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Ion transport blockers inhibit human rhinovirus 2 release

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Summary

Picornavirus replication causes leakage of cytoplasmic K^+ and an influx of Na^+ and Ca^{2+}. In this study, we have explored the possibility that a blockade of Ca^{2+} and Na^+ influx would reduce rhinovirus production and/or release. The Ca^{2+}-channel blockers, verapamil and diltiazem, as well as the blocker of Na^+/H^+ exchange and the epithelial Na^+ channel, EIPA, inhibited both virus production and release. The effect on virus release was more pronounced than the effect on production, thus raising the possibility that rhinovirus release may serve as a target for antiviral agents. Unexpectedly, our results also showed that the antiviral activity of the Ca^{2+}-channel blockers was not due to the block of Ca^{2+} influx. Similarly, the antiviral activity of EIPA appeared to be unrelated to the blockade of cellular Na^+/H^+ exchanger or the epithelial Na^+ channel. Potential alternative mechanisms of the antiviral activity of these compounds are discussed.

Keywords: Ion transport blockers; Rhinovirus

1. Introduction

The *Picornaviridae* are a family of non-enveloped animal viruses. They are currently divided into 9 genera, three of which are significant causes of human disease, the *Enteroviruses*, *Rhinoviruses* and *Hepatoviruses*. To date, no antiviral agent has been approved for the treatment of these infections. Drug development has been targeting steps of the replication cycle such as virus entry, RNA replication, protein synthesis and cleavage, and virion assembly. Here, we present evidence that progeny virus release from infected cells may have the potential to serve as an antiviral target.

It is assumed that the release of picornavirus progeny occurs via cell lysis, with the exception of hepatitis A virus. The mechanism of this process is largely unknown. Studies conducted on the *Enteroviruses* and *Cardioviruses* have demonstrated alterations in transport of ions and small molecules across the plasma membrane of infected cells, coinciding with virus production. Virus replication causes leakage of cytoplasmic K^+ and influx of Na^+ into the cytoplasm (Carrasco and Smith, 1976; Egberts et al., 1977; Lopez-Rivas et al., 1987; Nair, 1981; Nair et al., 1979; Schaefer et al., 1982). At the same time Ca^{2+} content of the ER and the Golgi is reduced and an influx of extracellular Ca^{2+} into the cytoplasm takes place (Irurzun et al., 1995; van Kuppeveld et al., 1997a). These events result in the collapse of ion gradients across the plasma membrane, and Na^+ and Ca^{2+} overload of infected cells. In addition to ions, non-permeant molecules under 750 Da become permeant whereas molecules over 10 kDa remain impermeant (Carrasco, 1978; Contreras and Carrasco, 1979). A hypothesis has been formulated that the changes in membrane permeability lead to cell lysis and release of virus progeny (Carrasco, 1995), which is partly supported by the data showing that the increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) facilitates enterovirus release (van Kuppeveld et al., 1997a).

We have explored the possibility that blockade of Ca^{2+} and Na^+ influx would reduce rhinovirus production and/or release. The blockers of Ca^{2+} channels, verapamil and dilti-

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azem, and the blocker of Na^+/H^+ exchange and the epithelial Na^+ channel, EIPA, inhibited both rhinovirus 2 production and release. However, the antiviral activity of these compounds was not due to the blockade of Ca^{2+} influx, or Na^+ influx via Na^+/H^+ exchanger or the epithelial Na^+ channel.

2. Materials and methods

2.1. Chemicals

Ion transport inhibitors were purchased from Sigma. Verapamil, (+)-*cis*-diltiazem and benzamil were dissolved in water; 5-(*N*-ethyl-*N*-isopropyl)amiloride and nifedipine in ethanol; and amiloride in DMSO.

2.2. Cells and viruses

HeLa T cells (picornavirus-susceptible HeLa line) were obtained from Victorian Infectious Diseases Reference Laboratory (Melbourne, Australia) and maintained in minimal essential medium (MEM, Gibco), supplemented with 5% heat-inactivated fetal bovine serum (FBS, Gibco).

Human rhinoviruses 2 and 14 were obtained from American Type Culture Collection (Manassas, USA) and propagated in HeLa T cells.

2.3. Virus infections

Cell monolayers in 24-well plates (Falcon) were incubated with the virus at the indicated multiplicity of infection (MOI) for 1 h at 34 °C, in MEM containing 1% FBS, except for the experiments conducted in Ca^{2+}-free medium. In that case Ca^{2+}-free MEM (S-MEM, Gibco) supplemented with 1% FBS was used for virus absorption. After the absorption the cells were washed once with the incubation medium, supplied with fresh medium, and further incubated at 34 °C for the indicated periods of time (calculated starting from the moment of virus addition to the cells).

