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MUSCARINIC RECEPTOR COUPLING TO INOSITOL PHOSPHOLIPID METABOLISM IN GUINEA-PIG CEREBRAL CORTEX, PAROTID GLAND AND ILEAL SMOOTH MUSCLE

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Abstract—Inositol phospholipid hydrolysis induced by agonist-stimulation of muscarinic receptors has been examined in slices of guinea-pig cerebral cortex, parotid gland and ileal smooth muscle. An assay measuring ^{3}H-inositol phosphate formation from prelabelled lipids in the presence of LiCl, allowed marked stimulation by agonists to be followed.

The pD_2-value of carbachol differed markedly, between tissues being more than 10-fold lower in cerebral cortex than in parotid gland. The partial agonist oxotremorine showed the largest relative maximal responsiveness in parotid gland, followed by ileum and cortex. Atropine suppressed the phosphoinositide response to carbachol with an almost similar affinity in each tissue, but pirenzepine was found to have a 20-fold higher affinity in cerebral cortex, $pK_i = 7.7$ than in parotid gland, $pK_i = 6.3$.

Carbachol, even in the presence of guanosine triphosphate (GTP), displayed complex binding against ^{3}H-*N*-methylscopolamine (^{3}H-NMS) in cortical and ileal membranes, though in membranes from the parotid gland a single homogeneous population was found. Atropine inhibition of ^{3}H-NMS parallelled its suppression of the phosphoinositide response, the affinities in each tissue studied being similar. Pirenzepine inhibited binding from two components in cerebral cortex, the high affinity value being similar to that obtained in the phosphoinositide assay. In parotid gland, however, only low affinity pirenzepine binding sites were observed, closely resembling the affinity found for this antagonist in the functional assay.

These experiments suggest (a) that there are differences between agonist occupation of muscarinic receptors and phosphoinositide hydrolysis within the different tissues, (b) that both high and low affinity pirenzepine binding sites appear to be linked to phosphoinositide metabolism, and (c) that low affinity pirenzepine sites may be more efficiently coupled to the hydrolysis of phosphoinositides.

A large number of recent studies suggest that muscarinic receptors do not form a single homogenous group and M_1- and M_2-muscarinic receptor subtypes have been proposed on the basis of ligand binding and functional pharmacological assays. M_1 receptors, showing high affinity for pirenzepine, are apparently located in forebrain areas and M_2 receptors, with low affinity for pirenzepine, in the hindbrain and in a variety of peripheral effector tissues [1, 2]. Unfortunately, much of this subclassification relies on the differential affinity described by one single compound, pirenzepine. Accordingly, it should be emphasized that a further subclassification of M_2 sites is warranted on the basis of the differential ileal/atrial affinity of the antagonist 4-diphenylacetoxy-*N*-methyl piperidine, 4-DAMP [1], and AFDX-116 [3, 4].

Muscarinic receptor stimulation induces a rapid hydrolysis of inositol phospholipids in many tissues [5] and an inhibition of adenylate cyclase in several cell types [6, 7]. The ability of pertussis toxin to selectively suppress the effects on adenylate cyclase suggests that these are separate effector systems [7–11]. The possibility that different muscarinic receptor subtypes could be linked to these separate effector systems was suggested with the proposal that M_1 receptors are linked to phosphoinositide hydrolysis and M_2 receptors to inhibition of adenylate cyclase [12]. Although there is some evidence supporting this contention [13], a completely opposite picture emerges in chick heart cells in which pirenzepine more potently blocks the adenylate cyclase response [14]. The coupling of M_2 muscarinic receptors to hydrolysis of phosphoinositides is also apparent in tracheal smooth muscle [15] and in guinea-pig colon [16]. Studies from our own group [17] and elsewhere [18] have shown different cerebral regional affinities for pirenzepine, but not for atropine, using muscarinic receptor phosphoinositide assays. In the present study, we have examined these phenomena in more detail by comparing muscarinic receptor-induced phosphoinositide hydrolysis in various tissues that have been proposed to possess predominantly "M_1" or "M_2" receptors.

