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MENADIONE INHIBITS THE α_1-ADRENERGIC RECEPTOR-MEDIATED INCREASE IN CYTOSOLIC FREE CALCIUM CONCENTRATION IN HEPATOCYTES BY INHIBITING INOSITOL 1,4,5-TRISPHOSPHATE-DEPENDENT RELEASE OF CALCIUM FROM INTRACELLULAR STORES

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Abstract—In order to establish the mechanism of perturbation of hormonally regulated calcium homeostasis in hepatocytes caused by menadione, the effects of menadione on hepatic α_1-adrenergic receptors and on α_1-adrenergic receptor-mediated increase in cytosolic free calcium concentration were determined. Menadione had no detectable effect on the α_1-adrenergic receptor but significantly inhibited (−)-epinephrine-dependent increases in intracellular free calcium concentration in Quin2 acetoxymethyl ester-loaded hepatocytes. The hormonally induced increase in intracellular free calcium concentration is caused by formation of inositol 1,4,5-trisphosphate (IP_3) which binds to a specific receptor and causes a release of intracellular ATP-dependently sequestrated calcium. The IP_3-stimulated release of calcium from intracellular pools in hepatocytes was inhibited to a great extent after treatment with menadione. This inhibition could also be observed after treatment of hepatocytes with *p*-benzoquinone and *N*-ethylmaleimide and could not be reversed by the thiol-reducing reagent dithiothreitol which indicated covalent binding to an essential free sulfhydryl group. The inhibition of IP_3-dependent release of intracellular calcium was accompanied by a large increase in the number of detectable IP_3 receptors without any change in the dissociation constant as determined in permeabilized hepatocytes. The increase in IP_3 receptors caused by menadione could be reversed by dithiothreitol which suggests the involvement of free sulfhydryl groups. It is concluded that the IP_3 receptor plays an important role in the mechanism of menadione-induced perturbation of hormonally regulated calcium homeostasis in rat hepatocytes.

Incubation of hepatocytes with toxic compounds such as carbon tetrachloride and menadione has been reported to lead to a sustained rise in intracellular free calcium concentration [1, 2], which has been suggested to play an important role in mediating toxic cell damage and ultimately in cell death [3, 4]. Many hormones and neurotransmitters elicit their responses by inducing rapid transient increases in intracellular free calcium concentration [5, 6]. A sustained increase in intracelllular free calcium concentration caused by toxic compounds might not only induce cell damage but might also lead to a perturbation of hormonally regulated calcium homeostasis.

In rat hepatocytes stimulation of the α_1-adrenergic receptor by (−)-epinephrine leads to increased breakdown of inositol phospholipids [7]. The two major metabolites formed by this breakdown are diacylglycerol [8] and inositol 1,4,5-trisphosphate (IP_3)† [9]. IP_3 induces a rapid release of calcium from intracellular stores by interacting with a specific receptor and this causes a rise in intracellular free calcium concentration [10].

The signal-transduction from the binding of (−)-epinephrine to the α_1-adrenergic receptor to the increase of intracellular free calcium concentration contains multiple target sites for oxidative stress or sulfhydryl reagents.

It has been shown that the hepatic α_1-adrenergic receptor protein is vulnerable to oxidative stress and can be damaged by sulfhydryl reagents [11–13]. Also the α_1-adrenergic receptor-stimulated breakdown of inositol phospholipids can be inhibited by oxidative stress or sulfhydryl reagents [14]. Recently, it was shown that the IP_3-receptor is sensitive to incubation with sulfhydryl reagents or menadione [15, 16] which is consistent with the observation that sulfhydryl reagents inhibit IP_3-dependent release of calcium in bovine adrenal cortex microsomes [15].

In this study we investigated the effects of treatment of hepatocytes with menadione on (−)-epinephrine-induced rises in intracellular free calcium concentration. We tried to elucidate the mechanism of menadione-induced perturbation of α_1-adrenergic receptor-mediated calcium homeostasis by focusing on the α_1-adrenergic receptor

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† Abbreviations: IP_3, inositol 1,4,5-trisphosphate; DTT, dithiothreitol; PBS, phosphate buffered saline; NEM, *N*-ethylmaleimide; DMSO, dimethyl sulfoxide; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; Quin2/AM, Quin2 acetoxymethyl ester; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; BSA, bovine serum albumin.

protein, on IP_3-dependent release of calcium from intracellular stores and on the IP_3-receptor protein.

