mutation is likely to be peculiar to yeast. The possibility remains that high concentrations of aspirin may affect mtDNA adversely in mammalian cells which could be a source of toxicity.

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REFERENCES

1. M. J. H. Smith and S. W. Jeffrey, *Biochem. J.* **64**, 589 (1956).
2. T. M. Brady, *J. Pharmac. exp. Ther.* **117**, 39 (1956).
3. A. R. Hughes and D. Wilkie, *Biochem. Pharmac.* **19**, 2555 (1970).
4. G. Bernardi, *TIBS* **4**, 197 (1979).
5. V. Hial, Z. Horakova, R. E. Shaff and H. Beavan, *Eur. J. Pharmac.* **37**, 367 (1976).
6. G. J. Roth and P. W. Majerus, *J. clin. Invest.* **56**, 624 (1975).
7. M. J. H. Smith and P. D. Dawkins, *J. Pharm. Pharmac.* **23**, 731 (1971).

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Inhibition of agonist-induced hydrolysis of phosphatidylinositol and muscarinic receptor binding by the calcium antagonist 8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethopybenzoate-HCl (TMB-8)

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Agonist-stimulated hydrolysis of phosphatidylinositol (PI) may be an essential step leading to an increase in intracellular Ca^{2+} concentration [1], although in some cells the hydrolysis apparently follows rather than precedes Ca^{2+} entry [2, 3]. The breakdown of PI in the exocrine pancreas is stimulated by agonists in the absence of extracellular Ca^{2+} [4] but, since these cells have intracellular stores from which Ca^{2+} can be released by agonists [5], this result does not eliminate a role for Ca^{2+} as the activator of PI breakdown in the pancreas. 8-(*N*,*N*-Diethylamino)-octyl-3,4,5-trimethoxybenzoate-HCl (TMB-8) has been used as an intracellular Ca^{2+} antagonist [6–8]. The mode and site of action of this agent have not been defined, although it has been proposed to immbolise Ca^{2+} at the membrane storage sites [7]. We used TMB-8 in an attempt to study the role of intracellular stores of Ca^{2+} in the activation of PI hydrolysis in the pancreas and report the results of these studies here.

The hydrolysis of PI in mouse pancreas was measured *in vitro*, as described previously [4], using pancreatic slices in which the PI had been prelabelled by injection of the mice with *myo*-[2-^{3}H]inositol. The pancreas slices were washed and incubated in Ca^{2+}-free Krebs, pH 7.4, at 37°. Tissue was preincubated with TMB-8 for 5 min prior to a 30-min incubation with agonist.

The Ca^{2+}-free EGTA Krebs solution consisted of (mmoles/l): NaCl, 126; KCl, 4.7; glucose, 2.8; Na fumarate, 2.7; Na glutamate, 4.9; Na pyruvate, 4.9; Tris (hydroxymethyl) aminomethane (Tris), 3; $MgCl_2$, 1.13; ethyleneglycol-bis-(β-amino-ethyl ether) *N*,*N*′-tetraacetic acid (EGTA), 0.1; adjusted with HCl to pH 7.4 at 37° and bubbled with O_2.

The effect of TMB-8 on muscarinic receptor binding was investigated using [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding to mouse pancreatic acini rather than to pancreas slices due to difficulties encountered with estimating [^{3}H]QNB binding in pancreas slices (our results, unpublished; [9]). Similar binding studies were carried out on a preparation from submandibular gland.

Mouse pancreatic acini were isolated using the method of Williams *et al.* [10]. The acini were resuspended in a buffer consisting of (mmoles/l): NaCl, 118; KCl, 4.7; $NaHCO_3$, 25; NaH_2PO_4, 1.2; glucose, 14; $CaCl_2$, 2.5; and which also contained soybean trypsin inhibitor (0.1 mg/ml) and essential and non-essential minimal Eagle's medium amino acid supplement (1%, final concentration); equilibrated with 95% O_2/5% CO_2.

Mouse submandibular glands were homogenized in 10 vol. of buffer consisting of (mmoles/l): NaCl, 137; KCl, 5.4; Na_2HPO_4, 0.34; KH_2PO_4, 0.44; $NaHCO_3$, 4.2; glucose, 5.6; Tris, 20; adjusted to pH 7.4 with HCl.

