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Effects of Na-Coupled Alanine Transport on Intracellular K Activities and the K Conductance of the Basolateral Membranes of *Necturus* Small Intestine

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Summary. Intracellular electrical potentials and K activity, $(K)_c$, were determined simultaneously in *Necturus* small intestine before and after the addition of alanine to the mucosal solution. As noted previously (Gunter-Smith, Grasset & Schultz, 1982), the addition of alanine to the mucosal solution resulted in a prompt depolarization of the electrical potential difference across the apical membrane (ψ^{mc}) and a decrease in the slope resistance of that barrier (r^m). This initial response was followed by a slower repolarization of ψ^{mc} associated with a decrease in the slope resistance of the basolateral membrane (r^s) so that when the steady state was achieved (r^m/r^s) did not differ significantly from control values in the absence of alanine.

In the absence of alanine, ψ^{mc} averaged -32 mV and $(K)_c$ averaged 67 mM. When a steady state was achieved in the presence of alanine these values averaged -24 mV and 50 mM, respectively. The steady-state *electrochemical potential differences* for K across the basolateral membrane in the absence and presence of alanine did not differ significantly.

Inasmuch as the rate of transcellular active Na transport or "pump activity" was increased two- to threefold in the presence of alanine, it follows that, *if* active Na extrusion across the basolateral membrane is coupled to active K uptake across that barrier with a fixed stoichiometry then, the decrease in r^s must be due to an increase in the conductance of the basolateral membrane to K that parallels the increase in "pump activity". This "homocellular" regulatory mechanism serves to (i) prevent an increase in $(K)_c$ due to an increase in pump activity; and, (ii) repolarize ψ^{mc} and thus restore the electrical driving force for the rheogenic Na-coupled entry processes.

Key Words *Necturus* small intestine · intracellular K activity · alanine · Na-coupled transport · basolateral membrane conductance

Introduction

Recent studies dealing with the effects of sugars and amino acids on the electrophysiologic properties of villus cells of *Necturus* small intestine (Gunter-Smith et al., 1982) disclosed a two-phase response following the addition of these nonelectrolytes to the mucosal solution. Immediately after the addition of alanine or galactose there is a rapid and marked depolarization of the electrical potential difference across the apical membrane (ψ^{mc}) and a decrease in the ratio of the slope resistances of the apical to basolateral membranes (r^m/r^s). These events are followed by a slow repolarization of ψ^{mc} which is paralleled by an increase in (r^m/r^s) to, or slightly above, the control value. The initial events can be attributed, in part[1], to the activation of rheogenic (conductive) Na-coupled influx mechanisms for these nonelectrolytes at the apical membrane which result in a decrease in r^m; these events are not directly dependent upon the availability of metabolic energy. The later events are the results of a decrease in r^s which parallels the increase in "Na-pump activity" at the basolateral membrane as reflected by the increase in the short-circuit current (I_{sc}); these events are abolished by metabolic inhibitors.[2]

The origin of the decrease in r^s was not established in these earlier studies, but it seemed most likely that it was due to an increase in the conductance of the basolateral membrane to K (G^s_K). The present studies were undertaken in order to test this notion directly by determining intracellular K activities under control conditions and following the addition of alanine to the mucosal bathing solution.

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[1] The other factor that contributes to the depolarization of ψ^{mc} is the "insertion" of a new electromotive force across the membrane which represents the chemical driving force of the co-transport process (Gunter-Smith et al., 1982).

[2] For an excellent discussion of the distinction between slope and chord conductances the reader is referred to Finkelstein and Mauro (1963, 1977).

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Materials and Methods

Mudpuppies, *Necturus maculosa* (Riverside, Somerset, WI) were stored in tap water containing methylene-blue at 4 °C. The animals were either stunned by a blow to the head or anesthetized by immersion in tap water containing tricaine methanesulfonate (Sigma) until reflexes were suppressed. The proximal one-third of the small intestine was excised, stripped of its underlying musculature by blunt dissection (White, 1977), opened along the mesenteric border, and mounted in the chamber described by Gunter-Smith et al. (1982).