2.4. Measurement of $[Ca^{2+}]_i$, virus yield and metabolic activity

$[Ca^{2+}]_i$ was measured using the cell-permeant fluorescent Ca^{2+} indicator fluo-4 AM (Molecular Probes). Cells in 24-well plates were washed with MEM, incubated with 5 μM fluo-4 AM in MEM for 30 min at 34 °C, then washed twice to remove extracellular indicator, and supplied with fresh MEM. The fluorescence of Ca^{2+}-bound fluo-4 was then measured on a Wallac 1420 VICTOR plate reader (Perkin-Elmer Life Sciences) using 485 nm excitation and 520 nm emission filters. Culture supernatants from separate wells were then transferred to Eppendorf tubes, and cells were lysed by freeze-thawing in an equal volume of fresh MEM. The HRV2 titres in cell lysates and culture supernatants were subsequently measured by plaque assay.

Metabolic activity of cells was measured using the indicator Alamar Blue (Serotec) that fluoresces as a result of its chemical reduction by living cells. Cells were washed with MEM and incubated with 10% Alamar Blue in MEM for 20 min at 34 °C. The plates were then read on the Wallac 1420 VICTOR plate reader using 530 nm excitation and 590 nm emission filters.

2.5. Confocal microscopy

Cells grown on coverslips were loaded with fluo-4 AM and ethidium bromide as follows. Thirty-five minutes before measurement, cells were washed once with MEM and incubated with 5 μM fluo-4 AM in MEM for 20 min at 34 °C. Ethidium bromide was then added to the medium to a final concentration of 50 μM, and incubation continued for another 10 min at 34 °C. The cells were subsequently washed with MEM twice, and the coverslip was placed into the chamber of an MRC-1024 confocal microscope (Bio-Rad). The cells were illuminated with the 488 nm line of a 100 mW argon ion laser. Fluorescence signals from Ca^{2+}-bound fluo-4 and ethidium ion were separated using a 565 nm long-pass filter, and collected using a 522 nm emission filter for fluo-4 and a 580 nm emission filter for ethidium. The data were collected using LaserSharp software (Bio-Rad).

3. Results

3.1. Ca^{2+} increase in HRV2-infected HeLa cells precedes membrane permeation to ethidium bromide

The increase in cell membrane permeability to cations and small molecules coinciding with virus production has been shown in studies conducted on enteroviruses and a cardiovirus. There are no published data regarding the membrane permeability of rhinovirus-infected cells. Therefore, we examined the effects of human rhinovirus 2 (HRV2) infection on the intracellular Ca^{2+} content of HeLa cells.

HeLa cells were infected with HRV2 at an MOI of 15 plaque-forming units (PFU) per cell, and intracellular Ca^{2+} concentration and virus titre were measured at 2-h intervals. $[Ca^{2+}]_i$ was measured using the cell-permeant fluorescent Ca^{2+} indicator fluo-4 AM and a fluorescence plate reader. The HRV2 titres were measured by plaque assay. The results demonstrated that HRV2 infection caused an increase in $[Ca^{2+}]_i$ which coincided with virus production (Fig. 1A), similar to the previously published data on poliovirus (Irurzun et al., 1995).

To examine whether the $[Ca^{2+}]_i$ increase in HRV2-infected cells coincided with the increase in membrane permeability to small molecules, HeLa cells grown on coverslips were infected with HRV2 as above and co-loaded with fluo-4 AM and ethidium bromide at 2-h intervals. The increase in $[Ca^{2+}]_i$ and the uptake of ethidium bromide were compared using confocal fluorescent microscopy.

