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NAFENOPIN, A HYPOLIPIDEMIC AND NON-GENOTOXIC HEPATOCARCINOGEN INCREASES INTRACELLULAR CALCIUM AND TRANSIENTLY DECREASES INTRACELLULAR pH IN HEPATOCYTES WITHOUT GENERATION OF INOSITOL PHOSPHATES

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Abstract—Addition of nafenopin (30–300 μM to $^{45}Ca^{2+}$ preloaded cultured hepatocytes caused a rapid and concentration-dependent increase in $^{45}Ca^{2+}$ efflux in a manner similar to vasopressin, as evidenced by the loss of radioactivity from the cells. In contrast to vasopressin, addition of nafenopin to [^{3}H]inositol prelabelled hepatocytes in culture did not increase [^{3}H]inositol phosphate production. When added simultaneously with vasopressin, nafenopin inhibited the vasopressin-stimulated [^{3}H]inositol phosphate production. In hepatocyte suspensions isolated from rats treated for 1 week with a carcinogenic dose of nafenopin (1000 ppm in their daily food) the incorporation of [^{3}H]inositol into the phosphoinositide fraction, particularly phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate, was much less than that in hepatocytes isolated from untreated rats. The vasopressin-stimulated [^{3}H]inositol phosphate production was also decreased. Experiments with hepatocyte suspensions preloaded with Ca^{2+} or pH sensitive fluorescent indicators demonstrated that addition of nafenopin caused an increase in intracellular free Ca^{2+} and transient acidification of the cells. The increase in $[Ca^{2+}]_i$ was decreased by only about 25% when extracellular calcium was removed indicating that nafenopin mainly mobilizes Ca^{2+} from intracellular stores. The recovery to basal pH was amiloride-sensitive indicating the importance of Na^+/H^+ exchange in pH recovery after intracellular acidification. Amiloride also inhibited DNA synthesis induced by nafenopin and by epidermal growth factor in cultured hepatocytes; but this effect occurred concomitantly with inhibition of basal DNA synthesis. We suggest that hepatic Ca^{2+} mobilization induced by nafenopin may play an important role in the mechanism by which nafenopin exerts its physiological as well as its tumour promotive activity upon chronic treatment with carcinogenic doses.

Nafenopin (Su-13437, 2-methyl-2-(*p*-1,2,3,4-tetrahydronaphthyl)phenoxypropionic acid) is a hypolipidemic agent structurally related to the drug clofibrate [1]. It also belongs to a class of structurally dissimilar peroxisome proliferators. When given subchronically, nafenopin like all of these agents induces hepatomegaly which is characterized by hypertrophy and hyperplasia [2, 3]. The nafenopin-induced hypertrophy is associated with proliferation of the peroxisomal and the smooth endoplasmic reticulum compartments (Refs 4 and 5 for review). The nafenopin induced hyperplasia occurs in the absence of hepatic necrosis and is regarded as a replicative rather than reparative hyperplasia [6].

The long term administration of nafenopin to rats and mice has been shown to produce liver tumours [7–9] and to accelerate liver tumour formation after pretreatment with an initiating carcinogen, i.e. it possesses tumour promoting activity [10, 11]. The lack of obvious genotoxicity of nafenopin and similar agents led to the suggestion that the hepatocarcinogenicity is linked to metabolic disturbances resulting from the sustained increase in peroxisomal activity and oxidative stress which may activate or alter oncogenes involved in the regulation of cell growth [7, 12]. A slightly higher mitotic activity remaining in livers of rats chronically treated with these agents [13] and a greater replicative rate in early 'preneoplastic' lesions as compared to surrounding tissue [14] have been reported. The mechanisms by which nafenopin and other peroxisome proliferators affect cell replication and cell growth control leading to hepatocarcinogenesis is not completely understood.

Nafenopin stimulates DNA synthesis *in vivo* after a single dose [6] and *in vitro* in hepatocyte cultures [15]. It has also been shown to stimulate DNA synthesis in hepatocyte culture in the absence of serum [16]. In addition, hepatocyte cultures maintained in the absence of serum can be stimulated to undergo DNA synthesis by the same factors which play an important role in liver cell

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proliferation after partial hepatectomy [17]; e.g. epidermal growth factor (EGF‖), insulin and glucagon and various hormones such as norepinephrine, vasopressin, angiotensin II (see Ref. 18 for review). The action of these mitogens have been associated in the early events, for example with the stimulation of phosphatidylinositol (PI) turnover, increases in intracellular free calcium levels $[Ca^{2+}]_i$, activation of protein kinase C, stimulation of Na^+/H^+ exchange and increased transcription of c-myc and c-fos and other mitogenesis-associated genes through receptor transduction pathways in a variety of cells (reviewed in Ref. 19). Thus, we used this culture system as well as hepatocyte suspensions to investigate the mechanism by which nafenopin affects some of these pathways of intracellular signalling in the liver. The hypothesis being that such effects, if prolonged during continuous treatment, could lead to alterations in the proteins, enzymes or genes involved in the signal transduction cascade which, by disrupting the normal regulation of cell proliferation, may give rise to unrestrained cell growth [19].

MATERIALS AND METHODS

Adult male Sprague–Dawley-derived rats (180–220 g) were obtained from The Ciba-Geigy breeding station (Basel, Switzerland). Collagenase was obtained from Boehringer (Mannheim, F.R.G.), (arginine)-vasopressin and angiotensin II from the Sigma Chemical Co. (Poole, U.K.). [^{3}H]Thymidine (18–20 Ci/mmol), *myo*-[2-^{3}H]inositol (20 Ci/mmol) and $^{45}CaCl_2$ (10–40 mCi/mg calcium) were purchased from Amersham (Amersham, U.K.). BCECF:AM (2′,7′-bis-(2-carboxyethyl)-5-(and -6-carboxyfluorescein, acetoxymethyl ester), antifluorescein Ig-G antibodies (0.75 units/mL), Fluo-3:AM and Fluo-3 were from Molecular Probes Inc. (OR, U.S.A.).

Fluo-3:AM and nafenopin were dissolved in DMSO. A similar volume of DMSO was added to control incubations. The final concentration of DMSO did not exceed 0.1%.

All rats had free access to food and water. Nafenopin-treated rats received a control diet (nafag 80) containing 1000 ppm nafenopin for 7 days whereas the matched controls received only the control diet.

Cell isolation, culture and incubation procedure. Hepatocytes were isolated by *in situ* collagenase perfusion as previously described [16]. For experiments where hepatocyte suspensions were used, the isolated hepatocytes were finally washed and resuspended in 20 mM Hepes buffer, pH 7.4, containing 7.85 g NaCl, 1 g KCl, 1 g $NaHCO_3$, 0.246 g $MgSO_4.7H_2O$, 36 mg NaH_2PO_4, 1 g bovine serum albumin (fraction V, Sigma) and 1 g glucose per liter and 2.0 mM $CaCl_2$. In all experiments involving hepatocyte suspensions, incubations were carried out in this buffer under an atmosphere of O_2/CO_2 (95:5) at 37° with constant shaking (80 cycles/min). Cell viability was assessed by the Trypan blue exclusion test and was always between 85–95%.

For experiments where hepatocyte cultures were used, the isolated hepatocytes were washed, resuspended and maintained in culture for several days in a chemically defined and serum free medium (HCD) supplemented as previously described [20]. Hepatocytes were cultured on rat tail collagen-coated 24-well plates (2 cm^2/well) at a density of 0.12×10^6 cells/cm^2.

DNA synthesis at various times in culture was assessed either by incorporation of [^{3}H]thymidine in trichloroacetic acid-precipitable material after 3 hr labelling period [15] or by measuring the proportion of radiolabelled nuclei, after exposure to [^{3}H]thymidine (1 μCi/mL) for the last 24 hr as estimated by autoradiography [20].

