically labeled 90- to 96-hr PI.

DNA. It may be noteworthy that restricted CMV infections are associated with stimulation of cellular DNA synthesis and an increase in the mitotic index (22, 23). On balance, it appears that some of the same cell activation responses that are necessary for cellular DNA synthesis may be involved in efficient CMV DNA synthesis.

Goodheart *et al.* (16) demonstrated that the CMV

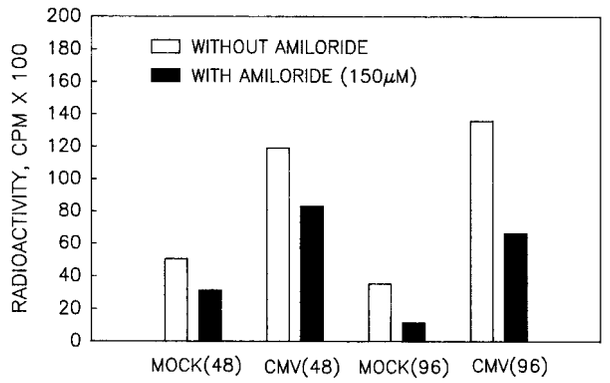

Figure 6. The effect of amiloride (150 μM) on total protein synthesis in CMV (5 PFU/cell) and mock-infected SM cells. Cells were pulse labeled from 24- to 48 (48)- or 72- to 96 (96)-hr PI with [^{3}H]leucine (10 μCi/ml) as described in Materials and Methods. Open bars, no amiloride; closed bars, amiloride (150 μM).

NI consists of virus DNA and protein. Thus, the development and progression of NI could be inhibited by several mechanisms, including inhibition of either viral DNA or protein synthesis, or both. Since late CMV protein synthesis is dependent on CMV DNA synthesis (24) and since CMV DNA replication is inhibited at late times by about 70% in amiloride-treated cells, substantial reductions in protein synthesis as shown in Figures 5 and 6 are consistent with the amiloride effect on DNA synthesis. Considering the amiloride effect on *in vitro* protein synthesis (25), however, it is possible that inhibition of CMV protein synthesis in addition to that resulting from reduction of CMV DNA synthesis also contributes to the reduction of CMV yields. This component is probably relatively minor though, at best, since immediate early and early protein synthesis, which are independent of CMV DNA synthesis, was not substantially affected in amiloride-treated, CMV-infected cells (Fig. 5).

The reduction in CMV yields by amiloride (approximately 100-fold at 150 μM, $ED_{90} = 46 \pm 9.2$ μM), while substantial, does not approach the remarkable reductions observed with the cyclic nucleotide modulator, papaverine (approximately 100,000-fold at 30 μM, $ED_{90} = 1.4$ μM), or the nucleoside analogue DHPG (approximately 100,000-fold at 30 μM, $Ed_{90} = 1.6$ μM), yet is greater than that observed for the Ca^{2+} influx blocker, verapamil (approximately 10-fold at 100 μM, $ED_{90} = 61.1$ μM) (7). Although, the exact mechanism of papaverine inhibition of CMV yields has not been described, both Na^+ (8) and Ca^{2+} (6) responses to CMV infection are inhibited by papaverine, possibly explaining its greater potency. The LD_{50}/ED_{90} ratio for these compounds indicate that amiloride ($LD_{50}/ED_{90} = 4.35$) is more effective than verapamil ($LD_{50}/ED_{90} = 0.3$) yet not as effective as papaverine ($LD_{50}/ED_{90} = 120$). Taken together, these results indicate that the Na^+ response to CMV infection, like the Ca^{2+} response, is important for efficient CMV replication.

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