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EPIDERMAL GROWTH FACTOR STIMULATES MONOVALENT CATION TRANSPORT IN ISOLATED RAT HEPATOCYTES

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SUMMARY : Epidermal growth factor (EGF) binds specifically to freshly isolated rat hepatocytes. Analysis of binding data at steady state at 20°C yields an apparent K_D of 1 nM and a total number of approximately 930,000 sites per cell. EGF stimulates $^{22}Na^+$ influx and the transport activity of the (Na^+-K^+)ATPase, measured by the ouabain-sensitive uptake of $^{86}Rb^+$. These effects of EGF on monovalent cation transport in hepatocytes may be relevant to the mitogenic action of the growth factor in liver.

Epidermal growth factor (EGF) stimulates growth and proliferation in a wide variety of cells in culture (1). Like for many polypeptide hormones, the first step in EGF biological action appears to be its binding to specific receptors on the plasma membrane of target cells (1). The effect of EGF in the liver has not been extensively studied ; there are numerous EGF specific binding sites in liver plasma membranes (2), but no direct biological effect of EGF has been demonstrated in liver preparation in vitro. A possible role for EGF in the control of liver cell proliferation has been postulated based on the observation that a mixture of EGF, insulin and glucagon was capable of initiating DNA synthesis in adult rat liver in vivo (3) and in primary cultured rat hepatocytes (4,5,6). On the other hand, recent studies have led to the hypothesis that the mitogenic signal required to initiate DNA replication may involve the stimulation of Na^+ influx (reviewed in 7). These observations prompted us to study the effect of EGF on Na^+ transport in isolated rat hepatocytes.

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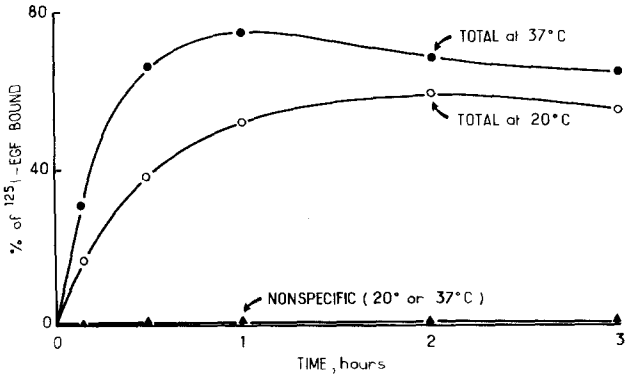

FIGURE 1. Time course of ^{125}I-EGF binding. Hepatocytes (1 x 10^6 cells /ml) were incubated with 0.3 ng/ml ^{125}I-EGF (20,000 cpm/tube) at 20°C or 37°C. At the indicated time points the cell-bound radioactivity was determined in triplicate as indicated in Materials and Methods. The nonspecific binding was determined at each time point in separate tubes containing 6 µg/ml of unlabeled EGF in addition to ^{125}I-EGF.

MATERIALS AND METHODS

Hepatocytes were isolated from adult male Wistar rats as previously described (8). In all experiments cells were incubated in microfuge tubes (Eppendorf, 1.5 ml capacity) at a final cell concentration of 1.0 x 10^6/ ml in 250 µl of Krebs-Ringer bicarbonate (KRb) buffer, pH 7.4, containing 10 mg/ml bovine serum albumin (Fraction V), gentamycin (50 µg/ ml) and bacitracin (0.8 mg/ml). Tubes were gassed with a mixture of 5% CO_2- 95% O_2, capped and incubated in a thermostated water bath. Assays (transport and binding) were terminated by adding 1.2 ml of chilled saline. Cells were immediately collected by a 5-s centrifugation at 2000 x g, resuspended in chilled saline and centrifuged again. In $^{22}Na^+$ uptake experiments cell pellets were washed once more. The cell pellets were directly counted in a gamma spectrometer for ^{125}I or ^{22}Na, or resuspended in 100 µl of water and 1.5 ml of scintillation liquid for $^{86}Rb^+$ radioactivity determination.

EGF (Collaborative Research) was iodinated as described for insulin (referred to as the second modification in 9) ; the ^{125}I-labeled hormone was purified by chromatography on Sephadex G-50. Calculated specific activity of ^{125}I- EGF ranged from 200 to 250 µCi/µg.

^{22}NaCl and ^{86}RbCl were from the Radiochemical Centre, Amersham (England), Na^{125}I from the Commissariat à l'Energie Atomique, Saclay (France).