Measurement of $^{45}Ca^{2+}$ efflux from prelabelled hepatocytes monolayer cultures. These experiments were performed according to the procedure described in Ref. 21. Hepatocyte monolayers in 24-well plates were used 2 or 3 days after plating. The medium was removed and the cells were washed three times with phosphate buffered saline (PBS). The cells were then loaded with $^{45}Ca^{2+}$ by incubation in 5 mM Hepes buffer pH 7.4, containing 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2.6H_2O$, 1.2 mM $CaCl_2$, and 10 mM glucose (PSS buffer) in the presence of $^{45}Ca^{2+}$ (30 μCi/mL) for 30 min at 37° under an atmosphere of 95% O_2/5% CO_2. The medium containing $^{45}Ca^{2+}$ was then aspirated and the monolayers were washed three times with ice-cold PSS buffer. Calcium efflux was initiated by incubating the cells in PSS at 37° in the absence of $^{45}Ca^{2+}$. At zero time or after various periods of time the medium was rapidly removed and the cultures were quickly rinsed with cold PSS. The cells were then solubilized with hot (95°) 0.1% sodium dodecylsulphate SDS/10 mM EDTA and the radioactivity remaining in the cells was determined by scintillation counting.

Measurement of inositol phosphate production and polyphosphoinositide content. These experiments were conducted essentially as described previously [22, 23]. Hepatocyte suspensions ($5–6 \times 10^6$ cells/mL), of similar viability from control and nafenopin-treated rats, were labelled with [^{3}H]inositol by incubation for 120 min in incubation buffer containing 15–25 μCi/mL [^{3}H]inositol. During the labelling period the viability of the cells was monitored and found to be stable. At the end of the labelling period, the hepatocytes were washed by centrifugation and resuspended at $2–3 \times 10^6$ cells/mL in incubation buffer containing 20 mM LiCl. Li^+ inhibits some inositol phosphate phosphatases, thereby allowing the accumulation of mainly Ins(1,3,4)-P_3, Ins(1,4)-P_2 and various isomers of $InsP_1$ [24]. The cell suspensions were then preincubated for 15 min before vasopressin addition. After another 15 min the reaction was stopped with $HClO_4$ (0.54 M, final concentration). The extract was then vortex mixed and centrifuged. For determination of inositol lipids, the precipitate was washed twice with water

‖ Abbreviations: BCECF:AM, 2′,7′-bis-(2-carboxyethyl)-5-(and -6-)carboxyfluorescein, acetoxymethyl ester; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; $InsP_n$, inositol polyphosphate; $[Ca^{2+}]_i$, intracellular free calcium; EGF, epidermal growth factor; DMSO, dimethyl sulfoxide; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid.

and the inositol lipids were extracted with chloroform : methanol : HCl (100 : 50 : 1). The extracted lipids were deacylated and the resulting inositol-labelled glycerophosphoinositol esters separated on small columns of Dowex 1 anion exchange resin [23]. For the determination of inositol phosphates, the supernatants were neutralized with 1.5 M KOH containing 75 mM HEPES. The water soluble derivatives of [^{3}H]inositol were separated by loading the neutralized cell extract after 5-fold dilution in water onto a Dowex column (×10; 100–200 mesh, formate form) and by sequential elution as previously described [25].

In hepatocyte cultures, labelling of phosphoinositide was performed by supplementing the medium with [^{3}H]inositol (5 μCi/mL) 5 hr after plating. Seventy-two hours after plating the labelled cells were washed three times with PSS buffer and preincubated with 20 mM LiCl in PSS buffer for 15 min before addition of hormones or nafenopin. After 15 min, the reaction was terminated by aspiration of the buffer and the immediate addition of hot (95°) 0.1% SDS/10 mM EDTA. The lysates were sonicated and analysed for the presence of [^{3}H]inositol-1-phosphate by anion exchange and scintillation counting [21, 25].

In the hepatocyte culture experiments, the amount of cellular protein was 135.7 ± 8 (SE)/well within individual experiments and varied between 120 and 160 μg/well in various independent cultures.

Measurement of cytosolic free calcium. In order to investigate changes in intracellular Ca^{2+} on a subsecond time scale, the Ca^{2+}-sensitive fluorescence indicator Fluo-3 was used [26]. Compared to the first and widely used Quin-2, Fluo-3 possesses an improved selectivity for Ca^{2+} and better spectroscopic qualities (i.e. brighter fluorescence, higher photostability). Fluo-3 has, in contrast to Fura-2 and Indo-1, an absorption maximum in the visible and an ester which is not fluorescent until hydrolysed by the cell. Spectral perturbations caused by autofluorescence and/or unhydrolysed ester can therefore be neglected. In addition to this, Fluo-3 has the smallest affinity to Ca^{2+} (K_d = 400 nM [27]) and causes only a small intracellular trapping of Ca^{2+}. Loading of the hepatocytes was performed essentially as described [27] with some modifications. Hepatocyte suspensions (10^6 cells/mL) were incubated for 30 min at 37° in the presence of Fluo-3 : AM (10 μM), FCS (10 μL/mL) and Pluronic (non-ionic detergent; 0.1 μg/mL). The cells were then centrifuged at 70 *g*, resuspended in a Fluo-3 : AM free buffer and incubated for another 30 min to complete the ester hydrolysis. The cells were then washed twice and preincubated for 15 min at 37° before measurements were performed. The viability after this procedure ranged between 85% and 95%. Immediately before starting the measurements, an aliquot of the loaded hepatocytes was diluted to a final concentration of 10^5 cells/mL.

All drug induced fluorescence changes of Fluo-3 loaded hepatocytes were measured at 25° with a Perkin-Elmer MPF-66 spectrofluorimeter. The excitation wavelength was fixed at 508 ± 5 nm and the emission wavelength set to 535 ± 7 nm. The intracellular Ca^{2+} levels and changes were calibrated at the end of each individual scan [28]. Addition of antifluorescein Ig-G antibodies (15 μL/mL) quenched the extracellular fluorescence but did not interfere with the intracellular changes in the fluorescence signal obtained upon the addition of substances. The observed leakage rate of the indicator was below 5% within 30 min. When tested, none of the substances used were found to interfere with the fluorescence spectra of the free or Ca^{2+} bound Fluo-3.

Measurement of intracellular pH. The intracellular pH of hepatocytes was measured fluorometrically using the pH-sensitive carboxyfluorescein derivative BCECF [29]. The loading procedure was analogous to that with Fluo-3 : AM. An aliquot of the washed cell suspension was diluted to a final concentration of 10^5 cells/mL and transferred to a thermostated cuvette (25°). Excited at 505 ± 2 nm, the drug-induced fluorescence changes of BCECF loaded cells were measured at 530 ± 5 nm in the presence of antifluorescein Ig-G antibodies (15 μL/mL) which quenched the extracellular BCECF fluorescence. The addition of this antibody was found necessary since hepatocytes are known to secrete BCECF. Extracellular BCECF thus contributes a large and steadily increasing proportion of the total fluorescence signal [30]. In its presence and in the absence of test substances the evaluated leakage rate was below 10% in 30 min. The BCECF fluorescence was calibrated at the end of each individual scan. After an equilibration of pH between intracellular and extracellular space with nigerin in the presence of high extracellular K^+ [31]; HCl and NaOH were added stepwise and the pH measured against a calibrated pH-electrode. As shown in Fig. 5, the fluorescence signal at 530 nm was linear within the range of 6.50 and 8.10 pH units.

A FORTRAN program based on non-linear regression-theory was developed to analyse the BCECF fluorescence curves. It is assumed that two independent mechanisms are responsible for the release and the elimination of H^+. The intracellular release of H^+ is characterized by τ_{rel}, the H^+-extrusion process by τ_{rec}.

$$pH_{measured}(t) = pH_{basal} - dpH_{max}(2^{-t/\tau_{rec}} - 2^{-t/\tau_{rel}})$$

where dpH_{max} = maximal decrease of pH in the absence of recovery processes; $\tau_{rec} = pH_i$ recovery half-time (sec); $\tau_{rel} = pH_i$ release half-time (sec).