The bathing solutions contained (mM): Na, 100; Cl, 105; K, 2.5; Ca, 1.2; Mg, 1.2; HPO_4, 1.2; H_2PO_4, 0.3; and mannitol, 10. For solutions containing alanine, 10 mM L-alanine replaced mannitol. The pH of this solution when gassed with air at 23 °C is 7.4.

The experimental set-up and the preparation of conventional (KCl-filled) and K-selective liquid, ion-exchanger microelectrodes have been described in detail previously (Gunter-Smith & Schultz, 1982; Gunter-Smith et al., 1982; Fromm & Schultz, 1981). Briefly, the stripped tissues were mounted, mucosal surface up, between two halves of a Lucite chamber exposing 0.13 cm^2 of tissue. Both surfaces of the tissue were superfused by gravity-feed from reservoirs at a rate sufficient to replace the volumes of the mucosal and serosal chambers every second. The transepithelial electrical potential difference with respect to the mucosal solution, ψ^{ms}, was monitored using an automatic voltage-current clamp in contact with the solutions via calomel cells and Ringer-agar bridges. Bipolar current pulses of ± 150 $\mu A/cm^2$ were passed across the tissue at 10-sec intervals; the duration of each pulse was 2 sec. Tissue resistance, r_t, was calculated from the deflection of ψ^{ms} in response to these constant current pulses. The short-circuit current is given by $I_{sc} = \psi^{ms}/r_t$.

The electrical potential difference across the apical membrane with respect to the mucosal solution, ψ^{mc}, was determined using conventional microelectrodes filled with 0.5 M KCl, having a resistance of approximately 100 MΩ when the tip is immersed in 0.5 M KCl. The ratio of the slope resistances of the apical to basolateral membranes (r^m/r^s) was determined from the deflections in ψ^{mc} and ψ^{ms} resulting from the current pulses as described previously (Gunter-Smith et al., 1982).

K-selective microelectrode were fabricated using the Corning liquid-ion exchanger (477317) and calibrated as described previously (Gunter-Smith & Schultz, 1982). The intracellular K activity, $(K)_c$ was calculated from the relation:

$$\Delta V_K = S \log\{(K)_c/[(K)_m + k_{K,Na}(Na)_m]\} + \psi^{mc} \quad (1)$$

where ΔV_K(mV) is the change in the output of the microelectrode when the tip passes from the mucosal solution into the cell; S(mV) is the change in the output of the microelectrode that corresponds to a tenfold change in K activity determined from calibrations carried out immediately before and after each experiment; $k_{K,Na}$ is the selectivity coefficient of the electrode for Na with respect to K; and $(K)_m$ is the activity of K in the mucosal (and serosal) bathing solutions. In these studies: S averaged 58.3 ± 0.5 mV (a value identical to that expected for an ideally cation selective electrode); $k_{K,Na}$ averaged 0.023 ± 0.02 which is in excellent agreement with values reported previously by this and other laboratories (*cf.* Gunter-Smith & Schultz, 1982); and $(K)_m$ was 1.9 mM.

Because alanine elicits a biphasic response in ψ^{mc} it was desirable to determine ψ^{mc} and ΔV_K simultaneously before and after the addition of the amino acid to the mucosal solution. Although this could be accomplished using double-barrelled microelectrodes, we chose to employ a simpler and perhaps safer approach; namely, impaling one cell with a conventional KCl-filled microelectrode and another, nearby, cell with a K-selective microelectrode. This approach is justified by the results of preliminary studies in which a villus cell was impaled with a conventional KCl-filled microelectrode and then, with the aid of a stereomicroscope at 30× magnification, a second cell, in close proximity to the first, was impaled with another KCl-filled microelectrode; the recorded values of ψ^{mc} and (r^m/r^s) were essentially identical. Further, in our earlier studies we observed that if the microelectrode was spontaneously dislodged from the cell during a recording of ψ^{mc}, simply repositioning the microelectrode and impaling a nearby cell restored the value of ψ^{mc}. These findings are consistent with the notion that, as in other epithelia (*cf.* Lowenstein, 1981), the villus cells are electrically coupled by intercellular communications and thus form a syncytium.