Muscarinic receptor binding was measured in isolated pancreatic acini and in submandibular gland homogenates incubated in the presence or absence of TMB-8 (2×10^{-5} and 2×10^{-4} moles/l) with [^{3}H]QNB (6×10^{-10} moles/l) in a volume of 2 ml. Submandibular gland homogenates were incubated for 60 min at room temperature; pancreatic acini were incubated at 37° for 120 min. TMB-8 was added 5 min before the addition of [^{3}H]QNB. Bound [^{3}H]QNB was separated by rapid filtration through Whatman GF/B glass fibre filters followed by two washes with 4 ml of ice-cold phosphate buffer (0.1 mole/l, pH 7.4). Non-specific binding was estimated from ^{3}H bound in the presence of atropine (10^{-5} moles/l) and represented 35 and 10% of total bound ^{3}H in pancreatic acini and submandibular gland homogenates respectively. Total ^{3}H bound represented less than 5% of ^{3}H present in the incubation medium.

The DNA content of the pancreatic acini was measured using the fluorescent dye Hoechst 33258 as described by Labarca and Paigen [11].

An unpaired *t*-test was used to determine the significance of differences in two group comparisons. For multiple group comparisons, results were analysed using a two-way analysis of variance to determine the significance between groups. To test if there was an interaction between the agonist and TMB-8, factorial analysis [12] was used.

Activation of muscarinic receptors or cholecystokinin (CCK) receptors produces secretion by the exocrine pan-

creas, apparently by a common pathway of stimulus–secretion coupling that may involve the hydrolysis of PI [4]. In an attempt to determine whether the breakdown of PI occurred prior to an increase in the intracellular calcium concentration rather than as a result of this calcium increase, the intracellular calcium antagonist TMB-8 was used.

TMB-8 (2×10^{-4} moles/l) inhibited completely the hydrolysis of PI induced by carbachol (Table 1). However, the breakdown of PI produced by an equipotent concentration of cholecystokinin-octapeptide (CCK-8) was reduced significantly but not inhibited completely by a higher concentration of TMB-8 (10^{-3} moles/l) (Table 1). TMB-8 (2×10^{-4} moles/l) did not reduce CCK-8-stimulated PI breakdown significantly (results not shown).

If PI hydrolysis was activated by release of a Ca^{2+} from intracellular stores, and if TMB-8 acted as an intracellular Ca^{2+} antagonist, one might expect TMB-8 to be equally effective in inhibiting the effects of equiactive concentrations of carbachol and CCK-8. The difference in the inhibitory potencies of TMB-8 toward the two agonists suggested that the antagonism may have been at a site that was not shared by carbachol and CCK-8; one possibility is that TMB-8 blocked the muscarinic receptor. It has been shown recently that a wide range of local anaesthetic and other substances with hydrophobic regions inhibit muscarinic receptor binding [13, 14].

The results in Table 2 show that [^{3}H]QNB binding to muscarinic receptors was inhibited in mouse pancreas by TMB-8 at a concentration (2×10^{-4} moles/l) which inhibited carbachol-induced PI hydrolysis. We do not know whether the inhibition by TMB-8 of CCK-8-stimulated PI breakdown in the pancreas can also be explained by a less potent action of TMB-8 on CCK receptors since we have not measured CCK receptor binding. However, a non-specific inhibitory action of TMB-8 on both types of receptors is a possibility since there is evidence from a recent study by Mürer *et al.* [15] that TMB-8 may be a general membrane-disrupting agent.

In pancreatic acini, non-specific binding of [^{3}H]QNB was reduced significantly in the presence of TMB-8 at 2×10^{-4} moles/l, but not in the lower concentration of TMB-8 (Table 2). We have no explanation of the effect of high concentrations of TMB-8 on non-specific binding of [^{3}H]QNB. However, a similar effect has been observed in pancreatic acini with high concentrations (2×10^{-4} moles/l) of tetracaine (J. A. Kennedy, unpublished), an agent which also inhibits the specific binding of [^{3}H]QNB to muscarinic receptors [16].