RESULTS

Time course of calcium efflux in cultured hepatocytes

The kinetics of $^{45}Ca^{2+}$ efflux from preloaded hepatocyte cultures is shown in Fig. 1(a). In the absence of stimulation two phases can be distinguished. A rapid initial phase causing an efflux of ~50% of cellular $^{45}Ca^{2+}$ within 4 min and a slower second phase where a further 15–20% loss of cellular $^{45}Ca^{2+}$ occurs in the next 15 min. This is similar to earlier reports on $^{45}Ca^{2+}$ efflux from rat liver cells [32]; where the initial phase represents the rapidly exchangeable $^{45}Ca^{2+}$ from the external surface of the cell membrane and the second slower phase

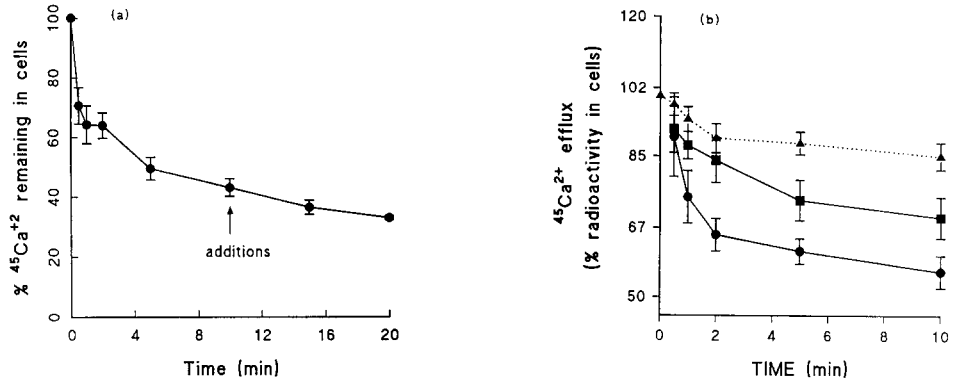

Fig. 1. (a) Time course of calcium efflux in cultured hepatocytes: cells were loaded with $^{45}Ca^{2+}$, washed at 4° and efflux initiated at 37° in PSS buffer. After various time periods, the buffer was aspirated and the calcium remaining in the cells determined as described in Materials and Methods. (b) Time course of calcium efflux in cultured hepatocytes in the presence of nafenopin and vasopressin. Cells were loaded with $^{45}Ca^{2+}$. Ten minutes after the initiation of $^{45}Ca^{2+}$ efflux, as described in Fig. 1(a), solvent, (▲), nafenopin, 150 μM (●), or vasopressin, 10^{-7} M (■) were added and efflux continued for various periods of time. 100% represents the radioactivity remaining in the cells at zero time prior to the additions.

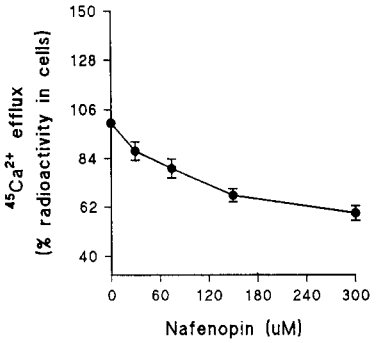

Fig. 2. Effect of various concentrations of nafenopin on $^{45}Ca^{2+}$ efflux. Addition of nafenopin and measurement of $^{45}Ca^{2+}$ efflux for a 10 min period were as described in the legend of Fig. 1(b). 100% represents the radioactivity remaining in the control incubation after 10 min.

represents the exchangeable $^{45}Ca^{2+}$ from intracellular pools. Thus, in order to eliminate the interference from the rapidly exchangeable pool, $^{45}Ca^{2+}$ efflux was allowed to proceed for 10 min before the test substance was added, as indicated in Fig. 1(a).

Effect of vasopressin and nafenopin on $^{45}Ca^{2+}$efflux in hepatocyte culture

In agreement with previously published reports using intact liver or $^{45}Ca^{2+}$ preloaded hepatocytes [33], vasopressin stimulated $^{45}Ca^{2+}$ efflux from the slowly exchangeable $^{45}Ca^{2+}$ pool in the $^{45}Ca^{2+}$ preloaded hepatocytes as evidenced by the loss of radioactivity from the cells (Fig. 1b). Nafenopin (250 μM), also stimulated the rate of $^{45}Ca^{2+}$ efflux. Figure 2 shows that the effect of nafenopin (30–300 μM) on $^{45}Ca^{2+}$ efflux was concentration dependent. In the presence of low extracellular calcium concentration (0.12 mM), both nafenopin and vasopressin stimulated $^{45}Ca^{2+}$ efflux (data not shown). Early studies on the stimulation of $^{45}Ca^{2+}$ exchange and loss in hepatocytes by Ca^{2+} mobilizing agonists in the presence of physiological as well as low extracellular Ca^{2+} concentration were interpreted as an indication of a decrease in the size of intracellular calcium pools and an increase in the size of the cytoplasmic exchangeable calcium pool mediated by the release of intracellular Ca^{2+} [34]. These results therefore indicate that nafenopin may exert its effect by mobilizing calcium from intracellular stores in a manner similar to vasopressin [30].

Comparison of the effect of nafenopin and Ca^{2+}mobilizing agents on $^{45}Ca^{2+}$ efflux and accumulation of inositol monophosphate in hepatocyte cultures

It is widely accepted that the Ca^{2+} response to vasopressin and other Ca^{2+} mobilizing agents is a consequence of receptor mediated breakdown of phosphatidylinositol-4,5-bisphosphate leading to the formation of Ins(1,4,5)-P_3 which releases Ca^{2+} from sensitive pools located in the endoplasmatic reticulum (e.g. see reviews 35–37). In order to investigate whether nafenopin also evokes the breakdown of this phosphoinositide, we measured the production of $InsP_1$ in nafenopin-stimulated cultured hepatocytes prelabelled with [^{3}H]inositol and compared it to the response to vasopressin and angiotensin II (Table 1). As expected, both vasopressin (10^{-7} M) and angiotensin II (10^{-6} M) caused the accumulation of $InsP_1$. However, nafenopin at concentrations up to 600 μM did not affect $InsP_1$ production; although the $^{45}Ca^{2+}$ efflux induced by 250 μM nafenopin was greater than that induced by 10^{-7} M vasopressin. These results indicate that the increase in $^{45}Ca^{2+}$ efflux elicited by nafenopin does not appear to be mediated through inositol phosphate production. In fact when added simultaneously with various concentrations of vasopressin to hepatocyte cultures nafenopin inhibited the vasopressin mediated accumulation of $InsP_1$ (Fig. 3).

Table 1. Comparison of the effects of nafenopin, vasopressin and angiotensin II on $^{45}Ca^{2+}$ efflux and [^{3}H]inositol phosphate production in hepatocyte cultures*

		[^{3}H]$InsP_1$ (% of control)	$^{45}Ca^{2+}$ efflux (% of control)
Vasopressin	(10^{-7} M)	182 ± 11‡ (7)	75 ± 3‡ (8)
Angiotensin II	(10^{-6} M)	148 ± 7‡ (6)	69 ± 6‡ (5)
Nafenopin	(30 μM)	106 ± 6 (4)	
	(150 μM)	116 ± 5† (5)	65 ± 4‡ (9)
	(300 μM)	111 (2)	
	(600 μM)	106 (2)	

* Inositol phosphate production and calcium efflux measurements were performed as described in Materials and Methods. Values for calcium efflux experiments are expressed as the percentage of radioactivity remaining in the cells treated with hormones or nafenopin to that in the control incubations (100%) as described in the legend of Fig. 2. Values for $InsP_1$ production are expressed as percentage of [^{3}H]$InsP_1$ radioactivity in cells in the absence of test substance. Data are the mean ± SE of the number of independent experiments shown in parentheses.

† $P < 0.05$ and ‡ $P < 0.001$ indicate statistical significance as compared to respective controls by two-tailed *t*-test.

