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Antiviral agents alter ability of HSV-2 to disrupt gap junctional intercellular communication between mammalian cells in vitro

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Abstract

In cultured mammalian cells (Vero), different antiviral agents change to differing degrees the ability of HSV2 to down-regulate gap junctions, each agent having a specific effect. Measured by intracellular electrodes, control cell populations showed 49–51% coupling, uninfected populations treated with acyclovir or SDS averaged 43–51% coupling while populations infected with HSV2 had coupling reduced to 8%. The antiviral agent acyclovir (1 μg/ml), which suppresses viral replication, failed to prevent this down regulation (final coupling ratio of 11%). A plant extract (250 μg/ml) from *Pilostigma thonningii* offered slightly more protection (final coupling ratio of 22%), while sodium dodecyl sulfate (SDS) (50 μM) afforded nearly complete protection (final coupling ratio of 40%). With SDS there was an initial down regulation to only 16% coupling by 3 h post infection, followed by a recovery of intercellular communication to near control levels by 24 h. While SDS was originally believed to alter the viral coat and prevent entry into the cell, our data are in agreement with recent studies which indicate that SDS treated viruses can enter into host cells, but in a severely diminished condition. Our data also suggest that the gap junction antagonist is brought into the cells as part of the entering virus.

Keywords: Acyclovir; SDS; Electrical coupling

1. Introduction

Quantification of the virulence of a viral infection, and the degree to which an antiviral agent may protect cells are most commonly determined by cytopathic effects or by standard plaque reduction techniques. However, these are not manifest until 24–72 h following infection. Techniques used to study the initial time-dependent events of viral infection have included examinations using electron microscopy (Piret et al., 2000) or pulse-chase radio-labeling of virally directed nucleic acid and/or protein synthesis (Achenheimer and Roizman, 1972; Frenkel and Roizman, 1972; Jacquemont and Roizman, 1975; Roberts et al., 1991; Mardassi et al., 1996), none of which allow monitoring of viral activity in real time. It

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has long been known that certain viruses cause down-regulation of gap junctions among infected cells (Atkinson et al., 1981; Azarnia and Loewenstein, 1984; Denis et al., 1989; Crow et al., 1990; Danave et al., 1994; Ennaji et al., 1995; Faccini et al., 1996), demonstrated in most cases by the loss of dye coupling. Recently, we have shown that virally induced down-regulation of gap junctions can be followed by electrophysiological techniques, allowing in real time, observation of ongoing changes caused by viral activity. Furthermore, the degree to which any two cells are communicating can be quantified. This also allows regular and precise monitoring of differences in the protection from gap junction down-regulation afforded cells by antiviral agents (Fischer et al., 2001).

Antiviral agents may act to suppress the virus at many stages in infection, each individual agent having efficacy at a particular stage. Agents such as the drug acyclovir are known to suppress viral infection by blocking replication of the viral DNA (Elion et al., 1977; Bernstein et al., 2000; Franchetti et al., 2000; Stevenson et al., 2000), while others are known or assumed to suppress attachment or entry of the virus. Recent reports have shown the detergent sodium lauryl sulfate (sodium dodecyl sulfate) (SDS) may be a highly effective antiviral agent (Howett et al., 1999; Krebs et al., 1999; Piret et al., 2000). This detergent is regularly used to denature proteins, and it has been proposed that SDS attacks both lipids in the viral coat and structural viral proteins (Howett et al., 1999).

In this report we have tested one suspected and two known agents with anti-viral activity. Our purpose was three-fold. Firstly, we wished to determine the degree to which electrophysiological monitoring of gap junction closure was an effective means of distinguishing among anti-viral agents. Secondly, we wished to gain insight into the specific mechanism by which HSV2 effected host cell gap junctional intercellular communication. Thirdly, because of its potential for prevention of sexually transmitted viral infection, we wished to accumulate more information of the effects of SDS upon an infecting virus, specifically upon the ability of the virus to down-regulate gap junctions. We show that electrophysiological monitoring of gap junctions can reveal differences in anti-viral agents. Acyclovir, the methanol extract from the African plant *Pilostigma thonningii*, and SDS each produced different effects upon HSV2 induced changes in coupling. Our investigations have also added information concerning details of HSV2 induced down-regulation of gap junctions. Finally, we have shown that the anionic detergent SDS does not prevent early down-regulation of gap junctions, but does allow infected cells to recover.