The results of an experiment in which ψ^{mc} and ΔV_K were determined simultaneously, in the manner described above, before and after the addition of alanine to the mucosal perfusate are illustrated in Fig. 1. The values of (r^m/r^s) for the conventional microelectrode (●) and the K-selective microelectrode (○) are shown in the lower panel and, in most instances, are essentially identical. It is also of interest to note that insertion of the K-selective microelectrode did not result in a change in ψ^{mc} recorded by the conventional microelectrode from a nearby cell (*cf.* Fig. 1). This finding strongly argues against significant impalement damage to the apical membrane by the microelectrodes.

Results are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was carried out using the Student t test, and a value of $P < 0.05$ was considered significant.

Results

In a series of studies on nine tissues in the absence of alanine, ψ^{mc} averaged -31 ± 7 mV (54 impalements) and ΔV_K averaged 43 ± 2.5 mV (47 impalements); the value of $(K)_c$ calculated using Eq. (1) averaged 67 ± 6 mM. This value of $(K)_c$ is significantly greater than that of 41 mM reported by White (1976) for *Amphiuma* small intestine bathed in the presence of 2.5 mM K. Garcia-Diaz, O'Doherty and Armstrong (1978) reported a value for $(K)_c$ of 108 mM in *Necturus* small intestine, but these studies were carried out in the presence of 5.4 mM K.

The results of studies on seven tissues in which ψ^{mc} and ΔV_K were recorded simultaneously before and after the addition of alanine to the mucosal solution are tabulated in Table 1; in five of these studies, the mucosal perfusate was switched back to an alanine-free solution after a steady-state in the presence of alanine was achieved.

The salient points are:

(1) The addition of alanine to the mucosal solution resulted in a rapid (<10 sec) depolarization of ψ^{mc} from an average value of -32 mV to a value of -6 mV at the "peak" of the response. This was followed by a slow (>4 min) repolarization to an average steady-state value of -24 mV.

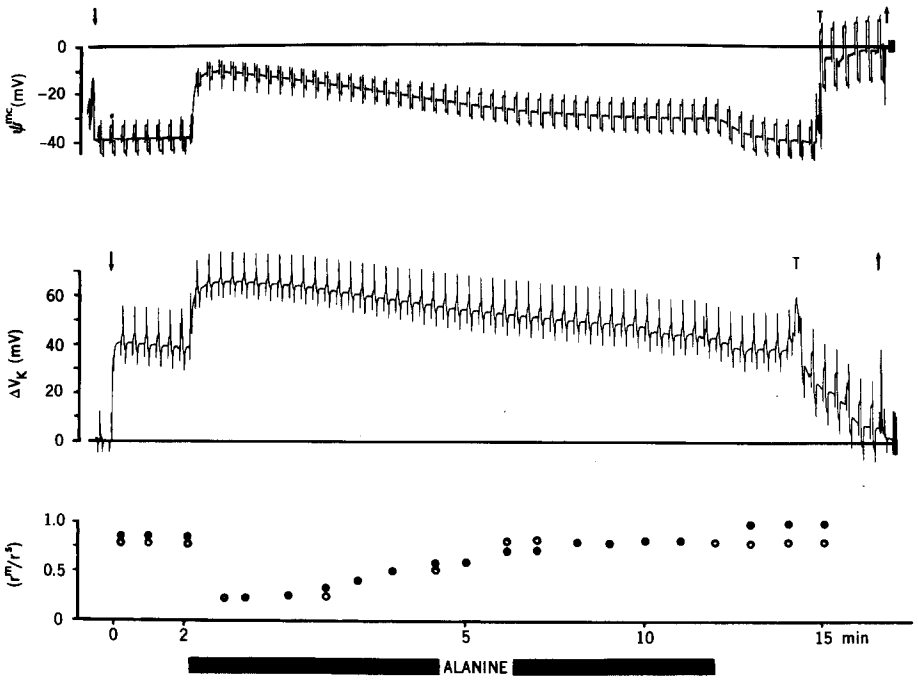

Fig. 1. Simultaneous determinations of ψ^{mc} and ΔV_K before and after the addition of 10 mM alanine to the mucosal solution. The solid circles (•) designate values of (r^m/r^s) determined using a conventional microelectrode, and the open circles (o) the values obtained with a K-selective microelectrode. Arrows indicate impalement of and withdrawal from the cell; *T* indicates that the microelectrode tip was advanced through the cell into the underlying tissue for the determination of r_t and (r^m/r^s). The rapid decrease in (r^m/r^s) with depolarization and the gradual increase in (r^m/r^s) with repolarization are self-evident. (Note that between 2 and 5 min the speed of the recorder was doubled to increase the resolution of the transient immediately after switching the mucosal solution to one containing alanine.)