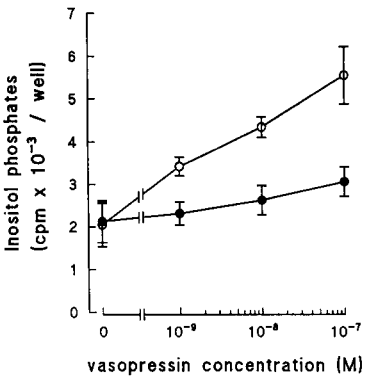

Fig. 3. Effect of nafenopin on $InsP_1$ accumulation induced by increasing concentration of vasopressin in hepatocyte cultures. [^{3}H]Inositol prelabelled hepatocyte cultures were incubated for 15 min without (○) or with 150 μM nafenopin (●) in the absence or presence of various concentrations of vasopressin as indicated in the figure. [^{3}H]$InsP_1$ accumulation was determined as described in Materials and Methods. Data are means ± SE of four separate cultures.

Inositol lipid metabolism in hepatocytes isolated from nafenopin-pretreated rats and their response to vasopressin

Hepatocyces were isolated from control rats and rats treated for 7 days with a carcinogenic dose of nafenopin (1000 ppm in their daily feed, equivalent to ~100 mg/kg body wt/day). The inositol lipids were then labelled for 2 hr *in vitro* by incubation with [^{3}H]inositol, and the production of polyphosphates in response to vasopressin was determined. [^{3}H]Inositol incorporation into both phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2) was markedly reduced in hepatocytes prepared from nafenopin-treated animals (Table 2). There was also clearly less radioactivity in PI, $InsP_1$, $InsP_2$ and $InsP_3$, but this difference was not statistically significant which may be due to the low number of determinations (N = 3). The nafenopin-treated hepatocytes were also unable to generate $InsP_3$ and $InsP_2$

Table 2. [^{3}H]Inositol incorporation into polyphosphoinositides and inositol phosphates in hepatocytes prepared from control and nafenopin-treated rats

	[^{3}H]Inositol incorporation (% of control hepatocytes)*
PI	75 ± 18
PIP	55 ± 6†
PIP_2	38 ± 10†
$InsP_1$	77 ± 31
$InsP_2$	56 ± 19
$InsP_3$	66 ± 22

* The preparation of hepatocytes labelled *in vitro* with [^{3}H]inositol, and the separation and measurement of [^{3}H]phosphoinositides and [^{3}H]inositol phosphates were performed as described in Materials and Methods. For each experiment conducted, parallel incubations of hepatocytes from a control and a treated animal were used. The amount of radioactivity present in any given fraction derived from the hepatocytes of pretreated animals was expressed as a percentage of the value obtained from the hepatocytes of control animals. The mean values for the amount of radioactivity present in the PI, PIP, PIP_2, $InsP_1$, $InsP_2$ and $InsP_3$ fractions eluted from the anion exchange columns for samples from control hepatocytes were 27,988 ± 7314, 1273 ± 318, 879 ± 222, 1827 ± 153, 612 ± 186 and 176 ± 69 dpm/10^6 cells (mean ± SE, N = 3 independent experiments), respectively. The values shown are the means ± SE of three experiments each conducted with a separate preparation of cells from control and treated animals.

† $P < 0.01$ compared with the respective untreated control by paired *t*-test.

Table 3. Vasopressin (2×10^{-7} M) stimulated inositol phosphate production in hepatocytes from control and nafenopin-treated animals

	Radioactivity in inositol phosphates (% of values in the absence of vasopressin)*	
	Hepatocytes from control rats	Hepatocytes from Nafenopin-treated rats
$InsP_1$	206 ± 53	122 ± 6
$InsP_2$	236 ± 26†	107 ± 10
$InsP_3$	218 ± 9‡	87 ± 14

* [^{3}H]Inositol labelled hepatocytes (same as those described in Table 2) were incubated in the presence of Li^+ with or without vasopressin for 15 min. Extraction and separation of inositol phosphates were performed as described in Materials and Methods. The radioactivities of inositol phosphate fractions in the absence of vasopressin are as described in the legend of Table 2.

† $P < 0.05$ and ‡ $P < 0.01$ indicate statistical significance as compared to values in the absence of vasopressin by two-tailed t-test.

in response to vasopressin treatment (Table 3). When tested between 0.5 and 10 min, the uptake of [^{3}H]inositol into hepatocytes from nafenopin-treated rats was found to be similar to that into hepatocytes from control rats (data not shown). Thus, nafenopin appears to interfere with inositol lipid synthesis and abolishes inositol phosphate production in response to vasopressin.

Changes in intracellular Ca^{2+} *in hepatocyte suspensions*

In agreement with previously published results [38], vasopressin (100 nM) caused an elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) from a basal level of about 200 nM to a sustained maximum of 350 nM (Fig. 4). Addition of nafenopin to the hepatocyte suspension also caused an immediate rise in $[Ca^{2+}]_i$ (e.g. an increase of ~105 nM at 80 μM; an average of two independent experiments). This effect of nafenopin was concentration dependent within the range tested (40–400 μM). A representative measurement is shown in Fig. 4. Similar to vasopressin the fluorescence decay curves obtained with nafenopin revealed non-exponential decay characteristics indicating that the intracellular Ca^{2+} stabilizes at an elevated steady state level. In the presence of 5 mM EGTA, the nafenopin-induced Ca^{2+} signal was reduced by about 25% indicating that the rise in $[Ca^{2+}]_i$ resulted mainly from a discharge from intracellular stores (Fig. 4).

When tested, ciprofibrate and clofibrate, structural analogues of nafenopin, also caused elevation of $[Ca^{2+}]_i$ in hepatocyte suspension at comparable concentrations (data not shown).

Changes in intracellular pH *in hepatocyte suspensions*

Using BCECF loaded hepatocytes and the pH calibration curve (Fig. 5, see Materials and Methods), a basal intracellular pH [pH_i] of 7.1 ± 0.1 was obtained (Fig. 6). This value is in good agreement with previously published reports [39]. The addition of 200 μM nafenopin to the BCECF-loaded hepatocytes caused an immediate, but reversible drop in pH_i by about 0.2 pH units (Fig. 6). Using the computer program described in Materials and Methods, the calculated half-life recovery time (τ_{rec}) to basal pH_i in response to 200 μM nafenopin was 160 ± 2 (SE) sec from three independent experiments.

The recovery to basal pH_i was completely repressed by 0.1 mM amiloride, an inhibitor of the Na^+/H^+ exchange in intact hepatocytes, indicating

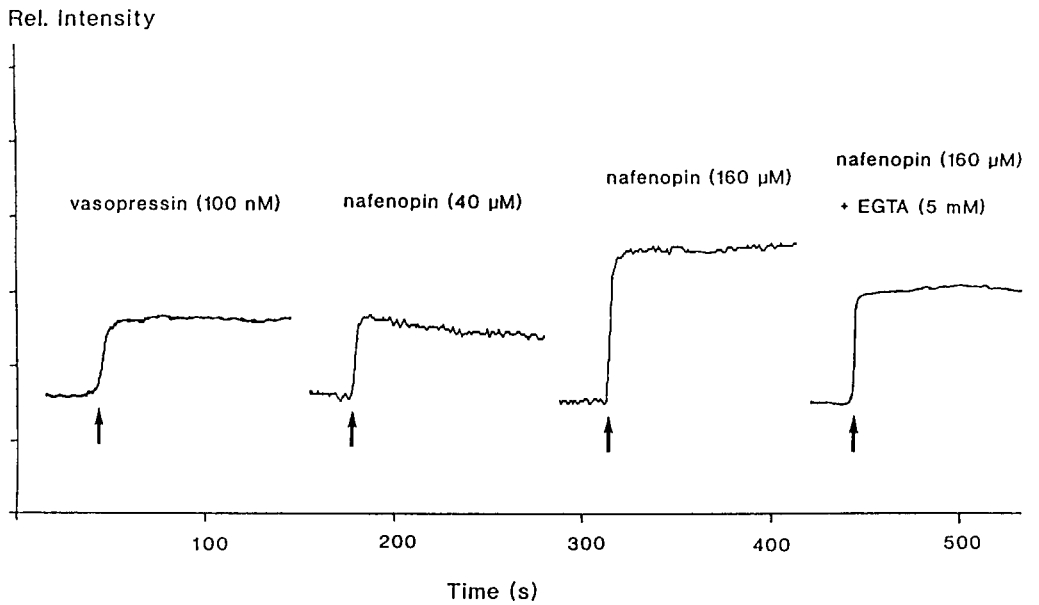

Fig. 4. Fluorescence response to vasopressin (100 nM), and nafenopin (40, 160 μM) of hepatocytes loaded with Fluo-3:AM. In one experiment 5 mM EGTA was added prior to the addition of nafenopin (160 μM). The x-axis corresponds to the time scale and the ordinate gives the relative fluorescence intensity, which can be directly related to $[Ca^{2+}]_i$.

