Glucagon-Stimulated Adenylate Cyclase detects a Selective Perturbation of the Inner Half of the Liver Plasma-Membrane Bilayer achieved by the Local Anaesthetic Prilocaine

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Prilocaine can increase the fluidity of rat liver plasma membranes, as indicated by a fatty acid spin-probe. This led to the activation of the membrane-bound fluoride-stimulated adenylate cyclase activity, but not the Lubrol-solubilized activity, suggesting that increased lipid fluidity can activate the enzyme. With increasing prilocaine concentrations above 10 mM, the membrane-bound fluoride-stimulated activity was progressively inhibited, even though bilayer fluidity continued to increase and the activity of the solubilized enzyme remained unaffected. Glucagon-stimulated adenylate cyclase was progressively inhibited by increasing prilocaine concentrations. Prilocaine (10 mM) had no effect on the lipid phase separation occurring at 28°C and attributed to those lipids in the external half of the bilayer, as indicated by Arrhenius plots of both glucagon-stimulated adenylate cyclase activity and the order parameter of a fatty acid spin-probe. However, 10 mM-prilocaine induced a lipid phase separation at around 11°C that was attributed to the lipids of the internal (cytosol-facing) half of the bilayer. It is suggested that prilocaine (10 mM) can selectively perturb the inner half of the bilayer of rat liver plasma membranes owing to its preferential interaction with the acidic phospholipids residing there.

Sheetz & Singer (1974) have suggested that, owing to the now well-documented asymmetry of the lipid composition of plasma membranes (Patton & Keenan, 1975; Rothman & Lenard, 1977; Zwaal, 1978; Sandra & Pagano, 1978; Patzer *et al.*, 1978; Higgins & Evans, 1978), where negatively charged phospholipids predominate at the cytosol-facing surface, drugs of opposite charge may act preferentially at one or other side of the bilayer. One might then expect such drugs to act selectively on functioning proteins that are themselves asymmetrically orientated in the bilayer.

In liver plasma membranes, when the hormone glucagon is absent the catalytic unit of adenylate cyclase is able to migrate independently in the plane of the bilayer (Houslay *et al.*, 1977, 1980) and senses the lipid environment of the inner half of the bilayer only (Houslay, 1979; Houslay & Palmer, 1978). In the presence of glucagon, its receptor physically couples with the catalytic unit of adenylate cyclase to form a transmembrane complex

(Houslay *et al.*, 1977, 1980) that is sensitive to the lipid environment of both halves of the bilayer (Houslay, 1979; Houslay & Palmer, 1978). Such a system allows us to monitor perturbations of the inner and outer halves of the bilayer of rat liver plasma membranes. The lipid bilayer of rat liver plasma membranes has been shown to be asymmetric with respect to chemical composition, where the acidic phospholipids are localized at the cytosol surface of the bilayer (Higgins & Evans, 1978) and also with respect to the physical properties of the two halves of the bilayer (Houslay, 1979; Houslay *et al.*, 1976*b,c*). In the present study we investigate the effect of the positively charged amine local anaesthetic prilocaine on the bilayer, by using adenylate cyclase activity and the e.s.r. method with a fatty acid spin-label as probes to monitor bilayer perturbations.

Materials and Methods

The *N*-oxyl-4,4-dimethyloxazolidine derivative of

Fig. 1. The structure of prilocaine [*N*-(2-propylaminopropionyl)-*o*-toluidine]

5-oxostearic acid, I(12,3), was obtained from Syva Co., Palo Alto, CA, U.S.A.; 50 μ l micro-pipettes were from Drummond Scientific Co., St. Louis, MO, U.S.A.; and Crito-seal was from Sherwood Medical Co., St. Louis, MO, U.S.A.

Creatine kinase, phosphocreatine, ATP, cyclic AMP and triethanolamine HCl were from Boehringer (U.K.) Ltd., Lewes, East Sussex, U.K. Sucrose was of A.R. grade from either Calbiochem, La Jolla, CA, U.S.A., or from BDH Chemicals, Poole, Dorset, U.K. Glucagon was a kind gift from Dr. W. W. Bromer, Lilly Research Laboratories, Indianapolis, IN, U.S.A. Prilocaine [*N*-(2-propylaminopropionyl)-*o*-toluidine] (Fig. 1) was a kind gift from Astra Chemicals, Watford, Herts., U.K. Lubrol 12A9 was a kind gift from ICI Pharmaceuticals, Macclesfield, Cheshire, U.K. All other chemicals were of A.R. grade from BDH Chemicals.