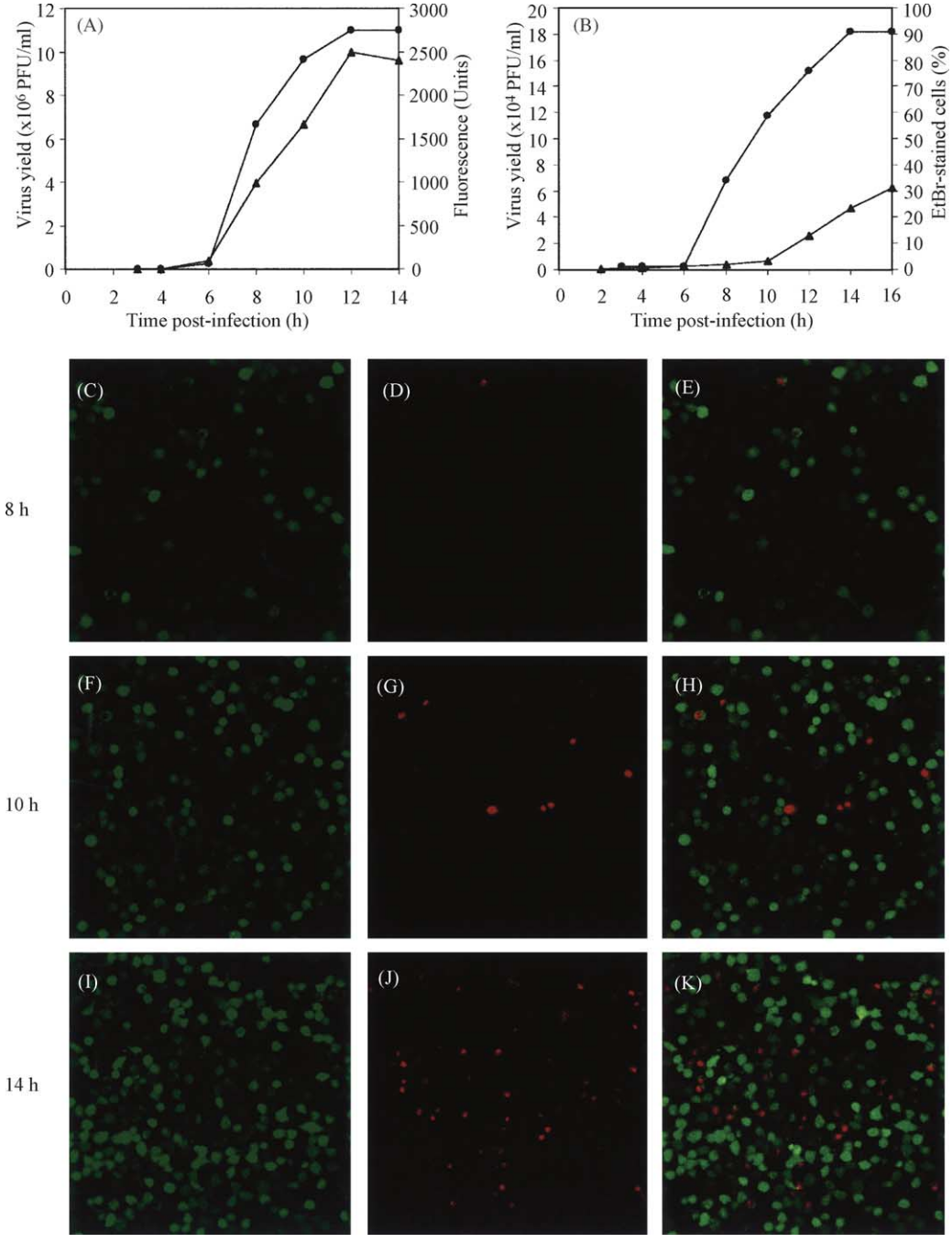

Fig. 1. Kinetics of HRV2 production and release, $[Ca^{2+}]_i$ increase and membrane permeation to ethidium bromide. (A) Time-courses of HRV2 production (●) and $[Ca^{2+}]_i$ increase (▲) in HeLa cells. Cells growing in 24-well plates were infected with HRV2 at MOI of 15 or mock-infected. The cells were loaded with the fluorescent Ca^{2+} indicator fluo-4 AM (5 μM) for 30 min before the indicated times. At each time the fluorescence of Ca^{2+}-bound fluo-4 was measured on a fluorescence plate reader, and HRV2 titre in a separate well measured by plaque assay. (B–K) HeLa cells growing on coverslips were infected with HRV2 at MOI of 15. The cells were loaded with 5 μM fluo-4 AM and 50 μM ethidium bromide, and visualised by confocal microscopy at the indicated times. HRV2 titres in culture supernatants from separate wells were measured by plaque assay. (B) Time-courses of HRV2 release (●) and accumulation of ethidium-stained cells (▲). The percentage of ethidium-stained cells at each time was calculated in four separate areas on a coverslip. (C, F and I) Fluo-4 fluorescence. (D, G and J) Ethidium fluorescence. (E, H and K) Merged images.

The first cells with increased $[Ca^{2+}]_i$ could be seen at 6 h post-infection (data not shown). The proportion of cells with elevated $[Ca^{2+}]_i$ increased with time (Fig. 1C, F and I), consistent with the time-course of $[Ca^{2+}]_i$ increase measured using the plate reader (Fig. 1A). The time-course of ethidium bromide uptake was different: very few cells were stained up to 10 h post-infection; after that the proportion of ethidium-stained cells started to increase more rapidly (Fig. 1B). These results demonstrated that $[Ca^{2+}]_i$ increase in HRV2-infected cells preceded membrane permeation to ethidium bromide.

The precedence of $[Ca^{2+}]_i$ increase in HRV2-infected cells to membrane permeation to ethidium bromide is also evident from the fact that the majority of the ethidium-stained cells showed little or no fluo-4 fluorescence (Fig. 1E, H and K). This is because the fluorescent form of fluo-4 AM, fluo-4, which is produced by intracellular de-esterification of fluo-4 AM, is retained by cells with intact plasma membrane (Handbook of Fluorescent Probes and Research Products, Molecular Probes). Plasma membrane permeation by HRV2 causes leakage of small molecules of fluo-4 (MW = 731.6) from the cell, coincidental with the uptake of ethidium ions. Therefore, the increase in $[Ca^{2+}]_i$ caused by the infection is only detected by fluo-4 if it takes place before the membrane permeation to small molecules.