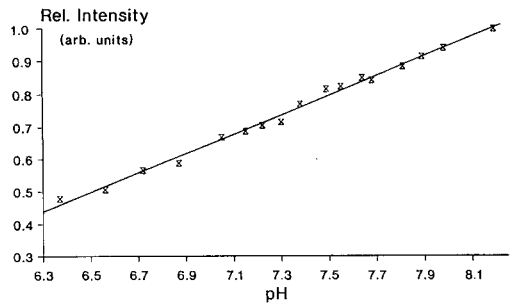

Fig. 5. Calibration curve for pH_i measurements with BCECF. For details on the calibration procedure see text. After excitation at 505 ± 2 nm, the fluorescence signal at 530 ± 5 nm grows linearly with pH.

the importance of membrane Na^+/H^+ exchange in the mechanism of pH recovery after cell acidification as was shown by others [39]. It should be noted, however, that the maximal drug-induced pH_i drop was not strongly affected by amiloride and the small additional H^+ release can be entirely explained by the amiloride dependent suppression of the pH_i recovery reaction pathway [30].

Clofibric acid and clofibrate ester showed a similar but a weaker effect on pH_i (data not shown).

Inhibition of nafenopin induced DNA synthesis by amiloride

Calcium mobilization and Na^+/H^+ exchange activation have been shown to be essential components for the proliferation of liver cells after partial hepatectomy [17, 40–42]. In hepatocyte cultures amiloride has been reported to block ^{22}Na uptake and inhibit basal DNA synthesis as well as that induced by EGF [41, 43, 44]. Since nafenopin indirectly stimulated amiloride-sensitive Na^+/H^+ exchange activity (Fig. 6), we investigated whether nafenopin-induced stimulation of DNA synthesis is also sensitive to amiloride. Figure 7 shows that nafenopin and EGF increased [^{3}H]thymidine incorporation in hepatocytes by 3.4- and 5.5-fold, respectively. A greater response to EGF was obtained when it was added to nafenopin treated cultured hepatocytes. However, this stimulation was dependent upon the order in which the mitogens were added and the time at which DNA synthetic activity was determined (data not shown). At 8 μM, amiloride did not inhibit basal DNA activity, but inhibited nafenopin induced DNA synthesis by only 25%. At higher concentrations amiloride inhibited both the basal and the induced activities. The inhibition by amiloride of nafenopin-induced DNA synthesis was confirmed by autoradiography performed after exposure to [^{3}H]thymidine for 24 hr commencing at day 2 after plating; but we could not observe an amiloride dependent inhibition of either EGF or vasopressin-stimulated DNA synthesis in the absence of inhibition of basal DNA synthesis (data not shown). This indicates that stimulation of Na^+/H^+ exchange activity by these agents is not the only essential component by which they exert their effect. Due to the lack of specific inhibitors of intracellular calcium release it was not possible to evaluate whether the

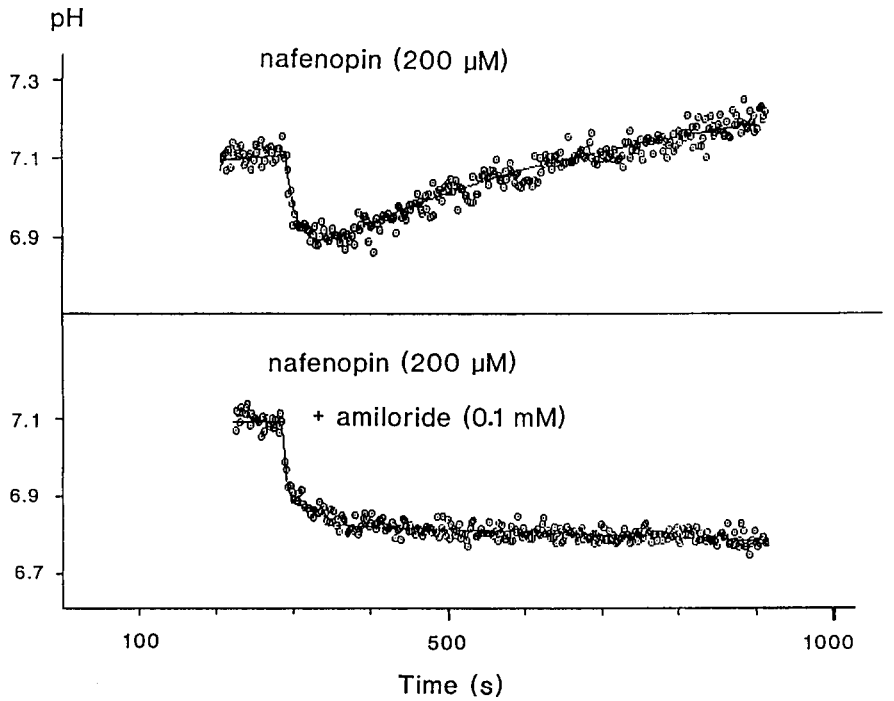

Fig. 6. Changes in intracellular pH after addition of 200 μM nafenopin to hepatocytes loaded with BCECF:AM in the absence and presence of 0.1 mM amiloride. The *x*-axis determines the time scale and the ordinate gives relative pH changes, which can be directly calculated from the emitted fluorescence intensity. Dotted points correspond to the experimentally observed data, whereas the line corresponds to the theoretically predicted spectrum by using the model function described in Materials and Methods.

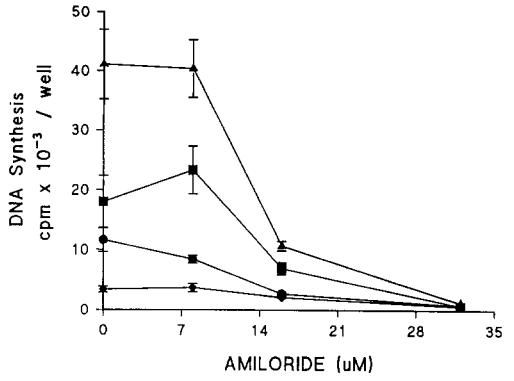

Fig. 7. Inhibition by amiloride of the EGF and nafenopin induced stimulation of DNA synthesis in hepatocyte cultures. Four hours after plating hepatocytes were fed HCD medium supplemented with or without various concentrations of amiloride. Nafenopin (30 μM) and EGF (50 ng/mL) were added 4 and 24 hr after plating, respectively. Two days after plating, DNA synthesis in the hepatocytes was assessed from the incorporation of [^{3}H]thymidine in the cells following a 3 hr pulse of radioactive thymidine determined, as described in Materials and Methods. Control, diamonds; nafenopin, circles; EGF, squares and EGF + nafenopin, triangles.

increase in $[Ca^{2+}]_i$ caused by nafenopin is essential for the induction of DNA synthesis.

DISCUSSION

Our results demonstrate that direct addition of nafenopin to hepatocytes causes an immediate and sustained increase in intracellular cytosolic calcium levels which results in increased calcium efflux. This effect appears to be independent of phosphoinositide hydrolysis. The rise in intracellular calcium evoked by nafenopin was only partially inhibited when extracellular calcium was removed. This indicates that calcium influx across the plasma membrane is not the major mechanism by which nafenopin exerts its effect. Since nafenopin increased calcium efflux, the rise in intracellular calcium is not a consequence of impaired calcium extrusion. The data therefore suggest that nafenopin causes mobilization of calcium from intracellular stores which may result in an increased Ca^{2+} cycling across the plasma membrane [45]. In the liver it is generally accepted that the second messenger of the Ca^{2+} mobilizing agonists is Ins(1,4,5)-P_3 which releases Ca^{2+} from a hormone sensitive pool by interacting with a specific recognition site that controls a Ca^{2+} channel in the membrane of the endoplasmic reticulum (ER) [46] (most likely on a site associated with the plasma membrane [47]). On the other hand thapsigargin, a tumour promotor which increases intracellular Ca^{2+} in intact hepatocytes was found to specifically inhibit the ER-Ca^{2+} ATPase [48], the microsomal Ca^{2+} sequestering system [49]. Our results clearly show that nafenopin does not mobilize calcium via $InsP_3$ mediated mechanism. However it is unclear whether the compound directly releases Ca^{2+} or inhibits Ca^{2+} sequestration. In this regard it is interesting to note that there are striking similarities between nafenopin and Ins(1,4,5)-P_3 when the orientation of the polar groups and the non-polar portions of the molecule are considered (see Fig. 8 and its legend).

