and the apparent affinity seemed to decrease also. Together these account for the lack of changes in total uptake. Low affinity binding sites and dissociation constants increased with age. Functional changes in vascular smooth muscle with increasing age may be due to altered calcium binding.

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Forskolin effects on the beta-adrenergic responsiveness of rat hepatocytes

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Forskolin, a diterpene isolated from the Indian plant *Coleus forskohli*, activates adenylate cyclase in both intact cells and plasma membranes [1]. Furthermore, it has been observed that low concentrations of forskolin can magnify the action of hormones that activate adenylate cyclase [2].

Liver cells from rats contain three types of adrenoceptors, i.e. alpha$_1$, alpha$_2$- and beta-adrenoceptors. In hepatocytes from normal adult rats, the metabolic actions of epinephrine are mediated through a calcium-dependent cyclic AMP-independent alpha$_1$-adrenergic mechanism [3–5]. Beta-adrenoceptors seem to play, if any, a minor role, and the physiological significance of alpha$_2$-adrenoceptors is unknown. However, there are certain conditions in which beta-adrenoceptors play a significant role in the actions of epinephrine in liver cells. Among these conditions are the age of the animals [6], hypothyroidism [7], adrenalectomy [8], cholestasis [9], liver regeneration [9, 10], and dedifferentiation during cell culture [11]. In most of these conditions it has been observed that the increased beta-adrenergic responsiveness is associated with an increased number of beta-adrenoceptors [7–11]. In the present paper, we report that low concentrations of forskolin can enhance the beta-adrenergic responsiveness of liver cells from adult normal rats which indicates that an increase in the number of beta-adrenoceptors is not required to observe beta-adrenergic actions in liver cells.

Materials and methods

l-Epinephrine, *l*-isoproterenol, urease, glutamine and ornithine were obtained from the Sigma Chemical Co. Bovine serum albumin (fraction V) and collagenase (type II) were obtained from the Reheis Chemical Co. and Worthington respectively. Forskolin (7β-acetoxy-8,13-epoxy-12, 6β, 9α-trihydroxylabd-14-ene-11-one) was obtained from Calbiochem. [^{3}H]Cyclic AMP was obtained from the New England Nuclear Corp.

Female Wistar rats weighing between 180 and 200 g were employed. Hepatocytes were isolated by the method of Berry and Friend [12] and incubated in Krebs–Ringer bicarbonate buffer supplemented with 1% albumin, 10 mM glucose, 10 mM glutamine and 2 mM ornithine. Cells (≈40 mg wet weight) were incubated in 1 ml of buffer for 60 min at 37°. Urea was determined by the method of Gutman and Bergmeyer [13]. Cyclic AMP accumulation 2 min after the addition of the agents was determined by the method of Gilman [14].

Results and discussion

In normal rat hepatocytes, activation of beta-adrenoceptors did not result in stimulation of ureogenesis (Ref. 5 and Fig. 1) although a clear 2-fold increase in cyclic AMP levels was produced (Fig. 2). Forskolin (10^{-7} M) did not elevate by itself urea production or cyclic AMP levels (Table 1). However, it clearly magnified the accumulation of cyclic AMP produced by isoproterenol (Fig. 2). This marked increase in cyclic AMP levels resulted in activation of ureogenesis (Fig. 1). The effects of isoproterenol (10^{-6} M) were blocked by the beta-adrenergic antagonist propranolol (10^{-5} M). Forskolin alone at higher concentrations markedly increased cyclic AMP levels and ureogenesis (Table 1).

Our results clearly show that forskolin can markedly magnify the beta-adrenergic responsiveness of adult rat hepatocytes. In addition, the data also show that an increase in the number of beta-adrenoceptors is not an absolute requirement to observe metabolically significant beta-adrenergic responsiveness in hepatocytes. Recently, it has been observed that forskolin can potentiate the action of glucagon on liver adenylate cyclase [15].