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MATERIALS AND METHODS

Tissue preparations. Guinea-pigs weighing 300–400 g were killed by cerebral dislocation. The whole ileum from the proximal part of caecum to the treitz ligament was dissected, cut longitudinally, cleaned, and kept in warm Krebs–Henseleit buffer (NaCl 118 mM, KCl 4.7 mM, $CaCl_2$ 1.3 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 25 mM and glucose 11.7 mM) equilibrated with 95% O_2 and 5%

CO_2 to a final pH of 7.4 at 37°. The lobes of the parotid gland were gently dissected free of fat and kept in buffer. Before the ileum was cut into 1–2 cm sections, the mucosa was gently scraped off and the smooth muscle was kept for 10–15 min in warm buffer solution. The slices were cut in two directions with a McIlwain tissue chopper, in sections of 150 × 350 μm for parotid gland, 500 × 500 μm for ileal smooth muscle and 350 × 350 μm for cerebral cortex. Immediately after cutting, the slices were transferred to bottles with Krebs–Henseleit buffer at a temperature of 37° and shaken gently for 90 min. The buffer solution was changed and the slices simultaneously oxygenated (95% O_2, 5% CO_2) four times during this period.

Phosphoinositide assay. After preincubation including oxygenation and several washings in Krebs–Henseleit buffer, 50 μl of packed slices containing 1.2–1.6 mg protein from the parotid gland, ileal smooth muscle and cerebral cortex were incubated for 60 min together with ^{3}H-myo-inositol (NEN, 0.5–1 μCi; 0.1–0.2 μM) in a final volume of 300 μl buffer solution with 5 mM LiCl. All drugs were added in a volume of 10 μl and the tissue was exposed to antagonists for 15 min prior to the agonist. Phosphoinositide hydrolysis was monitored as previously described [19] by measuring ^{3}H-inositol phosphates in the presence of 5 mM lithium [20]. Inositol phosphate formation, which was linear for at least 90 min under these conditions, was initiated by addition of agonist and terminated by addition of chloroform and methanol (2:1, v/v). Chloroform and water were then used to obtain a two-phase solution, which was centrifuged in order to clear the separation. The aqueous phase was then used to separate ^{3}H-inositol phosphates using anion exchange chromatography, and samples of the organic phase were taken to assess the labelling of phospholipids [19].

Radioligand binding assay. Binding assays were prepared with membranes from guinea-pig cerebral cortex, parotid gland and ileal smooth muscle in a total volume of 250 μl. The membranes were homogenized (polytron PTA 10–35) for 2 × 10 sec in a 20 mM Tris buffer solution containing 154 mM NaCl, 2 mM $MgCl_2$ at a pH of 7.4 at 30°. The homogenization was followed by two separate centrifugation steps involving resuspension in buffer solution for 10 min at 30,000 *g*. The membranes were incubated with ^{3}H-NMS* for 30 min at 30° in the Tris buffer solution containing 100 μM GTP. Parotid gland and ileal smooth muscle were used in a 1:30 dilution with buffer solution, while cerebral cortex was diluted 1:110. These dilutions were calculated from the tissue wet wt estimated before homogenization and gave a percentage bound ligand of approximately 8–12% in all tissues. Non-specific binding was defined in the presence of 1 μM atropine and was determined to be less than 4% at the K_d concentration of ^{3}H-NMS in all tissues.

