pharmacology

The discovery of a diversity of neuro- and hormonal polypeptides has increased interest in exploiting peptides and their analogues as therapeutic drugs. In general, the plasma half-lives of polypeptides are very short, which becomes a major stumbling block in the development of polypeptide drugs. To aid in the design of effective polypeptide drugs, knowledge of the factors involved in determining their disposition *in vivo* is essential. One of the most remarkable features of the pharmacokinetics of polypeptides is the contribution of specific binding sites (receptors) to peptide distribution and elimination in the body (1–9). Receptor-mediated endocytosis is now well recognized as a general mechanism employed by many cells in the uptake of biologically important polypeptide hormones (1, 2, 6, 10, 11), and the concept of "transport (or clearance) receptor" is now well established (7). The liver and the kidney have been widely accepted as the most important organs in clearing polypeptides from the circulation (1–4, 12–17).

We have been analyzing the kinetics of the hepatic handling of a model polypeptide, epidermal growth factor (EGF), in rats *in vivo* (1, 2, 4), in perfused liver (9, 14, 15), in isolated hepatocytes (18, 19), and in isolated liver plasma membrane vesicles (20). We have shown that liver plays a major role in the elimination of EGF from the circulating blood (1, 2) and suggested that the liver may regulate the plasma concentration of EGF via receptor-mediated endocytosis (1, 2, 15). In the presence of excess EGF, the binding of EGF to specific plasma membrane receptors is rapidly followed by the internalization of the complex, leading to a decrease in the number of receptors on the cell surface (down-regulation) (2, 5, 15, 16, 21). However, most of the internalized receptors recycle to the cell surface, leading to a recovery in the number of cell-surface receptors ($t_{1/2} \approx 30$ min) (1, 2, 15).

We have also determined the internalization rate constants of the EGF–receptor complex in perfused liver and isolated hepatocytes (mean time = 2–9 min) (14, 19) and have shown that the rate constant is independent of receptor occupancy (14). On the other hand, McClain and Olefsky (22) have reported that the insulin–receptor internalization rate in hepatoma cells depends on the insulin concentration in the medium and suggested that there exist two pathways for insulin–receptor internalization.

Relatively high doses of EGF (40–400 μg/kg of body weight) seem to be required to drive pharmacological activities such as promotion of hepatic growth (23) and enhancement of DNA synthesis in several organs (24) after its administration to experimental animals. Under such high dose conditions, the EGF concentration in the circulating plasma was estimated to be >50 nM, which may be high enough to saturate the high-affinity receptor-mediated hepatic uptake. To examine how the hepatic uptake clearance will be changed when saturation occurs is, therefore, essential to understanding EGF disposition in the body after a pharmacologically effective dose is administered. In the present study, as the first step in analyzing the EGF clearance mechanism at high doses, we attempted to clarify whether the internalization pathways may differ depending on the EGF concentration in the medium (isolated rat hepatocytes) and in the perfusate (perfused rat liver). For this purpose, we used phenylarsine oxide (PAO), a well-known inhibitor of polypeptide internalization (25–27).

## MATERIALS AND METHODS

**Materials.** PAO was purchased from Wako Pure Chemical (Osaka) and rotenone from Sigma. All other reagents were

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Abbreviations: EGF, epidermal growth factor; PAO, phenylarsine oxide.

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commercial products of analytical grade. EGF (human; Wakunaga Pharmaceutical, Osaka) was radiolabeled with $^{125}I$ by the chloramine-T method (9). $^{125}$I-EGF thus obtained had a specific activity of 220–330 Ci/g (1 Ci = 37 GBq).

**Determination of Surface-Bound and Internalized EGF in Perfused Liver.** The method reported previously (14) was used. After a preperfusion in the presence or absence of PAO for 30 min, the single-pass perfusion of EGF was initiated by switching to a perfusate containing $^{125}$I-EGF (0.1 $\mu$Ci/ml; 0.03 nM) alone or $^{125}$I-EGF with 20 nM unlabeled EGF. The samples (0.4 ml) drawn from the inlet and outlet of the liver were collected and the amounts of intact peptide and degraded product were determined by trichloroacetic acid precipitation (14). Twenty minutes after the start of perfusion the perfusate was switched to the acid buffer (pH 3.0) and the amount of $^{125}$I-EGF bound to liver cell surfaces, $LR_s$, was estimated from the trichloroacetic acid-precipitable radioactivity recovered in the outflow (14). After the acid wash, the liver was excised and its associated radioactivity was determined and regarded as the amount of internalized $^{125}$I-EGF.

