8. H. Nakata, J. W. Regan and R. J. Lefkowitz, *Biochem. Pharmac.* **35**, 4089 (1986).
9. F-Z. Chung, K-U. Lentes, J. Gocayne, M. Fitzgerald, D. Robinson, A. R. Kerlavage, C. M. Fraser and J. C. Venter, *Fedn Eur. Biochem. Soc. Lett.* **211**, 200 (1987).
10. B. K. Kobilka, H. Matsui, T. S. Kobilka, T. L. Yang-Feng, U. Francke, M. G. Caron, R. J. Lefkowitz and J. W. Regan, *Science* **238**, 650 (1987).
11. D. Gurwitz and M. Sokolovsky, *Biochemistry* **24**, 8086 (1985).

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Mediation of norepinephrine effects on free cytosolic calcium in rat parotid acinar cells by α_1 adrenergic receptors

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Stimulation of sympathetic nerves or injection of norepinephrine (NE*) produces a pronounced increase in parotid salivary amylase secretion and weakly stimulates salivary fluid secretion, relative to parasympathetic nerve stimulation [1, 2]. The contributions of different adrenergic receptors to these effects have been studied extensively *in vitro*. Adrenergic effects on amylase exocytosis are primarily mediated via β receptors and involve a cyclic AMP (cAMP) dependent pathway [2]. However, a small activation of protein exocytosis is provided by α_1 receptor agonists, similar to that observed with muscarinic agonists and substance P, and apparently results from protein kinase C activation [3]. NE effects on parotid salivary fluid secretion appear absolutely dependent on an increase in the intracellular free calcium concentration (Ca_i^{2+}) in these cells, which stimulates vectorial ion movements [4, 5]. Although initial studies of Ca^{2+}-dependent ion movements in parotid and submandibular cells suggested that the α_2 receptor was primarily responsible for adrenergic increases in salivary fluid secretion [6, 7], more extensive characterization of ion fluxes in salivary glands has established the α_1 receptor as the primary adrenergic mediator [8–10]. Moreover, mobilization of Ca^{2+} via phospholipase C-activated phosphatidylinositide (PI) turnover also is mediated by α_1 (but not α_2 or β) adrenergic receptors in parotid cells [11, 12].

The development of fluorescent Ca_i^{2+}-indicator dyes has allowed direct examination of the effects of the different adrenergic receptors on Ca_i^{2+} in dispersed parotid cell or acini preparations. Initial studies with quin 2 provided evidence for an α receptor-mediated increase in Ca_i^{2+} but there were conflicting reports as to the effects of β agonists [13–15]. Recently Nauntofte and Dissing have re-examined the contribution of the different parotid adrenergic receptor types to the increase in Ca_i^{2+} using the more sensitive indicator Fura 2 [16]. They found that the β agonist isoproterenol (ISO) is only 10% as effective as epinephrine and that the selective α_1 agonist phenylephrine (PE) is only 60% as effective. There was no additivity between ISO and PE effects on parotid Ca_i^{2+}, and these authors concluded that ISO was acting through α_1 receptors. The difference between epinephrine and PE effects was ascribed to additional α_2 effects of epinephrine [16]. An alternative explanation for the latter finding is that PE is a partial agonist at parotid α_1 receptors. In the present study, we examined this possibility and closely studied the effects of various adrenergic agonists and antagonists on Ca_i^{2+}.

* Abbreviations used: NE, norepinephrine; cAMP, cyclic AMP; Ca_i^{2+}, intracellular free calcium concentration; ISO, isoproterenol; PE, phenylephrine; Met, Methoxamine; Yoh, yohimbine; Cor, corynanthine; Aten, atenolol; Phent, phentolamine; and Clon, clonidine.

Materials and methods

Prazosin, phentolamine, methoxamine, clonidine, and atenolol were gifts from Pfizer (New York, NY), Ciba Geigy (Summit, NJ), Burroughs-Wellcome (Research Triangle Park, NC), Boehringer Ingelheim (Ridgefield, CT) and Stuart Pharmaceuticals (Wilmington, DE) respectively. NE, PE, ISO, propranolol, corynanthine and yohimbine were from Sigma (St. Louis, MO). All drugs were dissolved in distilled water (except prazosin which was dissolved in ethanol). In some experiments, NE and ISO were added with equimolar ascorbate. Male Sprague–Dawley rats (200–350 g) were obtained from Charles River Laboratories (Kingston, NY). Fura 2 acetoxymethylester was purchased from Molecular Probes (Eugene, OR) and [^{3}H]inositol (15 Ci/mmol) from American Radiolabeled Chemicals (St. Louis, MO).