The mechanism through which forskolin potentiates hormonal stimulation of adenylate cyclase is obscure. Hormonal modulation of adenylate cyclase activity is mediated through the interaction of the hormone-receptor complexes with the guanine nucleotide regulatory proteins (Ns and Ni for stimulation and inhibition of the enzyme respectively) and the catalytic subunit of adenylate cyclase. We have shown previously that administration of pertussis toxin to

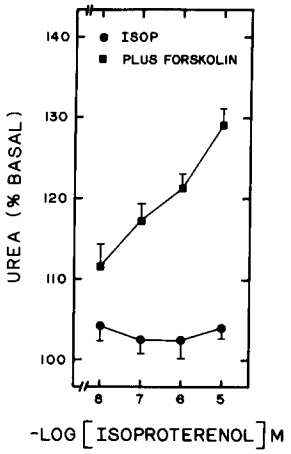

Fig. 1. Dose–response curves for the effect of isoproterenol on urea synthesis in the presence and absence of forskolin. Hepatocytes were incubated in 1 ml of Krebs–Ringer bicarbonate buffer containing 1% albumin, 10 mM glucose, 10 mM glutamine and 2 mM ornithine for 60 min at 37°. Basal urea production was 24.3 ± 1.9 nmoles/mg cells. Results are the means, and the vertical bars represent S.E.M. of triplicate determinations from three cell preparations. ISOP: isoproterenol.

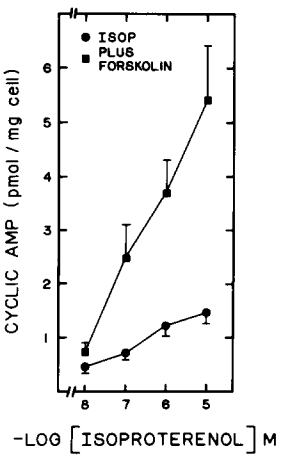

Fig. 2. Dose–response curves for the effect of isoproterenol on the accumulation of cyclic AMP in the presence and absence of forskolin. Hepatocytes were incubated in Krebs–Ringer bicarbonate buffer for 2 min with the agents. Results are the means, and the vertical bars represent S.E.M. of triplicate determinations from three cell preparations. ISOP: isoproterenol.

rats, which blocks the action of inhibitory receptors [16], enhances the beta-adrenergic responsiveness of hepatocytes probably by releasing a constraint exerted by Ni on adenylate cyclase [17]. However, the action of forskolin does not seem to be due to blockade of Ni; inhibition of forskolin-activated adenylate cyclase through receptor-mediated processes has been observed [18, 19]. It has been suggested that forskolin may act on Ns or by altering the interaction of the Ns with a catalytic subunit of the cyclase [20].

All the observations with liver cells suggest that their beta-adrenergic sensitivity may be modulated by modifying the number of beta-adrenoceptors or the interaction of the components of the adenylate cyclase system (Ns-C-Ni).

Table 1. Effect of forskolin on cyclic AMP levels and ureogenesis*

Forskolin (M)	Urea (% basal)	Cyclic AMP (pmoles/mg cells)
None	100 ± 3	0.54 ± 0.03
10^{-7}	102 ± 2	0.63 ± 0.06
10^{-6}	105 ± 2	1.08 ± 0.18
10^{-5}	121 ± 3	1.69 ± 0.24
10^{-4}	127 ± 3	12.36 ± 6.00

* Hepatocytes were incubated in the presence or absence of forskolin for 2 min (cyclic AMP levels) or for 60 min (ureogenesis). Basal urea production was 24.3 ± 1.9 nmoles/mg cells. Results are the means ± S.E.M. of duplicate determinations of at least three experiments using different cell preparations.

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Hepatic microsomal oxidation of styrene to 4-hydroxystyrene 7,8-glycol via 4-hydroxystyrene and its 7,8-oxide as short-lived intermediates

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4-Hydroxystyrene **3** is a urinary metabolite of the plastic monomer, styrene, in the human [1] as well as in the rat [2–4] and has recently been demonstrated to be a sole specific rearrangement product of the highly reactive and mutagenic epoxide, styrene, 3,4-oxide (1-vinylbenzene 3,4-oxide) **2**, which has a half-life of 4.3 sec at 37° and pH 7.4 in an aqueous solution, and has been considered as a putative intermediate in the biotransformation of **1** to **3** [5, 6] (Fig. 1). However, nothing has been reported on the hepatic formation of **3** from **1** *in vitro*. In the present communication, we wish to report that the radioactive metabolite **3** formed from ^{14}C-labelled **1** is undetectable in the hepatic microsomal incubation system fortified with NADPH without using a large amount of unlabelled **3** as a trapping agent for the metabolite, because it is rapidly oxidized by monooxygenase to yield 4-hydroxystyrene 7,8-glycol (4′-hydroxyphenylethane-1,2-diol) **5** via the highly reactive intermediate, 4-hydroxystyrene 7,8-oxide (4′-hydroxyphenyloxiran) **4**.