Table 1. Effects of alanine on electrophysiologic parameters and intracellular potassium

	ψ^{ms} (mV)	I_{sc} ($\mu A/cm^2$)	r_t ($\Omega\ cm^2$)	ψ^{mc} (mV)	(r^m/r^s)	$(K)_c$ (mM)
Control	1.5±0.4	22± 5	73±3	32±2	0.95±0.20	67±7
Peak	3.2±0.2	43± 4	70±3	−6±3	0.45±0.16	45±5
Steady state	5.0±1.0	77±13	66±3	−24±1	1.07±0.20	50±6
Alanine-free	2.0±0.0	33±10	64±2	−40±3	1.63±0.20	63±4

(2) These changes in ψ^{mc} were paralleled by an initial, rapid decline in (r^m/r^s) from an average value of 0.95 to that of 0.45 ("peak") which was followed by a slow increase to an average steady-state value of 1.07; the latter does not differ significantly from the control value.

(3) The steady-state values of ψ^{ms} and I_{sc} in the presence of alanine were each increased approximately 3.5-fold over the control values. As discussed previously, these increases paralleled the repolarization of ψ^{mc} and the increase in (r^m/r^s) from their values at the "peak" of the response (Gunter-Smith et al., 1982).

(4) The average value of $(K)_c$ under control conditions was 67 mM and it declined significantly ($P<0.05$) to a value of 50 mM when the steady-state was reached. At the "peak", $(K)_c$ was lower than the value observed when the steady state was achieved, but the averages do not differ statistically at the $P<0.05$ level.[3]

Discussion

The electrophysiological responses of villus cells of *Necturus* small intestine following the addition

[3] The values of $(K)_c$ at the "peak" displayed the widest spread of all of the values obtained in this study. In two experiments it scarcely changed at all from control (*cf.* Fig. 1); in two experiments it fell by approximately 50 mM; and in four experiments the decline ranged between 12–16 mM. We have no explanation for this except to raise the possibility that during the transient the value of $(K)_c$ may depend upon the location of the microelectrode tip within the cell.

of alanine (or galactose) to the mucosal solution have been described in detail (Gunter-Smith et al., 1982); the effects of alanine on ψ^{mc}, (r^m/r^s), ψ^{ms} and I_{sc} observed in the present studies are very similar to those earlier findings. The aim of the present studies was to determine $(K)_c$ before and after the addition of alanine to the mucosal perfusate and to correlate these observations with our earlier findings and conclusions.

The value of $(K)_c$ determined in these studies, in the absence of alanine, is in reasonably good agreement with values reported for a number of other epithelia determined using liquid, ion-exchanger microelectrodes. This value is much greater than that predicted for a passive distribution of K across the basolateral membrane (7 mM), leaving no doubt that K is actively accumulated by these cells against a considerable electrochemical potential difference. This observation is not surprising.

The finding that the steady-state value of $(K)_c$ in the presence of alanine is significantly lower than that observed under control conditions is also not surprising. Lee and Armstrong (1972) have reported that exposure of bullfrog small intestine to galactose results in a significant decline in $(K)_c$ determined using K-selective microelectrodes, and several groups have found that the "apparent" intracellular *concentration* of K in small intestine, determined using chemical techniques, declines in the presence of actively transported sugars or amino acids (Brown & Parsons, 1962; Schultz, Fuisz & Curran, 1966; Koopman & Schultz, 1969; Armstrong, Muselman & Reitzug, 1970; Okada, 1979). This decrease in $(K)_c$ has been attributed to an increase in cell water content secondary to the intracellular accumulation of the sugar or amino acid in an osmotically active form. However, in retrospect, this explanation is untenable and should be abandoned. In the final analysis, the steady-state value of $(K)_c$ is *uniquely* determined by the rate at which K is pumped into the cell across the basolateral membrane, the K conductances of the two limiting membranes, and the electrical potential difference across those barriers; a change in the volume of the cell is irrelevant unless it *directly* affects one or more of those parameters.