RESULTS

Characteristics of EGF binding to isolated hepatocytes : The time course of total and nonspecific ^{125}I-EGF binding to isolated hepatocytes at 20°C and 37°C is shown in Figure 1. Maximal binding was reached after 30 min and 2 h at 37°C and 20°C, respectively, and remained stable up to 3

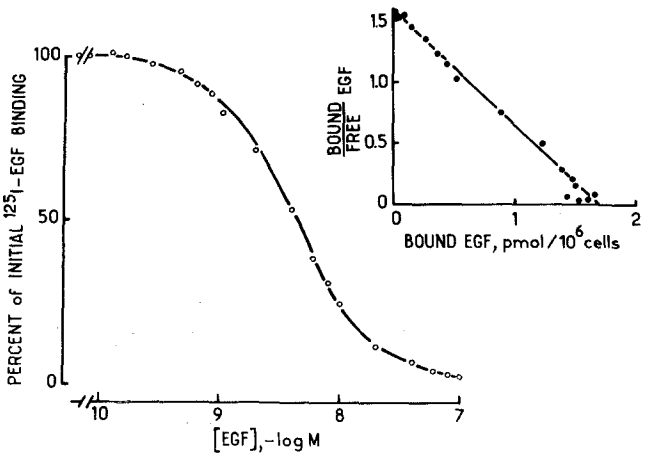

FIGURE 2. EGF binding at steady state. Hepatocytes (1 x 10^6 cells/ml) were incubated for 2 h at 20°C in the presence of 0.3 ng/ml ^{125}I-EGF and varying concentrations of unlabeled EGF. Inset : Scatchard analysis of the data.

h. At steady state, 60 to 80% of total ^{125}I-EGF (0.3 ng/ml, 0.05 nM) was bound ; the nonspecific binding was about 1% of total binding. Varying concentrations (from 0.01 to 1000 nM) of unlabeled EGF inhibited the binding of ^{125}I-EGF at steady state at 20°C in a dose-related fashion (Fig. 2). Scatchard analysis of the data yielded a linear plot (Fig. 2, inset), indicating that the binding of EGF can be accounted for by a single class of receptors. A mean number of 930,000 sites per cell and an apparent dissociation constant (K_D) of 0.95 nM were calculated from 3 separate experiments.

Effect of EGF on $^{86}Rb^+$ uptake : As shown in Figure 3, the basal uptake of $^{86}Rb^+$ (a K^+ tracer) increased rapidly during the first 15 min, and followed a slower near-linear rate between 30 and 90 min. The inhibition of basal $^{86}Rb^+$ uptake in the presence of ouabain indicated that this uptake was accounted for mainly by the transport activity of the (Na^+-K^+) ATPase. Active (ouabain-sensitive) $^{86}Rb^+$ uptake was enhanced by EGF (100 nM) within 30 min ; a 50% increase was observed after 60 min, no effect was detected in the presence of ouabain (Fig. 3). Half-maximal stimulation

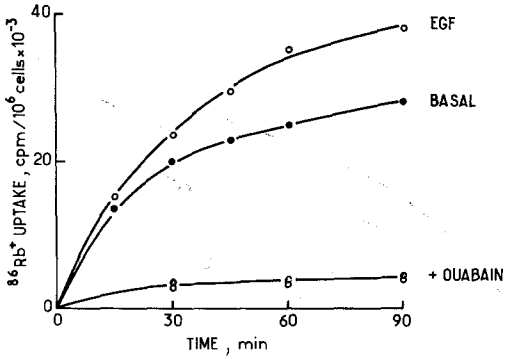

FIGURE 3. Effect of EGF on the time course of $^{86}Rb^+$ uptake by isolated hepatocytes. The transport assay was initiated by adding 150 µl of a cell suspension (3 x 10^6 cells/ml) to microfuge tubes containing 50 µl of $^{86}Rb^+$ (400,000 cpm/tube), 25 µl of 1 µM EGF or buffer (basal), and 25 µl of 20 mM ouabain or buffer, in KRb buffer containing 6 mM K^+. The tubes were incubated at 37°C with intermittent shaking and assays were terminated at the indicated time points as described in Materials and Methods.

(EC_{50}) and maximal effect occured with EGF at about 1 nM and 10 nM, respectively (Fig. 4).

Effect of EGF on $^{22}Na^+$ uptake : To investigate whether EGF could affect Na^+ entry in isolated hepatocytes, the uptake of $^{22}Na^+$ by cells previously exposed to ouabain (to inhibit active Na^+ efflux through the

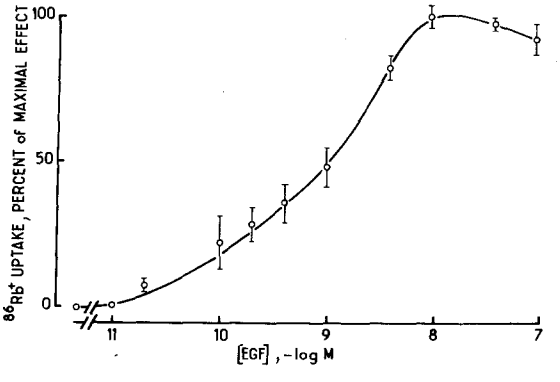

FIGURE 4. Dose response of EGF stimulation of $^{86}Rb^+$ transport. Hepatocytes (3 x 10^6 cells/ml) were incubated for 1 h at 37°C in KRb buffer with varying concentrations of EGF in the presence of $^{86}Rb^+$ (400,000 cpm/tube). Each determination was run in triplicate within each experiment. Data are means ± SE of 6 separate experiments.