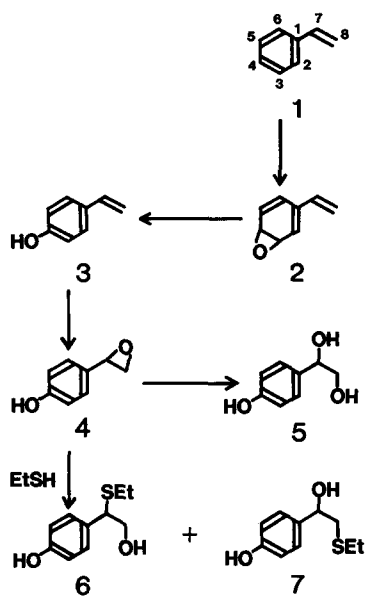

Fig. 1. Oxidation of styrene to 4-hydroxystyrene and 4-hydroxystyrene 7,8-glycol via highly reactive epoxides. **1**: Styrene, **2**: styrene 3,4-oxide, **3**: 4-hydroxystyrene, **4**: 4-hydroxystyrene 7,8-oxide, **5**: 4-hydroxystyrene 7,8-glycol, **6**: 2-(4′-hydroxyphenyl)-2-ethylmercaptoethanol, **7**: 1-(4′-hydroxyphenyl)-2-ethylmercaptoethanol.

Styrene **1** (0.1 mM), redistilled before use and dissolved in acetone (1.2% v/v), was aerobically incubated at 37° for 30 min in a final volume of 5 ml of 0.1 M phosphate buffer, pH 7.4, with liver microsomes (25 mg protein/ml) from male Wistar rats (100–120 g), pretreated with phenobarbital as previously reported [7] in the presence of an NADPH-generating system consisting of NADP (1 mM), glucose 6-phosphate (10 mM), glucose 6-phosphate dehydrogenase (1 IU/ml) and magnesium chloride (5 mM) in an air-tight flask.

The mixture was extracted with an equal volume of ethyl acetate in the presence of a saturating amount of sodium chloride. From the organic phase separated by centrifugation, the solvent was evaporated through a fractional condenser to obtain a condensate, with minimal loss of the volatile metabolites. A g.l.c.–m.s. study showed the condensate not to contain any detectable amount of the phenol **3**. However, it contained the triol **5** which had a retention time of 6 min as a tri-TMS ether on a 1.5% OV-101 column (coated on 80–100 mesh Chromosorb W, 2 m × 3 mm) eluted at 200° with 40 ml He/min; chemical-ionization m.s., recorded with isobutane as a reagent gas at 0.5–1 Torr, *m/z* (relative intensity, %) at an ionization voltage of 100 eV with 100 A ionization current at an ion-source temperature of 250°: 371 ($M^+ + 1$, 18), 355 (25), 282 (40), 269 (15), 268 (30), 267 (100) and 147 (20). The mass spectrum and the retention time were identical with those of the corresponding authentic specimen synthesized as previously reported [8].

[7-^{14}C] **1** [9] (4 μCi/μmole, 0.1 mM) was incubated in the presence of unlabelled **3** (0.8 mM) in a final volume of 250 ml of the mixture under the aforementioned conditions in order to trap radioactive **3** formed during incubation. Using the liquid scintillation counting-absorptiometry-method, **3** isolated as a phenolic fraction from the incubation mixture was found to contain 243 pCi/μmole of radioactivity at 260 nm after it was purified to the constant radioactivity to absorbance ratio by twice successive use of an octadecylsilicone column (ODS, Nucleosil 7C_{18}, 5 μ in particle size, 30 cm × 4 mm) and a silica column (Spherisorb silica, 5 μ, 30 cm × 4 mm) eluted at 1 ml/min by MeOH–H_2O (1:1) and by *n*-hexane-*iso*-PrOH (200:1), respectively. Under the h.p.l.c. conditions used, **3** was eluted at 11.6 min together with its isomers, 2- and 3-hydroxystyrenes [10], as an inseparable peak from the ODS column as far as the authentic phenols were co-chromatographed. The phenol **3**, however, was separated from the eluted phenolic fraction by subsequent h.p.l.c. on the silica column; retention times of 2-hydroxystyrene, 3-hydroxystyrene and **3** were 24, 33 and 38 min respectively. No detectable amount of radioactivity was incorporated into the phenolic fraction when boiled microsomes were used.