In parallel to the observed Ca^{2+} release, a transient drop of pH_i occurred. Although, physicochemical buffering reactions are expected to reach completion within a fraction of seconds [50], the possibility that the acidification could be caused by nafenopin itself cannot be excluded. On the basis of an intracellular H^+-buffering capacity of 35 mM/pH unit [39], the addition of 200 μM nafenopin may explain the observed change in pH of 0.2 pH units, if one assumes a concentration factor of 2000 (derived from the ratio between extracellular and intracellular space assuming a single cell volume of 4940 μm^3 [51]).

Although it is difficult to determine the sequence of the observed physiological reactions (i.e. Ca^{2+} and H^+ release), a liberation of Ca^{2+} as a result of acidification is unlikely, because indirect modulation of cytosolic pH within the range from 6.8 to 7.5 was not associated with increased cytosolic Ca^{2+} concentrations (data not shown). This observation is in good agreement with the results of Joseph and Williamson [52], who demonstrated that, contrary

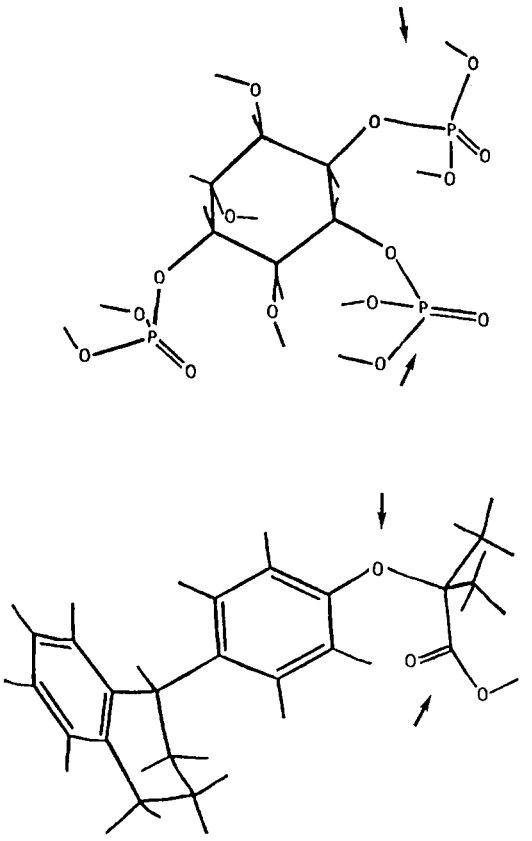

Fig. 8. A comparison of the 3-dimensional structures of Ins(1,4,5)-P_3 and nafenopin using computer-assisted molecular modelling (MM2 force field). The structure of the physiologically active Ins(1,4,5)-P_3 isomer is shown [73], and it is known that the marked polar groups are required for binding to the receptor. It is of interest that the two polar regions of the nafenopin molecule have similar positions with respect to the common hydrophobic region.

to other cell types, alteration of intracellular pH between pH 6.5 and 8.0 did not affect $InsP_3$-induced Ca^{2+} release in permeabilized hepatocytes.

On the other hand, the intracellular acidification following nafenopin exposure may be the result of the rise in intracellular calcium to an extent that activation of H^+ efflux from intracellular locations occurs. This mechanism has been suggested to explain the transient acidification caused by several agents that increase $[Ca^{2+}]_i$ in 3T3 cells e.g. bradykinin, PDGF, bombesin and ionomycin [53] or following direct injection of Ca^{2+} into snail neurons [54–56]. Further studies are necessary to determine the relationship between Ca^{2+} release and intracellular acidification.

An association between both Na^+/H^+ exchange activity [44, 57], Ca^{2+} mobilization [17, 40, 42, 58] and hepatocyte growth and regeneration have been reported. Our results demonstrate that nafenopin mobilizes intracellular calcium and indirectly activates the Na^+/H^+ exchanger and may therefore shed some light on the mechanism by which it induces DNA synthesis in the liver. The exact role in the activation of Na^+/H^+ exchange in inducing DNA synthesis by nafenopin cannot be ascertained for two reasons, (i) only a weak inhibition (~25%) by amiloride of the nafenopin response was obtained in the absence of inhibition of basal DNA synthesis activity and (ii) amiloride may affect protein synthesis and Na^+/K^+-ATPase [59, 60]. In support of the important role of increased $[Ca^{2+}]_i$ in the mechanism of induction of DNA synthesis by nafenopin is the observation that nafenopin can overcome the block imposed on the DNA synthetic activity of neonatal hepatocytes by extracellular Ca^{2+} deprivation [61].

Our results also indicate that subchronic treatment with a carcinogenic dose of nafenopin appears to impair inositol lipid metabolism as evidenced by the reduced incorporation of [^{3}H]inositol into PIP and PIP_2 fractions (Table 2). The mechanism of such an effect is unclear. It is possible that a chronic elevation of $[Ca^{2+}]_i$ by nafenopin caused down regulation of the phosphoinositides through a negative feedback control or that nafenopin directly inhibited the PI-kinases. The observed loss of response to vasopressin in nafenopin treated rats could be a consequence of either a poorly labelled pool of phosphoinositides or impairment in the vasopressin receptor activation pathway, possibly through interaction with phospholipase C. We have noted in preliminary experiments that the binding of [^{3}H]vasopressin to intact hepatocytes and microsomal membranes isolated from nafenopin treated animals was reduced compared to that from control rats. Recently, the presence of a calcium-dependent cytosolic protein which can bind to the hepatocyte membrane and reversibly inactivates vasopressin and angiotensin II binding to hepatocyte membranes has been reported [62]. It is possible that the increase in intracellular Ca^{2+} caused by nafenopin may activate this protein resulting in an inactivation of vasopressin binding. Alteration in steady state PIP_2 levels may also be physiologically significant [63] because this phospholipid has been implicated as a regulator of a plasma membrane Ca^{2+} ATPase [64, 65]. It is interesting to note that ATPase levels appear to be reduced in liver foci induced by peroxisomal proliferators [66]. There is considerable interest in the role that phosphatidylinositol turnover plays in regulating cell growth and the possibility that derangement of the phosphoinositide messenger system may be important in the development of uncontrolled growth in transformed cells has been discussed [19].

It has been reported that thapsigargin, a non-TPA like tumour promotor, discharges Ca^{2+} from internal stores leading to increase in Ca^{2+} without hydrolysis of inositol lipids [48]. The authors argued that a steady increase in Ca^{2+} may be sufficient for promotion of carcinogenesis. The striking similarities between the effects of thapsigargin and nafenopin on $[Ca^{2+}]_i$ tempt us to speculate that similar mechanisms may be responsible for tumour promotion by nafenopin. Furthermore nafenopin treatment did not cause the translocation of protein kinase C from the cytosol to the plasma membrane (unpublished observations) nor cellular alkalization as is the case of TPA-like tumour promotors. The role of elevation of Ca^{2+} in cell injury, tumour promotion and carcinogenesis has been well discussed [67–69].

The involvement of Ca^{2+} mobilization in various aspects of lipid and carbohydrate metabolism in the liver [70] suggests that some of the physiological effects of nafenopin on lipid and carbohydrate metabolism [1] may be mediated by the increase in $[Ca^{2+}]_i$. For example the glycogen depletion that occurs after subchronic treatment of rodents with nafenopin and clofibrate [1, 71] may be associated with the effect of nafenopin on $[Ca^{2+}]_i$ in a manner similar to that of other calcium mobilizing agents [70, 72].