MATERIALS AND METHODS

Isolation of hepatocytes. Rat hepatocytes were prepared by EDTA-dissociation according to Meredith [17]. In brief, male Wistar rats (Harlan CPB, Zeist, The Netherlands) of 180–222 g were anaesthetized by i.p. injection of Nembutal®. The portal vein was cannulated and an incision in the heart was made to allow outflow of the perfusate. The flow rate was adjusted to 50–55 mL/min. The perfusion buffer contained 140 mM NaCl, 5 mM KCl, 0.8 mM $MgCl_2$, 1.6 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$ and 2 mM EDTA, pH 7.4. The buffer was saturated with O_2 by leading carbogen through the solution and the temperature was adjusted to 37°. The perfusion was terminated after 30 min. The liver was removed, washed and minced in wash buffer. The wash buffer was the perfusion buffer without $NaHCO_3$ and EDTA, but with 1 mM $CaCl_2$. The wash buffer was stored at 4°. The isolated hepatocytes were allowed to sediment under gravity and subsequently washed twice by centrifugation at 50 *g* for 1 min. After the last centrifugation step the cells were resuspended in 20 mL wash buffer and diluted with 34 mL buffered Percoll®. The Percoll solution was made by adding 10 mL 10× stock solution to 90 mL Percoll. The 10× stock solution contained 1.4 M NaCl, 50 mM KCl, 8 mM $MgCl_2$, 16 mM Na_2HPO_4 and 4 mM KH_2PO_4. The buffered Percoll solution was stored at 4°. The hepatocytes were pelleted by centrifugation at 50 *g* for 5 min. In some experiments cells were isolated by using the collagenase perfusion method [18]. Collagenase-isolated cells were washed three times in PBS followed by centrifugation in buffered Percoll solution.

Cells were resuspended in Leibowitz L-15 medium supplemented with 20 mM Hepes, 5.5 mM glucose, 25 mM $NaHCO_3$ and 2 mM L-glutamine and stored at 4°. In some experiments cells were resuspended in Krebs–bicarbonate buffer containing 1.8 mM $CaCl_2$ supplemented with 5.5 mM glucose, 5.5 mM fructose and 20 mM Hepes, pH 7.4 at 37° under carbogen atmosphere. Cell viability was checked by Trypan blue exclusion and was always higher than 95%.

Incubations. Cells were diluted to 2.5×10^6 cells/mL in supplemented Leibowitz L-15 medium and preincubated for 40 min at 37° under carbogen atmosphere. In some experiments incubations were performed in supplemented Krebs–bicarbonate buffer. Incubations were performed as indicated and in some experiments the incubations were followed by an incubation with freshly prepared DTT (5 mM) for 30 min. All incubations were terminated by washing the hepatocytes with ice-cold medium followed by storage on ice. The incubations were always without effect on cell viability as checked by Trypan blue exclusion (data not shown). When the cells were to be used for IP_3-binding studies, the medium consisted of 25 mM Na_2HPO_4, 100 mM KCl, 20 mM NaCl, 1 mM EDTA, pH 7.4 at 0°. The cell concentration was about $10–15 \times 10^6$ cells/mL. The cells were permeabilized with saponin (100 μg/mL) at 0° for about 20 min. Permeability was checked by Trypan blue exclusion. The cells were subsequently used for IP_3-binding studies. When the cells were to be used for measurement of IP_3-induced calcium release, the buffer consisted of 115 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4, 20 mM Hepes, pH 7.2 at 37°. The cells were stored on ice at a cell concentration of about 2×10^6 cells/mL without permeabilization with saponin.

IP_3 binding to hepatocytes. Saponin-permeabilized cells were diluted to about 1.5×10^6 cells/mL in a final volume of 300 μL. Incubations were performed for 40 min at 0° with [^{3}H]IP_3 (20,000 dpm; 0.8 nM) and increasing amounts of unlabeled IP_3. Non-specific binding of [^{3}H]IP_3 was determined in the presence of 1 μM IP_3. Incubations were terminated within 2 sec by diluting the samples with 3 mL ice-cold incubation medium, followed by immediate filtration through presoaked glass-fiber filters (Whatman GF/C) and washing the filters with 3 mL ice-cold incubation medium.

IP_3-induced calcium release in hepatocytes. Hepatocytes (2×10^6 cells/mL) were incubated in 115 mM KCl, 10 mM NaCl, 4 mM $MgCl_2$, 1 mM KH_2PO_4, 20 mM Hepes, pH 7.2 at 37° in a final volume of 2 mL in a Perkin–Elmer MPF-2A fluorescence spectrophotometer equipped with a thermostatted cuvette holder. Quin2 (50 μM) was added to measure the free calcium concentration and to buffer the free calcium concentration in the hepatocyte suspension within the physiological range (350–450 nM). The hepatocytes were permeabilized in the cuvette by addition of 100 μg/mL saponin. Loading of the intracellular calcium pools was initiated by addition of 2 mM ATP. When the calcium sequestration was completed (about 4 min), IP_3-induced calcium release was determined by addition of 0.67 μM IP_3. The total amount of sequestered calcium was determined after addition of the calcium ionophore ionomycin (2 μM), which released all vesicular bound calcium. Instrumental settings were an excitation wavelength of 339 nm (10 nm slit) and an emission wavelength of 500 nm (9 nm slit).

[^{3}H]*Prazosin binding to hepatocytes.* Cells were isolated with collagenase and resuspended in Krebs–bicarbonate buffer (1.8 nM Ca^{2+}) supplemented with 20 mM Hepes, 2% BSA, 5.5 mM glucose, pH 7.4 at 37° under carbogen atmosphere at a cell concentration of $2–3 \times 10^6$ cells/mL. After an equilibration period of 40 min, cells were incubated as indicated and subsequently washed and resuspended in supplemented Krebs medium containing 0.2% BSA. Hepatocytes were incubated for 30 min at a cell concentration of 1×10^6 cells/mL in a final volume of 1 mL in order to minimize uptake and metabolism of the ligands [19] with increasing concentrations of [^{3}H]prazosin in saturation experiments. In competition experiments hepatocytes were incubated with 0.27 nM [^{3}H]prazosin for 30 min at 37° or for 180 min at 0° in the presence of increasing concentrations of (−)-epinephrine. At the end of the incubations the samples were diluted with 4 mL ice-cold incubation buffer and filtered rapidly under vacuum through Whatman GF/C filters, and test tubes were washed twice with 4 mL buffer. Non-

specific binding of [^{3}H]prazosin was determined in the presence of 10 μM phentolamine.