**Determination of Surface-Bound and Internalized $^{125}$I-EGF in Isolated Hepatocytes.** Hepatocytes were prepared as reported previously (19). After incubation of $^{125}$I-EGF with the isolated hepatocytes at 37°C for the specified time in the buffer described previously (19), the binding reaction was terminated by diluting an aliquot of cell suspension with a large volume of ice-cold buffer. When necessary, the hepatocytes were preincubated for 10 min or 30 min with various concentrations of rotenone or PAO, respectively. Rotenone and PAO were prepared as 0.4 mM and 12 mM stock solutions in dimethyl sulfoxide, respectively. The stock solution was diluted with the buffer to give specified concentrations of rotenone or PAO. The surface-bound and internalized $^{125}$I-EGF were separated by the acid washing method (18, 19).

**ATP Determination.** A modified luciferin–luciferase assay was used to determine the cellular ATP content (28).

**Calculation of Internalization Rate Constants.** At elapsed times before intracellular degradation has occurred, the internalization rate of ligand–receptor complexes is given by

$$d[LR_i]/dt = k_{int}[LR_s], \quad [1]$$

where, for the EGF–receptor complex, $[LR_i]$ is the internalized amount, $k_{int}$ is the internalization rate constant, and $[LR_s]$ is the amount on the cell surface. Integration of Eq. **1** from time 0 to $t$ yields the following equation:

$$[LR_i]_t = k_{int}\int_0^t [LR_s]dt. \quad [2]$$

The integral was calculated numerically by the trapezoidal rule. The $k_{int}$ value could then be estimated from the initial slope of a plot of $[LR_i]_t$ vs. $\int_0^t [LR_s]\,dt$ from Eq. **2** (see Fig. 2). This is designated an "integration plot." If $d[LR_s]/dt = 0$—that is, if the amount of the EGF–receptor complex on the cell surface is at steady state—Eq. **1** yields the following equation:

$$[LR_i]_t = k_{int,app}[LR_s]t. \quad [3]$$

Thus, the approximated $k_{int}$ value ($k_{int,app}$) could be estimated by dividing $[LR_i]_t$ by $[LR_s]t$ (see Table 1; Figs. 4 and 5). Our experience is that 20 min is enough time for $[LR_s]$ to reach steady state both in perfused rat liver (14) and in isolated rat hepatocytes (Fig. 1).

## RESULTS

**Effect of PAO on $^{125}$I-EGF Internalization by Perfused Rat Liver.** The effect of PAO on $^{125}$I-EGF internalization was examined in both the presence and the absence of excess unlabeled EGF (20 nM) in the perfusate (Table 1). The amount of surface-bound $^{125}$I-EGF after perfusion of tracer $^{125}$I-EGF for 20 min was ≈5 times greater in the presence of PAO than that in its absence (Table 1). However, the internalized $^{125}$I-EGF in the presence of PAO was less than one-sixth of that in the absence of PAO. As a result, in the presence of PAO, the $k_{int}$ value calculated based on Eq. **3** was only 3.5% of that in its absence. On the other hand, after perfusion of $^{125}$I-EGF with excess unlabeled EGF, surface-bound and internalized $^{125}$I-EGF in the presence of PAO showed minimal change compared with that in the absence of PAO. That is, the $k_{int}$ value decreased only to 40% of the control value (Table 1).