Liver plasma membranes were isolated from male Sprague-Dawley rats weighing 200–300 g by the method of Pilkis *et al.* (1974) as modified by Houslay *et al.* (1976a). Membranes were stored in 1 mM-KHCO₃, pH 7.2, at protein concentrations between 8 and 14 mg of protein/ml in liquid N₂. To investigate lipid fluidity with the spin-label technique, routinely membranes were freeze-dried to dryness, stored in sealed ampoules under N₂ gas, and then rehydrated with 50 mM-triethanolamine hydrochloride/8% (w/v) sucrose, pH 7.6, before use. Identical results were obtained with membranes that had not been freeze-dried but were stored at –70°C.

Adenylate cyclase activity was assayed as described in detail previously (Houslay *et al.*, 1976a). Prilocaine was shown to have no effect on the method of determination of cyclic AMP. Assays were routinely carried out at 30°C in the presence of either 1 μ M-glucagon or 15 mM-NaF, at protein concentrations of 0.4 mg of membrane protein/ml and a final assay pH 7.6 with all constituents added. Activities of adenylate cyclase reflect initial rates obtained from 10 min time-courses.

Methods for e.s.r. studies were essentially as described previously in some detail (Gordon *et al.*, 1978, 1980). Samples (70 μ l) of liver plasma membranes (4.3 mg of protein/ml) were added to the I(12,3) spin-label that had initially been deposited on the side of the tube by evaporation from aqueous ethanol to yield an experimentally determined low

probe ratio of 9 μ g of I(12,3)/mg of membrane protein (Gordon & Sauerheber, 1977). Spectra were recorded on a Varian E-104A Century series e.s.r. spectrometer, and the cavity temperature calibrated as described previously (Gordon *et al.*, 1978).

The hyperfine splittings of labelled liver plasma membranes were determined from 'unexpanded spectra with magnified wings' (Gordon *et al.*, 1978) to give an outer hyperfine splitting, $2T_{\parallel}$ and an inner hyperfine splitting, $2T_{\perp}$. The following order parameter expressions (Gordon & Sauerheber, 1977) may be used to evaluate the flexibility of the liver plasma-membrane-incorporated fatty acid spin label:

$$S(T_{\parallel}) = \frac{1}{2} \left[\frac{3(T_{\parallel} - T_{xx})}{T_{zz} - T_{xx}} - 1 \right] \quad (1)$$

$$S(T_{\perp}) = \frac{1}{2} \left[\frac{3[(T_{zz} + T_{xx}) - 2T_{\perp}]}{(T_{zz} - T_{xx})} - 1 \right] \quad (2)$$

and

$$S = \frac{(T_{\parallel} - T_{\perp})(a_N)}{(T_{zz} - T_{xx})(a_N')} \quad (3)$$

Here, T_{xx} and T_{zz} are the hyperfine splitting elements of the static interaction tensor (**T**) parallel to the static Hamiltonian (**H**) principal nuclear hyperfine axes *x* and *z* respectively. The *x* axis is parallel to the N–O bond direction, and the *z* axis is parallel to the nitrogen 2p π orbital. The elements of **T** used in this study were previously determined by incorporating nitroxide derivatives into host crystals as substitutional impurities: (T_{xx} , T_{zz}) = (6.1, 32.4) G (Seelig, 1979). a_N and a_N' are the isotropic hyperfine coupling constants for the probe in the membrane and crystal states respectively [i.e. $a_N' = 1/3(T_{\parallel} + 2T_{\perp})$ and $a_N = 1/3(T_{zz} + 2T_{xx})$].