Calculations and statistics. The relationship between the concentration of agonist and its ability to stimulate, and that of antagonists to inhibit hydrolysis of phosphoinositides, is presented as pD_2 values and pK_i values, respectively, whereas inhibition of ^{3}H-NMS is presented as pK_d values. All concentration–response curves or binding inhibition curves were analyzed by means of a computer-assisted least-squares non-linear program [21, 22]. More complex curves were determined by comparing the sum of squares from a multiple site analysis with that from a single site analysis. The inhibition constants, pK_i, for pirenzepine and atropine were estimated from the inhibition of the carbachol- and oxotremorine-induced increase in phosphoinositide hydrolysis and the inhibition constants, pK_d, in binding experiments were calculated by using the equation of Cheng and Prusoff [23]. A dose ratio method [$\log K_i = -\log (DR - 1) - \log B$] was also used for calculation of pirenzepine and atropine affinity values in parotid gland. In the same set of slices a dose–response curve for carbachol and an antagonist inhibition curve was performed. The dose ratio was estimated from the carbachol EC_{50} value and the concentration of carbachol present when performing the antagonist inhibition curve and the antagonist IC_{50} value was used as antagonist concentration. A Student's *t*-test for unpaired samples was used to test for significant differences.

RESULTS

In the present experiments from guinea-pig cerebral cortex, ileal smooth muscle and parotid gland, ^{3}H-inositol were linearly incorporated into phospholipids for at least 2 hr in all tissues (data not shown). The formation of ^{3}H-inositol phosphates in the presence of lithium and carbachol also increased linearly for at least 90 min. Agonist stimulation for 60 min increased the accumulation of ^{3}H-inositol phosphates in each tissue, and this was amplified substantially in the presence of lithium. In parotid gland a 20-fold and in cerebral cortex a 15-fold stimulation in the presence of lithium was observed, while in the ileal smooth muscle only a 5-fold stimulation was seen. Under these conditions of long stimulation in the presence of lithium and using aqueous extractions to separate individual phosphates, > 90% of labelled phosphates were eluted as inositol monophosphate (data not shown).

The data in Fig. 1 demonstrate that the muscarinic agonists carbachol and oxotremorine stimulated the accumulation of ^{3}H-inositol phosphates in a dose-related manner in all tissues, though in all cases the former agonists was more effective. However, the pD_2 value for carbachol was substantially higher in the parotid gland that in the cerebral cortex. Furthermore, these data were also associated with the relative maximal responsiveness of oxotremorine, which was greatest in parotid gland, followed by ileal smooth muscle and cerebral cortex (Fig. 1 and Table 1). A linear relationship between the three tissues was established, where a high pD_2 value for the full agonist carbachol was followed by a high maximal response of the partial agonist oxotremorine (Fig. 1 insert). To distinguish whether the partial agonist oxotremorine and the full agonist carbachol acted on the same population of muscarinic receptors, 10 and 100 μM of oxotremorine were

* Abbreviations used: GTP, guanosine triphosphate; ^{3}H-NMS, ^{3}H-*N*-methylscopolamine.

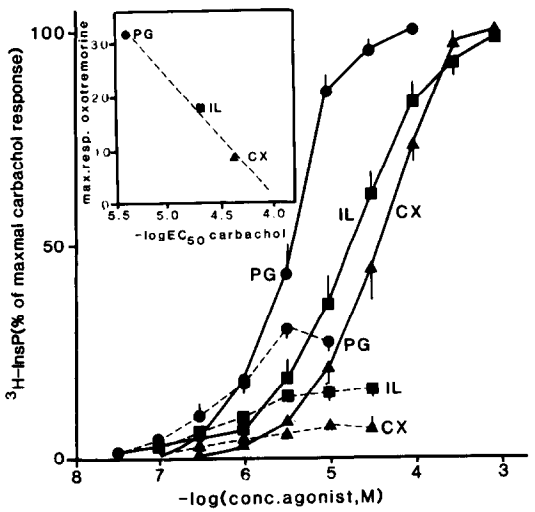

Fig. 1. Stimulation of hydrolysis of phosphoinositides induced by increasing concentrations of carbachol (solid lines), and by oxotremorine, partial agonist (dotted lines), in parotid gland (circles), ileal smooth muscle (squares) and cerebral cortex (triangles). The insert shows a linear relationship between the maximal stimulation induced by oxotremorine on the ordinate and the potency (pD_2 = $-\log EC_{50}$) of carbachol on the abscissa in the three examined tissues. Data are presented as percentage of maximal carbachol response. The maximal response by carbachol in parotid gland corresponds to a 20-fold increase in inositol phosphates while that in ileal smooth muscle and cerebral cortex corresponds to a 5- and 15-fold stimulation, respectively, see Results. The presented data are mean values ± SE from five experiments performed in triplicate.

incubated together with 1 mM carbachol in all tissues. Ten μM of oxotremorine reduced the carbachol response by approximately 30% and 100 μM by approximately 60% (data not shown).