Cytosolic calcium concentration in hepatocytes. Cells were isolated with collagenase and resuspended in Krebs–bicarbonate buffer (1.8 mM Ca^{2+}) supplemented with 20 mM Hepes, 2% BSA, 5.5 mM glucose, pH 7.4 at 37° under carbogen atmosphere at a cell concentration of 2–3 × 10^6 cells/mL. After an equilibrium period of 40 min, cells were incubated as indicated and subsequently washed and resuspended in supplemented Krebs medium. Cells were then incubated for an additional 10 min and loaded for 5 min with 50 μM Quin2/AM (the membrane permeable acetoxymethyl ester of Quin2) added from a 50 mM stock solution in DMSO. After the loading procedure the cells were washed and resuspended to a cell concentration of approximately 1.5 × 10^6 cells/mL in about 3 mL fresh buffer without BSA because of the impairment of Ca^{2+}–Quin2 fluorescence emission by this compound.

The fluorescence of Quin2-loaded hepatocytes was measured at an excitation wavelength of 339 nm (5 nm slit) and an emission wavelength of 492 nm (5 nm slit) using a Perkin–Elmer MPF-2A spectrofluorometer fitted with a thermostatted cell holder.

Hormonal rises in free calcium concentration were measured after adding cumulative concentrations of (−)-epinephrine. Following completion of these measurements the cells were made permeable by addition of 4 μM digitonin followed by addition of 0.25 mM EGTA and 0.25 mM Ca^{2+} to correct the maximal fluorescence for heavy metal contamination. Minimal fluorescence was measured in the presence of 4 mM EGTA (pH > 8.0). Calcium concentrations were calculated from the equation: $[Ca^{2+}]_i = K_D(F - F_{min})/F_{max} - F)$ in which F, F_{min} and F_{max} are the fluorescence signal to be calibrated, the minimal fluorescence signal and the maximal fluorescence signal respectively and K_D is the dissociation constant of the Ca^{2+}–Quin2 complex which is 115 nM [20, 21].

Plasma membranes. Rat liver plasma membranes were prepared as described previously [11]. Incubations were performed in buffer containing 125 mM KCl, 25 mM Hepes, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.4 at 37° for 10 min at a protein concentration of about 2 mg/mL. To avoid interaction of the drugs used in the incubations on [^{3}H]prazosin binding, the protein was diluted five times with fresh buffer and washed by centrifugation for 30 min at 240,000 *g*. The pellet was resuspended in fresh buffer and stored on ice until use. Binding experiments were performed using increasing concentrations [^{3}H]-prazosin (sp. act. 60 Ci/mmol) added to plasma membranes in a final volume of 350 μL. Protein concentration was about 680 μg/mL. After 30 min incubation at 37° samples were diluted with 3 mL ice-cold buffer and filtered rapidly under vacuum through Whatman GF/C filters. The filters were washed twice with 3 mL ice-cold washing medium and dried. Subsequently, the filters were transferred to scintillation vials containing 5 mL Dynagel® scintillation liquid and counted in a Hewlett–Packard tri-carb 460 CD scintillation counter. Non-specific binding of [^{3}H]prazosin was determined by performing the binding experiment in the presence of 1 μM phentolamine. Protein concentration was measured with Biorad-reagent® using BSA as standard [22].

Analysis of radioligand binding data. Data were analysed with the non-linear curve fitting program LIGAND [23] by estimating the maximum number of binding sites (B_{max}) and the dissociation constant (K_D). The model of ligand–receptor interaction (one or more binding sites) was determined with the *F*-test. Treatments and their respective controls were first evaluated individually and then simultaneously in one calculation. A significant simultaneous fit allows the controls and treatments to be treated as a homogenous receptor population and the receptor densities to be compared. Data are expressed as mean ± SD as estimated by LIGAND.

Chemicals. [^{3}H]IP_3 and [7-*methoxy*-^{3}H]prazosin were obtained from Amersham Inc. IP_3, NEM, menadione, BSA (fraction V), (−)-epinephrine, Quin2/AM, paraquat, DTT, ATP, Quin2, Percoll, 4α-phorbol, phorbol 12-myristate 13-acetate, dibutyryl-cyclic AMP, saponin and phenylmethylsulfonyl fluoride were obtained from Sigma. Other chemicals were obtained as follows: collagenase and leupeptin (Boehringer); L-glutamine and *p*-benzoquinone (Merck); heparin (Organon Teknika); cianidanol-3 (Zyma Switzerland); Nembutal (Sanofi); carbon tetrachloride (Baker); ionomycin (Calbiochem); Leibowitz L-15 medium (Flow Labs); phentolamine (Ciba-Geigy); and digitonin (Koch-Light Labs). All other chemicals were of the highest grade of purity available.