The order parameters S , $S(T_{\parallel})$ and $S(T_{\perp})$ reflect the membrane fluidity (or, more accurately, the flexibility of the membrane-incorporated probe). S , $S(T_{\parallel})$ and $S(T_{\perp})$ may each assume values between 0 and 1; these extreme order parameters indicate that the probe samples fluid and immobilized environments respectively. The order parameter S , which requires both hyperfine splittings, corrects for small polarity differences between the membrane and reference crystal. Although $S(T_{\parallel})$ and $S(T_{\perp})$ do not include corrections for polarity contributions, these expressions have been found to be useful approximate measures of the fluidity in those cases where only one hyperfine splitting is usable (Gordon & Sauerheber, 1977). $S(T_{\parallel})$ was used in the constant-temperature studies to assess the effect of prilocaine on bilayer fluidity, or more accurately probe flexibility (Gordon & Sauerheber, 1977; Sauerheber *et al.*, 1977). This was because studies were also carried out at 1.4 mg of protein/ml, where

the probe ratio increased to 11 μg of label/mg of membrane protein. At this point the evaluation of $2T_{\perp}$ was less precise, owing to the overlap of liquid lines (Gordon & Sauerheber, 1977), thus precluding an accurate evaluation of S .

A Lubrol-solubilized enzyme was prepared as described previously (Dipple & Houslay, 1978).

Arrhenius-plot data were handled as described previously to assign break points (Houslay & Palmer, 1978).

Protein was determined by a modified micro-biuret method (Houslay & Palmer, 1978) of Goa (1953).

Results

Concentrations of prilocaïne above 4 mM led to an augmentation of fluoride-stimulated adenylate cyclase activity, up to a maximum of 150% of its original activity at 10 mM-prilocaïne (Fig. 2). Further increases in prilocaïne concentration led to a progressive inhibition of adenylate cyclase activity

Fig. 2. Effect of prilocaïne on the adenylate cyclase activity of rat liver plasma membranes

This Figure demonstrates the change in adenylate cyclase activity (as % of original activity) of liver plasma membranes expressed as a function of log prilocaïne concentration. Results are means $\pm$ S.E.M. for six determinations of adenylate cyclase activity with three different plasma-membrane preparations. Data show the effect on the fluoride-stimulated activity ($\bullet$), glucagon-stimulated activity (Δ) and the fluoride-preactivated Lubrol-solubilized enzyme ($\circ$). The assay temperature was kept constant at 30°C.

(Fig. 2). This contrasted with the effect of prilocaïne on the glucagon-stimulated adenylate cyclase activity, which was progressively inhibited by increasing prilocaïne concentrations above 4 mM-prilocaïne (Fig. 2).

All of these effects were fully reversible by washing the membranes exposed to prilocaïne by repeated centrifugation (two or three times) at 14000g for 6 min and resuspension in 1 mM-KHCO₃, pH 7.2. The responses observed did not cause time-dependent changes in activity, as time-courses were linear over a 10 min period.

A Lubrol 12A9-solubilized fluoride-preactivated enzyme preparation showed no sensitivity to prilocaïne over the range of concentrations tested (Fig. 2).

At concentrations of prilocaïne greater than 4 mM the fluidity of liver plasma membranes was progressively augmented with increasing prilocaïne concentrations, as indicated by the decrease in $S(T_{\parallel})$ observed (Fig. 3). Such increases in fluidity were observed up to the highest concentration of prilocaïne tested, 33 mM. Essentially identical results were obtained with membrane concentrations of either 1.4 or 4.3 mg of protein/ml, with probe/protein ratios of 11 or 9 μg of probe/mg of protein respectively.

Arrhenius plots of the adenylate cyclase activity of native plasma membranes exhibited a well-defined

Fig. 3. Effect of prilocaïne on the fluidity of rat liver plasma membranes

This plot demonstrates the percentage change in order parameter, $\Delta S(T_{\parallel})$ as defined in the Materials and Methods section for rat liver plasma membranes labelled with the fatty acid spin-probe I(12,3) as a function of prilocaïne concentration. The assay temperature was kept constant at 30°C.