Rat parotid cells were prepared and Fura 2 studies performed as previously described [17]. If necessary, Ca_i^{2+} measurements were corrected for drug fluorescence or quenching. Cells were loaded with [^{3}H]inositol as reported [11, 17], but accumulation of total [^{3}H]inositol phosphates rather than of [^{3}H]inositol trisphosphate was measured.

Results and discussion

NE consistently produced a small elevation of Ca_i^{2+} in rat parotid acinar cells. The adrenergic effect on Ca_i^{2+} in this system and the effect on PI turnover appeared to be mediated solely by the α_1 adrenergic receptor, as follows: (1) the α_1-selective antagonist prazosin potently blocked NE effects on Ca_i^{2+} as well as on [^{3}H]inositol phosphate accumulation (Table 1 and Fig. 1C), (2) the beta blockers atenolol (10^{-5} M) and propranolol (10^{-6} M) had no effect on NE stimulation of Ca_i^{2+} or PI turnover (Fig. 1D and Table 1), and (3) yohimbine, an antagonist used at an α_2-selective concentration (10^{-6} M) also was without effect on NE actions (Fig. 1D and Table 1). Corynanthine, a structural analog which lacks the α_2 selectivity of yohimbine [18], was about 10-fold more potent than yohimbine in blocking NE effects and about 1000-fold less potent than prazosin (Table 1 and Fig. 1A, B and C). Importantly, no α_2 or β effects of NE on Ca_i^{2+} were apparent even under conditions where the α_1 receptor was mostly blocked (Fig. 1B and C).

In agreement with the findings of Nauntofte and Dissing [16], the selective α_1 agonist PE was much less effective than NE in elevating Ca_i^{2+} (Fig. 1D and E) and also as a stimulus for PI turnover (Table 1). However, this reflects the partial agonist property of PE in parotid cells, rather than an additional α_2 effect of NE. At a concentration supramaximal for effects on Ca_i^{2+} (300 μM), PE increased Ca_i^{2+} only 40% as effectively as NE (Fig. 1D and E and Table 1). PE also partially reversed NE effects on Ca_i^{2+} (Fig. 1 and Table 1), as would be expected of a partial

Table 1. Effects of adrenergic agonists and antagonists on NE stimulation of Ca_i^{2+} and PI turnover

	NE stimulation (%)	
	Ca_i^{2+}	PI
NE	100	100
NE + Prazosin 10^{-7} M	2 ± 3	0 ± 0
10^{-8} M	9 ± 6	34 ± 14
10^{-9} M	—	71 ± 8
NE + Yohimbine 10^{-4} M	—	45 ± 8
10^{-5} M	73 ± 9	96 ± 7
10^{-6} M	102 ± 5	99 ± 11
NE + Corynanthine 10^{-4} M	—	18 ± 10
10^{-5} M	15 ± 6	41 ± 13
10^{-6} M	66 ± 9	77 ± 11
NE + Atenolol 10^{-5} M (or propanolol 10^{-6} M)	99 ± 4	116 ± 13
PE	35 ± 5	41 ± 9
NE + PE	67 ± 4	53 ± 8
Methoxamine	48 ± 8	73 ± 9
NE + Methoxamine	87 ± 8	98 ± 5
ISO	3 ± 2	0 ± 3
NE + ISO	97 ± 3	102 ± 13
Clonidine	0 ± 1	3 ± 7
NE + Clonidine	14 ± 10	20 ± 14