RESULTS

In order to determine the effects of menadione treatment on hormonally regulated calcium homeostasis, hepatocytes were incubated in the presence of menadione and subsequently loaded with Quin2/AM to measure the α_1-adrenergic receptor-mediated rise in intracellular free calcium concentration. As can be seen from Fig. 1, (−)-epinephrine caused a concentration-dependent rise in intracellular free calcium concentration in rat hepatocytes. Treatment of hepatocytes for 10 min in the presence of 50 μM menadione depressed the maximal effect of (−)-epinephrine; 100 μM menadione caused a more substantial inhibition of the (−)-epinephrine-induced increase in intracellular free calcium concentration. Incubation of hepatocytes with 200 μM menadione for 10 min caused an increase in the basal free calcium concentration from 150 nM to about 230 nM and significantly depressed hormonal effects (Fig. 1).

To establish whether the observed inhibition of hormonal effects in hepatocytes after treatment with menadione was caused by changes in the α_1-adrenergic receptor, rat liver plasma membranes were incubated with menadione and subsequently [^{3}H]prazosin binding was determined. Partially purified rat liver plasma membranes bound [^{3}H]-prazosin with high affinity (K_D = 46 pM) and in a saturable way (B_{max} = 139 fmol/mg protein) similar to the values reported in literature (Table 1) [24]. Preincubation of liver plasma membranes in the presence of 200 μM menadione for 10 min at 37° had

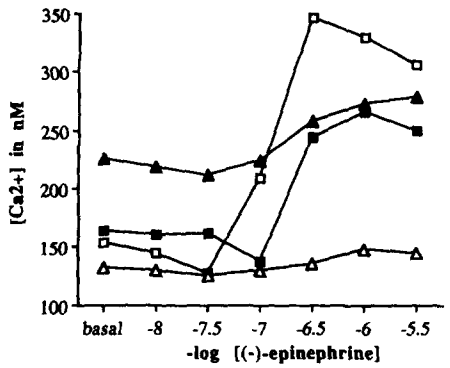

Fig. 1. Hepatocytes were incubated for 10 min at 37° without (□) or with 50 μM (■); 100 μM (△); 200 μM menadione (▲) and washed. Subsequently, the cells were loaded for 5 min at 37° with 50 μM Quin2/AM and washed. α_1-Adrenergic receptor-mediated rises in the intracellular free calcium concentration were measured at 37° by cumulative addition of (−)-epinephrine. The data represent the results of three independent experiments performed in triplicate.

no effect on the binding characteristics of [^{3}H]-prazosin to hepatic α_1-adrenergic receptors; neither the K_D nor the B_{max} were changed significantly (Table 1).

Incubation of intact hepatocytes in the presence of menadione induces changes in cellular thiol and calcium homeostasis and leads to cell death [3, 4]. Therefore, the effects of menadione on α_1-adrenergic receptors were studied in intact hepatocytes in addition to the studies performed on isolated plasma membranes.

The binding of [^{3}H]prazosin to intact hepatocytes reveals a dissociation constant of 0.36 nM and a maximal number of binding sites of 166 × 10^3 sites/cell. The affinity of the binding sites for prazosin did not change after preincubation of intact hepatocytes in the presence of 200 μM menadione for 10 min. Also the number of detectable binding sites remained unaltered (Table 1).

The interaction of (−)-epinephrine with hepatic α_1-adrenergic receptors was studied by displacement by (−)-epinephrine of [^{3}H]prazosin bound to intact hepatocytes at 37° (low-affinity state) or at 0° (high-affinity state) [24–26]. The high-affinity state of the α_1-adrenergic receptor was characterized by a dissociation constant for (−)-epinephrine of 38 nM and the low-affinity state of the receptor had a dissociation constant of 3.0 μM. Pretreatment of intact hepatocytes with 200 μM menadione for 10 min resulted in a decrease of the number of receptors which were able to exist in the high-affinity or in the low affinity state by 20 and 10%, respectively, without any changes in the dissociation constants (Table 1).

In order to establish the role of the IP_3 receptor in the mechanism of perturbation of hormonally regulated calcium homeostasis in hepatocytes by menadione, IP_3-dependent release of calcium from intracellular stores and IP_3-binding were studied in saponin-permeabilized hepatocytes.

Incubation of intact hepatocytes for 10 min in the presence of menadione had no effect on ATP-dependent Ca^{2+}-sequestration in subsequently permeabilized cells (Fig. 2). However, IP_3-induced release of calcium was significantly inhibited at 50 μM menadione and almost completely blocked by 100 μM menadione (Fig. 2). The radical scavenger cianidanol (400 μM) was not able to prevent the

Table 1. Effects of incubation for 10 min at 37° with 200 μM menadione on [^{3}H]prazosin binding to partially purified rat liver plasma membranes and intact rat hepatocytes or on displacement of 0.27 nM [^{3}H]prazosin by (−)-epinephrine on hepatocytes for 30 min at 37° (low affinity) or for 180 min at 0° (high affinity)