Fig. 4. Effect of 10 mM-prilocaine on Arrhenius plots of glucagon- and fluoride-stimulated adenylate cyclase activity

The logarithm of the reaction rate (v , μ units/mg) is plotted as a function of the reciprocal of the absolute temperature for assays of (●) fluoride- and (△) glucagon-stimulated adenylate cyclase activity carried out in the presence of 10 mM-prilocaine.

break at 28°C when glucagon was present in the assay, and were linear over the range 0–45°C when fluoride was present in the assay (Houslay *et al.*, 1976a; Dipple & Houslay, 1978). The addition of 10 mM-prilocaine to these assays had a profound effect on the form of the Arrhenius plots. That of the glucagon-stimulated activity (Fig. 4) exhibited two well-defined breaks at $28.3 \pm 0.4^\circ\text{C}$ and $10.8 \pm 1.2^\circ\text{C}$ with activation energies for the three segments (from the high- to low-temperature end) 25.2 ± 7.1 , 52.5 ± 5.5 and 102.5 ± 35.3 kJ/mol respectively (means $\pm$ S.D., $n = 5$, for five different plasma-membrane preparations), whereas the fluoride-stimulated activity (Fig. 4) exhibited a single break at $11.0 \pm 1.7^\circ\text{C}$ with activation energies of 55.9 ± 7.6 and 101.2 ± 20.6 kJ/mol above and below the break respectively (means $\pm$ S.D., $n = 4$, for four different plasma-membrane preparations). At 10 mM, prilocaine had no significant effect on Arrhenius

Fig. 5. Effect of 10 mM-prilocaine on the temperature-dependence of the order parameter S , $S(T_{\parallel})$ and $S(T_{\perp})$ for the spin-probe I(12,3) incorporated in rat liver plasma membranes

The order parameters S (▲), $S(T_{\parallel})$ (●) and $S(T_{\perp})$ (■) for the spin-probe I(12,3) incorporated into rat liver plasma membranes in the presence of 10 mM-prilocaine are plotted as a function of the reciprocal of the absolute temperature. Order parameters were calculated as described in the Materials and Methods section. Plasma membranes were loaded with $9 \mu\text{g}$ of probe/mg of membrane protein (see the Materials and Methods section).

plots of the Lubrol-solubilized fluoride-pre-activated adenylate cyclase activity.

Arrhenius plots of the order parameter S of the probe I(12,3) incorporated in rat liver plasma membranes showed a well-defined break at 28°C owing to the presence of a lipid-phase separation (Houslay *et al.*, 1976a,c; Gordon *et al.*, 1980). Fig. 5 demonstrates Arrhenius-type plots of S , $S(T_{\parallel})$ and $S(T_{\perp})$ versus $1/T(\text{K})$ for rat liver membranes labelled with the I(12,3) probe and in the presence of 10 mM-prilocaine. A high-temperature break at $28.4 \pm 1.4^\circ\text{C}$, $29.3 \pm 0.8^\circ\text{C}$ and $28.1 \pm 0.8^\circ\text{C}$ could be assigned to the plots of S , $S(T_{\parallel})$ and $S(T_{\perp})$ respectively (results are means $\pm$ S.D., with $n = 3$, for

three different plasma-membrane preparations). At temperatures below approx. 15°C, the probe no longer executed rapid anisotropic motion about its long molecular axis, hence $2T_{\perp}$ become unmeasurable and we were left with $2T_{\parallel}$ as an index of the flexibility of the probe. However, $2T_{\parallel}$ is a useful index of fluidity in these membranes (Gordon & Sauerheber, 1977). A low-temperature break occurring at $11.4 \pm 1.0^{\circ}\text{C}$ (means $\pm$ S.D., $n = 3$ for three different membrane preparations) was evident from the plot of $S(T_{\parallel})$ in membranes with 10mM-prilocaine present (Fig. 5), but this did not occur in native membranes in the absence of prilocaine (Houslay *et al.*, 1976b,c; Gordon *et al.*, 1980).

Discussion

In the absence of glucagon, the uncoupled catalytic unit of adenylate cyclase may be stimulated by fluoride ions, and is able to undergo free lateral diffusion in the plane of the bilayer (Houslay *et al.*, 1977), where it is sensitive to the lipid environment of the inner half of the bilayer only (Houslay & Palmer, 1978; Houslay, 1979).