**Time Course of Surface-Bound and Internalized $^{125}$I-EGF in the Presence and Absence of PAO.** In untreated cells (control cells), surface-bound $^{125}$I-EGF reached a maximum around 1 min in either the presence or the absence of excess (20 nM) unlabeled EGF (Fig. 1 *A* and *B*). In the presence of PAO, the amounts of internalized $^{125}$I-EGF were significantly lower than those of the control in either the presence or the absence of excess EGF. The internalization rate constants ($k_{int}$) were estimated based on Eq. **2** (Fig. 2). Under the "tracer" condition, the $k_{int}$ value decreased from 0.37 min$^{-1}$ (control) to 0.038 min$^{-1}$ in the presence of PAO. Under the "excess" condition, the decrease in $k_{int}$ value was smaller, in that it decreased from 0.31 min$^{-1}$ (control) to 0.093 min$^{-1}$ in the presence of PAO. Since PAO is known to reduce cellular ATP content (25), it was possible that the inhibition of $^{125}$I-EGF internalization by PAO was due to a decrease in cellular ATP. To examine this possibility, we lowered the cellular ATP content with rotenone (Fig. 3) and measured the

Table 1. Effect of PAO on cell-surface binding and internalization of $^{125}$I-EGF after a 20-min liver perfusion of $^{125}$I-EGF (0.03 nM) in the presence or absence of excess unlabeled EGF (20 nM)

| EGF, nM | Condition | $^{125}$I-EGF/infusion rate, (cpm per liver)/(cpm per min) Surface-bound | Internalized | $k_{int}$, min$^{-1}$ | $k_{int}$ ratio (+ PAO/control) |
|---|---|---|---|---|---|
| 0.03 | Control | 2.18 ± 0.13 | 9.94 ± 0.93 | 0.232 ± 0.031 | 0.035 |
| | + PAO | 10.4 ± 0.8* | 1.59 ± 0.26* | 0.0081 ± 0.0019* | |
| 20 | Control | 0.459 ± 0.137* | 2.42 ± 0.31* | 0.354 ± 0.103 | 0.395 |
| | + PAO | 0.666 ± 0.056† | 1.84 ± 0.42 | 0.140 ± 0.032† | |

After a preperfusion in the presence of 500 $\mu$M PAO for 30 min, the single-pass perfusion of EGF was initiated by switching to a perfusate containing $^{125}$I-EGF (0.03 nM) or $^{125}$I-EGF with unlabeled EGF (20 nM). Twenty minutes after the start of the perfusion, the amounts of surface-bound and internalized $^{125}$I-EGF were determined as described in the text. The $k_{int}$ values were calculated based on Eq. 3. Values are expressed as mean ± SE of three independent experiments.
*Significantly different from 0.03 nM control, $P < 0.05$.
†Significantly different from 20 nM control, $P < 0.05$.