2. Materials and methods

2.1. Cell culture and HSV-2 infection

Vero cells, African Green Monkey kidney cells, American Type Culture Collection, ATTC, Rockville, MD, were propagated as the continuous cell line to be used as the permissive cell type for these experiments. Cell culture and infection were carried out as previously described (Fischer et al., 2001). In all experiments in which an anti-viral agent was being tested, all incubation medium contained the agent.

2.2. Choice of antiviral agents

The antiviral agents used here were chosen for the following reasons. Acyclovir is a commonly used agent, the action of which is well understood (Elion et al., 1977). Its effectiveness in plaque reduction is well established, but little was known of its effect on viral down-regulation of gap junctions. The methanol soluble extract from *P. thonningii* is currently under study in one of our labs (G.N.K.M.), and served as a test of differential activity by a potential antiviral agent. SDS has recently been identified as having antiviral activity (Howett et al., 1999; Krebs et al., 1999; Piret et al., 2000), has been presumed to have a different mode of action from acyclovir, and has been proposed as being particularly promising in the prevention of sexually transmitted viruses.

2.3. Testing the effectiveness of antiviral agents

Data were gathered from: (a) uninfected controls, (b) uninfected controls in the presence of each of the antiviral agents, and (c) 1 mM octanol treated cultures. Octanol is a known down-regulator of gap junctions (Bohrmann and Haas-Assenbaum, 1993; Gho, 1994; Burghardt et al., 1995; Charles et al., 1996; Adler and Woodruff, 2000). (d) Cells infected with virus pre-treated with each of the separate agents being tested.

2.4. Treatment with anti-viral agents

2.4.1. Acyclovir

Prior to infection, cells were treated for 1 h with 1 μg/ml acyclovir. They were then exposed to the virus at 10 pfu/cell for an additional hour. The final concentration of acyclovir in the pre-treatment and incubation media was constant at 1μg/ml.

2.4.2. P. thonningii extract

Material from this plant was obtained in the following manner. Dried whole leaves and stems of *P. thonningii*, obtained from West Africa, courtesy of Dr. Mamadou Foula Barry, University of Conakry, Guinea, were used as the source of crude extracts. Leaves and stems were ground to a coarse powder using a standard Waring blender. The powder was then extracted in a soxhlet extractor using a 50% v/v methanol water solution, and allowed to continue until the return was clear. The extract was collected and dried at 60 °C. Finally, the extract was scraped from the evaporating dish and stored in airtight vials at room temperature. The dried extract was then weighed out and dissolved in 0.2 ml DMSO per 10 g extract prior to each assay. Once dissolved, it was mixed with serum-free media and sterilized by filtration, using a 0.45 μm filter. Final concentration of this extract used to treat cells was 250 μg/ml.

2.4.3. SDS

A stock of HSV-2 (10^8 pfu) in MAC 5A was diluted 1:1 with MAC 5A containing 100 μM SDS to reach a final SDS concentration of 50 μM and then incubated for 1 h at 37 °C to allow SDS to react with the virus. Following incubation, appropriate dilutions were made to deliver an m.o.i. of 10 per Vero cells in culture. Chamber slides with monolayers of confluent Vero cells were decanted, 1 ml of viral/antiviral mixture was added to each well as described above.

2.5. Electrical measurements

Made of proteins called connexins, gap junctions are patent channels which lead from the cytoplasm of one cell to that of a contiguous cell. The structures formed by these proteins are called connexons (for recent reviews of gap junction structure and function, see Kumar and Gilula, 1996; Lo, 1999). These channels allow the passage of molecules up to about 2000 da mol. wt. Both dye coupling and electrical coupling are regularly used to reveal intercellular communication through gap junctions, with electrical coupling being recognized as more sensitive and quantifiable (Loewenstein et al., 1978; Flagg-Newton et al., 1979). We have previously shown that monitoring electrical coupling may be useful in studying early events of viral infection (Fischer et al., 2001). Electrophysiological data were collected at 0 time (just prior to introduction of the virus) and at 3, 6 and 24 h post-infection. In unprotected cells at 3 h post infection, viral gene expression is known to be occurring (Honess and Roizman, 1974; Ackermann et al., 1984), and viral replication is about to commence. By 6 h post infection viral replication has been shown to be actively occurring (Roizman et al., 1963). At neither of these time periods are there any morphological signs of infection. Under the conditions of incubation used, at 24 h post infection cells had become multinucleate, but had not yet become rounded nor lost contact with surrounding cells. For electrophysiology, an Olympus CK-2 inverted microscope (Olympus Instruments, Japan) equipped with Single Sideband optics (Ellis, 1978) was used. Cells remained in the Tissue Tek chambers (Miles Scientific, Naperville, IL) in which they had been incubated. Following removal of their tops and sides, chambers were grounded through a Ag/AgCl wire. Standard 3 M KCl microelectrodes were attached to S-7071A

