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α_1-Adrenergic Receptor Subtypes and Formation of Inositol Phosphates in Dispersed Hepatocytes and Renal Cells

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SUMMARY

The ability of α_{1a}- and α_{1b}-adrenergic receptor subtypes to stimulate [^{3}H]inositol phosphate ([^{3}H]InsP) formation was examined in collagenase-dispersed hepatocytes and renal cells. α_1-Adrenergic receptor binding sites were labeled with ^{125}I-BE 2254, and the proportion of α_{1a} and α_{1b} subtypes was determined with chloroethylclonidine (CEC) and WB 4101. Hepatocytes contained only α_{1b}-adrenergic receptors, whereas renal cells had approximately equal proportions of both subtypes. Pretreatment of renal cells with CEC selectively inactivated the α_{1b} subtype, leaving a homogeneous population of α_{1a} receptors. Norepinephrine stimulated [^{3}H]InsP accumulation to a similar extent in both hepatocytes and renal cells. Pretreatment with CEC inactivated this response completely in hepatocytes but only partially in renal cells. WB 4101 was 1000-fold more potent in inhibiting the [^{3}H]InsP response in renal cells than hepatocytes; however, some of this difference was due to rapid metabolism of WB 4101 by hepatocytes. After correction for metabolism, WB 4101 was still 11-fold more potent in inhibiting norepinephrine-stimulated [^{3}H]InsP formation in hepatocytes (α_{1b}) than in CEC-pretreated renal cells (α_{1a}). These results demonstrate that both α_{1a}- and α_{1b}-adrenergic receptor subtypes activate formation of [^{3}H]InsP, although the molecular mechanisms by which these responses occur remain to be determined.

There are at least two pharmacologically distinct subtypes of α_1-adrenergic receptors, which can be distinguished using radioligand binding assays or contractile responses of smooth muscle (1–4). α_{1a}-Adrenergic receptors have a high affinity for the competitive antagonist WB 4101 and are not inactivated by the site-directed alkylating agent CEC. α_{1b}-Adrenergic receptors have a 20-fold lower affinity for WB 4101 and are potently inactivated by CEC. Comparing the proportion of each subtype determined by CEC inactivation or affinity for WB 4101 gives similar results in a variety of tissues (5), suggesting that these two methods are distinguishing two different receptor subtypes. These two receptor subtypes also have different affinities for the competitive antagonists phentolamine (1, 2) and 5-methylurapidil (6).

α_1-Adrenergic receptors are among those receptors thought to initiate cellular signals by hydrolysis of PIP_2. The resulting Ins(1,4,5)P_3 and diacylglycerol release stored intracellular calcium and activate protein kinase C, respectively. However, many different biochemical responses to α_1-adrenergic receptor activation have been described in different tissues. Activation of α_1-adrenergic receptors has been shown to increase cyclic AMP accumulation, increase cyclic GMP accumulation, potentiate cyclic AMP responses to activation of G_s-linked receptors, activate phospholipase A_2, activate cyclic AMP phosphodiesterase, release adenosine, and increase calcium influx in various tissues (see Ref. 7 for review). It is not clear which, if any, of these responses are subsequent to primary alterations in PIP_2 hydrolysis and generation of Ins(1,4,5)P_3 and diacylglycerol.

Previous studies from this laboratory have noted a correlation between the presence of the α_{1a} subtype and the requirement for influx of extracellular Ca^{2+} in α_1-adrenergic receptor-mediated contraction of smooth muscle (8). We hypothesized that α_{1b} receptors might selectively activate inositol phospholipid hydrolysis and release intracellular Ca^{2+}, whereas α_{1a} receptors might work through a different mechanism to control Ca^{2+} influx (8). This suggestion was supported by the recent work of Tsujimoto *et al.* (9) studying glycogen phosphorylase activity in rat aorta and by the work of Hanft and Gross (10) studying contractile responses in rat heart and vas deferens. However, we had shown previously that pretreatment of brain slices with CEC, which should inactivate the α_{1b} subtype, did

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ABBREVIATIONS: CEC, chloroethylclonidine; InsP, inositol phosphates; KRB, Krebs Ringer bicarbonate buffer; PBS, phosphate-buffered saline; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; BE, BE 2254; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ins(1)P, inositol 1-monophosphate; Ins(1,3,4)P_3, inositol 1,3,4-trisphosphate.

not decrease norepinephrine-stimulated [^{3}H]InsP formation (3). This would be inconsistent with selective coupling of the α_{1b} subtype to inositol phospholipid metabolism.

Slices and isolated tissues present diffusion barriers to drugs, which will vary in different tissues, and apparent pharmacological differences between receptors mediating responses in these preparations might be due to such pharmacokinetic factors rather than to distinct receptor subtypes. It would be better to perform these studies in dispersed cell preparations, where such tissue barriers will be minimized. We have shown previously that rat liver contains almost exclusively the α_{1b} subtype, whereas rat kidney contains a mixture of both subtypes (5). Collagenase dispersal of cells from these two tissues and selective inactivation of α_{1b} receptors with CEC pretreatment allow us to obtain cells primarily enriched in either the α_{1b} (control hepatocytes) or α_{1a} (renal cells pretreated with CEC) subtypes. We have used these preparations to determine which subtype(s) of α_1-adrenergic receptors is/are linked to formation of [^{3}H]InsPs.

Experimental Procedures

Materials. The following chemicals were used: CEC and WB 4101 (Research Biochemicals Inc., Natick, MA); (−)-norepinephrine bitartrate and Type IV collagenase (Sigma, St. Louis, MO); phentolamine mesylate (CIBA-Geigy, Summit, NJ); BE [2-β(4-hydroxyphenyl)ethylaminomethyl)-tetralone] (Beiersdorf, Hamburg, FRG); [^{3}H]inositol (20–40 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO); and carrier-free Na^{125}I (Amersham, Arlington Heights, IL).