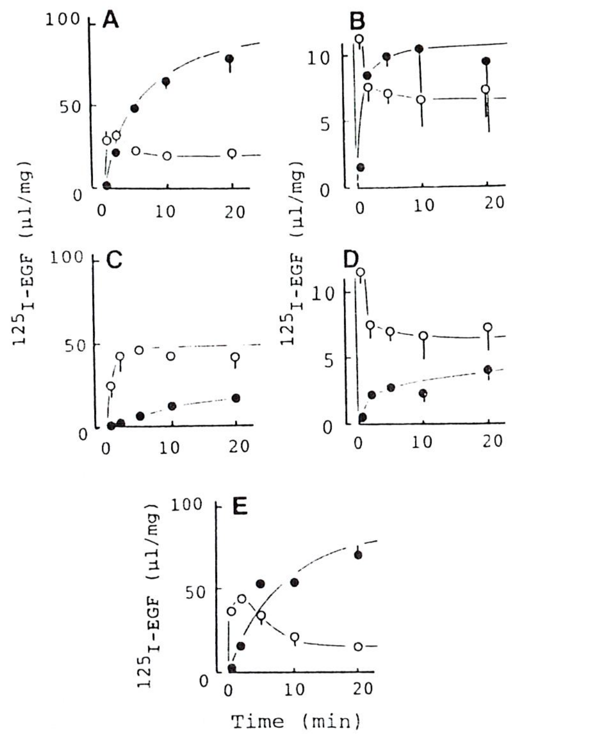

FIG. 1. Effect of PAO or rotenone on the time course of surface-bound (○) and internalized (●) $^{125}$I-EGF in isolated rat hepatocytes. The cells (2 mg of protein per ml) were incubated at 37°C with the tracer concentration of $^{125}$I-EGF with (*B* and *D*) or without (*A*, *C*, and *E*) 20 nM unlabeled EGF in the presence of 30 $\mu$M PAO (*C* and *D*), 1 $\mu$M rotenone (*E*), or neither PAO nor rotenone (*A* and *B*). Each point and vertical bar represent the mean and SE of two (*B*, *D*, and *E*), or three (*A* and *C*) independent experiments. Data are expressed as the values normalized by total applied $^{125}$I-EGF concentrations.

time courses of the surface-bound and internalized $^{125}$I-EGF (Fig. 1*E*). Rotenone (1 $\mu$M) decreased the cellular ATP content more rapidly than did PAO (30 $\mu$M), but the final ATP level was somewhat lower with PAO than with rotenone (Fig. 3). Although rotenone thus decreased the cellular ATP level, the time courses of surface-bound and internalized $^{125}$I-EGF in the presence of rotenone showed minimal change compared with the control (Fig. 1*E*). The $k_{int}$ values for rotenone-treated and control cells were also comparable (Fig. 2).

**Inhibition of $^{125}$I-EGF Internalization by Increasing Concentrations of PAO.** The concentration-dependent effect of PAO and rotenone on both the internalization of $^{125}$I-EGF and the ATP decrease was also examined. The decrease in surface-bound $^{125}$I-EGF with increasing PAO concentration was minimal in either the presence or the absence of excess EGF (20 nM, Fig. 4). The internalized $^{125}$I-EGF, however, decreased significantly with increasing concentrations of PAO and the maximum decrease, attained at 30 $\mu$M PAO, was constant up to 500 $\mu$M (Fig. 4 *A* and *B*). Internalized $^{125}$I-EGF decreased finally to approximately 17% and 40% of the control values in the absence and presence of excess EGF, respectively. The $k_{int}$ values calculated based on Eq. **3** decreased to 12% of the control under the "tracer" condition but decreased only to 50% of control under the "excess" condition (Fig. 4*C*). Like rotenone, PAO decreased the cellular ATP level in a concentration-dependent manner (Fig. 5), although the effect of rotenone was observed at a much lower concentration range. However, in the cells treated with increasing concentrations of rotenone, no significant difference from the control was observed in surface-bound $^{125}$I-EGF, internalized $^{125}$I-EGF, and $k_{int}$ value in either the presence or the absence of excess unlabeled EGF (Fig. 6).

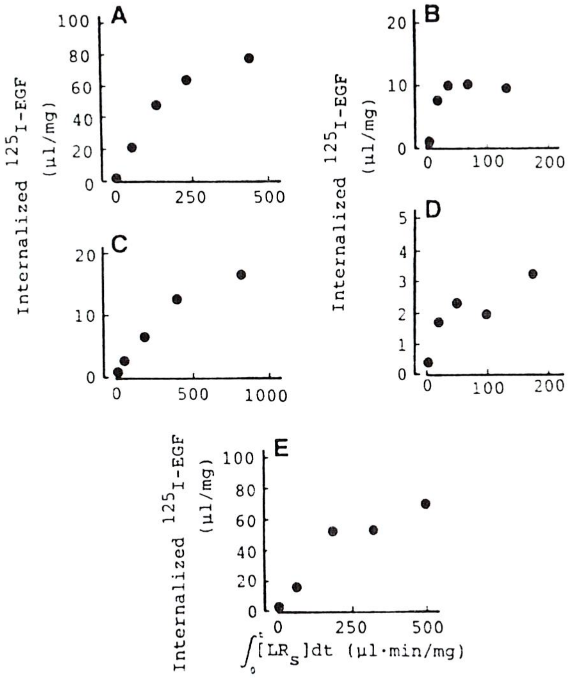

FIG. 2. Integration plots for estimating the internalization rate constants ($k_{int}$) according to Eq. **2**. Data shown in Fig. 1 were used and *A–E* correspond to those in Fig. 1 *A–E*. The slope obtained by the linear regression of the initial three points gives the $k_{int}$ value. The $k_{int}$ values obtained were 0.366 ± 0.011, 0.309 ± 0.044, 0.038 ± 0.004, 0.093 ± 0.008, and 0.282 ± 0.012 min$^{-1}$ in *A–E*, respectively.