The most revealing finding that emerges from these studies is that the steady-state electrochemical potential differences for K across the basolateral membrane $(\Delta\tilde{\mu}^s_K/\mathcal{F})$, in mV, before and after the addition of alanine to the mucosal solution do not differ significantly. Thus,

$$(\Delta\tilde{\mu}^s_K/\mathcal{F}) = RT/\mathcal{F}) \ln[(K)_c/(K)_s] - \psi^{cs} = E^s_K - \psi^{cs}$$

where $\psi^{cs} = \psi^{ms} - \psi^{mc}$. From the data given in Table 1, prior to the addition of alanine to the mucosal perfusate, $E^s_K = 90$ mV and $\psi^{cs} = 34$ mV so that $(\Delta\tilde{\mu}^s_K/\mathcal{F}) = 56$ mV; when a steady-state is achieved in the presence of alanine, $E^s_K = 82$ mV, $\psi^{cs} = 29$ mV and $(\Delta\tilde{\mu}_K/\mathcal{F}) = 53$ mV.[4]

Thus, the steady-state driving force for the diffusional flow of K out of the cell is essentially the same in the absence and the presence of alanine. However, the rate of transcellular Na transport in the presence of the amino acid is 2–3.5 times greater than that under control conditions. Thus, if active K uptake across the basolateral membrane is coupled to Na extrusion from the cell across that barrier, as is almost certainly the case, and if the stoichiometry of this exchange pump is fixed, a threefold increase in the rate at which Na is pumped out of the cell must be associated with a threefold increase in the rate at which K is pump into the cell and, under steady-state conditions, a threefold increase in the rate at which K leaves the cell across the basolateral membrane.[5] Inasmuch as the driving force for this exit process is unchanged, the most direct interpretation of our findings is that the K conductance of the basolateral membrane increases *pari passu* with the increase in pump rate. Although other explanations are possible they would involve mechanisms for which there is little or no evidence.

Thus, the present results provide strong support for the conclusions drawn in our earlier study; namely, that (i) the initial response to the addition of sugar or amino acid to the mucosal solution is the activation of new conductive pathways for Na across the apical membrane leading to a decrease in r^m (and r^m/r^s) and a depolarization of ψ^{mc};[6] and (ii) this is followed by an increase in G^s_K (which results in a decrease in r^s and the restoration of (r^m/r^s) to control values), leading to the partial repolarization of ψ^{mc}. This repolarization restores the electrical driving force for the Na-

[4] It should be noted that Gunter-Smith et al. (1982) calculated a value of 100 mV for the total electromotive force across the basolateral membrane in the absence of alanine. This value is in reasonably good agreement with E^s_K.

[5] There is no evidence for active K secretion by *Necturus* small intestine. Further, the equality between the increase in I_{sc} and the increase in the rate of active Na absorption following the addition of alanine to the mucosal solution (Gunter-Smith et al., 1982) strongly suggests that the increase in rate of K uptake across the basolateral membrane is matched by an increase in K efflux across the basolateral membrane alone. Thus, as appears to be the case in a variety of other epithelia, the apical membrane of *Necturus* small intestine appears to be essentially impermeable to K.

[6] *See* footnote 1, p. 89.

coupled entry process so that the rate of increase in transcellular Na transport, as reflected by the increase in I_{sc}, parallels the increases in G^s_K and ψ^{mc}. The new steady-state level of $(K)_c$ is, then, determined by the steady-state values of I_{sc}, ψ^{cs} and G^s_K.

As discussed previously (Gunter-Smith et al., 1982; Schultz, 1981), this homocellular regulatory process not only prevents an increase in $(K)_c$ with increasing pump activity but also energizes the Na-coupled entry mechanism.[7]

Increases in the conductance of the basolateral membranes associated with an increase in pump activity have also been observed in *Necturus* urinary bladder (Higgins, Gebler & Frömter, 1977; Schultz, 1981; R. Thomas, Y. Suzuki, S.M. Thompson, and S.G. Schultz, *unpublished observations*) and frog skin (Schultz, 1981). In addition, Nagel and Crabbé (1980) have reported that following prolonged (overnight) exposure of toad skin to aldosterone, there was the expected increase in the I_{sc} which was associated with a marked (80%) increase in the conductance of the basolateral membrane and a somewhat smaller (10%) but significant increase in the electromotive force across that barrier. These investigators entertained the possibility that the increase in G^s was directly related to the increase in I_{sc} but pointed out that the possibility that it is a delayed response to the effects of aldosterone could not be excluded.