The cationic local anaesthetic prilocaine began to activate the fluoride-stimulated adenylate cyclase activity at concentrations where it started to increase the fluidity of the lipid bilayer, as seen by a decrease in the order parameter $S(T_{\parallel})$ for the incorporated fatty acid spin-probe (Figs. 2 and 3). This activation of the fluoride-stimulated activity was not due to a direct effect of prilocaine on the protein, as the Lubrol-solubilized enzyme was apparently unaffected by these concentrations of prilocaine (Fig. 2). Furthermore, there are no significant permeability barriers in our membrane preparation that could account for these observations (Houslay & Palmer, 1979). The simplest explanation for this activation is that the increase in bilayer fluidity achieved by prilocaine relieved a physical constraint imposed on the enzyme by the bilayer, leading to an increase in the conformation flexibility of the protein, with concomitant activation. Consistent with such an hypothesis are our previous observations that the neutral local anaesthetic benzyl alcohol could similarly activate this membrane-bound enzyme at concentrations where it achieved an increase in bilayer fluidity, but it did not activate the Lubrol-solubilized enzyme activity (Dipple & Houslay, 1978; Gordon *et al.*, 1979, 1980).

Concentrations of prilocaine greater than 10mM led to a progressive inhibition of the fluoride-stimulated activity (Fig. 2), even though bilayer fluidity continued to increase (Fig. 3). Again this was clearly not due to a direct effect of prilocaine on the protein, as the detergent-solubilized enzyme was unaffected. It has been suggested that a certain

minimum rigidity of the bilayer might be essential for function (Dipple & Houslay, 1978), and thus a 'too-fluid' bilayer may lead to inhibition of enzyme activity. However, from our studies of benzyl alcohol action (Gordon *et al.*, 1980) we can discount this hypothesis for the extent of increased bilayer fluidity encountered here. For example, at 33mM-prilocaine, where a significant inhibition has ensued (Fig. 2), the total increase in bilayer fluidity achieved by prilocaine (Fig. 3) can be estimated as equivalent to that produced by a 6°C rise in temperature (see Gordon *et al.*, 1980). As fluoride-stimulated adenylate cyclase activity continued to rise over a temperature range from 30°C to at least 45°C (Houslay *et al.*, 1976a; Dipple & Houslay, 1978), in parallel with continuing increases in bilayer fluidity achieved by elevations in temperature over this range (Gordon *et al.*, 1980), such an hypothesis becomes untenable. For reasons discussed in detail previously (Dipple & Houslay, 1978; Gordon *et al.*, 1980) it is likely that the inhibition at the high prilocaine concentrations is due to a displacement of annular lipid from around the protein. Such annular lipid might well be essential for activity (Dipple & Houslay, 1978), or occupancy of such lipid-obscured sites by the anaesthetic may allow it to be inhibitory. It is tempting to offer such an Annular Displacement Mechanism (Dipple & Houslay, 1978; Richards *et al.*, 1978; Gordon *et al.*, 1980) for the phenomenon of local anaesthesia, where the protein in question is the sodium channel.

In the presence of glucagon, the receptor and catalytic unit couple to form a transmembrane complex (Houslay *et al.*, 1977, 1980) spanning the lipid bilayer and sensitive to the lipid environment of both halves of the bilayer (Houslay, 1979; Houslay & Palmer, 1978; Houslay *et al.*, 1976b,c). As the uncoupled catalytic unit of adenylate cyclase was activated by the increases in bilayer fluidity achieved by prilocaine, it was quite unexpected to observe an inhibition of the glucagon-stimulated adenylate cyclase activity ensuing at concentrations of prilocaine where the uncoupled catalytic unit began to be activated (Fig. 2). This is in contrast with benzyl alcohol, which activated both the uncoupled and coupled activities (Dipple & Houslay, 1978). It is highly likely that prilocaine perturbs the coupling interaction between the receptor and catalytic unit and that this is responsible for the observed inhibition. This coupling interaction has been suggested to occur at the cytosol surface of the bilayer (Iyengar *et al.*, 1980) and to involve acidic phospholipids (Rubaclava & Rodbell, 1973), which are localized at the cytosol half of the bilayer (Higgins & Evans, 1978). As there is good evidence from n.m.r. studies for a strong interaction between cationic anaesthetics and negatively charged phospholipids (Hauser *et al.*, 1969; Yeagle *et al.*, 1977),

an interaction between prilocaine and the acidic phospholipids involved in the coupling process might well be expected to yield an inhibitory response, masking any increase in activation caused by increased bilayer fluidity. Consistent with this is our observation that the cationic drug vinblastine can selectively inhibit the activity of the coupled state (Whetton & Houslay, 1980).