## DISCUSSION

We previously analyzed the kinetics of EGF binding to liver cell-surface receptors, the subsequent internalization and intracellular degradation of EGF, and receptor recycling by using isolated rat hepatocytes, perfused rat livers, and *in vivo* systems (1–4, 9, 14, 15, 18, 19). Following these studies, we attempted to elucidate the details of the internalization process of EGF. While we were examining the inhibitory effect of PAO on the internalization of EGF by perfused rat livers, we found that the internalization depended much on the EGF concentration in the perfusate (Table 1). That is, PAO decreased the internalization rate constant ($k_{int}$) to 1/30th of the control when a tracer concentration (0.03 nM) of $^{125}$I-EGF was perfused in the liver. Considering the dissociation constant (1–20 nM) for EGF binding to rat liver (9, 18), we estimated that <3% of the surface receptors may be occupied by EGF under this condition. On the other hand, PAO decreased the $k_{int}$ value only to 40% of the control when excess EGF (20 nM) was present in the perfusate, a condition under which most of the cell-surface receptors were occupied (Table 1). We then tried to confirm this EGF concentration-

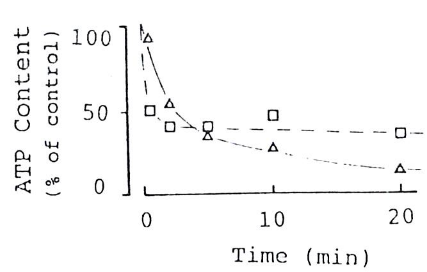

FIG. 3. Time course of the decrease in cellular ATP levels after addition of PAO or rotenone. Isolated hepatocytes (2 mg of protein per ml) were incubated at 37°C in the presence of 30 $\mu$M PAO (△) or 1 $\mu$M rotenone (□). At the indicated times, the cellular ATP content was determined as described in the text.

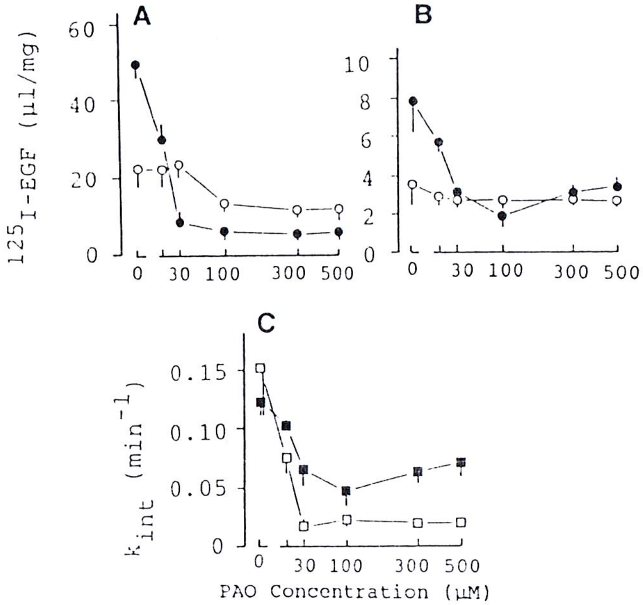


FIG. 4. Concentration-dependent effect of PAO on the amounts of surface-bound and internalized $^{125}$I-EGF in isolated rat hepatocytes. The cells (2 mg of protein per ml) were preincubated with various concentrations of PAO for 30 min at 37°C and incubated for an additional 20 min with $^{125}$I-EGF in the absence (*A*) or presence (*B*) of 20 nM unlabeled EGF. Each point and vertical bar represent the mean and SE of four (*B*) or five (*A*) independent experiments. Data are expressed as the values normalized by total applied $^{125}$I-EGF concentrations. The apparent internalization rate constants were estimated (*C*) by dividing the internalization rate of $^{125}$I-EGF by the amount of surface-bound $^{125}$I-EGF (Eq. 3). ○, Surface-bound $^{125}$I-EGF; ●, internalized $^{125}$I-EGF; □, $k_{int}$ of tracer $^{125}$I-EGF; ■, $k_{int}$ of tracer $^{125}$I-EGF in the presence of 20 nM unlabeled EGF.

dependent inhibition of internalization by PAO in isolated rat hepatocytes.