NE increased Ca_i^{2+} (nM) from a basal (zero) level of 225 ± 17 to a peak of 345 ± 45 which then declined to a stable plateau (within 2 min, see Fig. 1) of 306 ± 32 (N = six cell preparations). NE increased PI turnover (as determined by accumulation of [^{3}H]inositol phosphates after 60 min of incubation with 10 mM LiCl [11]) to 396 ± 48% of basal levels, which averaged 4.24 ± 0.53% of the total water-soluble [^{3}H]cpm (N = thirteen cell preparations). For PI turnover studies, cells were incubated with drugs + NE, and stimulation was compared to that of NE alone. For Ca_i^{2+} studies, peak agonist effects were determined and compared to the NE effect in the same preparation. Antagonist or partial agonist effects were determined during the plateau phase of NE stimulation, 2 min after drug addition. Each value (mean ± SE) represents results from three to eight cell preparations. Antagonist concentrations were as indicated. For Ca_i^{2+} studies, NE, ISO and clonidine were used at 30 μM, and PE and methoxamine were at 300 μM; 10-fold higher concentrations of these five agents were used for PI turnover studies. At the concentrations used, these adrenergic drugs had no effect on carbachol-induced increases in Ca_i^{2+} or PI turnover.

agonist competing with an agonist for the same set of receptors. These partial agonist effects were also apparent in PI turnover experiments using 10-fold higher concentrations of PE and NE (Table 1). Maximally effective concentrations of methoxamine were also less effective than NE at increasing Ca_i^{2+} and PI turnover (Table 1), though this agonist appeared to have greater efficacy than PE at parotid α_1 receptors, being a less effective blocker of NE effects (Table 1). At a concentration which mimicked maximal PE effects on Ca_i^{2+}, methoxamine was a weaker antagonist of NE effects (Fig. 1F and G). Clonidine, an α_2 agonist in many systems but an α blocker in rat salivary glands [7], acted as an antagonist or weak partial agonist at α_1 receptors (Fig. 1F and G, Table 1). The effects of clonidine were unaffected by yohimbine (Fig. 1G), arguing against a mechanism by which clonidine works through α_2 receptors to decrease Ca_i^{2+}.

No β adrenergic effect on Ca_i^{2+} was observed in the present study. Beta blockers had no apparent effect on Ca_i^{2+} responses to NE, and the β-selective agonist ISO had no significant effects (Fig. 1C, D and E and Table 1). While these findings are consistent with preliminary reports from others [15, 16], Takemura has reported pronounced ISO effects on Ca_i^{2+} using quin 2 in parotid cells, which differed qualitatively from effects of α adrenergic and muscarinic agonists [13]. Since the parotid is unusual in that cAMP rather than Ca^{2+} is the chief mediator of exocytosis [2, 19], a β adrenergic effect on Ca_i^{2+} would be interesting since Ca^{2+} might then be considered as a permissive or obligatory cofactor for secretion, as in most other systems. The ISO effect on Ca_i^{2+} which Takemura and Ohshika describe is unusual in that it is not mimicked by phosphodiesterase inhibitors, forskolin or dibutyryl cAMP [14]. If there is a cAMP-independent β adrenergic pathway in parotid cells which elevates Ca_i^{2+}, this pathway is lost in our cell preparation [although cAMP and amylase responses to ISO are retained (data not shown)]. Other possible explanations are that ISO stimulates release of other neurotransmitters from nerve terminals which may be retained in some dispersed cell preparations, or that ISO increases Ca_i^{2+} in a contaminating cell population, or that the apparent increase in Ca_i^{2+} is an artifact. In cells over-loaded with Fura 2 or quin 2, we have observed that NE frequently stimulates secretion of the indicator dye, which then fluoresces and artifactually appears as a pronounced increase in Ca_i^{2+}. This can be detected by quenching extracellular dye with Mn^{2+} which should be done routinely to screen for artifactual responses. Pretreatment with propranolol lessened this dye-secretion effect, but neither propranolol nor phentolamine reversed the increase in fluorescence once obtained. Surprisingly, in studies where β adrenergic increases in Ca_i^{2+} were reported in salivary gland cells, reversal of the ISO responses by α and β blockers was not attempted [13, 14, 20]. A strength of the Ca_i^{2+}-indicator technique is that reversal of agonist effects by antagonists (or partial agonists) can be directly examined (Fig. 1). Since the role of Ca^{2+} in β adrenergic stimulation of exocytosis in parotid cells is conceptually important and controversial [2, 19], further characterization of the purported β effects on Ca^{2+} is necessary.