Saturation experiments from 4–5 experiments using ^{3}H-NMS gave equilibrium constant values of 0.4 ± 0.01 nmol in cerebral cortex, 0.6 ± 0.04 nmol and 0.4 ± 0.03 nmol in ileal smooth muscle and parotid gland, respectively. Inhibition of specific ^{3}H-NMS binding by the agonists showed affinity values, pK_d, as presented in Table 1. The carbachol inhibition curves in cerebral cortex and ileal smooth muscle clearly showed biphasic interactions both in the absence and in the presence of 100 μM GTP with slope factors of 0.5 ± 0.08 and 0.5 ± 0.05, respectively (Fig. 2). In parotid gland, a biphasic interaction was shown in the absence of GTP, which was converted to a monophasic interaction in the presence of GTP now with a slope factor close to unity, 0.9 ± 0.07 (Table 1 and Fig. 2). In the case of oxotremorine, similar interactions were shown in the presence of GTP, with a clear biphasic inhibition curve in cerebral cortex and ileal smooth muscle and a monophasic curve in parotid gland observed in the presence of GTP (Table 1).

In parotid gland, carbachol showed a 126-fold higher potency on hydrolysis of phosphoinositides $pD_2 = 5.4$ than the estimated low binding affinity, $pK_d = 3.3$ (Table 1 and Fig. 3). Oxotremorine showed only a four-fold higher potency on the phosphoinositide response, $pD_2 = 6.2$, than in binding, $pK_d = 5.6$ (Table 1). Comparison of the potency values of carbachol on phosphoinositides with the low affinity binding site showed a ten-fold difference in cerebral cortex and a four-fold difference in ileal smooth muscle. Similarily expressed values for oxotremorine showed similar potency values in both cerebral cortex and ileal smooth muscle (Table 1).

The affinities of pirenzepine and atropine in the cerebral cortex and in the parotid gland on inhibition of the agonist-induced increase in phosphoinositides and on inhibition of binding with ^{3}H-NMS are presented in Table 2. The pirenzepine affinity in the phosphoinositide assay in cerebral cortex $pK_i = 7.7$ was approximately one order of magnitude higher than in parotid gland with a slope factor close to unity, 0.9 ± 0.08.

The affinity of pirenzepine in parotid gland was assessed when carbachol was used as agonist, pK_i = 6.3 with a slope factor close to unit, 0.9 ± 0.07, and

Table 1. Affinity values (pD_2) and maximum responses (per cent) of carbachol and oxotremorine on stimulation of hydrolysis of phosphoinositides (PI) and affinity values (pK_d) and proportion of muscarinic binding sites (per cent) on inhibition of binding with ^{3}H-NMS

	PI		^{3}H-NMS				Ratio
	max resp.		high aff.		low aff.		IC_{50} (low aff.)
	pD_2	(%)	pK_d	(%)	pK_d	(%)	/EC_{50} (PI)
Carbachol							
CX	4.4 ± 0.1	100	5.3 ± 0.2	27 ± 1	3.4 ± 0.4	73 ± 1	10
IL	4.8 ± 0.1	100	6.6 ± 0.2	46 ± 1	4.2 ± 0.3	54 ± 1	4
PG	5.4 ± 0.1	100	—	—	3.3 ± 0.3	98 ± 2	126
Oxotremorine							
CX	6.1 ± 0.1	9 ± 2	8.3 ± 0.1	14 ± 1	6.0 ± 0.1	86 ± 1	1
IL	6.2 ± 0.1	18 ± 2	8.2 ± 0.1	39 ± 3	6.1 ± 0.1	61 ± 3	1
PG	6.2 ± 0.1	32 ± 3	—	—	5.6 ± 0.1	96 ± 4	4

The values are presented as mean values ± SE, N = 3–6. The binding experiments were performed in the presence of 100 μM GTP.