electrometers for membrane potential measurements, or to an S-7061A iontophoresis unit to provide current pulses. Electrometers and iontophoresis unit operate within an S7100A mainframe (all from World Precision Instruments, Sarasota, FL). Signals were stored on a Tektronix (Beaverton, OR) 5116 dual-beam storage oscilloscope with a 5D10 waveform digitizer. Electrodes were carried by either precision Chambers-type micropositioners (Line Tool Co., Allentown, PA) or a Barber-type positioner (E. Slobotka Co., Farmingdale, NY) fitted with a Narishige MMO-203 hydraulic single axis manipulator (Narishige USA, Greenvale, NY) for the final advance.

Electrical coupling was tested by impaling one cell with both stimulating and recording microelectrodes. A contiguous cell was then impaled with a second recording electrode, the original cell was stimulated, and the change in membrane potential in each cell was recorded. The coefficient of coupling, a commonly used measure of cell-to-cell communication, was taken to be $\Delta V_2/\Delta V_1$ where ΔV_1 was the change in the stimulated cell and ΔV_2 was the change in the responding cell (Loewenstein, 1979). Where convenient and where it aids in clarity we have also expressed coupling coefficients as percent coupling (coupling ratio) ($[\Delta V_2/\Delta V_1]*100$).

3. Results

3.1. Electrophysiological measurements

3.1.1. Establishing the degree to which HSV2 down-regulates gap junctions

Uninfected controls and untreated HSV2-infected cell populations behaved as previously reported (Fischer et al., 2001) (Table 1). Control populations showed coupling coefficients of about 50%, while introduction of the virus caused gap junctions to be down-regulated in a predictable manner to about 8% by 24 h post infection. To determine how fully HSV2 down-regulated gap junctional communication, we treated cultures with the known gap junction antagonist, 1 mM octanol, which was fully effective within 15 min. This established that the "coupling ratio" for cells with fully down-regulated gap junctions was a mere 6.5%. Thus the 8% value of HSV2-treated cells must represent nearly complete closure of the connexon lumina.

3.1.2. The effectiveness of anti-viral agents

3.1.2.1. Acyclovir. As with each of the known or suspected anti-viral agents, we first tested for any toxic effects of acyclovir by regularly monitoring coupling coefficients while incubating cells for $>$ 30 min (and up to 24 h) in the presence of a 1 μg/ml solution dissolved in regular growth medium. The presence of acyclovir alone had no effect on the coupling ratio of the cultured Vero cells, as evidenced by the average 51% value recorded. During the first 24 h following introduction of the virus, this compound provided only minimal protection from viral down-regulation of gap junctions. The average coupling ratio at 24 h post infection for cells exposed to HSV2 treated with 1 μg/ml acyclovir was only 11% (Table 1). However, acyclovir at this concentration has been shown by plaque reduction assay at 72 h to provide 100% cell protection (Thompson, 1998; Edert et al., 2000).

3.1.2.2. Pilostigma extract. We wished to determine if our electrophysiological monitoring of viral invasion could distinguish among different anti-viral agents. Because it was already under investigation in one or our labs (G.N.K.M.), we gathered data from cells treated with a methanol extract from the African plant, *P. thonningii*. It should be noted that this compound is currently under study, and has not as yet been fully characterized. The agent was first tested in the absence of HSV2. In cells exposed to the agent for at least 30 min (and up to 24 h), the average coupling ratio was quickly reduced to 37%, where it remained constant (Table 1). With this extract, down-regulation by addition of the virus was at first even more rapid than with HSV2 alone. However, after initially falling to 18% by 3 h post-infection, the coupling ratio stabilized at about 22%, indicating a degree of protection considerably better than that afforded by acyclovir (Table 1). This also confirmed that our electro-

Table 1
Gap junctional coupling among cultured Vero cells and the effect upon it of the gap junction antagonist octanol, of infection with HSV-2 and how that is changed by selected anti-viral agents