To test whether the effect of TMB-8 on the binding of [^{3}H]QNB to specific and non-specific sites was unique to the pancreas or more widely spread, binding studies were carried out using the mouse submandibular gland. The results in the submandibular gland (Table 3) were similar to those obtained using pancreatic acini.

Table 2. Effect of TMB-8 on muscarinic receptor binding in mouse pancreatic acini*

Treatment	[^{3}H]QNB bound†	
	Specific	Non-specific
Control (N = 6)	2.92 ± 0.26	1.89 ± 0.18
TMB-8 (2×10^{-5} moles/l) (N = 10)	1.12 ± 0.12‡	1.45 ± 0.14
TMB-8 (2×10^{-4} moles/l) (N = 10)	0.08 ± 0.03‡	0.38 ± 0.02‡

* Acini were incubated, in the presence or absence of TMB-8, with 6×10^{-10} moles/l [^{3}H]QNB for 120 min at 37°. The number of samples is given in parentheses.

† Results are given as mean ± S.E. in moles $\times 10^{-12}$/mg DNA.

‡ $P < 0.001$, compared with control.

Table 3. Effect of TMB-8 on muscarinic receptor binding in mouse submandibular gland*

Treatment	[^{3}H]QNB Bound†	
	Specific	Non-specific
Control (N = 9)	5.42 ± 0.18	0.53 ± 0.06
TMB-8 (2×10^{-5} moles/l) (N = 6)	0.46 ± 0.10‡	0.46 ± 0.07
TMB-8 (2×10^{-4} moles/l) (N = 6)	0.05 ± 0.03‡	0.31 ± 0.04§

* Submandibular gland homogenates were incubated, in the presence or absence of TMB-8, with 6×10^{-10} moles/l [^{3}H]QNB for 60 min at room temperature. The number of samples is given in parentheses.

† Results are given as mean ± S.E. in moles $\times 10^{-15}$/mg tissue.

‡ P (TMB-8 vs control) < 0.001.

§ P (TMB-8 vs control) < 0.05.

These results suggest that inhibition of carbachol-induced PI hydrolysis was due to inhibition of the binding of the agonist to receptors rather than to any effect on the release of Ca^{2+} from stores and indicate that TMB-8 should be used with caution as an intracellular Ca^{2+} antagonist, since this agent acts, with reasonable potency, to block at least one class of membrane receptors.

Table 1. Effects of TMB-8 on carbachol and CCK-8-stimulated PI hydrolysis in mouse pancreas*

	PI hydrolysed (Bq released/kBq ^{3}H incorporated by the pancreas)	
	TMB-8	
	(−)	(+)
CCK-8 (10^{-8} moles/l)	133 ± 14 (13)	76 ± 13† (10)
Carbachol (10^{-5} moles/l)	130 ± 32 (4)	5 ± 10† (5)

* PI hydrolysis was measured as ^{3}H released from PI above control values. TMB-8 concentrations used were 10^{-3} moles/l for incubations with CCK-8 and 2×10^{-4} moles/l for incubations with carbachol; TMB-8 alone (2×10^{-4} and 10^{-3} moles/l) produced no significant effect on PI breakdown ($P > 0.05$). The control samples released 198 ± 8 Bq/kBq^{3}H incorporated by the pancreas during the 30-min incubation. Values are means ± S.E.; the number of samples in each group is given in parentheses. † P (secretagogue vs secretagogue + TMB-8) < 0.05.