In summary, the present results show that the α_1 receptor was the major and probably the only adrenergic receptor mediating rapid increases in Ca_i^{2+} in our dispersed rat parotid acinar cell preparation. In particular, our results demonstrate that the weak effect of PE relative to NE [16] was due to low efficacy of PE rather than to additional α_2 or β effects of NE on Ca_i^{2+}. This seems to explain the results reported by others. As with muscarinic and substance P receptors in these cells, parotid α_1 receptors are coupled to phospholipase C [3, 11, 12] and activation of this pathway is sufficient to account for adrenergic effects on Ca^{2+} mobilization (the present study), K^+ fluxes [8, 9] and parotid salivary fluid secretion.

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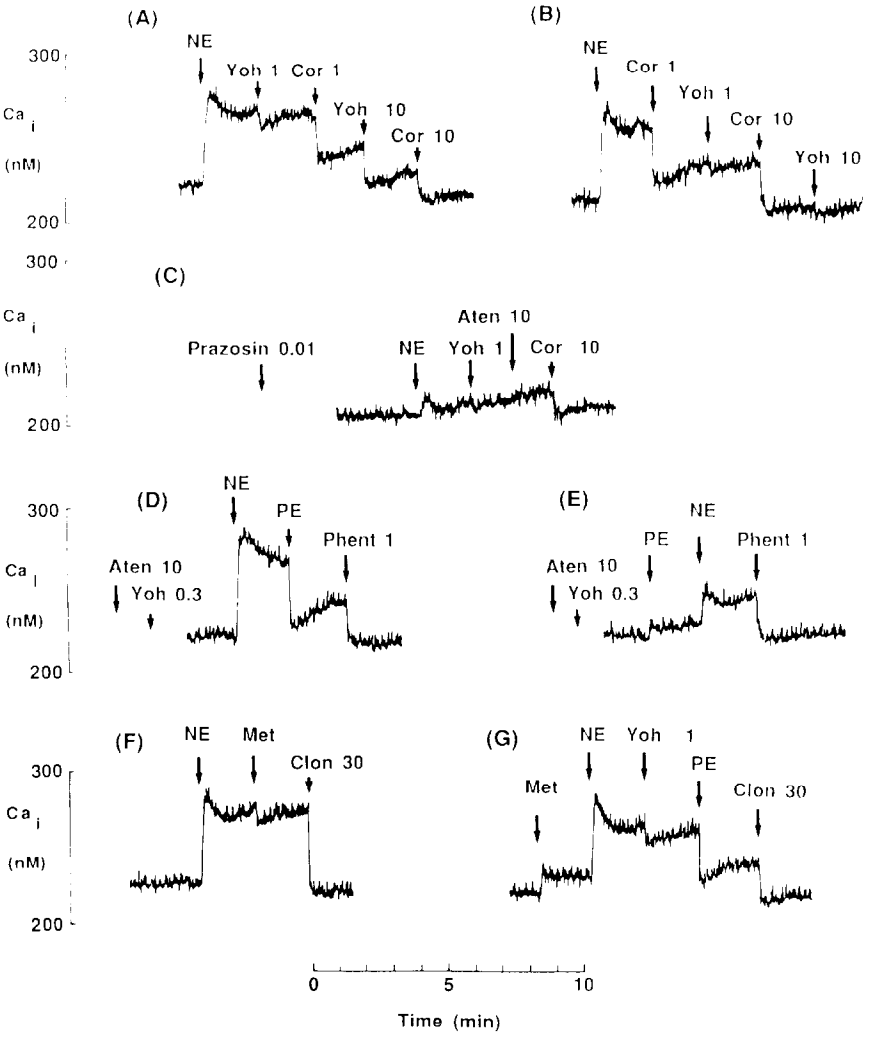

Fig. 1. Effects of adrenergic antagonists and agonists on NE-induced increases in Ca_i^{2+} in Fura 2-loaded rat parotid cells. These seven traces are from one preparation of cells and are typical of the results presented in Table 1. Numbers indicate concentration (μM). In this preparation, NE (30 μM in all traces) increased Ca_i^{2+} by 61 ± 3 nM in the absence of drugs. Concentrations of PE and Met (methoxamine) were 300 and 30 μM respectively. Other abbreviations: yohimbine (Yoh), corynanthine (Cor), atenolol (Aten), phentolamine (Phent) and clonidine (Clon). See text for discussion of traces.