Cell preparation. Renal cells and hepatocytes were prepared essentially as described by Ormstad *et al.* (11) and Moldeus *et al.* (12), respectively. Briefly, rats were anesthetized with 1 ml/kg of body weight of a xylazine (10 mg/ml)/ketamine (25 mg/ml) mixture. The peritoneal cavity was opened and 0.2 ml of heparin (2 mg/ml) was injected into the inferior vena cava. Either the portal vein (liver) or the aorta (kidney) was cannulated, and accessory vessels were tied off. The vena cava was cut and the tissues were perfused at a rate of 10 ml/min for 5 to 7 min with Hanks' solution (137 mM NaCl, 5.4 mM KCl, 0.9 mM $MgSO_4$, 0.4 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 26 mM $NaHCO_3$, and 12.6 mM HEPES) containing 0.5 mM EGTA and 2% bovine serum albumin. During this time, the tissue was dissected free from the visceral cavity and transferred to a beaker of Hanks' solution containing 4 mM $CaCl_2$ and 0.1% collagenase (Type IV). The perfusion was continued for another 7 min for liver cells and 15 min for renal cells. Hepatocytes were dispersed into a beaker by gentle rubbing, filtered through gauze, and washed three times with KRB (120 mM NaCl, 5.5 mM KCl, 2.5 mM $CaCl_2$, 20 mM $NaHCO_3$, 11 mM glucose, 0.029 mM $CaNa_2EGTA$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4). Renal cells were filtered through a 210-μm nylon mesh sieve to remove large tissue fragments and glomeruli and then washed with the same buffer. These methods routinely result in fully dispersed cells, because undispersed elements are removed by filtration. Renal cells are predominantly (>80%) from tubular epithelium (11) and are spherical and of apparently uniform size. Viability, as estimated by trypan blue exclusion, was about 90% for both hepatocytes and renal cells.

CEC treatment. Cell preparations were divided in half, resuspended in 30 ml of KRB, and incubated with or without 100 μM CEC at 37° under O_2/CO_2 for 30 min, with stirring every 3–5 min. This appeared to be a maximal concentration of CEC, because similar receptor inactivation was observed after treatment with a 10-fold lower concentration (10 μM; data not shown). The reaction was terminated by addition of 20 ml of KRB and centrifugation at 500 × *g* for 5 min. The cells were washed three times and finally resuspended in either PBS for binding studies or 10 mM KRB containing 10 mM LiCl for accumulation of [^{3}H]InsPs.

^{125}I-BE Binding. BE was radioiodinated to theoretical specific activity and stored in methanol at −20° (13). Renal cells were homogenized with a Polytron in 20 ml of PBS (20 mM $NaPO_4$, 154 mM NaCl, pH 7.6), centrifuged at 20,000 × *g* for 10 min, and resuspended in PBS (0.1 ml of original packed cells/ml of final resuspension). Purified plasma membranes were made from hepatocytes by Percoll gradient centrifugation, as described previously for whole liver (4), and finally resuspended in PBS (0.1 ml of original packed cells/ml of final resuspension). Specific ^{125}I-BE binding was measured by incubating 0.1 ml of tissue with ^{125}I-BE, in a final volume of 0.25 ml of PBS for 20 min at 37° in the presence or absence of competing drugs (14). The incubation was stopped by addition of 10 ml of 10 mM Tris·HCl (pH 7.4) and filtration over glass fiber filters (no. 30; Schleicher and Schuell) under vacuum. Filters were washed with an additional 10 ml of buffer and dried, and radioactivity was determined in a γ-counter. Nonreceptor binding was determined in the presence of 10 μM phentolamine.

Saturation curves were determined by incubating tissue with six increasing concentrations of ^{125}I-BE (25–800 pM), and data were analyzed by Scatchard analysis. The potency of WB 4101 in competing for specific ^{125}I-BE binding sites was determined by incubating a single concentration of ^{125}I-BE (40–50 pM) in either the presence or absence of 16 concentrations of the competing drug (3×10^{-11} to 1.8×10^{-7} M). IC_{50} values were determined from the *x*-intercept of a Hill plot, and K_i values were calculated by the method of Cheng and Prusoff (15). The best two-site fit for a binding curve was calculated by minimizing the sum of squares of the errors, using nonlinear regression analysis. A partial *F* test was used to determine whether the increase of the goodness of fit was significantly more than would be expected on the

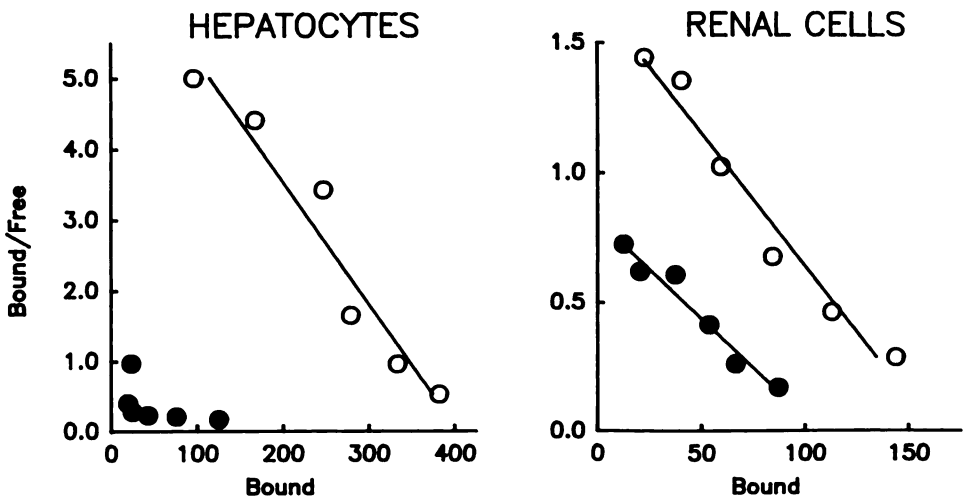

Fig. 1. Effect of CEC pretreatment on ^{125}I-BE binding sites in Percoll-purified membranes from hepatocytes or a crude particulate fraction from renal cells. Membrane preparations were incubated for 10 min with (●) or without (○) 10 μM CEC and were washed three times. ^{125}I-BE binding was determined as described in Experimental Procedures. Each *point* is the mean of duplicate determinations from six experiments.