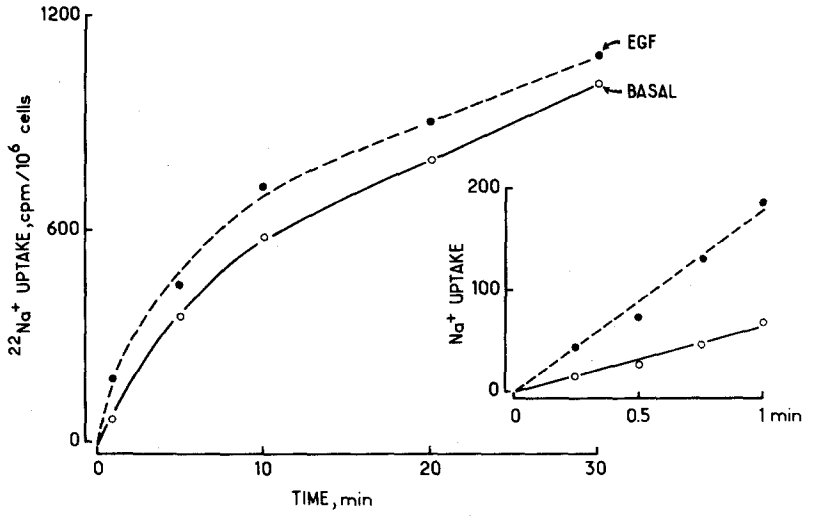

FIGURE 5. Effect of EGF on $^{22}Na^+$ influx. Hepatocytes (3 x 10^6 cells/ml) were preincubated for 10 min at 37°C with 2 mM ouabain. The transport assay was initiated by adding 150 µl of $^{22}Na^+$ (100,000 cpm, 10 cpm/nmole) and 50 µl of buffer (basal) or 0.5 µM EGF. Assays were terminated at the indicated time points as described in Materials and Methods. Each determination was run in triplicate and data represent the means of 2 separate experiments.

(Na^+-K^+) pump) was measured in the absence or presence of EGF. Figure 5 shows that EGF enhanced $^{22}Na^+$ entry within 15 s ; this effect was maximal after 1 min, but was also observed at later time points. The stimulatory effect of EGF on Na^+ influx was dose-dependent ; half-maximal stimulation and maximal effect occurred with EGF at about 2 nM and 10 nM, respectively (Fig. 6).

DISCUSSION

A possible growth-promoting role for EGF in the liver has been suggested by studies performed *in vivo* (3) and *in vitro* (4-6). Koch and Leffert (5) observed that amiloride, a specific inhibitor of passive Na^+ influx, prevented the stimulation of hepatocyte proliferation by a mixture of insulin, glucagon and EGF .These authors proposed that the stimulation of Na^+ influx by growth factors such as EGF could represent an early mitogenic signal in the liver. It was therefore of interest to investigate EGF binding and effect on Na^+ influx in hepatocytes.

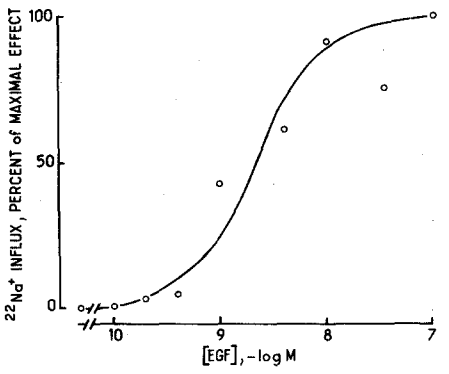

FIGURE 6. Dose-response of EGF stimulation of $^{22}Na^+$ influx. Initial rates of $^{22}Na^+$ transport were determined in 1-min assays in the presence of varying concentrations of EGF. Data are means of triplicate determinations in one experiment.

In the present study, we have shown that isolated hepatocytes bind specifically EGF both at 37°C and 20°C. Although the interpretation of the binding data is complicated by the fact that EGF is rapidely internalized by the cells (J.L. Carpentier, P. Gorden, P. Freychet, B. Canivet and L. Orci, submitted), steady-state binding experiments at 20°C have revealed that the peptide binds to a single class of high affinity receptors in isolated hepatocytes. On the other hand, EGF rapidly stimulates Na^+ entry into these cells, in a dose-dependent fashion.

It has been suggested that the stimulation of cell proliferation (insofar as it is related to an increase in Na^+ influx) may involve an activation of the (Na^+-K^+) pump (5,10). In agreement with this hypothesis, our data indicate that EGF enhances the transport activity of the (Na^+-K^+) ATPase, as measured by the ouabain-sensitive uptake of Rb^+. The dose-dependence of EGF stimulation of both Na^+ influx and active Rb^+ uptake (EC_{50} of about 1 nM) was close to that observed for EGF binding (apparent K_D of about 1 nM), suggesting that both types of effects are mediated by the peptide's interaction with the observed receptors.

In conclusion, we have presented evidence that adult rat hepatocytes possess specific receptors for EGF and respond to this growth factor by an

increase in Na^+ influx and in the activity of the (Na^+-K^+) pump. These findings may be relevant to the mitogenic effect of EGF in liver.

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