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REFERENCES

1. Hess R and Bencze WL, Hypolipidemic properties of a new tetralin derivative (Ciba 13,437-Su). *Experientia* **24/5**: 418–419, 1968.
2. Beckett RB, Weiss R and Stitzel RE, Studies on the hepatomegaly caused by the hypolipidemic drug nafenopin and clofibrate. *Toxicol Appl Pharmacol* **23**: 41–53, 1972.
3. Hess R, Stäubli W and Riess W, Nature of the hepatomegalic effect produced by ethyl-chlorophenoxy-isobutyrate in the rat. *Nature* **208**: 856–859, 1965.
4. Reddy JK, Svoboda DJ and Azarnoff DL, Microbody proliferation in liver induced by nafenopin, a new hypolipidemic drug: comparison to CPIB. *Biochem Biophys Res Commun* **52**: 537–543, 1973.
5. Hawkins JM, Jones WE, Bonner FW and Gibson GG, The effect of peroxisome proliferators on microsomal, peroxisomal and mitochondrial enzyme activities in the liver and kidney. *Drug Met Rev* **18**: 441–516, 1987.
6. Levin WG, Ord MG and Stocken LA, Some biochemical changes associated with nafenopin-induced liver growth in the rat. *Biochem Pharmacol* **26**: 939–942, 1977.
7. Reddy JK, Azarnoff DL and Hignite CE, Hypolipidemic hepatic peroxisome proliferators form a novel

class of chemical carcinogens. *Nature* **283**: 397–398, 1980.
8. Reddy JK and Lalwani ND, Carcinogenesis by hepatic peroxisome proliferators: evaluation of the risk of hypolipidemic drugs and industrial plastisizers to humans. *CRC Crit Rev Toxicol* **12**: 1–58, 1983.
9. Reddy JK and Rao MS, Malignant tumours in rats fed nafenopin, a hepatic peroxisome proliferator. *J Natl Cancer Inst* **59**: 1645–1650, 1977.
10. Schulte-Herman R, Timmerman-Trosiener I and Schuppler J, Promotion of spontaneous preneoplastic cells in rat liver as a possible explanation of tumour production of non-mutagenic compounds. *Cancer Res* 43: 839–844, 1983.
11. Schulte-Herman R, Ohde G, Schuppler J and Timmerman-Trosiener I, Enhanced proliferation of putative preneoplastic cells in rat liver following treatment with the tumour promotors phenobarbital, hexachlorocyclohexane, steroid compounds and nafenopin, *Cancer Res* **41**: 2556–2562, 1981.
12. Rao MS and Reddy JK, Peroxisome proliferations and hepatocarcinogenesis. *Carcinogenesis* **8**: 631–636, 1987.
13. Moody DE, Rao MS and Reddy JK, Mitogenic effect in mouse liver by hypolipidemic drug, nafenopin. *Virchows Arch Abt B* **23**: 291–296, 1977.
14. Popp JA, Marsman DS, Cattley CC and Conway JC, Hepatocarcinogenecity and peroxisome proliferation. *CIIT Act* **9**: 1–9, 1989.
15. Bieri F, Bentley P, Waechter F and Stäubli W, Use of primary culture to investigate mechanism of action of nafenopin, a hepatocarcinogenic peroxisome proliferator. *Carcinogenesis* **5**: 1033–1039, 1984.
16. Muakkassah-Kelly SF, Bieri F, Waechter F, Bentley P and Stäubli W, The use of primary cultures of adult rat hepatocytes to study induction of enzymes and DNA synthesis: effect of nafenopin and electroporation. *Experientia* **44**: 823–828, 1988.
17. Fausto N and Mead JE, Biology of disease: regulation of liver growth: protooncogenes and transforming growth factors. *Lab Invest* **60**: 4–13, 1989.
18. McGowan JA, Hepatocyte proliferation in culture. In: *Isolated and Cultured Hepatocytes* (Eds Guillouzo A and Guguen-Guillouzo C), pp. 13–38. John Libbey & Co. Ltd, London, 1986.
19. Whitman M and Cantley L. Phosphoinositide metabolism and the control of cell proliferation. *Biochim Biophys Acta* **948**: 327–344, 1988.
20. Muakkassah-Kelly SF, Jans DA, Lydon N, Bieri F, Waechter F, Bentley P and Stäubli W, Electroporation of cultured adult rat hepatocytes with the c-mye gene potentiates DNA synthesis in response to epidermal growth factor. *Exp Cell Res* **178**: 296–306, 1988.
21. Doyle VM and Ruegg UT, Vasopressin induced production of inositol trisphosphate and calcium efflux in smooth muscle cell line. *Biochem Biophys Res Commun* **131**: 469–476, 1985.
22. Thomas AP, Alexander J and Williamson JR, Relationship between inositol polyphosphate production and the increase in cytosolic free calcium induced by vasopressin in isolated hepatocytes. *J Biol Chem* **259**: 5574–5584, 1984.
23. Creba JA, Downes CP, Hawkins PT, Brewster G, Michell RH and Kirk CJ, Rapid breakdown of phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate in rat hepatocytes. *Biochem J* **212**: 473–482, 1983.
24. Maccallum SH, Barker CJ, Hunt PA, Wong NS, Kirk CJ and Michell RH, The use of cells doubly labelled with [^{14}C]inositol and [^{3}H]inositol to search for a hormone-sensitive inositol lipid pool with atypically rapid metabolic turnover. *J Endocrinol* **122**: 379–389, 1988.
25. Berridge MJ, Dawson RMC, Downes CP, Heslop JP and Irvine RF, Changes in the levels of inositol phosphates after agonist dependent hydrolysis of membrane phosphatidylinositides. *Biochem J* **212**: 473–482, 1983.
26. Minta A, Harootunian AT, Kao JPY and Tsien RY, New fluorescent indicators for intracellular sodium and calcium. *J Biol Chem* **105**: 89a, 1987.
27. Kao JPY, Haroutonian AT and Tsien RY, Photochemically generated cytosolic calcium pulses and their detection by Fluo-3. *J Biol Chem* **262**: 8179–8184, 1989.
28. Tsien RY, Pozzan T and Rink TJ, Calcium homeostasis in intact lymphocytes: Cytoplasmic free Ca^{2+} monitored with a new intracellularly trapped fluorescent indicator. *J Cell Biol* **94**: 325–334, 1982.
29. Rink TJ, Tsien RY and Pozzan T, Cytoplasmic pH and free Mg^{2+} in lymphocytes. *J Cell Biol* **95**: 189–196, 1982.
30. Renner EL, Lake JR, Scharschmidt BF, Zimmerli B and Meier PJ, Rat hepatocytes exhibit basolateral Na^{+}/HCO_3^- cotransport. *J Clin Invest* **83**: 1225–1235, 1989.
31. Thomas JA, Buchsbaum RN, Zimniak A and Racker E, Intracellular pH measurements in Ehrlich ascites tumour cells utilizing spectroscopic probes generated *in situ*. *Biochemistry* **18**: 2210–2218, 1979.
32. Claret-Berthon B, Claret M and Mazet JL, Fluxes and distribution of calcium in rat liver cells. Kinetic analysis and identification of pools. *J Physiol* **227**: 529–552, 1977.
33. Altin JG and Bygrave FL, Second messengers and the regulation of Ca^{2+} mobilizing hormones. *Biol Rev* **63**: 551–611, 1988.
34. Barritt GJ, Parker JC and Wadworth JC, A kinetic analysis of the effect of adrenaline on calcium distribution in isolated rat liver parenchymal cells. *J Physiol (Lond)* **312**: 29–55, 1981.
35. Berridge MJ, Inositol triphosphate and diacylglycerol as second messengers. *Biochem J* **220**: 345–360, 1984.
36. Berridge MJ and Irvine RF, Inositol triphosphate a novel second messenger in cellular signal transduction. *Nature* **312**: 315–321, 1984.
37. Downes CP and Michell RH, Inositol lipid breakdown receptor-controlled generator of second messengers. In: *Molecular Mechanisms of Transmembrane Signalling* (Eds Cohen P and Houslay MD), pp. 3–56. Elsevier, Amsterdam, 1985.
38. Williamson JR, Cooper RH, Joseph SK and Thomas AP, Inositol trisphosphate and diacylglycerol as intracellular second messengers in liver. *Am J Physiol* **248**: C203–C216, 1985.
39. Henderson RM, Graf J and Boyer JL, Na–H exchange regulates intracellular pH in isolated rat hepatocyte couplets. *Am J Physiol* **252**: G1109–G113, 1987.
40. Whitfield JF, Boynton AL, MacManus JP, Rixon RH, Sikorska M, Tsang B, Walker PR and Swierenga SH, The role of calcium and cyclic AMP in cell proliferation. *Ann NY Acad Sci* **339**: 216–240, 1980.
41. Koch SK and Leffert HL, Increased sodium influx is necessary to initiate rat hepatocyte proliferation. *Cell* **18**: 153–163, 1979.
42. Cruise J, Houck KA and Michalopoulos G, Induction of DNA synthesis in cultured rat hepatocytes through stimulation of α_1 adrenoreceptor by norepinephrine. *Science* **227**: 749–751, 1985.
43. Hasegawa K, Namai K and Koga M, Induction of DNA synthesis in adult rat hepatocytes cultured in serum free medium. *Biochem Biophys Res Commun* **95**: 243–249, 1980.
44. Leffert HL and Koch KS, Ionic events at the membrane initiate rat liver regeneration. *Ann NY Acad Sci USA* **339**: 201–215, 1980.