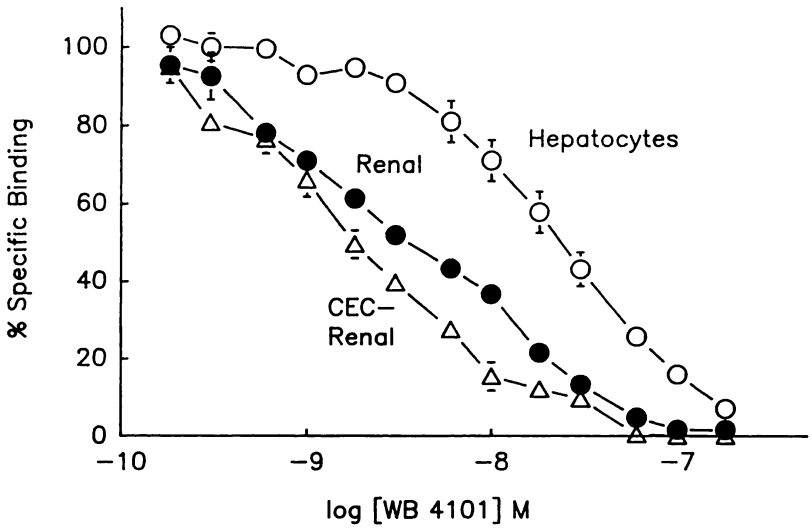

Fig. 2. Inhibition of specific ^{125}I-BE binding by WB 4101 in membranes from hepatocytes, renal cells, or renal cells pretreated with 100 μM CEC for 30 min (*CEC-Renal*). Each *point* is the average of duplicate determinations from six (hepatocytes, renal cells) or 11 (CEC-Renal) experiments.

TABLE 1

WB 4101 displacement of specific ^{125}I-BE binding in membranes prepared from collagenase-dispersed hepatocytes and renal cells

Collagenase-dispersed hepatocytes and renal cells were prepared as described in Experimental Procedures. Renal cells were preincubated with or without 100 μM CEC for 30 min at 37° in KRB. Renal cells were homogenized and centrifuged at 30,000 × *g* for 10 min, washed once, and resuspended in PBS for ^{125}I-BE binding. Each value is the mean ± standard error of the indicated number (*n*) of experiments. Equilibrium binding constants (K_D) and Hill coefficients (n_H) were determined from Hill plots, as described in Experimental Procedures.

	K_D	n_H	*n*
	nM		
Control hepatocytes	11 ± 1.2	1.15 ± 0.099	6
Control renal cells	1.7 ± 0.32[a]	0.70 ± 0.044[a]	6
CEC-pretreated renal cells	1.1 ± 0.15[a]	0.90 ± 0.062[b]	11

[a] $p < 0.01$ compared with control hepatocytes.
[b] $p < 0.05$ compared with control renal cells.

TABLE 2

Effect of CEC pretreatment on ^{125}I-BE binding sites in collagenase-dispersed hepatocytes and renal cells

Hepatocytes and renal cells were dispersed as described in the text and whole cells were preincubated for 30 min in the presence or absence of 100 μM CEC, at 37° in KRB. At the end of the incubation, renal cells were diluted in 20 ml of PBS and homogenized with a Polytron. Following centrifugation, the pellet was washed once and resuspended in PBS for ^{125}I-BE binding. Percoll-purified membranes were prepared from the hepatocytes and resuspended in PBS for ^{125}I-BE binding. Each value is the mean ± standard error from four experiments.

	Hepatocytes		Renal cells	
	K_D	B_{max}	K_D	B_{max}
	pM	*fmol/mg*	*pM*	*fmol/mg*
Control	64 ± 14.1	426 ± 66.5	89 ± 15.4	161 ± 23.8
CEC	81	22 ± 22[a] (−95%)	121 ± 14.0	102 ± 7.0[b] (−37%)

[a] $p < 0.001$ compared with control.
[b] $p < 0.05$.

basis of chance alone in the comparison of one- and two-site models (16) and *p* values less than 0.05 were considered significant.

Stability of ^{125}I-BE binding sites was examined after 0, 30, 60, and 120 min of incubation of intact cells under conditions used for measurement of [^{3}H]inositol metabolism. No losses of binding sites occurred during any of these incubation times (data not shown). The density of ^{125}I-BE binding sites in membranes prepared by homogenization in hypotonic medium (10 mM NaHEPES, pH 7.4) was not different from the density in those prepared by homogenization in PBS (not shown), suggesting that cells were fully disrupted.