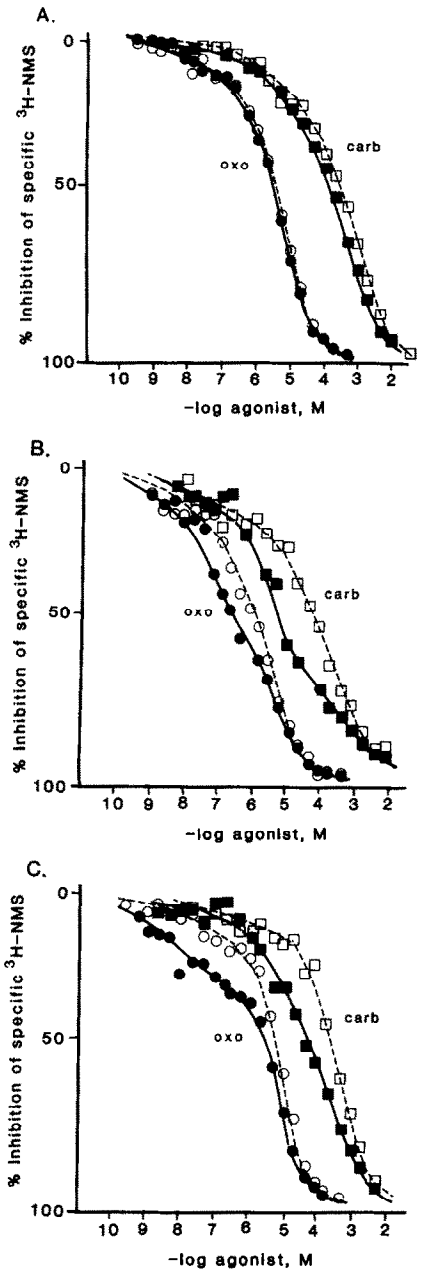

Fig. 2. Inhibition of ^{3}H-NMS by carbachol (carb) and oxotremorine (oxo) in the absence (closed symbols) and in the presence (open symbols) of 100 μM GTP in cerebral cortex (A), in ileal smooth muscle (B) and in parotid gland (C). One representative experiment out of three is shown, each value having been determined in duplicate.