The mechanism responsible for the increase in G^s_K is unclear. In our previous paper (Gunter-Smith et al., 1982) we suggested that it may be inherently linked to an increase in pump activity. But, at the same time, it is also *indirectly responsible* for the increase in pump activity through its effect on Na entry. Thus, it is entirely possible that the immediate mechanism responsible for the increase in G^s_K is independent of the "pump" and that the increase in the activity of the pump is entirely *secondary* to the increase in G^s_K. In this respect it is of interest that Nagel, Eigler and Fruchtel (1981) have recently reported that the saliuretic agent tizolemide markedly decreases the conductance of the basolateral membrane of frog skin, which results in depolarization of ψ^{mc} and thus decreases the rate of Na entry across the apical (outer) membrane and, *pari passu,* transcellular Na absorption. In any event, the decrease in G^s_K in response to sugars or amino acids is not observed in metabolically-poisoned tissues, suggesting that, whatever the underlying mechanism, it is dependent upon metabolic energy.[8]

Finally, these findings have important implications regarding the electrophysiologic criteria for a "rheogenic Na–K exchange pump." In 1971 Rose and Schultz (1971) argued that the increase in the transepithelial electrical potential difference, ψ^{ms}, across rabbit ileum in response to the presence of sugars or amino acids in the mucosal solution could not be attributed entirely to the depolarization of ψ^{mc} which, because of the very low resistance parallel shunt pathway, has hardly any effect on ψ^{ms}. Instead, it was argued that the increase in ψ^{ms} is the result of an event at the basolateral membrane that is blocked by ouabain and/or metabolic inhibitors. Later, Schultz (1973) suggested that this event could be an increase in the activity of a "rheogenic" Na pump at the basolateral membrane which increases the electromotive force across that barrier (E^s), the electrical potential difference across that barrier (ψ^{cs}) and, in turn, ψ^{ms}. Schultz (1977) also suggested that such a "rheogenic" pump mechanism at the basolateral membrane could be part of an "electro-chemical" feedback that energizes the Na-coupled entry step at the basolateral membrane.

It is now clear that an energy-dependent, ouabain-sensitive hyperpolarization of ψ^{cs} (and ψ^{mc}) in response to and/or responsible for an increase in Na entry across the apical membrane and transcellular Na transport can be due to an increase in G^s_K *alone* (regardless of the underlying mechanism) and cannot be considered evidence for a "rheogenic" pump mechanism; that is, an increase in G^s_K could bring about all of these changes even if the pump mechanism were neutral.

In short, although there is no reason to doubt that the Na–K pump at the basolateral membrane of epithelial cells is "rheogenic" (*cf.* Schultz, 1981) and, therefore, must contribute to E^s and ψ^{cs}, an increase in these parameters can also be solely the result of an increase in G^s_K. If, as appears to be the case for a variety of Na-transporting epithelia, the basolateral membrane is predominantly permselective for K, the most compelling electrophysiological evidence for a rheogenic pump would be the finding that ψ^{cs} transiently exceeds E^s_K follow-

[7] Kristensen (1980) has demonstrated an increase in K permeability of isolated rat hepatocytes associated with Na-coupled alanine uptake and has drawn the same conclusion.

[8] Anner (1981) has recently reported that incorporation of ATP-free Na–K–ATPase into phosphatidylcholine liposomes results in a large increase in the K permeability of these artificial membranes and suggested that this ATPase "...may contribute to the K-selectivity of the cell membrane." This finding is consistent with the notion (Schultz, 1981) that the parallel increases in pump activity and G^s_K are the results of the recruitment of additional pump-leak "units" into the basolateral membrane.

ing stimulation of the pump. Evidence of this nature has been presented by Nagel (1980) in support of the rheogenicity of the Na pump in frog skin.

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