Measurement of [^{3}H]inositol metabolism. InsP accumulation was determined in the presence of 10 mM LiCl by the method of Berridge *et al.* (17), as described previously (3). An aliquot of 80 μl of gravity-packed cells was added to 420 μl of Li-KRB (110 mM NaCl, 10 mM LiCl, 5.5 mM KCl, 2.5 mM $CaCl_2$, 20 mM $NaHCO_3$, 11 mM glucose, 0.029 mM $CaNa_2EGTA$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4) containing either 0.5 μCi (kidney) or 1.0 μCi (liver) of [^{3}H]inositol and appropriate drugs. Samples were incubated for 2 hr at 37° under 95% O_2/5% CO_2, in a shaking water bath. [^{3}H]Inositol prelabeling occurred mainly during the first hour of incubation in renal cells, and incorporation into lipid increased only 16% during the second hour of incubation (data not shown). The reaction was terminated by addition of 5 ml of ice-cold Li-KRB and centrifugation at 500 × *g* for 5 min. The supernatant was discarded and renal cells were washed twice more in the same manner as described previously for kidney slices (18). Both hepatocytes and renal cells were resuspended in 0.35 ml of Li-KRB, 0.94 ml of chloroform/methanol (1:2; v/v) was added, and the mixture was sonicated for 10 sec. Water (0.31 ml) and chloroform (0.31 ml) were added, and samples were centrifuged at 10,000 × *g* for 5 min to separate aqueous and organic phases. Aliquots of the aqueous phase (0.9 ml) were added to prepared Dowex columns (13 mm of 1 × 8–200 anion exchange region in 8 × 200-mm Kontes columns), which had been previously washed with 40 ml of 10 mM Tris-formate buffer (pH 7.4). Free [^{3}H]inositol was washed off the column with 40 ml of 5 mM *myo*-inositol and [^{3}H]InsPs were eluted with 1 ml of 1 M ammonium formate/0.1 N formic acid. Three milliliters of Scintiverse (Fisher) were added to each vial and after vigorous shaking, the samples were counted on a scintillation counter with 40% efficiency. The columns were regenerated with 4 ml of 1.0 N formic acid and stored in 0.1 N formic acid.

Results

Hepatocytes contain only the α_{1b} subtype, whereas renal cells contain both subtypes. Pretreatment of membrane preparations with CEC caused an almost complete inactivation of ^{125}I-BE binding sites in hepatocyte membranes but only about a 35% loss in renal cell membranes (Fig. 1). WB 4101 inhibition of ^{125}I-BE binding in hepatocyte membranes was characterized by a steep slope and a relatively low affinity, whereas in renal cell membranes the inhibition curve was shallower with a higher affinity (Fig. 2; Table 1). Two-site analysis of WB 4101 inhibition curves showed a one-site fit in hepatocytes (K_D = 11 ± 0.8 nM), but a significantly better two-site fit in renal cells ($p < 0.01$). In renal cells, 41 ± 6.8% of the sites had a K_D for WB 4101 of 5 ± 0.5 nM, whereas the rest

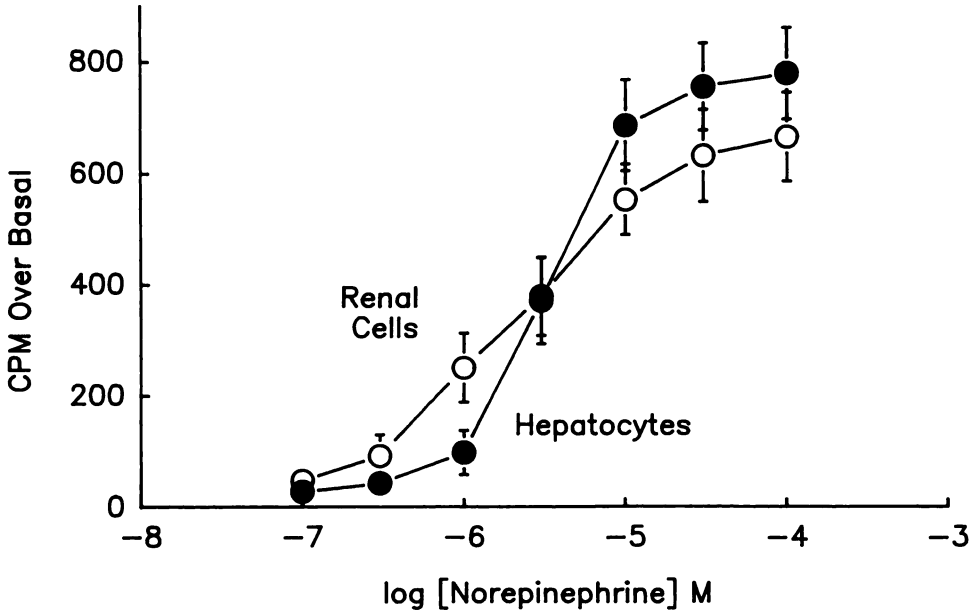

Fig. 3. Stimulation of [^{3}H]InsP formation by norepinephrine in intact hepatocytes and renal cells. Experiments were performed as described in Experimental Procedures. Results are expressed as the cpm increase over basal. Basal cpm were 831 ± 44 in renal cells and 595 ± 29 in hepatocytes. Each *point* is the mean ± standard error of triplicate determinations from three or four experiments.

TABLE 3

Norepinephrine-stimulated [^{3}H]InsP accumulation in hepatocytes and renal cells

Cells were prepared and renal cells were preincubated with or without 100 μM CEC for 30 min. After washing, [^{3}H]InsP formation was determined as described in Experimental Procedures. Each value is the mean ± standard error of the indicated number of experiments (*n*).

	n	EC_{50}	Maximal response
		μM	*fold of basal cpm*
Hepatocytes	3	2.2 ± 0.6	2.4 ± 0.1
Control renal cells	4	3.2 ± 0.5	2.0 ± 0.1
CEC-pretreated renal cells	3	4.4 ± 0.7	1.7 ± 0.1

showed a K_D of 0.45 ± 0.08 nM. These data demonstrate that hepatocytes contain only the α_{1b} subtype (inactivated by CEC and with a low affinity for WB 4101), whereas renal cells contain approximately 60–65% of the α_{1a} subtype.