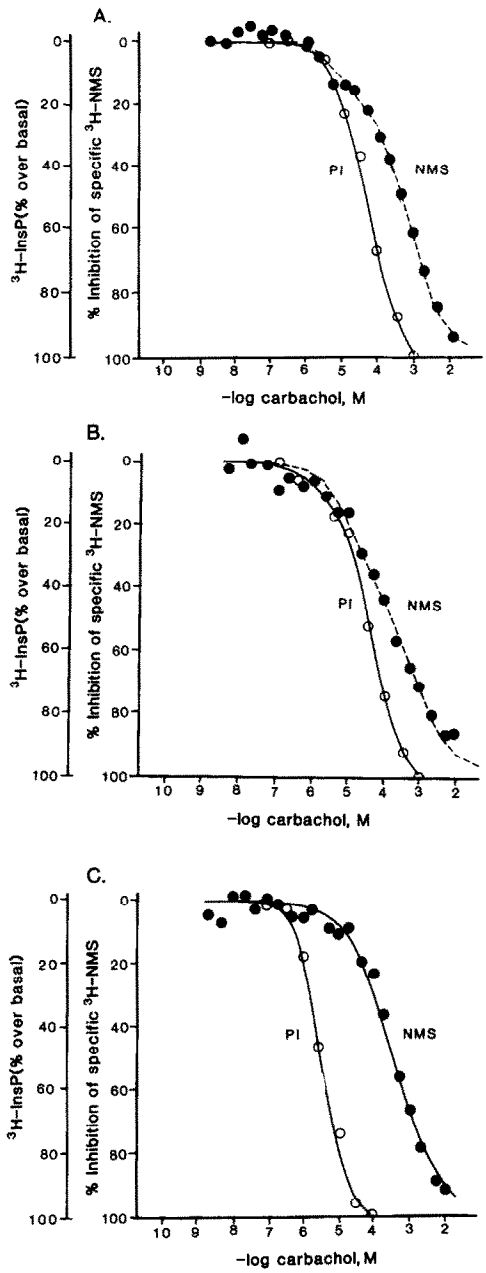

Fig. 3. Stimulation of hydrolysis of phosphoinositides (PI, open symbols) in tissue slices and inhibition by carbachol of ^{3}H-NMS binding (NMS, closed symbols) to membranes, from cerebral cortex (A), ileal smooth muscle (B) and parotid gland (C). The binding experiments were performed in the presence of 100 μM GTP. Dotted lines represent biphasic analysis and solid lines monophasic analysis. The PI values are mean values from five experiments and the binding values are derived from one representative experiment out of three, each value having been determined in duplicate.

when oxotremorine was used as agonists, $pK_i = 6.7$. In the phosphoinositide assay with both agonists almost similar affinity values were demonstrated for atropine in the cerebral cortex and parotid gland (Table 2). Furthermore, a dose ratio method (see Calculations and statistics) were used for the estimation of pirenzepine and atropine affinity values, $pK_i = 6.3$ and $pK_i = 8.8$, respectively (Table 2).

The inhibition of ^{3}H-NMS by pirenzepine in cerebral cortex showed a biphasic interaction and in parotid gland, a one-component interaction was established (Table 2). The affinity of atropine was almost the same in both tissues with slope factors close to unity (Table 2).

Table 2. Affinity values (pK_i) of pirenzepine and atropine against agonist induced hydrolysis of phosphoinositides (PI) and affinity values (pK_d) and percentage proportion of muscarinic receptors on inhibition of binding with ^{3}H-NMS

	PI	^{3}H-NMS			
		High aff.		Low aff.	
	pK_i	pK_d	(%)	pK_d	(%)
Pirenzepine					
CX	7.7 ± 0.1	7.8 ± 0.2	55 ± 5	6.6 ± 0.2	45 ± 5
PG	(a) 6.3 ± 0.2†	—	—	6.5 ± 0.1	100
	(b) 6.7 ± 0.1*				
	(c) 6.3 ± 0.3†				
	PI	^{3}H-NMS			
	pK_i	pK_d		Slope factor	
Atropine					
CX	9.1 ± 0.1	8.8 ± 0.1		0.9 ± 0.1	
PG	(a) 8.8 ± 0.3	8.9 ± 0.1		0.8 ± 0.1	
	(b) 9.1 ± 0.2				
	(c) 8.8 ± 0.4				

(a) Carbachol (100 μM) was used as agonist.
(b) Oxotremorine (100 μM) was used as agonist.
(c) Dose ratio method (see Calculations and statistics).
The values are presented as means ± SE, $N = 3$–5. The binding experiments were performed in the presence of 100 μM GTP. The affinity of pirenzepine in the PI assay in cerebral cortex was significantly different from that obtained in the parotid gland (*$P < 0.05$, †$P < 0.01$).

DISCUSSION

It is now known that several receptors transform membrane signals via a guanine nucleotide binding protein to a phospholipase phosphodiesterase that leads to hydrolysis of phosphoinositides (particularly phosphatidylinositol-4,5-bisphosphate) to generate inositol-1,4,5-trisphosphate and diacylglycerol [24–26]. Although recent studies suggest that the subsequent metabolism is more complicated than first thought [27, 28], it is assumed here that accumulation of label as inositol monophosphates in the presence of lithium faithfully reflects receptor–phospholipase C interaction.