Treatment	Uninfected cells	3 h	6 h	24 h
Untreated cells (%)	49.5±1.4 $n=149$	(n.a.)	(n.a.)	49±1 $n=149$
Octanol (%)	50±2 $n=140$	[a]6.5±0.7 $n=50$	(n.a.)	(n.a.)
	Pre-infection	3 h (HSV)	6 h (HSV)	24 h (HSV)
HSV2 (%)	51.3±1 $n=30$	35±6 $n=8$	25±2 $n=17$	8±1 $n=27$
Acyclovir (%)	51±2.3 $n=17$	23.2±2.6 $n=24$	24.4±3 $n=24$	10.8±0.9 $n=44$
[b]Plant extract (%)	37±3 $n=24$	18±1.4 $n=21$	27±2.6 $n=17$	21.9±1.7 $n=13$
SDS (%)	43±0.6 $n=32$	16±1.2 $n=36$	20±1.3 $n=27$	40±1 $n=22$ [c]$P=0.0001$

n.a. = not applicable.
[a] Octanol treated cells reacted within 15 min.
[b] Plant extract is a methanol soluble extract from *P. thonningii*.
[c] Compared to 24 h untreated, HSV2 infected cells.

physiological monitoring allowed us to see differing modes of action and/or levels of protection conferred by differing anti-viral agents.

3.1.2.3. SDS. By plaque reduction assay, pretreatment of virus with 50 μM SDS has been reported to be nearly 100% effective in protecting cultured cells from viral infection (Howett et al., 1999; Piret et al., 2000). Before testing the protection against gap junction down-regulation afforded by this detergent, we first tested for toxic effects of SDS. This was done as for other agents by incubating the cells in the presence of the agent, but without the virus, for at least 30 min, and up to 24 h. As seen in Table 1, 50 μM SDS alone reduced the average coupling ratio only slightly to 43%. This change took place within minutes and was then stable for extended periods of up to 24 h.

In the presence of the virus, electrophysiological measurements revealed that by 3 h post-infection the coupling ratio had dropped to only 16%, the lowest 3 h coupling ratio recorded for any of our treatments. Coupling then began to increase, up to an average of 20% by 6 h and had fully recovered to 40% by 24 h, nearly identical to SDS-treated uninfected controls (Table 1).

4. Discussion

Data presented here show that: (1) Electrophysiological monitoring of infected cells can sharply distinguish among different antiviral agents dependent upon their mode of action upon the virus. (2) But: while commonly used antiviral agents such as acyclovir ultimately confer protection from viral replication, they may have little or no effect upon virus induced changes in cell physiology, particularly down-regulation of gap junctions. Therefore, monitoring electrical coupling would not serve well as a general screening technique for anti-viral agents. (3) The down-regulation of gap junctions is an early effect of viral infection, beginning before, and not dependent upon, the first round of viral replication. And finally, (4) SDS treated virus was still able to initially cause down-regulation. Over time the infected cells were able to reverse this condition and reopen intercellular communication. Thus, it is most probable that the initial ability of

the virus to down-regulate gap junctions depends upon some expendable viral component, supply of which the SDS treated virus retained, but could not replenish.

Our data show both useful features and caveats of electrophysiological monitoring as a tool for determining the efficacy and aspects of the mode of action of antiviral agents. Differences in the effect upon gap junctional intercellular communication seen when comparing cells infected by virus treated with acyclovir, with the plant extract, or with infected SDS were striking. In each case, treatment-specific differences were clearly and reproducibly discernible. While highly effective in suppressing plaque formation (Thompson, 1998; Edert et al., 2000), acyclovir was completely ineffective in protecting cells against down-regulation of gap junctions. This emphasized that, as a general screening technique for antiviral agent effectiveness, monitoring of gap junctional communication by either dye injection or electrophysiological techniques would fail to properly assay any agent, activity of which occurred at a point downstream of junction down-regulation. However, because it does not require damaging short wavelength illumination of fluorescent dye and is highly quantifiable, the technique remains an excellent assay specifically for viral and/or antiviral agent effects upon intercellular communication through gap junctions.

In HSV2 infected cells, early expression of viral genes peaks at 2–4 h (Honess and Roizman, 1974; Ackermann et al., 1984), while viral DNA replication begins at about 3 h after cells are infected, and continues thereafter for about 9 h (Roizman et al., 1963). At the concentration of acyclovir used in our experiments, reduced, but not eliminated, plaque formation attested to DNA replication being suppressed but not totally eliminated. For down-regulation of gap junctions by acyclovir-treated virus reported here, if the initial fall in coupling was caused by an expendable gap junction antagonist carried into the cell as part of the virus, then continued suppression of intercellular communication would require additional synthesis of the antagonist. Synthesis directed by the original viral DNA could account for the lack of connexon recovery. This synthesis would be increasingly augmented by reduced but still existent viral DNA replication, which would account for the increasing down-regulation of intercellular communication. By 24 h post-infection, although the amount of viral DNA replicated in the presence of acyclovir would be suppressed, it was apparently still sufficient over time to down-regulate gap junctions almost to the level seen with untreated virus.