REFERENCES

1. Young JA and Schneyer CA, Composition of saliva in mammalia. *Aust J Exp Biol Med Sci* **59**: 1–53, 1981.
2. Butcher FR and Putney JW, Jr, Regulation of parotid gland function by cyclic nucleotides and calcium. In: *Advances in Cyclic Nucleotide Research* (Eds. Greengard P and Robison GA), pp. 215–246. Raven Press, New York, 1980.
3. Tennes KA and Putney JW, Jr, Phosphoinositides and exocrine gland receptors. In: *Phosphoinositides and Receptor Mechanisms* (Ed. Putney JW, Jr), pp. 363–382. Alan R. Liss, New York, 1986.
4. Nauntofte B and Poulsen JH, Effects of Ca^{2+} and furosemide on Cl^- transport and O_2 uptake in rat parotid acini. *Am J Physiol* **251**: C175–C185, 1986.
5. Melvin JE, Kawaguchi M, Baum BJ and Turner RJ, A muscarinic agonist-stimulated chloride efflux pathway is associated with fluid secretion in rat parotid acinar cells. *Biochem Biophys Res Commun* **145**: 754–759, 1987.
6. Miller GD and Davis JN, Potassium release by α_2-adrenergic receptor stimulation of rat parotid acinar cells. *Biochem Pharmacol* **31**: 2197–2199, 1982.
7. Davis JN and Maury W, Clonidine and related imidazolines are postsynaptic alpha adrenergic antagonists in dispersed rat parotid cells. *J Pharmacol Exp Ther* **207**: 425–430, 1978.
8. Ito H, Hoopes MT, Baum BJ and Roth GS, K^+ release from rat parotid cells: An α_1-adrenergic mediated event. *Biochem Pharmacol* **31**: 567–573, 1982.

9. Danielsson A and Sehlin J, Effects of selective α_1 and α_2 adrenoceptor active drugs on $^{86}Rb^+$ efflux from pieces of rat parotid gland. *Acta Physiol Scand* **117**: 561–566, 1983.
10. Bylund DB, Martinez JR and Pierce DL, Regulation of autonomic receptors in rat submandibular gland. *Mol Pharmacol* **21**: 27–35, 1982.
11. Berridge MJ, Dawson RMC, Downes CP, Heslop JP and Irvine RF, Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem J* **212**: 473–482, 1983.
12. Aub DL and Putney JW, Jr, Properties of receptor-controlled inositol trisphosphate formation in parotid acinar cells. *Biochem J* **225**: 263–266, 1985.
13. Takemura H, Changes in cytosolic free calcium concentration in isolated rat parotid cells by cholinergic and β-adrenergic agonists. *Biochem Biophys Res Commun* **131**: 1048–1055, 1985.
14. Takemura H and Ohshika H, Effects of Ca^{2+} agonists and antagonists on cytosolic free Ca^{2+} concentration: Studies on Ca^{2+} channels in rat parotid cells. *Comp Biochem Physiol* **C88**: 219–224, 1987.
15. Aub DL and Putney JW, Jr, Mobilization of intracellular calcium by methacholine and inositol 1,4,5-trisphosphate in rat parotid acinar cells. *J Dent Res* **66**: 547–551, 1987.
16. Nauntofte B and Dissing S, Stimulation-induced changes in cytosolic calcium in rat parotid acini. *Am J Physiol* **253**: G290–G297, 1987.
17. McMillian MK, Soltoff SP and Talamo BR, Rapid desensitization of substance P- but not carbachol-induced increases in inositol trisphosphate and intracellular Ca^{2+} in rat parotid acinar cells. *Biochem Biophys Res Commun* **148**: 1017–1024, 1987.
18. McGrath JC, Evidence for more than one type of post-junctional α-adrenoceptor. *Biochem Pharmacol* **31**: 467–484, 1982.
19. Gomperts BD, Calcium shares the limelight in stimulus-secretion coupling. *Trends Biochem Sci* **11**: 290–292, 1986.
20. Helman J, Ambudkar IS and Baum BJ, Adreno-receptor mobilization of calcium in rat submandibular cells. *Eur J Pharmacol* **143**: 65–72, 1987.