Treatment	K_D	B_{max}
	Plasma membranes	
[^{3}H]Prazosin saturation		
Control	46 ± 6 pM	139 ± 10 fmol/mg protein
200 μM menadione	idem*	158 ± 13 fmol/mg protein
	Hepatocytes	
[^{3}H]Prazosin saturation		
Control	0.36 ± 0.06 nM	166 ± 35 × 10^3 sites/cell
200 μM menadione	idem*	158 ± 40 × 10^3 sites/cell
Displacement by (−)-epinephrine at 37°		
Control	3.0 ± 1.3 μM	100%
200 μM menadione	idem*	90 ± 1%
Displacement by (−)-epinephrine at 0°		
Control	38 ± 6 nM	100%
200 μM menadione	idem*	80 ± 1%

The K_D and B_{max} values of the [^{3}H]prazosin saturation curves refer to the K_D and B_{max} values of [^{3}H]prazosin binding. The K_D and B_{max} values of the displacement curves refer to the K_D and B_{max} values of (−)-epinephrine binding. Data are the mean of three independent experiments ± SD as estimated by the non-linear curve fitting program LIGAND.

* Control groups and menadione-treated groups have been fitted simultaneously in one fit; see Materials and Methods.

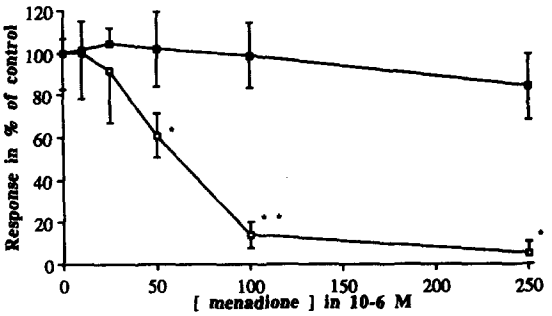

Fig. 2. Concentration-dependent effect of treatment of intact hepatocytes for 10 min at 37° with menadione on ATP-dependent sequestration (■) (100% is 6.3 ± 1.3 nmol/10^6 cells) and calcium release by 0.67 μM IP_3 (□) (100% is 1.9 ± 0.2 nmol/10^6 cells) in hepatocytes permeabilized after incubation with menadione. Data are expressed as mean ± SD of three independent experiments performed in triplicate. Significance was tested by the Student's *t*-test; * $P < 0.005$; ** $P < 0.001$, compared to control.

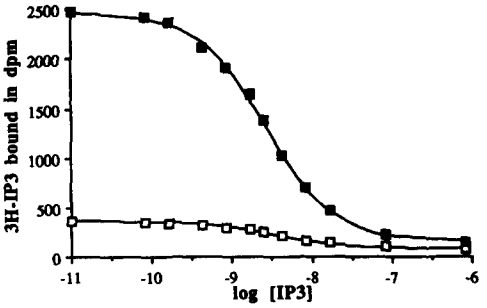

Fig. 3. Displacement of 0.8 nM (20,000 dpm) [^{3}H]IP_3 by unlabelled IP_3 at 0° on 1.5×10^6 hepatocytes permeabilized with saponin after preincubation for 10 min at 37° with (closed symbols) or without (open symbols) 100 μM menadione. The solid lines are the fitted curves according to non-linear regression analysis by LIGAND. One typical experiment is shown.

menadione-induced inhibition of IP_3-dependent calcium release (data not shown).

Incubation of hepatocytes for 10 min in the presence of 100 μM *p*-benzoquinone, which can only arylate sulfhydryls, did not cause any inhibition of IP_3-dependent release of calcium or of ATP-dependent sequestration of calcium (Table 2). Also incubation of hepatocytes with the hepatotoxic compounds carbon tetrachloride (100 μM) or paraquat (1 mM) did not affect IP_3-dependent responses (Table 2).

Treatment of hepatocytes with the sulfhydryl alkylator NEM (100 μM) resulted in a decrease of IP_3-induced calcium release and ATP-dependent Ca^{2+}-sequestration of 57 and 39%, respectively (Table 2).

DTT did not reverse the inhibition of IP_3-induced release of calcium caused by menadione or NEM (Table 2). Incubation of hepatocytes followed by treatment for 30 min with 5 mM DTT resulted in a small but insignificant decrease of IP_3 effects compared to incubation without treatment with DTT (not shown).

It has been reported previously that IP_3-receptors can be detected in permeabilized hepatocytes [27]. The effects of menadione treatment of intact hepatocytes on IP_3-receptors were therefore determined in saponin-permeabilized cells. IP_3 bound to permeabilized hepatocytes with high affinity (K_D = 2.7 ± 0.6 nM, N = 3). and in a saturable way (B_{max} = 12 ± 2 fmol/10^6 cells, N = 3). Preincubation of hepatocytes for 10 min in the presence of 100 μM menadione remarkably increased IP_3-binding in subsequent permeabilized cells by about 600% (B_{max} = 76 ± 28 fmol/10^6 cells, N = 3) without a change in the dissociation constant. A typical experiment is shown in Fig. 3.

Altering the isolation procedure (using collagenase instead of EDTA) or the incubation medium (using Krebs–bicarbonate buffer instead of Leibowitz L-15 medium) did not affect the observed increase in IP_3-binding to hepatocytes induced by pretreatment with menadione (data not shown).