These results demonstrated that the increase in intracellular Ca^{2+} concentration in HRV2-infected cells was not due to a non-specific plasma membrane permeation or cell lysis. This suggested that rhinoviruses may utilize cellular ion transport mechanisms to create ion changes in infected cells.

Also of interest is the observation that more than a half of the amount of HRV2 released in the course of a replication cycle was released by 10 h post-infection (Fig. 1B), at a time when no significant membrane damage occurred. This suggests that rhinoviruses may exit the cell using a mechanism other than cell lysis.

3.2. Ion transport blockers inhibit replication of rhinoviruses

If Ca^{2+} and Na^+ influx to picornavirus-infected cells is due to activation of cellular ion transport mechanisms, then blocking these mechanisms may inhibit virus replication. To test this hypothesis we selected a number of inhibitors of Ca^{2+} and Na^+ influx: verapamil, (+)-*cis*-diltiazem, nifedipine, amiloride, 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA), and benzamil. Verapamil, diltiazem and nifedipine are Ca^{2+}-channel blockers, all inhibiting voltage-gated Ca^{2+} channels, some ligand-gated Ca^{2+} channels, and some nonselective cation channels at μM concentrations (Diochot et al., 1995; Lenz and Kleineke, 1997; Taylor and Broad, 1998). Amiloride is a strong inhibitor of epithelial Na^+ channel (IC_{50} ~0.35 μM) and a weaker inhibitor of Na^+/H^+ exchanger (IC_{50} ~84 μM). In comparison to amiloride, EIPA is a much more potent inhibitor of Na^+/H^+ exchanger and a weaker inhibitor of Na^+ channel; whereas benzamil is a stronger Na^+-channel blocker, but a weaker blocker of Na^+/H^+ exchanger (Kleyman and Cragoe, 1988).

We examined the potential antiviral activity of the selected compounds on multiple cycles of HRV2 replication. HeLa cells were infected at an MOI of 0.01 and treated for 70 h with different concentrations of the compounds in MEM, 1% FBS, or left untreated. Virus titre in each sample (cells plus culture supernatant) was then determined by plaque assay. Cytotoxicity of the compounds was determined in parallel, by incubating mock-infected cells with the same concentrations of the compounds for 70 h and then measuring cell metabolism using the fluorescent indicator Alamar Blue.

The three Ca^{2+}-channel blockers had similar inhibitory effects on HRV2 production, with IC_{50} of ~7–12 μM (Fig. 2A). Ninety-nine percent reduction of virus yield was achieved with 50 μM verapamil, 75 μM nifedipine and 100 μM diltiazem. Antiviral effects of amiloride and its derivatives were much more varied. The antiviral activity of EIPA was similar to that of the Ca^{2+}-channel blockers, with IC_{50} of ~7 μM and 99% inhibitory concentration of 25 μM (Fig. 2B). Benzamil was a significantly weaker inhibitor of HRV2 replication than EIPA, with IC_{50} of ~40 μM; and amiloride had even less antiviral effect (Fig. 2B).

At 99% inhibitory concentration, diltiazem caused less than 15% reduction in cell metabolism; the effects of 50 μM verapamil, 75 μM nifedipine and 25 μM EIPA on cell metabolism were more pronounced: 35, 39 and 44% reduction respectively (Fig. 2C and D).

These results demonstrated that Ca^{2+}-channel blockers and EIPA had antiviral activity against HRV2. We also tested verapamil and EIPA against human rhinovirus 14 (HRV14), which belongs to a different species of the *Rhinovirus* genus, and observed very similar inhibitory activity as against HRV2 (data not shown).

3.3. Stages of HRV2 replication cycle affected by Ca^{2+}-channel blockers and EIPA

To determine which stages of the rhinovirus replication cycle are inhibited by Ca^{2+}-channel blockers and EIPA, time-of-addition experiments were performed on HeLa cells infected with HRV2 at an MOI of 1.5. Verapamil or EIPA were added to the culture medium (MEM, 1% FBS) 1 h before the infection, at the time of HRV2 addition, or at various times thereafter, and incubated until 12 h post-infection. Final concentrations of verapamil and EIPA in the culture medium were 50 and 25 μM, respectively. When the compounds were added before the infection, both virus and cells were pretreated. Guanidine hydrochloride (2 mM), an inhibitor of picornaviral RNA replication (Caliguiri and Tamm, 1968), was used as a control. The effects of the compounds on cell metabolism were measured in the same experiments after 13 h of treatment of uninfected cells. Verapamil and EIPA were not cytotoxic under these conditions, with cell metabolism in both cases 96% (±6% standard deviation) of that of untreated cells.