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The cytotoxicity of menadione in hepatocytes isolated from streptozotocin-induced diabetic rats

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Alterations in the activity of both the cytochrome P-450 dependent mixed function oxidase (MFO*) system [1, 2] and the enzymes of conjugation [3, 4] have been shown to occur in experimentally-induced diabetes. In male streptozotocin treated diabetic rats the glucuronic acid conjugation of several substrates is deficient and the activity of glutathione-*S*-transferase is decreased [4]. These alterations in detoxification pathways will alter the balance between the processes of activation and detoxification of xenobiotics in diabetic animals and may result in increased susceptibility to xenobiotic-induced cytotoxicity. To investigate this possibility the cytotoxicity of menadione (2-methyl-1,4-naphthoquinone) was assessed in hepatocytes isolated from streptozotocin-induced diabetic rats.

Quinones can undergo either one-electron reduction to yield semiquinone free radicals or two electron reduction directly to the more stable hydroquinone which is then readily conjugated and excreted from the cell. Many semiquinones are readily re-oxidised in aerobic conditions and can enter redox cycles with molecular oxygen forming deleterious reactive oxygen species, causing oxidation of reduced glutathione (GSH) and ultimately cell death [5, 6]. The toxicity of menadione in isolated hepatocytes is influenced by the activities of the competing one- and two-electron reduction pathways, the glucuronic acid and glutathione conjugation reactions and the enzymes which afford cellular protection from oxidative challenge (e.g. glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase) and by intracellular GSH concentrations. The flavoprotein NAD(P)H:(quinone acceptor) oxidoreductase (also known as DT-diaphorase) catalyses the two-electron reduction of menadione directly to the hydroquinone, whereas NADPH-cytochrome *c*- and NADH-cytochrome b_5- reductases catalyse the one-electron reduction to the semiquinone radical [5].

* *Abbreviations*: MFO, mixed function oxidase; GSH, reduced glutathione.

Materials and methods

1. *Treatment of animals and assessment of the induced diabetes.* Male Sprague–Dawley rats (180–220 g) were used and were starved for 24 hr prior to receiving 60 mg/kg streptozotocin, intravenously, in acetate buffer, pH 4.5, on day 1. The animals were used for experiments on day 6 after treatment. The induced diabetes was assessed as described previously [3, 4]. On day 6 after treatment, streptozotocin-treated rats had blood glucose concentrations over 250 mg/100 ml compared with 80–100 mg/100 ml in control rats. Between 4 and 6 days after treatment diabetic rats excreted approximately 16 g glucose/24 hr in the urine.

2. *Preparation and incubation of hepatocytes.* Hepatocytes were prepared by collagenase perfusion as described previously [7] and viability was assessed by Trypan Blue exclusion. Control rat preparations were 90 ± 1% (N = 12) viable and diabetic rat preparations 87 ± 2% (N = 14). Incubations were carried out at 10^6 viable cells/ml in Krebs–Henseleit buffer, pH 7.4, containing 10 mM Hepes, in 50 ml round bottomed flasks at 37° under 95% O_2/5% CO_2. Cytotoxicity was evaluated by cell membrane damage (Trypan Blue exclusion) and depletion of GSH.

3. *Preparation of hepatic cytosol and microsomal fractions.* Rat livers were washed in ice-cold 0.01 M Tris buffer, pH 7.4, containing 1.15% (w/v) KCl, homogenised in 4 vol. of ice-cold 0.1 M Tris buffer, pH 7.4, containing 1.15% (w/v) KCl and 15% (v/v) glycerol and centrifuged at 15,000 *g* for 20 min at 0–4°. The supernatant was centrifuged again at 105,000 *g* for 50 min at 0–4° to separate the cytosolic (supernatant) and microsomal fractions. The cytosol was stored in 1 ml aliquots at −80° until required. The microsomal pellet was washed with homogenising buffer, recentrifuged at 105,000 *g* for 30 min at 0–4° and finally resuspended at 1 ml/g liver in 0.1 M Tris buffer, pH 7.4. Aliquots (0.5 ml) of the microsomal fraction were stored at −80° until required.