Two alternative explanations exist. One is that the antagonist may be more stable in host cells infected with acyclovir treated HSV2 than those infected with HSV2 treated with other agents. However, to reach the final extent of down-regulation required nearly 24 h, and thus, it seems likely that increasing down-regulation required increasing amounts of antagonist. A second alternative is that down-regulation was caused by products synthesized by input viral DNA, and which suppress host cell transcription/translation of molecules required for maintenance of connexons. While we cannot at present rule this out, connexons are usually stable unless acted upon by an antagonist. Thus, even blocking synthesis of the connexin proteins of which connexons are made would not alone cause progressive down-regulation.

As an antiviral agent, SDS was at first presumed to prevent the virus from entering the cells by effecting the lipids and proteins in the viral coat (Howett et al., 1999; Piret et al., 2000). However, electron microscopy has revealed capsids in the nuclei of cells infected with SDS treated virus, indicating that at least part of the virus had found entry into the cell (Piret et al., 2000). Our data, also, suggest that at least part of the virus had entered the cells. If gap junctions were effected by external attachment of the virus, one would expect the change to occur in a very short time. But our data suggest nearly 3 h was required for the virus to significantly down-regulate gap junctions in unprotected cells, as well as those protected with acyclovir, the plant extract or SDS. As with acyclovir treatment, SDS treated viruses were initially able to down-regulate the gap junctions, but the cells were able to recover, presumably by synthesizing new connexin proteins. The virus, disabled by the SDS treatment, was incapable of

responding, and the cells repaired their gap junctions. Thus, change wrought upon HSV2 by SDS must have left the virus with sufficient reserves to be able to initially down-regulate gap junctions, but without the ability to direct new synthesis of this material.

As to the mechanism of HSV2 induced down-regulation, the junction antagonist of other viruses has been found to ultimately cause change in a tyrosine protein kinase (Fletcher et al., 1987) which phosphorylates tyrosine residues in the gap junctional protein connexin-43 (Cx43) (Crow et al., 1990; Filson et al., 1990; Brissette et al., 1991; Goldstein et al., 1991). That Cx43 has an essential role in the proper functioning of gap junctions has been demonstrated (Ennaji et al., 1995; Vrionis et al., 1997), post-transcriptional modification of the protein resulting in reduction of gap junctional communication (Kalimi et al., 1992). Phosphorylation of Cx43 prevents the necessary localization of the protein into cell membranes, thus blocking the proper configuration of connexon hemichannels (McMasters et al., 1998).

Whether or not HSV2 utilizes this same antagonist and causes down-regulation via phosphorylation of Cx43, the antagonist most likely was already a part of the pre-entry virus. This is indicated by the ability of the virus to initially down-regulate gap junctions to as low as 16% within 3 h while being incapable of maintaining the condition as cells repaired their junctions. If the antagonist was synthesized within the infected cells by the activity of viral genes, the initial viral infection would be expected to produce either of two effects. The initial population of viral DNA might have been able to synthesize only small amounts of the antagonist and thus cause minimal down-regulation of gap junctions. Greater degrees of down-regulation would require higher levels of the antagonist, which in turn would require replication of the virus. Alternatively, the initial viral population might be able to continue to produce the antagonist, eventually achieving full down-regulation of gap junctions. But neither of these scenarios occurred. Our data are most consistent with an antagonist which is already present prior to entry into the cells.

Electron microscopy and radio-labeling evidence from Piret et al. (2000) suggest one result of SDS treatment is that the virus was unable to properly replicate. They reported that capsids were present in cells infected with SDS treated virus. While their presence demonstrated that the virus had been able to enter the cell, the capsids seen in the nuclei of cells were few in number, of abnormal appearance and lacked a DNA core. Nor were any seen in the cytoplasm. Their results from radio-labeling experiments revealed reduced viral DNA production in cells well protected by SDS. Our data are in agreement with their findings, suggesting that the virus initially entered the cells, bringing with it an intact antagonist, accounting for the early down-regulation of the gap junctions. The reopening over time of the gap junctions suggests that the antagonist was not an enzyme, but rather bound irreversibly to its target. If this interpretation is correct, as the cells repaired their gap junction complexes, the disabled virus would be unable to produce more of the antagonist and gap junctions would reopen in the manner seen in our experiments. Thus, our data are consistent with the hypothesis that at least one action of SDS upon HSV2 results in severely suppressing transcriptional gene activity by the originally infecting viral DNA.

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