The time-courses of HRV2 production and release were measured in parallel to the time-of-addition. The production of progeny virus started 6 h after virus addition to the cells (Fig. 3A), with the release of the produced virus starting 2 h later (Fig. 3B). At 12 h post-infection around 5% of progeny virus was released into the culture medium (Fig. 3A and B).

Guanidine was fully effective when added to the cells before the start of new virus production. Its addition during the course of virus synthesis resulted in a time-dependent decrease of the antiviral effect, as expected (Fig. 3C). The

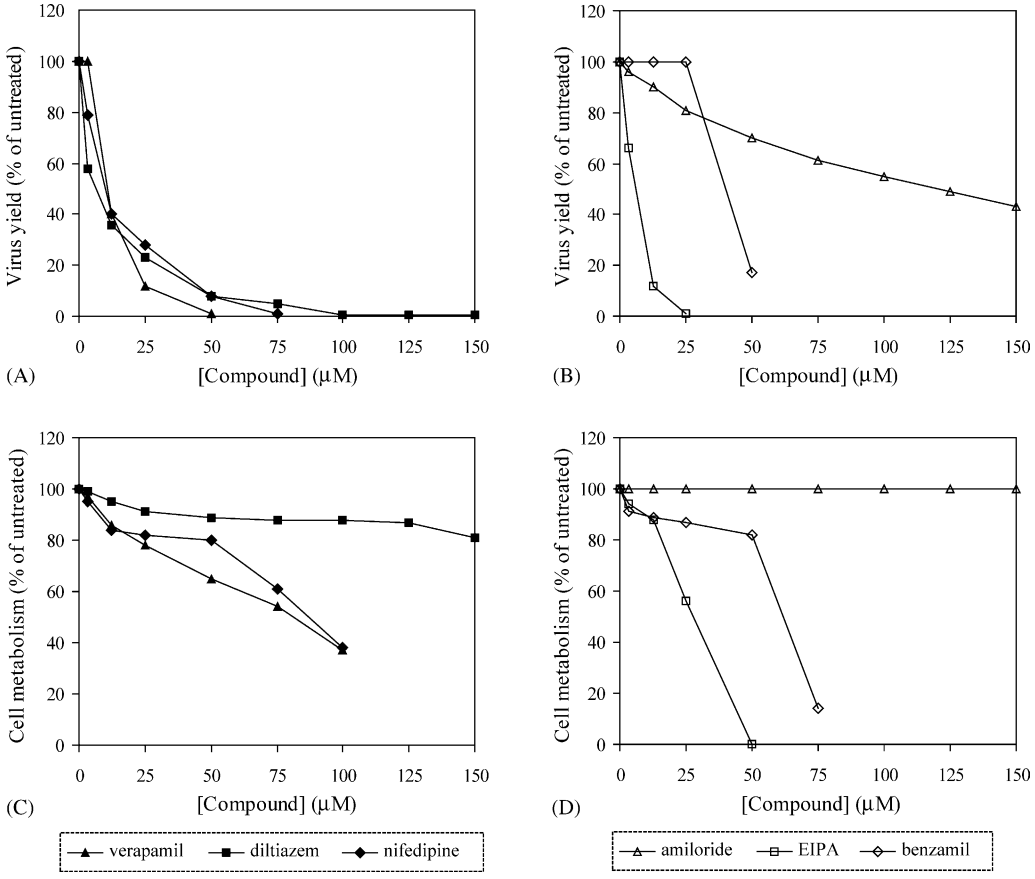

Fig. 2. Antiviral and cytotoxic effects of ion transport blockers. HeLa cells growing in 24-well plates were infected with HRV2 at MOI of 0.01 or mock-infected. After virus absorption, cells were incubated with the indicated concentrations of verapamil (▲), diltiazem (■), nifedipine (♦), amiloride (Δ), EIPA (□), or benzamil (◊) for 70 h, or left untreated. Infected cells were then lysed into culture medium by freeze-thawing, and virus titre was measured by plaque assay. Cytotoxicity of the compounds after 70 h of incubation was measured on mock-infected cells, by incubating the cells with fluorescent indicator Alamar Blue (10%) for 20 min, and measuring fluorescence on a plate reader. (A and B) HRV2 yield at different concentrations of the compounds. (C and D) Cell metabolism at different concentrations of the compounds. The data are the average of three independent experiments.

yield of the extracellular virus was inhibited by guanidine to the same extent and with the same time-dependence as that of the intracellular virus (Fig. 3D), consistent with the fact that guanidine has no effect on virus release from the cells.

EIPA had a modest inhibitory effect on HRV2 production in a single cycle. An average of 2.3-fold reduction of intracellular virus yield was observed when EIPA was added between 1 h before and 6 h after infection (Fig. 3E). There was some variability in virus yield between these time-points; however, consistent decrease of the antiviral effect occurred only when the compound was added after 6 h of infection, suggesting that it affects one or more steps in virus synthesis: RNA replication, protein synthesis or virus assembly.