CEC-pretreated renal cells contain only the α_{1a} subtype. Intact cell suspensions were pretreated with a high concentration of CEC (100 μM) for 30 min, to inactivate the α_{1b} subtype. This treatment caused a 95% inactivation of ^{125}I-BE binding sites in hepatocytes but only a 37% inactivation in renal cells (Table 2). The ^{125}I-BE binding sites remaining after CEC pretreatment of renal cells showed a higher affinity for WB 4101 (Table 1), and two-site analysis showed only a single-site fit.

Norepinephrine stimulates [^{3}H]InsP accumulation in both hepatocytes and renal cells. Norepinephrine stimulated formation of [^{3}H]InsPs in both hepatocytes and renal cells, in a concentration-dependent manner (Fig. 3). The EC_{50} and maximal stimulation were similar for both cell types (Table 3).

CEC pretreatment inactivates the [^{3}H]InsP response completely in hepatocytes but only partially in renal cells. Pretreatment of intact hepatocytes with 100 μM CEC for 30 min caused a complete abolition of norepinephrine-stimulated [^{3}H]InsP accumulation (Fig. 4). However, CEC pretreatment of renal cells only partially reduced the [^{3}H]InsP response to norepinephrine (Fig. 4). In control renal cells, norepinephrine caused a 127 ± 8% increase over basal levels, whereas CEC pretreatment reduced this by about half, to a 60 ± 3% increase.

WB 4101 is much more potent in inhibiting the [^{3}H]InsP response in renal cells than in hepatocytes. WB 4101 was about 1000-fold more potent in blocking norepinephrine-stimulated [^{3}H]InsP formation in renal cells than it was in

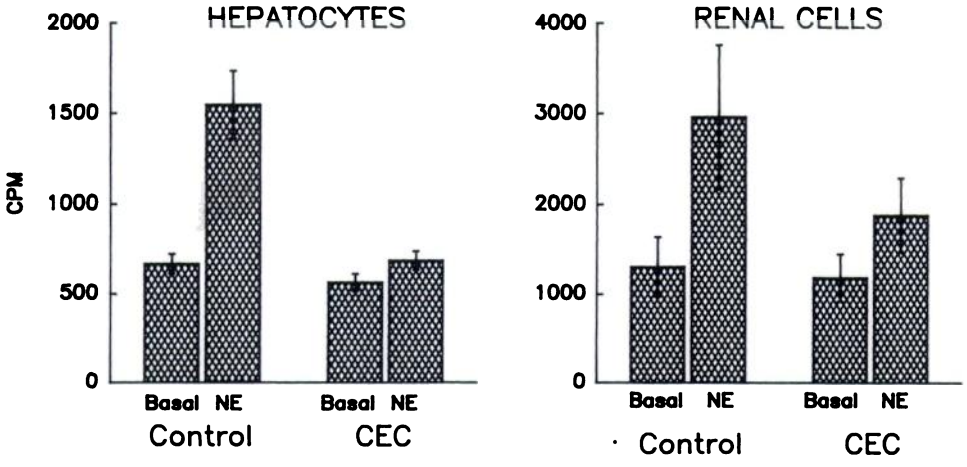

Fig. 4. Effect of CEC pretreatment on norepinephrine-stimulated [^{3}H]InsP formation in hepatocytes and renal cells. Cells were preincubated without (*Control*) or with (*CEC*) 100 μM CEC for 30 min, and [^{3}H]InsP accumulation in response to 100 μM norepinephrine (*NE*) was determined as described in Experimental Procedures. Each *bar* represents the mean ± standard error of triplicate determinations from four experiments. The response to norepinephrine was significantly reduced ($p < 0.01$) in CEC-treated renal cells compared with norepinephrine controls.

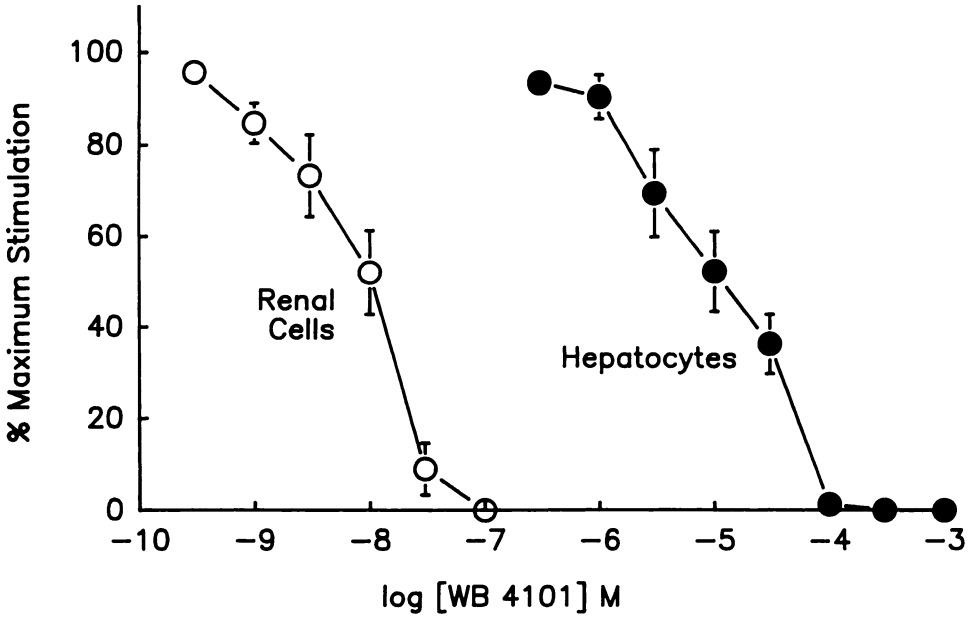

Fig. 5. Inhibition by WB 4101 of norepinephrine-stimulated [^{3}H]InsP formation in renal cells and hepatocytes. The concentration of norepinephrine was 10 μM. Each *point* is the mean ± standard error of triplicate determinations from four experiments.