It has been shown in various tissues that carbachol acts as full agonists on phosphoinositide hydrolysis with similar maximal formation of inositol phosphates as acetylcholine [5, 13, 19, 29–31]. However, although it has been demonstrated in some cases that dose–response curves for phosphoinositide hydrolysis are closely associated with the curve for agonist receptor occupancy, the present study reveals that the potency of carbachol to enhance the hydrolysis of phosphoinositides differs markedly in the three tissues examined. Both in the presence and in the absence of lithium, a larger maximal stimulation by carbachol was observed in parotid gland and cerebral cortex than in ileal smooth muscle. Although the maximal capacity of the carbachol response differs among different tissues, it appears that responses to the partial agonist oxotremorine are related to the sensitivity of carbachol to increase the hydrolysis of phosphoinositides. This is clearly shown in Fig. 1 and Table 1, where carbachol is more potent and oxotremorine shows a higher maximal response in parotid gland than in cerebral cortex and ileal smooth muscle. This difference in response suggests that muscarinic receptor stimulation in parotid gland may be more effectively linked to the formation of inositol phosphates and therefore possesses a larger receptor reserve quantified as the difference between the ability to produce inositol phosphates and the ability to occupy muscarinic receptors.

A higher carbachol sensitivity on phosphoinositide metabolism has also been reported in guinea-pig neostriatum compared to cerebral cortex and hippocampus and has been suggested to reflect the presence of a larger receptor reserve for this response in the former brain area [18]. A recent study by these authors [32] has provided more evidence for this hypothesis by inactivating a fraction of the muscarinic receptors in these brain regions, as well as in two neuroblastoma cell lines, with the alkylating agent propylbenzilyl choline mustard. Preliminary work from our own laboratory with benzilylcholine mustard similarly supports a difference in the receptor reserve between guinea-pig cerebral cortex and parotid gland [33 and in preparation].

The apparent difference in coupling of muscarinic receptors to hydrolysis of phosphoinositides may depend on the different stoichiometric relationships between the receptors, guanine nucleotide binding proteins and phospholipase C within the tissue. Another possibility may relate to muscarinic receptors in different tissues being linked to different routes of metabolism of phosphoinositides (phospholipase C and/or phospholipase A_2), with the possible secondary generation of receptor-active agonists being associated with various tissue responses. In relation to the first possibility it is tempting to link the higher sensitivity of carbachol

on hydrolysis of phosphoinositides in parotid gland to the pronounced sensitivity of GTP on agonist binding in the same tissue. However, it is considered unwise to be too speculative concerning the relationship between the affinity of agonists in the membrane binding assays in the presence and absence of GTP and the activity obtained in the functional phosphoinositide system. It should be emphasized that ^{3}H-NMS would also be expected to label muscarinic receptors linked to effector systems other than phosphoinositide metabolism, and there is no reason to assume that similar receptor G-protein interactions occur at each of these effectors. Even if this was the case, it is not certain that a ternary complex model can be applied to each of the systems with the extent of the GTP-shift related to agonist activity.

The present studies also indicate that there is a good agreement between the binding affinities of pirenzepine and inhibition of carbachol or oxotremorine-induced stimulation of inositol phosphate accumulation in the cerebral cortex and the parotid gland. Our results strongly support the suggestion that the two muscarinic receptor subclasses M_1 in cerebral cortex and M_2 in parotid gland defined by the antagonist pirenzepine are both linked to phosphoinositide hydrolysis. As demonstrated in rat cerebral cortex [17] we were unable to show a low slope factor for pirenzepine in guinea-pig cerebral cortex, suggesting that only M_1-muscarinic receptors are associated with phosphoinositide hydrolysis in this tissue. Our data support the contention [32] that, whereas M_1-receptors are linked to phosphoinositide metabolism with little or no receptor reserve, M_2-receptors are coupled in tissues which display spare receptors for this response. This could relate to the cholinergic innervation of the tissues, in which the sensitivity of the cholinergic phosphoinositide response is proportional to the availability of released acetylcholine. Whether these differences in receptor coupling may be related to this fact is under investigation.

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