The maximal effect of EIPA on extracellular virus yield in a single cycle was significantly larger than that on intracellular virus yield: around 8-fold reduction when the compound was added up to 8 h post-infection (Fig. 3F). This result suggested that, unlike guanidine, EIPA inhibited virus release.

Verapamil, when added to the cells before or together with the virus, caused a 2.5-fold inhibition of HRV2 production (Fig. 3G), similar to the effect of EIPA. However, time-dependence of its inhibitory effect was different to that of EIPA. Addition of verapamil at 1 h post-infection resulted in a two-fold decrease of the antiviral effect, signalling that verapamil affects HRV2 entry (Fig. 3G). Then no change in the activity of the compound was observed until the start of virus synthesis at 6 h post-infection, after which it began to diminish again (Fig. 3G). These results suggested that verapamil inhibited virus entry and virus synthesis.

Like EIPA, verapamil had an effect on virus release. Maximal inhibition of extracellular virus yield was 10-fold (Fig. 3H) in comparison with 2.5-fold inhibition of intracellular virus yield (Fig. 3G). The two-fold increase in virus production between 0 and 1 h addition of verapamil resulted in a similar increase in extracellular virus yield between these time-points (Fig. 3H). This higher virus yield was sustained, with some variability, between 1 and 8 h time-of-addition. Addition of verapamil after 8 h post-infection, when both virus synthesis and release were already taking place, resulted in further decrease of its effect on extracellular virus yield.

The time-dependence of inhibitory effects of 100 μM diltiazem on HRV2 was very similar to that of verapamil (data