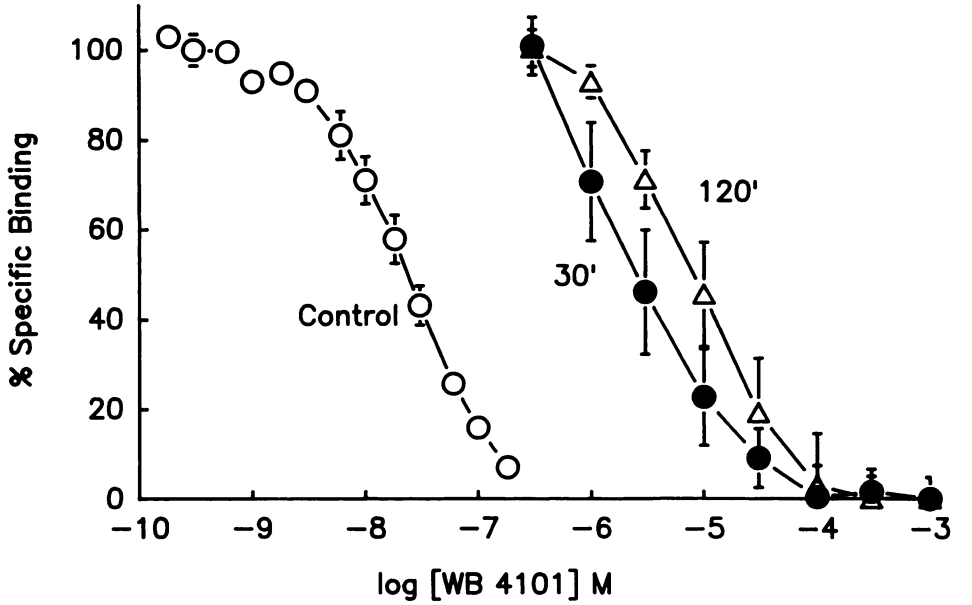

Fig. 6. Metabolism of WB 4101 by hepatocytes. Hepatocytes (80 μl) were incubated with increasing concentrations of WB 4101 in a final volume of 0.55 ml LIKRB. After 30 min, cells were centrifuged briefly and a 50-μl aliquot of supernatant was removed. Cells were then resuspended, and incubated an additional 90 min, and then centrifuged again, and another 50-μl aliquot of supernatant was removed. The supernatants were monitored for displacement of specific ^{125}I-BE binding in Percoll-purified membranes from control hepatocytes and compared with known concentrations of authentic WB 4101 (*Control*). The concentration of WB 4101 plotted on the *x*-axis is the concentration initially added to the incubation mixture, corrected for the dilution in the binding assay (5-fold). Each *point* is the mean ± standard error of duplicate determinations from four experiments.

TABLE 4

Metabolism of WB 4101 by hepatocytes

Methods were as described in the legend to Fig. 6. The fold shift was calculated as the ratio of the IC_{50} after preincubation divided by the IC_{50} of the control value. Each value is the mean ± standard error of data from four experiments.

	IC_{50}	n_H	Fold shift
	nM		
Control	20.4 ± 4.2	1.01 ± 0.061	
30-min preincubation	2570 ± 283	0.94 ± 0.111	126
120-min preincubation	7943 ± 612	0.92 ± 0.161	389

hepatocytes (Fig. 5). In renal cells, the potency of WB 4101 in blocking this response correlated with its potency at competing for ^{125}I-BE binding sites (compare Figs. 2 and 5). This was not true in hepatocytes, however, where WB 4101 was about 100-fold less potent at blocking the [^{3}H]InsP response than in competing for ^{125}I-BE binding sites.

WB 4101 is rapidly metabolized by hepatocytes. The extremely low potency of WB 4101 in blocking the [^{3}H]InsP response in hepatocytes suggested that the drug might be inactivated during the 2-hr incubation period. Therefore, we monitored the concentration of WB 4101 in the incubation mixture by radioreceptor assay. Hepatocytes were incubated with increasing concentrations of WB 4101, and aliquots of the supernatant were removed after 30 or 120 min of incubation. The concentration of WB 4101 remaining was determined by the ability of these supernatant aliquots to inhibit specific ^{125}I-BE binding to control hepatocyte membranes (Fig. 6). The potency of WB 4101 in competing for ^{125}I-BE binding sites was reduced dramatically following preincubation with hepatocytes (Table 4) under conditions that were identical to those used for the [^{3}H]InsP measurements (except for the omission of norepinephrine). However, most metabolism occurred within the first 30 min, when a 130-fold decrease in potency occurred. Only an additional 3-fold decrease in potency occurred during the remaining 90 min. Attempts to block metabolism of WB 4101 with 0.5 mM metyrapone were unsuccessful (data not shown).