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REFERENCES

1. R. H. Michell, *Biochim. biophys. Acta* **415**, 81 (1975).
2. S. Cockcroft, J. P. Bennett and D. Gomperts, *Biochem. J.* **200**, 501 (1981).
3. R. L. Bell and P. W. Majerus, *J. biol. Chem.* **255**, 1790 (1980).
4. K. A. Tennes and M. L. Roberts, *Aust. J. exp. Biol. med. Sci.* **59**, 791 (1981).
5. J. A. Williams, *Am. J. Physiol.* **238**, G269 (1980).
6. I. F. Charo, R. D. Feinman and T. C. Detwiler, *Biochem. biophys. Res. Commun.* **72**, 1462 (1976).
7. S. Rittenhouse-Simmons and D. Deykin, *Biochim. biophys. Acta* **543**, 409 (1978).
8. J. O. Shaw, *Prostaglandins* **21**, 571 (1981).
9. R. T. Jensen and J. D. Gardner, *Fedn Proc.* **40**, 2486 (1981).
10. J. A. Williams, P. Cary and B. Moffat, *Am. J. Physiol.* **231**, 1562 (1976).
11. C. Labarca and K. Paigen, *Analyt. Biochem.* **102**, 344 (1980).
12. G. W. Snedecor and W. G. Cochran, *Statistical Methods*, 6th Edn. Iowa State University Press, Ames, IA (1967).
13. A. S. Fairhurst, M. L. Whittaker and F. J. Ehlert, *Biochem. Pharmac.* **29**, 155 (1980).
14. J. S. Aguilar, M. Criado and E. De Robertis, *Eur. J. Pharmac.* **68**, 317 (1980).
15. E. H. Mürer, G. J. Stewart, K. Davenport and E. Siojo, *Biochem. Pharmac.* **30**, 523 (1981).
16. W. J. Taylor, A. Wolf and J. M. Young, *Br. J. Pharmac.* **71**, 327 (1980).

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Adenylate cyclase activity of circular and longitudinal muscle layers of rat myometrium*

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The uterus is composed of two smooth muscle layers, an outer longitudinal and an inner circular layer which are separated by a well-developed vascular bed [1]. Most physiological studies that have measured effects of catecholamines on uterine contractility used the longitudinal axis, although Nesheim [2] found that the circular and longitudinal layers responded quite differently. He found the circular layer was less sensitive to isoprenaline and, since then, the circular and longitudinal layers have been shown also to differ in their motility patterns [3] and in their sensitivities to indomethacin and prostaglandins [4].

We separated the longitudinal from the circular muscle layer of the rat myometrium using two different methods. Adenylate cyclase activity of the outer longitudinal layer was greater and more responsive to isoproterenol than that of the inner circular layer. Since cAMP mediates the function of the beta-adrenergic receptor in the smooth muscle cell [5], there are important anatomical differences in the distribution of adenylate cyclase in the myometrium.

Materials and methods

The circular and longitudinal layers of the myometrium were separated either by mechanical or enzymatic manipulations.

Mechanical separation. The uteri of eight Sprague–Dawley rats (250 g) were removed and placed in warm (25°) Hanks' solution (HS), without Ca^{2+} or Mg^{2+}, buffered with Na^+, K^+ phosphate (1.5 mM) and PIPES† (5 mM), gassed with carbogen (95% O_2, 5% CO_2), and adjusted to pH 7.5 with NaOH. The uteri were cut open, and the endometrium was scraped off with a microscope slide. The circular (inner) layer was then separated from the longitudinal (outer) layer with the edge of a microscope slide firmly applied on the luminal side and moved from the cervical to the fallopian end of each horn. The different layers were transferred to 5 vol. of cold (4°) adenylate cyclase homogenization buffer (CHB): 0.05 M Na^+-HEPES, 0.001 M EGTA, 10% dimethyl sulfoxide, pH 7.6. The tissue was homogenized at 4° with a polytron PT10 (Brinkmann Instruments, Westbury, NY) for 30 sec at a setting of 4.5. The homogenates were filtered through glass wool and centrifuged at 20,000 *g* for 30 min at 4°. The pellets were washed twice, resuspended in 5 vol. of CHB, and used fresh.

Enzymatic separation. The uteri of twelve Sprague–Dawley rats (250 g) were removed and placed in warm (25°) HS. Six uteri were tied at the cervix and at both fallopian ends with surgical silk while the rest were turned inside out and then tied in an identical manner. Each group of uteri was transferred to Erlenmeyer flasks containing 1 ml of HS with 0.02% EDTA, 0.30 mg/ml trypsin (Type III from bovine pancreas, Sigma Chemical Co., St. Louis, MO), 0.30 mg/ml collagense (Type II, 320 units/mg, Sigma), and 0.10 mg/ml deoxyribonuclease I (bovine pan-

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† Abbreviations: PIPES, piperazine-*N*,*N*′-bis[2-ethane sulfonic acid] disodium salt; EDTA, [ethylene dinitrilo]-tetraacetic acid, disodium salt; EGTA, ethylene glycol-bis-(β-aminoethyl ether) *N*,*N*′-tetraacetic acid; and HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.