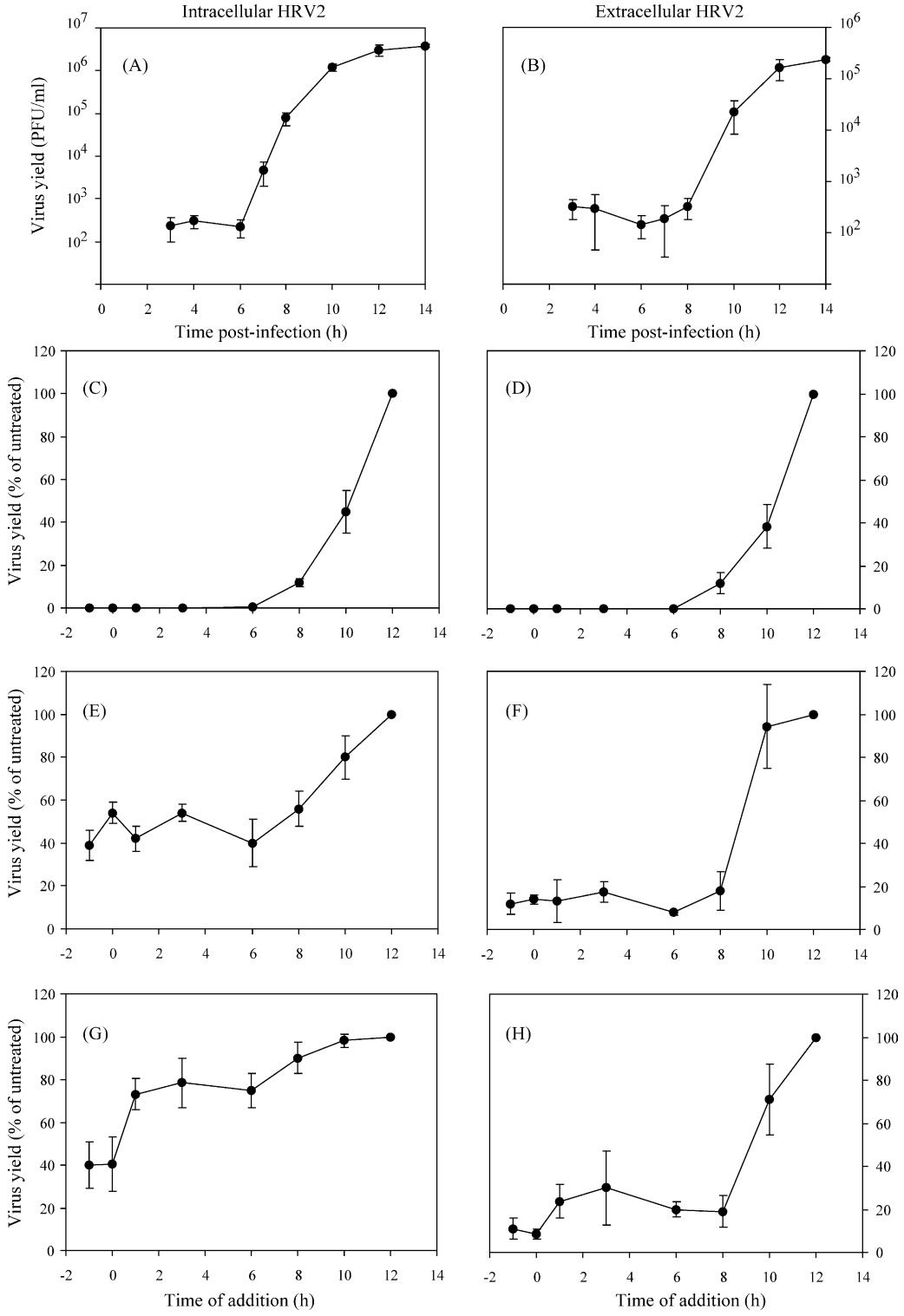

Fig. 3. Dependence of antiviral effects of guanidine, EIPA and verapamil on time of addition. (A and B) Time-courses of HRV2 production and release. HeLa cells growing in 24-well plates were infected with HRV2 at MOI of 1.5, and incubated for the indicated periods of time. Culture supernatants were then collected, cells lysed in equal volumes of fresh medium by freeze-thawing, and HRV2 titres in cell lysates (A) and culture supernatants (B) were measured by plaque assay. (C–H) The cells were infected as above, and 2 mM guanidine, 25 μM EIPA, or 50 μM verapamil were added to the culture medium at the indicated times (calculated from the time of virus addition). At 12 h post-infection virus titres in cell lysates (C, E and G) and culture supernatants (D, F and H) were determined as above. (C and D) Guanidine. (E and F) EIPA. (G and H) Verapamil. The data points are averages + standard deviations from four independent experiments using duplicate samples.

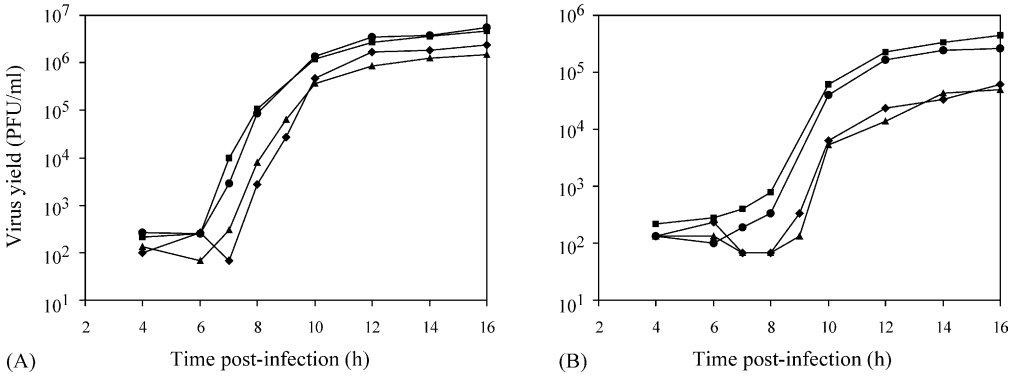

Fig. 4. Effects of Ca^{2+}-free medium, EIPA and verapamil on the kinetics of HRV2 production and release. HeLa cells growing in 24-well plates were infected with HRV2 at MOI of 1.5, and incubated in nominally Ca^{2+}-free MEM, 1% FBS (■); MEM, 1% FBS (●); MEM, 1% FBS, supplemented with 25 μM EIPA (♦); or MEM, 1% FBS, supplemented with 50 μM verapamil (▲) from 1 h before the infection until the indicated times post-infection. Then culture supernatants were collected, cells lysed in equal volumes of fresh medium by freeze-thawing, and HRV2 titres in cell lysates (A) and culture supernatants (B) were measured by plaque assay. One representative experiment of three is shown.

not shown), suggesting that Ca^{2+}-channel blockers have a common mechanism of activity.

The reduction of extracellular virus yield in a single replication cycle by EIPA and verapamil (Fig. 3F and H) was about 10 times lower than the reduction of the total virus yield after multiple cycles of infection achieved by the same concentrations of the compounds (Fig. 2A and B). The difference is likely to be due to a cumulative effect after multiple cycles of infection. However, we cannot exclude the possibility that the cytotoxic effects of the compounds may have contributed to the reduction in virus yield after longer incubation times.

3.4. $[Ca^{2+}]_i$ increase does not enhance HRV2 production or release

Earlier work has shown that $[Ca^{2+}]_i$ increase facilitates enterovirus release as incubation of CVB3-infected HeLa cells in Ca^{2+}-free medium slowed the release without affecting virus production (van Kuppeveld et al., 1997a). Therefore, we examined the effects of verapamil, EIPA, and Ca^{2+}-free medium on the kinetics of HRV2 production and release.

HeLa cells were infected with HRV2 at MOI of 1.5 and incubated in nominally Ca^{2+}-free MEM supplemented with 1% FBS, or in MEM, 1% FBS. The latter samples were either treated with 25 μM EIPA or 50 μM verapamil starting from 1 h before the infection, or left untreated.