Neither the presence of protease inhibitors (phenylmethylsulfonyl fluoride 0.2 mM and leupeptin 10 μg/mL) during the incubation with 100 μM menadione, nor washing the hepatocytes after permeabilization in order to remove the cytosolic

Table 2. Effects of pretreatment of intact hepatocytes with the indicated compounds and subsequent incubation with DTT on calcium release induced by 0.67 μM IP_3 and on ATP-dependent sequestration of calcium in hepatocytes permeabilized after the indicated treatments

Pretreatment (10 min)	no DTT		5 mM DTT (30 min)	
	IP_3-induced release*	Ca^{2+}-sequestration*	IP_3-induced release*	Ca^{2+}-sequestration
Control	100 ± 20 (9)	100 ± 7 (9)	100 ± 22 (6)	100 ± 14 (6)
100 μM menadione	28 ± 14 (9)‡	85 ± 12 (9)†	23 ± 11 (5)‡	84 ± 20 (6)
100 μM *p*-benzoquinone	108 ± 35 (6)‡	99 ± 8 (6)	108 ± 30 (5)	102 ± 19 (5)
100 μM NEM	43 ± 33 (6)‡	61 ± 23 (6)‡	25 ± 12 (6)‡	78 ± 28 (4)
100 μM CCl_4	88 ± 23 (6)	94 ± 9 (6)	ND	ND
1 mM paraquat	88 ± 29 (6)	98 ± 9 (6)	ND	ND

Data are expressed as mean ± SD in % of the respective control and the numbers between parentheses denote the number of experiments.

* Significance is tested by the Student's *t*-test; † $P < 0.01$; ‡ $P < 0.001$, compared to control.

ND, not determined.

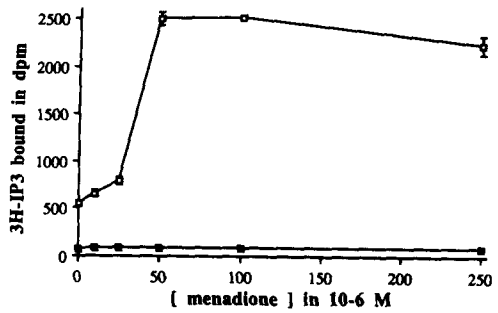

Fig. 4. Concentration-dependent effect of treatment of intact hepatocytes for 10 min at 37° with menadione on total binding (open symbols) and non-specific binding (closed symbols) of 0.8 nM (20,000 dpm) [^{3}H]IP_3 to 1.5×10^6 hepatocytes at 0° permeabilized after treatment with menadione. Non-specific binding was determined in the presence of 1 μM labelled IP_3. Data are the results of three experiments ± SD.

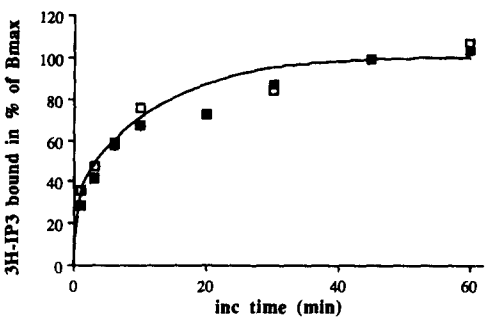

Fig. 5. Time-dependent association of 0.8 nM (20,000 dpm) [^{3}H]IP_3 at 0° to 1.46×10^6 hepatocytes permeabilized with saponin after preincubation for 10 min at 37° with (closed symbols) or without (open symbols) 100 μM menadione. The solid line has been obtained by fitting the data-points according to a mono-exponential function. The B_{max} of the control was 352 dpm and the B_{max} of the menadione treatment was 2034 dpm. One typical experiment is shown.

contents, had any effect on the increase in the number of IP_3 receptors caused by menadione (data not shown).

In Fig. 4, the concentration dependency of the stimulatory effects of menadione on the number of IP_3-binding sites of hepatocytes is depicted. As can be seen from Fig. 4, treatment of hepatocytes with menadione had no effects on non-specific binding of IP_3 to hepatocytes. However, the total binding of IP_3 was concentration-dependently increased by menadione with half-maximal increase between 25 and 50 μM menadione (Fig. 4).

In Fig. 5 it is shown that the kinetics of IP_3-binding to permeabilized hepatocytes were not changed by pretreatment of intact hepatocytes with 100 μM menadione for 10 min.

Treatment of hepatocytes with menadione in the presence of the radical scavenger cianidanol (400 μM) did not prevent the increase in the number of IP_3 receptors (data not shown).

Incubation of hepatocytes with *p*-benzoquinone or NEM also resulted in an increase of IP_3-binding in contrast to incubation with carbon tetrachloride or paraquat which did not change the amount of IP_3 bound to permeabilized hepatocytes (Table 3). Subsequent incubation for 30 min in the presence of DTT completely (NEM) or partially (menadione and *p*-benzoquinone) reversed the increase in IP_3-binding to hepatocytes (Table 3). Incubation in the presence of NEM at a concentration of 100 μM gave similar results compared to incubation at a concentration of 200 μM (data not shown).

DISCUSSION

In this paper we report that menadione inhibits α_1-adrenergic receptor-mediated increase in intracellular free calcium concentration in rat hepatocytes as determined by direct measurement of the intracellular free calcium concentration with Quin2.