The maximum inhibitory effect of PAO on the internalization of $^{125}$I-EGF was attained at 30 $\mu$M PAO (Fig. 4). In the presence of tracer $^{125}$I-EGF alone, PAO decreased the $k_{int}$ value by a factor of 8–10 compared with the control value (Figs. 2 and 4). On the other hand, PAO decreased the $k_{int.app}$ value to only half to one-third of the control value when an excess of EGF was present (Figs. 2 and 4). A much greater inhibition of $^{125}$I-EGF internalization by PAO under "tracer" conditions than with excess unlabeled EGF was thus obtained in the isolated hepatocytes as well as in the perfused livers.

In both perfused livers and isolated hepatocytes, PAO inhibited the bulk of the internalization process in the presence of tracer $^{125}$I-EGF alone, but only when an excess of EGF (20 nM) was added was significant PAO-insensitive internalization observed. These results indicated that there

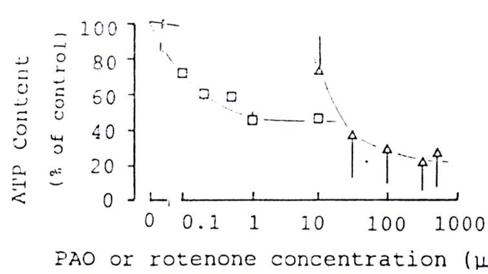


FIG. 5. Effect of PAO or rotenone on cellular ATP levels. Isolated hepatocytes (2 mg of protein per ml) were incubated at 37°C with various concentrations of PAO for 30 min (△) or various concentrations of rotenone for 10 min (□). ATP content was determined as described in the text.

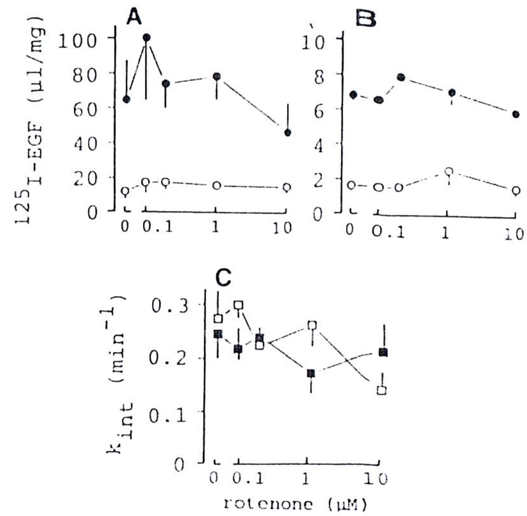


FIG. 6. Concentration-dependent effect of rotenone on the amounts of surface-bound and internalized $^{125}$I-EGF in isolated rat hepatocytes. The experimental protocol was as described for Fig. 4 except for the use of rotenone instead of PAO. Each point and vertical bar represent the mean and SE of two or three independent experiments. ○, Surface-bound $^{125}$I-EGF; ●, internalized $^{125}$I-EGF; □, $k_{int}$ of tracer $^{125}$I-EGF; ■, $k_{int}$ of tracer $^{125}$I-EGF in the presence of 20 nM unlabeled EGF.

existed at least two internalization pathways for EGF, in the form of a low-dose pathway and a high-dose pathway. These two internalization pathways may be explained if two classes of binding sites, high-affinity/low-capacity and low-affinity/high capacity, are taken into consideration. If we further assume that the internalization via high-affinity sites is PAO-sensitive and that the internalization via low-affinity sites is PAO-insensitive, the above findings can be easily understood.