Incubation in nominally Ca^{2+}-free medium prevented $[Ca^{2+}]_i$ increase in infected cells, as expected (data not shown). However, this had no negative effect on virus production or release. Instead, a small increase in virus release was observed (Fig. 4).

Verapamil and EIPA delayed the starts of virus production and release for 1 h in comparison with those in the untreated cells (Fig. 4). Once started, virus production and release in the presence of the compounds occurred at rates similar to those in untreated cells. However, at completion virus yields were lower (Fig. 4).

These results suggested that $[Ca^{2+}]_i$ increase during rhinovirus infection does not enhance virus production or release. Therefore, the antiviral effects of Ca^{2+}-channel blockers cannot be due to inhibition of Ca^{2+} influx to infected cells.

4. Discussion

Picornavirus replication causes leakage of cytoplasmic K^+ and influx of Na^+ and Ca^{2+} into the cytoplasm. In this study, we have explored the possibility that blockade of Ca^{2+} and Na^+ influx would reduce rhinovirus production and/or release.

We have demonstrated that $[Ca^{2+}]_i$ increase in HRV2-infected HeLa cells precedes plasma membrane permeation to ethidium bromide. This suggested that virus-induced cation fluxes may occur via cellular ion transport pathways, which could be blocked by ion transport blockers. Consistent with this hypothesis, three Ca^{2+}-channel blockers – verapamil, diltiazem and nifedipine – inhibited HRV2 replication at the concentrations often used to block Ca^{2+} influx. Verapamil was equally active against HRV14, belonging to another species of the *Rhinovirus* genus. Amiloride derivative EIPA, a blocker of Na^+/H^+ exchanger and epithelial Na^+ channel, inhibited replication of rhinoviruses 2 and 14 as effectively as the Ca^{2+}-channel blockers.

Verapamil, diltiazem and EIPA inhibited both HRV2 production and release, with the effect on release being more pronounced than the effect on production. To the best of our knowledge, this is the first report of rhinovirus release being a target of antiviral action.

Unexpectedly, further experiments demonstrated that the antiviral activity of the Ca^{2+}-channel blockers cannot be due to the block of Ca^{2+} influx. Incubation of infected cells in nominally Ca^{2+}-free medium, which prevented Ca^{2+} influx, had no effect on HRV2 production and slightly increased virus release. Thus, $[Ca^{2+}]_i$ increase during rhi-

novirus infection does not enhance virus production (similar to enteroviruses) or release (opposite to enteroviruses, van Kuppeveld et al., 1997a).

The antiviral activity of EIPA is also unlikely to be due to the inhibition of Na^+ influx. This conclusion is based on the result that the activity of amiloride derivatives against HRV2 decreased in the order EIPA > benzamil > amiloride (Fig. 2). This does not correspond to the relative potencies of these compounds as inhibitors of the Na^+/H^+ exchanger (EIPA ≫ amiloride > benzamil) or the epithelial Na^+ channel (benzamil > amiloride ≫ EIPA) (Kleyman and Cragoe, 1988).

Since the antiviral activity of the Ca^{2+}-channel blockers and EIPA appears to be unrelated to the blockade of cellular transporters responsible for the Ca^{2+} and Na^+ influx, what could be the alternative mechanisms of action? Two studies have suggested that EIPA could inhibit picornavirus replication due to its effects on the intracellular pH. Suzuki et al. (2001) have shown that EIPA inhibits HRV14 replication in human tracheal epithelial cells, and decreases the number of acidic endosomes. Therefore, they concluded that its antiviral activity is due to the block of rhinovirus RNA entry from acidic endosomes to the cytoplasm. However, our results contradict this conclusion, showing that in HeLa cells EIPA does not affect virus entry. In another study, Holsey et al. (1990) have shown that poliovirus infection causes an increase of cytoplasmic pH which promotes virus production. EIPA inhibited both the pH rise and poliovirus production when added after virus absorption. The effects of EIPA on virus release have not been examined in these studies; however, it is possible that the cytoplasmic and/or endosomal pH is important for this process.

A different possibility is that the antiviral activity of EIPA and/or the Ca^{2+}-channel blockers may be due to interactions with viral rather than cellular proteins. Recent studies have shown that amiloride derivatives similar to EIPA block ion channels formed by the HIV Vpu and HCV p7 proteins (Ewart et al., 2002; Premkumar et al., 2004), and inhibit HIV replication (Ewart et al., 2004). Picornaviral transmembrane protein 2B has some structural similarity with the Vpu and p7 proteins. It is crucial for viral RNA replication and may play a role in virus release (Aldabe et al., 1996, 1997; Doedens and Kirkegaard, 1995; Johnson and Sarnow, 1991; van Kuppeveld et al., 1995, 1997a, 1997b).

In conclusion, although the mechanisms of the antiviral activity of verapamil, diltiazem, nifedipine and EIPA remain to be elucidated, the inhibition of rhinovirus release by these compounds supports the idea that this stage of infection may become a target for antiviral agents.

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