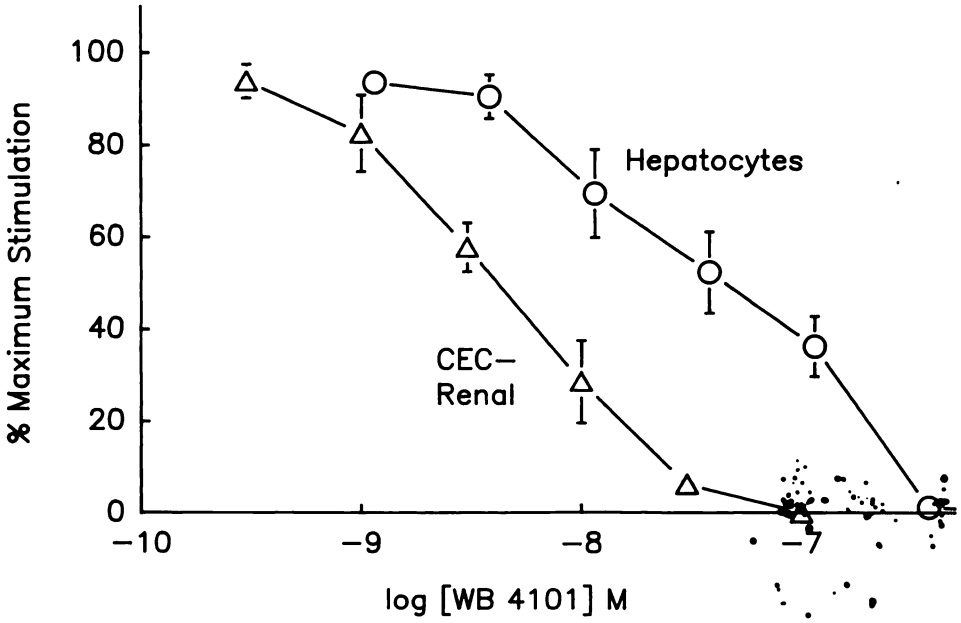

Fig. 7. Inhibition of norepinephrine-stimulated [^{3}H]InsP formation by WB 4101 in hepatocytes and CEC-pretreated renal cells (*CEC-Renal*). Renal cells were pretreated with 100 μM CEC for 30 min. The concentration of norepinephrine was 10 μM. In hepatocytes, norepinephrine was not added until 30 min into the incubation, and the concentration of WB 4101 was monitored in parallel samples, as described in the legend to Fig. 6. The concentration of WB 4101 indicated on the *x*-axis for the hepatocytes is corrected for metabolism. This was done by dividing the concentration added by the average of the metabolism at 30 and 120 min (262.5-fold from Table 4). This should reflect the average concentration of WB 4101, which varied by only 3-fold during the presence of agonist (30 to 120 min). Each *point* is the mean ± standard error of triplicate determinations from four experiments.

TABLE 5

Inhibition of norepinephrine-stimulated [^{3}H]InsP formation by WB 4101

Some renal cells were pretreated with 100 μM CEC for 30 min. WB 4101 inhibition of norepinephrine (10 μM)-stimulated [^{3}H]InsP formation was determined in control and CEC-pretreated renal cells as described in Experimental Procedures. In hepatocytes, norepinephrine (10 μM) was not added until 30 min into the incubation, and the concentration of WB 4101 was monitored in parallel samples as described. Each value is the mean ± standard error of the indicated number of experiments (*n*).

	n	IC_{50}	K_I
		nM	
Hepatocytes	4	46 ± 18.4[a]	8 ± 3.3
Control renal cells	3	8 ± 1.8[b]	2 ± 0.5[b]
CEC-pretreated renal cells	4	4 ± 0.8[c]	1 ± 0.2[c]

[a] Corrected by 262.5-fold (average of 30- and 120-min fold shift from Table 4).
[b] $p < 0.05$.
[c] $p < 0.01$ compared with hepatocytes.

The α_{1a} subtype mediates the [^{3}H]InsP response in CEC-pretreated renal cells. The potency of WB 4101 in blocking norepinephrine-stimulated [^{3}H]InsP formation was compared in CEC-pretreated renal cells (α_{1a}) and control hepatocytes (α_{1b}). Because of the rapid metabolism of WB 4101 in hepatocytes, norepinephrine was not added until 30 min after the incubation had started, when most metabolism has already occurred (Table 4). The concentration of WB 4101 remaining was determined by radioreceptor assay of aliquots of the supernatant of parallel samples, as described above. WB 4101 was still about 11-fold more potent in blocking norepinephrine-stimulated [^{3}H]InsP formation in CEC-treated renal cells than in hepatocytes (Fig. 7; Table 5), even after correction for metabolism of WB 4101 by hepatocytes. This suggests that both the α_{1a} and α_{1b} subtypes can activate this response in these tissues.

Different [^{3}H]InsPs are formed in hepatocytes and renal cells. Preliminary experiments were performed to determine the types of [^{3}H]InsPs formed following 2-hr stimulation with norepinephrine in hepatocytes and renal cells. High pressure liquid chromatography separation (19) showed that the primary [^{3}H]InsP formed in both tissues was [^{3}H]Ins(1)P (data not shown). This is not surprising, because incubations were performed in the presence of 10 mM LiCl to block breakdown of Ins(1)P. However, large (5-fold) increases in [^{3}H]Ins(1,3,4)P_3 were observed in hepatocytes, but not renal cells, after 2-hr stimulation with NE. This raises the possibility that the different α_1-adrenergic receptor subtypes may promote formation of different [^{3}H]InsPs.

Discussion

These experiments were designed to determine which of the two α_1-adrenergic receptor subtypes distinguished by CEC and WB 4101 are linked to [^{3}H]InsP formation. Collagenase-dispersed cell preparations were used to minimize tissue access barriers and ensure that any apparent pharmacological differences would be due to differences in receptor properties.