45. Rasmussen H, The cycling of calcium as an intracellular messenger. *Sci Am* **261**: 44–51, 1989.
46. Spat A, Bradford PG, McKinney JS, Rubin RP and Putney JW Jr, A saturable receptor for ^{32}P-inositol-1,4,5-trisphosphate in hepatocytes and neutrophils. *Nature* **319**: 514–516, 1986.
47. Guillemette G, Balla T, Baukal AJ and Catt KJ, Characterization of inositol 1,4,5-trisphosphate receptors and calcium mobilization in a hepatic plasma membrane fraction. *J Biol Chem* **263**: 4541–4548, 1988.
48. Thastrup O, Dawson AP, Scharf O, Foder B, Cullen PJ, Drobak BK, Bjerrum PJ, Christensen SB and Hanley MR, Thapsigargin, a novel molecular probe for studying intracellular calcium release and storage. *Agents Actions* **27**: 17–23, 1989.
49. Carafoli E, Intracellular calcium homeostasis. *Annu Rev Biochem* **56**: 395–433, 1987.
50. Anwer MS and Nolan K, Characterization of proton efflux pathways in rat hepatocytes. *Hepatology* **8**: 728–734, 1988.
51. Weibel ER, Stäubli W, Gnägi HR and Hess FA, Correlated morphometric and biochemical studies on the liver cell. *J Cell Biol* **42**: 68–91, 1969.
52. Joseph SK and Williamson JR, Characteristics of inositol trisphosphate-mediated Ca^{2+} release from permeabilized hepatocytes. *J Biol Chem* **261**: 14658–14664, 1986.
53. Ives HE and Daniel TO, Interrelationship between growth factor induced pH changes and intracellular Ca^{2+}. *Proc Natl Acad Sci USA* **84**: 1950–1954, 1987.
54. Meech RW and Thomas RC, Effect of measured calcium chloride injections on the membrane potential and internal pH of snail neurons. *J Physiol (Lond)* **298**: 119–129, 1980.
55. Vasington FD, Gazzoti P, Tiozzo R and Carafoli E, The effect of ruthenium red on Ca^{2+} transport and respiration in rat liver mitochondria. *Biochim Biophys Acta* **256**: 43–54, 1972.
56. Altin JG and Bygrave FL, Synergistic stimulation of Ca^{2+} uptake by glucagon and Ca^{2+}-mobilizing hormones in the perfused rat liver. A role of mitochondria in long term Ca^{2+} homeostasis. *Biochem J* **238**: 653–661, 1986.
57. Moseley RH, Barrett C and Boyer JL, Na^+/H^+ exchange activity is enhanced in liver plasma membrane (LPM) vesicles derived from partially hepatectomized rats (Abstract). *Gastroenterology* **90**: 1947, 1986.
58. Tsai WH, Cruise J and Michalopoulos GK, Blockade of α_1-adrenergic receptor inhibits hepatic DNA synthesis stimulated by tumour promotors. *Carcinogenesis* **10**: 73–78, 1989.
59. van Dyke RW and Ives HE, Na^+/H^+ exchange: What, where and why. *Hepatology* **8**: 960–965, 1988.
60. Holland R, Woodgett JR and Hardie DG, Evidence that amiloride antagonizes insulin-stimulated protein phosphorylation by inhibiting protein kinase activity. *FEBS Lett* **154**: 269–273, 1983.
61. Armato U, Romano F and Andreis PG, The tumour promotor TPA, phenobarbital, and nafenopin and the prostaglandins AE and F series overcome the G1/S block imposed by extracellular Ca deprivation on neonatal rat hepatocytes. *Prostaglandins Leukotrienes Med* **13**: 237–247, 1984.
62. Fishman JF, Dickey BF, McGory MF and Fine RE, Reversible inactivation of vasopressin and Angiotensin II binding to hepatocyte membranes by a calcium-dependent cytosolic protein. *J Biol Chem* **261**: 5810–5816, 1986.
63. Whitman M, Fleischman L, Chahwala SB, Cantely L and Rosoff P, Phosphoinositides, mitogenesis, and oncogenesis. In: *Phosphoinositides and Receptor Mechanism, Receptor Biochemistry and Methodology* (Ed. Putney JW), pp. 197–217. Alan R. Liss, Inc., New York, 1986.
64. Choquette D, Hakin G, Filoteo AG, Plishker GA, Bostwick JR and Pennington JT, Regulation of plasma membrane Ca^{2+} ATPases by lipids of the phosphatidylinositol cycle. *Biochem Biophys Res Commun* **125**: 908–915, 1984.
65. Smith CD and Wells WW, Solubilization and reconstitution of a nuclear envelope associated ATPase. *J Biol Chem* **259**: 11890–11894, 1984.
66. Cattley RC, Failure of the peroxisome proliferator WY-14'643 to initiate growth-selectable foci in rat liver. *Toxicology* **56**: 1–7, 1989.
67. Trump BF and Berezesky IK, Ion regulation, cell injury and carcinogenesis. *Carcinogenesis* **8**: 1027–1031, 1987.
68. Chang J, Musser JH and Mcgregor H, Phospholipase A_2: function and pharmacological regulation. *Biochem Pharmacol* **36**: 2429–2436, 1987.
69. Dena A, Hra H, Tsujiuchi T, Tsutsumi M, Eimoto H, Takashima Y, Kitazawa S, Kinugasa T and Konishi Y, Possible involvement of arachidonic acid metabolism in phenobarbital promotion of hepatocarcinogenesis. *Carcinogenesis* **10**: 1929–1935, 1989.
70. Exton JH, Role of phosphoinositides in the regulation of liver function. *Hepatology* **8**: 152–166, 1988.
71. Platt DS and Thorp JM, Changes in the weight and composition of the liver in the rat, dog and monkey treated with ethyl chlorophenoxyisobutyrate. *Biochem Pharmacol* **15**: 915–925, 1966.
72. Hutson NJ, Brumley FT, Assimacopoulos FD, Harper SC and Exton JH, Studies on the α-adrenergic activation of phosphorylase and gluconeogenesis and inactivation of glycogen synthase in isolated rat liver parenchymal cells. *J Biol Chem* **251**: 5200–5208, 1976.
73. Willcocks AL, Cooke AM, Potter BVL and Nahorski SR, Stereospecific recognition sites for [^{3}H]inositol-(1,4,5)-trisphosphate in particulate preparations of rat cerebellum. *Biochem Biophys Res Commun* **146**: 1071–1078, 1987.