It is shown in Fig. 1 that under conditions in which menadione does not cause any detectable change in basal free calcium concentration, (−)-epinephrine-induced increase in intracellular free calcium concentration is significantly inhibited in hepatocytes. The α_1-adrenergic receptor protein is vulnerable to sulfhydryl reagents and oxidative stress [11–13]. To investigate whether the α_1-adrenergic receptor protein is involved in the inhibition of the (−)-epinephrine-induced increase in intracellular free calcium concentration in hepatocytes caused by menadione, we studied the binding of [^{3}H]prazosin in partially purified plasma membranes and in intact hepatocytes. The observed binding characteristics were in good agreement with literature data [24–26]. However, menadione did not affect [^{3}H]prazosin binding to the hepatic α_1-adrenergic receptor (Table 1). Moreover, we studied the binding of (−)-epinephrine to intact hepatocytes. It has been reported that hepatic α_1-adrenergic receptors can bind agonists [e.g. (−)-epinephrine] with high (0°) or low (37°) affinity [24–26]. Agonists are thought to induce a conformational change of the receptor protein, a process in which a GTP-binding regulatory protein (G-protein) might be involved, which leads to the high-affinity state of the agonist–receptor complex [28, 29]. However, treatment of intact hepatocytes with menadione had only minor effects on the high- or low-affinity binding of (−)-epinephrine (Table 1). Recently, it has been reported that the purified α_1-adrenergic receptor was still able to bind agonists with high or low affinity after proteolytic cleavage of the coupling domain (coupling to a G-protein) from the binding domain (binding of agonists) and that the formation of the high-affinity state of the receptor at 2° did not require a G-protein [30]. We can therefore not exclude the possibility that menadione treatment has indeed damaged the α_1-adrenergic receptor, which cannot be detected by ligand-binding studies.

Ca^{2+}-mobilizing receptors induce a rapid breakdown of phosphatidyl inositol in the plasma

Table 3. Effects of pretreatment of intact hepatocytes with the indicated compounds at 37° and subsequent incubation with DTT on the amount of specifically bound [^{3}H]IP_3 after 40 min incubation at 0° of permeabilized hepatocytes in the presence of 0.8 nM [^{3}H]IP_3

Pretreatment (10 min)	[^{3}H]IP_3 bound (dpm/10^6 cells)*		Significance compared to treatment without DTT‡
	no DTT	5 mM DTT (30 min)	
Control	316 ± 65 (7)	237 ± 63 (4)	NS
100 μM menadione	1609 ± 113 (4)†	525 ± 15 (4)†	$P < 0.001$
100 μM *p*-benzoquinone	1397 ± 127 (3)†	667 ± 39 (3)†	$P < 0.001$
200 μM NEM	866 ± 28 (3)†	290 ± 28 (3)	$P < 0.001$
100 μM CCl_4	414 ± 22 (4)§	ND	
1 mM paraquat	373 ± 45 (4)	ND	

Data are expressed as mean ± SD and the numbers between parentheses denote the number of experiments.

* Significance is tested by the Student's *t*-test; † $P < 0.001$, compared to respective control.

‡ Significance is tested by the Student's *t*-test compared to control treatment for only 10 min.

§ Not significant compared to treatment with 0.1% ethanol (379 ± 106 dpm/10^6 cells).

NS, not significant; ND, means not determined.

membrane by stimulation of phospholipase C, which has been suggested to be mediated by a G-protein [31–33]. One metabolite generated by this breakdown is IP_3 [8], which mobilizes calcium from intracellular stores causing an increase in intracellular free calcium concentration as observed after stimulation of α_1-adrenergic receptors in hepatocytes by (−)-epinephrine [34–36].

It has been reported that treatment of hepatocytes with 100 μM menadione for 10 min leads to a decrease in the amount of inositol phosphates formed after stimulation of the cells by (−)-epinephrine [14]. This might be a likely explanation for the observed inhibition of (−)-epinephrine-induced increase in intracellular free calcium concentration caused by menadione (Fig. 1). We extended these studies by investigating the IP_3 receptor and IP_3-induced release of calcium in hepatocytes after treatment with menadione.

Under the applied experimental conditions, menadione had only marginal effects on ATP-dependent loading of calcium in intracellular stores in permeabilized hepatocytes (Table 2 and Fig. 2). This is consistent with the absence of a menadione-induced effect on basal free calcium concentration in intact hepatocytes up to 100 μM menadione for 10 min (Fig. 1). At a concentration of 200 μM, menadione induced a sustained increase in intracellular free calcium concentration (Fig. 1), but failed to inhibit ATP-dependent Ca^{2+}-loading. However, incubation for 40 min in the presence of 250 μM menadione resulted in a profound decrease of ATP-dependent Ca^{2+}-sequestration of about 60% (data not shown). Moreover, it should be noted that basal intracellular free calcium concentration is buffered by the concerted action of ATP-dependent Ca^{2+}-sequestration in intracellular stores, ATP-dependent extrusion of calcium by Ca^{2+}-ATPases located in the plasma membrane and by uptake of calcium in mitochondria, which can all be affected by menadione [1, 3, 4].