Radioligand binding data show that collagenase-dispersed hepatocytes, like whole liver (5), contain almost exclusively the α_{1b} subtype. ^{125}I-BE binding sites are almost completely inactivated by CEC pretreatment and have a uniformly low affinity for WB 4101. As expected, pretreatment with CEC essentially abolished norepinephrine-stimulated [^{3}H]InsP formation in hepatocytes, consistent with mediation by the α_{1b} subtype. However, WB 4101 had an extremely low potency in blocking this response, much lower than its potency in competing for the receptor binding sites. This appeared to be due to rapid destruction of WB 4101 by the intact hepatocytes. Quantitation by radioreceptor assay showed that 99.2% of the drug was destroyed in the first 30 min of incubation with hepatocytes. However, a further 90 min of incubation resulted in a much smaller loss in remaining drug, probably due to the greatly reduced drug substrate concentration, enzyme inactivation, or depletion of essential cofactors. After correction for drug metabolism, WB 4101 showed the expected relatively low potency in blocking norepinephrine-stimulated [^{3}H]InsP formation in hepatocytes, and the K_I for this response was similar to the K_D for the α_{1b} receptor subtype. All of these data are consistent with a stimulation of [^{3}H]InsP formation by the α_{1b} subtype in hepatocytes.

Collagenase-dispersed renal cells appeared to contain a predominance of the α_{1a} subtype but also some α_{1b} receptors, as

shown previously in whole kidney (5). Based on CEC inactivation (35–37%) and the ratio of high and low affinity WB 4101 binding sites (59:41), we conclude that these renal cell preparations contained both α_{1a} and α_{1b} subtypes in about a 60:40 ratio. As previously reported for whole kidney (18), norepinephrine stimulated [^{3}H]InsP accumulation in collagenase-dispersed renal cells. The maximum response was similar to that observed in parallel preparations of hepatocytes. This response appeared to involve both α_{1a} and α_{1b} receptors, based on two lines of evidence. First, almost total inactivation of the α_{1b} population by CEC pretreatment caused only about a 50% reduction in the norepinephrine response. Second, WB 4101 was substantially more potent in inhibiting this response in renal cells (containing both subtypes) than in hepatocytes (containing only the α_{1b} subtype). This is consistent with involvement of both subtypes in the [^{3}H]InsP response to norepinephrine in renal cells.

To provide further evidence that the α_{1a} subtype stimulated increased [^{3}H]InsP accumulation, we examined the potency of WB 4101 in blocking this response in CEC-pretreated renal cells. These cells contain essentially only the α_{1a} subtype, and WB 4101 potently inhibited the [^{3}H]InsP response to norepinephrine. The K_I for this inhibition was similar to the K_D for the high affinity (α_{1a}) binding site. Finally, WB 4101 was approximately 11-fold more potent in inhibiting the response in CEC-treated renal cells than in hepatocytes. These data all support the hypothesis that both receptor subtypes can increase formation of [^{3}H]InsPs in the presence of lithium.

We have previously suggested that the α_{1b} subtype might stimulate hydrolysis of PIP_2, formation of $Ins(1,4,5)P_3$, and mobilization of intracellular Ca^{2+}, whereas the α_{1a} subtype might control the influx of extracellular Ca^{2+} by some mechanism not involving formation of InsPs (8). This hypothesis was based on the correlation between the presence of the α_{1a} subtype and the requirement for extracellular Ca^{2+} in contraction of smooth muscle (7). Similar results were obtained by Tsujimoto *et al.* (9), who showed that α_{1a} receptors in rat aorta also require calcium influx for activation of glycogen phosphorylase. Hanft and Gross (10) also reported that inotropic responses in rat heart mediated mainly by the α_{1b} subtype are insensitive to calcium channel blockers, whereas contractile responses of vas deferens mediated by the α_{1a} subtype are potently blocked by nitrendipine.

Our current results show clearly that α_{1a} receptor activation can also increase [^{3}H]InsP accumulation. Thus, our previous hypothesis that only the α_{1b} subtype activates formation of inositol phosphates (8) is incorrect. However, this does not necessarily eliminate the possibility that α_{1a} receptors specifically activate influx of extracellular Ca^{2+}. Because we only measured production of total [^{3}H]InsPs during relatively long incubations in the presence of LiCl, it is possible that the two subtypes activate formation of different products from different precursors. The α_{1b} subtype is thought to activate a GTP-binding protein, which then activates a PIP_2-specific phospholipase C to form $Ins(1,4,5)P_3$ and diacylglycerol. It is possible that the α_{1a} subtype might primarily activate calcium influx and that the increased intracellular Ca^{2+} might subsequently increase hydrolysis of PIP_2 or other inositol phospholipids. Alternatively, α_{1a} receptor activation might activate a different GTP-binding protein (possibly sensitive to inactivation by pertussis toxin) (20, 21), which would either directly or indirectly activate formation of as yet undefined InsPs.

Although both receptor subtypes appear to be capable of increasing [^{3}H]InsP formation, the types of InsPs formed, their source, and the mechanism by which receptor activation is linked to increased formation remain to be determined. Preliminary experiments showed that the primary [^{3}H]InsP formed after 2 hr of stimulation was Ins(1)P in both tissues, as expected after long incubations in the presence of lithium. However, large increases in [^{3}H]$Ins(1,3,4)P_3$ were observed in hepatocytes but not renal cells, suggesting that each receptor subtype may activate formation of different inositol phosphate isomers. We are currently examining the time course of formation of different types of [^{3}H]InsPs in these two cell preparations and their sensitivity to CEC. It will also be interesting to determine the Ca^{2+} dependence and pertussis toxin sensitivity of the [^{3}H]InsP responses to each receptor subtype (7).

In conclusion, we have shown that both α_{1a}- and α_{1b}-adrenergic receptor subtypes can activate formation of [^{3}H]InsPs. Whether the two subtypes act through the same molecular pathways remains to be established.

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