In rat liver plasma membranes there is a lipid phase separation occurring at 28°C (Houslay *et al.*, 1976*b*; Sauerheber *et al.*, 1977; Dipple & Houslay, 1978; Luly & Shinitzky, 1979) that can be localized to those lipids in the outer half of the bilayer, whereas no lipid phase separation occurs in the inner half of the bilayer over a temperature range of 0–42°C (Houslay *et al.*, 1976*b,c*; Houslay, 1979). The neutral local anaesthetic benzyl alcohol, which increases bilayer fluidity, decreases the temperature at which this lipid phase separation occurs by about 6°C at 40 mM-benzyl alcohol (Dipple & Houslay, 1978; Gordon *et al.*, 1980). This is because small lipophilic molecules like benzyl alcohol and prilocaine partition into fluid lipid, but are excluded from the crystalline-phase lipid, forming below the lipid phase-transition temperature. The net effect of this is that these molecules will depress the lipid phase-transition/separation temperature (Colley & Metcalfe, 1972; Papahadjopoulos *et al.*, 1975; Lee, 1976*a,b*) by a classical depression-of-freezing-point mechanism (see Hill, 1974; Lee, 1977). At 10 mM, prilocaine caused an increase in the activity of fluoride-stimulated adenylate cyclase (Fig. 2) and an increase in bilayer fluidity (Fig. 3); however, it had no significant effect on the lipid phase separation occurring at 28°C that could be detected by either the fatty acid spin-probe (Fig. 5) or Arrhenius plots of glucagon-stimulated adenylate cyclase activity (Fig. 4). We suggest that 10 mM-prilocaine does not perturb the lipids of the external half of the bilayer of liver plasma membranes. Prilocaine at such a concentration appears selectively to perturb the lipids of the inner half of the bilayer by increasing their fluidity. Consistent with this are our observations that the Arrhenius plots of the fluoride-stimulated adenylate cyclase activity, which were normally linear over the temperature range studied (Houslay *et al.*, 1976*a*), exhibited a well-defined break at around 11°C when 10 mM-prilocaine was present in the assays. The occurrence of such a prilocaine (10 mM)-induced break was also apparent in Arrhenius plots of glucagon-stimulated adenylate cyclase activity, which, unlike the fluoride-stimulated activity, is sensitive to the lipid environment of both halves of the bilayer. The break in the Arrhenius plots occurring at around 11°C does not appear to be due to an effect of prilocaine on the protein, as Arrhenius plots for the solubilized enzyme were unaffected, but is due to a lipid phase

separation that can be detected by the incorporated fatty acid spin-probe (Fig. 5). This prilocaine (10 mM)-induced lipid phase separation appears to be localized in the inner (cytosol-facing) half of the bilayer of liver plasma membranes.

Our experiments thus support the contention of Sheetz & Singer (1974), who suggested that cationic drugs may act preferentially at the cytosol-facing half of the bilayer of plasma membranes. This is presumably because the negatively charged phospholipids that predominate at this surface (Higgins & Evans, 1978) can in model systems allow cationic drugs to increase the fluidity of lipid bilayers containing them at much lower concentrations than those necessary to increase the fluidity of neutral bilayers, providing a basis for their selective effect (see, e.g., Hauser *et al.*, 1969; Papahadjopoulos *et al.*, 1975; Lee, 1976*a*). It is likely that the presence of such asymmetrically distributed negatively charged species and also membrane-bound Ca^{2+} , which preferentially associates with them (Hope & Cullis, 1979) and can increase the rigidity of these plasma membranes (Gordon *et al.*, 1978), is likely to create domains of laterally segregated lipids of different chemical and physical properties (Papahadjopoulos *et al.*, 1975; Marinetti & Crain, 1978). The incorporation into the bilayer of a cationic hydrophobic molecule such as prilocaine, which can also displace membrane-bound Ca^{2+} (Hauser & Dawson, 1968; Papahadjopoulos, 1972; Low *et al.*, 1979), is likely to have profound effects on lateral lipid distribution. It is not perhaps too surprising then that prilocaine does not act in a relatively simple fashion like the neutral benzyl alcohol, which depresses the temperature of an existing lipid phase separation by about 6–8°C. Presumably prilocaine induces a lipid phase separation at 11°C in the inner half of the bilayer by virtue of its charge characteristics, which perturb the existing lateral organization of lipid species there.

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