IP_3-dependent release of calcium was significantly inhibited after treatment of hepatocytes with 50 μM menadione for 10 min and almost completely blocked by 100 μM menadione (Fig. 2). These results are consistent with the observed effects of menadione on (−)-epinephrine-induced increase in intracellular free calcium concentration in intact hepatocytes (Fig. 1).

Menadione has excellent redox cycling properties and is able to arylate sulfhydryls [37]. The radical scavenger cianidanol has been reported to prevent toxic events caused by menadione in perfused rat liver [38]. However, cianidanol did not protect against menadione-induced inhibition of IP_3-induced calcium release. Compared to menadione, *p*-benzoquinone is a better sulfhydryl arylator without redox cycling properties [37]. In contrast to menadione, *p*-benzoquinone did not alter IP_3-dependent calcium release in hepatocytes. Also radical stress applied by carbon tetrachloride [39–41] or the redox cycling compound paraquat [42] were without effect on IP_3-dependent calcium release (Table 2). The sulfhydryl alkylator NEM inhibited both IP_3-induced release of calcium and ATP-dependent Ca^{2+}-sequestration (Table 2). This hampers a clear interpretation because these two processes are interrelated and there is a large variation in the obtained data. Guillemette and Segui [15] established the inhibitory effect of NEM on IP_3-induced calcium release in bovine adrenal cortex microsomes by appliance of IP_3 shortly after addition of NEM. The inhibitory effect of NEM on IP_3-induced calcium release compared to the relatively smaller effect of NEM on ATP-dependent Ca^{2+}-sequestration in rat hepatocytes is more clearly observed in the results obtained by incubation with NEM followed by treatment for 30 min with DTT (Table 2).

The inability of the thio-reducing agent DTT to reverse the effects of menadione and NEM on IP_3-induced calcium release in hepatocytes (Table 2) suggests covalent attachment as a critical step in the mechanism of inhibition.

IP_3-dependent calcium release is initiated by binding of IP_3 to a specific receptor which has at least one essential free sulfhydryl group [15, 43]. Treatment of rat liver plasma membranes with NEM or menadione results in a decrease of the number of IP_3 receptors [16]. In bovine adrenal cortex microsomes both the amount of calcium released by IP_3 and the number of IP_3-receptors are decreased after incubation with sulfhydryl reagents [15].

Treatment of intact hepatocytes with menadione, *p*-benzoquinone or NEM resulted in an increase in the number of IP_3 receptors as measured in subsequently permeabilized hepatocytes without a change in the observed dissociation constant (Table 3 and Fig. 3). The menadione-induced increase in IP_3-binding was concentration-dependent (Fig. 4) and was not caused by alteration in the kinetics of IP_3-binding (Fig. 5). The increase of IP_3 receptors could be reversed by treatment with DTT (Table 3). This indicates that oxidation of sulfhydryl groups is an important step in the mechanism of increase of IP_3 receptors caused by menadione, *p*-benzoquinone or NEM.

It has been reported that menadione and NEM increase the activity of protein kinase C, in contrast to *p*-benzoquinone which inhibits protein kinase C activity [44]. Protein kinase C has been suggested to inhibit IP_3-dependent effects via negative feedback inhibition of phospholipase C [45–47]. Protein kinase A has been reported to decrease IP_3-dependent calcium release and to regulate IP_3-receptors [27, 48, 49]. However, neither stimulation of protein kinase C activity by incubation of hepatocytes with phorbol 12-myristate 13-acetate, nor stimulation of protein kinase A activity by dibutyryl-cyclic AMP could mimic the effects of menadione, *p*-benzoquinone or NEM on IP_3-dependent release of calcium or on the number of IP_3 receptors (data not shown). Neither radical stress induced by carbon tetrachloride or paraquat caused any changes in the number of IP_3 receptors (Table 3) nor did the radical scavenger cianidanol prevent menadione-induced increase in the number of IP_3 receptors. This is in agreement with the absence of any inhibitory effects of carbon tetrachloride or paraquat on IP_3-induced calcium release and with the inability of cianidanol to protect against menadione-induced inhibition of IP_3-dependent release of calcium in hepatocytes (Table 2).

In summary, it is concluded that menadione-induced inhibition of (−)-epinephrine-dependent increase in intracellular free calcium concentration in hepatocytes is not caused by a decrease in the number of α_1-adrenergic receptors nor by a change in the affinity of the α_1-adrenergic receptor protein for (−)-epinephrine. This inhibition can be explained by a decreased ability of IP_3 to release calcium from intracellular stores, in addition to the reported decrease in (−)-epinephrine-stimulated formation of IP_3 caused by menadione [14].

The inhibition of IP_3-dependent release of calcium from intracellular stores is not caused by a decrease of the number of IP_3 receptors nor by a change of the dissociation constant, but accompanied by a large increase in IP_3 receptor concentration.

It is suggested that critical thiols play an important role in the mechanism of inhibition of IP_3-dependent release of calcium and of increase in the amount of detectable IP_3 receptors in hepatocytes caused by menadione.

The increase in the number of IP_3 receptors in hepatocytes observed after treatment with menadione or NEM appears not to be consistent with the inhibition of IP_3-dependent calcium release. We are currently investigating this discrepancy in our laboratory.

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