On the other hand, the $k_{int}$ values determined in the absence of PAO for tracer and excess dose conditions were comparable (Figs. 2 and 4), indicating that the probability of an EGF–receptor complex on the cell-surface being internalized per minute is independent of the classes of binding sites, as we found previously (14). However, in other studies (9, 15, 18, 20), we could not find multiple binding sites in any experimental system, although nonspecific (unsaturable) binding was observed. We estimated that the equilibrium dissociation constant, $K_d$, was a few nanomolar in either isolated hepatocytes (18) or isolated liver membrane vesicles (20) and was 18 nM in perfused rat liver (9). Other laboratories also reported a single receptor population with $K_d$ values of 0.7–5 nM (29–31). On the other hand, Gladhaug and Christoffersen (21) reported that there were two EGF receptor classes on isolated rat hepatocytes, with $K_d$ values of 0.02 nM and 0.6 nM. Dunn *et al.* (16) also identified two classes of receptors ($K_d$ values of 12 nM and 200 nM) in the perfused rat liver. The reason for these discrepancies between the laboratories is not clear, but it may be related to the differences in EGF concentration range used in the binding studies or to the method of $^{125}$I-EGF preparation.

McClain and Olefsky (22) have reported that there exist two independent pathways for insulin internalization in hepatocytes and hepatoma cells. They found that hypotonic shock and hypokalemia, which arrest coated-pit formation, blocked internalization of insulin and its receptors at a low insulin concentration (1 ng/ml) but allowed internalization in response to a high concentration (100 ng/ml) of insulin (22). From these results, they speculated that the high-dose pathway, unlike the low-dose pathway, may proceed independently of coated pits. It is still unknown whether the high-dose pathway of EGF internalization in hepatocytes that we found in this study may proceed dependently or independently of coated pits. In either case, EGF is considered to be taken up by the liver via the low-dose (high-affinity) pathway in the physiological condition, if the normal EGF concentration (<0.3 nM) in the plasma is taken into consideration (32). When a high dose of EGF is administered, which has often been done to drive *in vivo* pharmacological effects (23, 24), the PAO-insensitive internalization pathway in the liver may play a significant role in taking up EGF from the circulation.

PAO is also known to decrease the cellular ATP content in some cell systems (25, 27). It is therefore possible that the inhibition of $^{125}$I-EGF internalization by PAO was due to a decrease in intracellular ATP. To examine this possibility, we measured the change in intracellular ATP level induced by PAO. Furthermore, we lowered the cellular ATP content with rotenone, a well-known ATP depleter, and examined whether or not the internalization of $^{125}$I-EGF was inhibited. As expected, the intracellular ATP content was decreased with increasing concentration of these agents (Fig. 5). The intracellular ATP level was finally decreased to 40% and 30% of the control value with rotenone (1 $\mu$M) and PAO (30 $\mu$M), respectively. On the other hand, PAO (30 $\mu$M) decreased the $k_{int}$ value of tracer $^{125}$I-EGF to one-eighth of the control value (Fig. 4), whereas rotenone decreased the $k_{int}$ value minimally (Fig. 6). These results indicate that (*i*) the decrease in cellular ATP caused by PAO did not contribute much to the inhibition of $^{125}$I-EGF internalization and (*ii*) the internalization of $^{125}$I-EGF is not affected by the decrease in cellular ATP contents (at least to one-third of the normal ATP level). So far, it has been controversial whether or not the internalization of peptides requires the energy supplied from ATP hydrolysis. Hertel *et al.* (33) and Schmidt and Carter (34) reported the ATP-dependent internalization of EGF and transferrin, respectively. On the other hand, it has been suggested that a single round of receptor-mediated endocytosis of asialoorosomucoid by hepatocytes (35) and of low density lipoprotein by fibroblasts (36) can occur in cells where ATP levels were decreased. Our present study supports the latter findings.

We conclude that a high concentration of EGF leads to $^{125}$I-EGF internalization through a pathway that is relatively PAO-insensitive and is distinct from the pathway taken by high-affinity receptors internalized by a low (physiologic) concentration of $^{125}$I-EGF. The latter pathway functioning at low concentrations of EGF is found to be PAO-sensitive.

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