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STIMULATION OF PHOSPHOINOSITIDE HYDROLYSIS BY MUSCARINIC RECEPTOR ACTIVATION IN THE RAT OLFACTORY BULB

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Abstract—The effect of muscarinic receptor activation on phosphoinositide hydrolysis in the rat olfactory bulb was investigated by determining either the inositol (1,4,5) trisphosphate ($Ins(1,4,5)P_3$) mass or the accumulation of [^{3}H]inositol phosphates ([^{3}H]InsPs). In miniprisms of rat olfactory bulb, carbachol produced an atropine-sensitive increase in $Ins(1,4,5)P_3$ concentration. In a membrane preparation, the formation of $Ins(1,4,5)P_3$ was stimulated by guanosine-5′-(3-*O*-thio) triphosphate (GTPγS), but not by carbachol. However, carbachol potentiated the GTPγS stimulation when the two agents were combined. In miniprisms prelabelled with [^{3}H]*myo*-inositol, carbachol increased the accumulation of [^{3}H]InsPs and this effect was significantly reduced by tissue treatment with either 1 μM phorbol 12-myristate 13-acetate or 1 mM dibutyryl cyclic AMP. Analysis of concentration–response curves indicated that carbachol (EC_{50} = 96 μM) and oxotremorine-M (EC_{50} = 8.2 μM) behaved like full agonists, whereas oxotremorine, BM5, arecoline and bethanechol were partial agonists. The carbachol stimulation of [^{3}H]InsPs accumulation was counteracted with high affinity by the M1 antagonist pirenzepine (pA_2 = 8.26), and less potently by the M3 antagonist *para*-fluorohexahydro-sila-difenidol (pA_2 = 6.7) and the M2 antagonist AF-DX 116 (pA_2 = 6.12). The biochemical and pharmacological properties of the muscarinic stimulation of phosphoinositide hydrolysis were compared with those displayed by the muscarinic stimulation of adenylate cyclase in the rat olfactory bulb.

The rat olfactory bulb receives an extensive cholinergic innervation from the most rostral part of the magnocellular forebrain nuclei and the medial part of the horizontal limb of the diagonal band of Broca [1]. Anatomical and biochemical studies have shown that in the olfactory bulb cholinergic fibers and muscarinic cholinergic receptors are concentrated more densely in the glomerular, external and internal plexiform layers [2, 3]. *In situ* hybridization studies have demonstrated that the olfactory bulb contains the messenger RNA for m1, m2, m3 and m4 receptor genes [4], indicating that all these molecular receptor subtypes are expressed in this brain region. Also quantitative autoradiographic studies using selective ligands have identified the presence of the pharmacologically defined M1 and M2 receptor subtypes [5, 6]. However, the functional activities of the muscarinic receptors of the olfactory bulb are not completely known. Previous studies from our and other laboratories [7, 8] have shown that, as in other brain areas, carbachol stimulates the accumulation of [^{3}H]inositol phosphates ([^{3}H]-InsPs†) in slices of rat olfactory bulb prelabeled with [^{3}H]*myo*-inositol, indicating the presence of muscarinic receptors coupled to phosphoinositide-phospholipase C. In addition, we have observed recently that, differently from the inhibitory effect on cyclic AMP formation elicited in corpus striatum [9, 10], muscarinic receptor stimulation increases basal adenylate cyclase activity in membranes of the rat olfactory bulb [9, 11, 12]. Because of the relevance of the second messengers generated by the hydrolysis of phosphoinositides in the control of a variety of neuronal cell functions [13], in the present study we have characterized the muscarinic stimulation of phosphoinositide hydrolysis in the olfactory bulb. Moreover, since in some cell types muscarinic receptors may increase the formation of cyclic AMP indirectly through the activation of phospholipid hydrolysis [14, 15], the pharmacological and biochemical properties of the muscarinic stimulation of phosphoinositide hydrolysis were compared with those displayed by the muscarinic stimulation of adenylate cyclase in the rat olfactory bulb.

MATERIALS AND METHODS

Materials. *myo*-[2-^{3}H]Inositol (13.8 Ci/mmol) was purchased from Amersham (U.K.). Oxotremorine-M and *para*-fluoro-hexahydro-sila-difenidol (*p*-FHHSiD) were purchased from Research Biochemicals Inc. (Weyland, MA, U.S.A.). Guanosine-5′-(3-*O*-thio)triphosphate (GTPγS) was from Boehringer Mannheim GmbH (Germany). Carbamylcholine (carbachol), oxotremorine, arecoline, bethanechol, phorbol 12-myristate 13-acetate (PMA), dibutyryl cyclic AMP (dBcAMP) and the chemicals used for the enzyme assays were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). BM5 was generously provided by Dr S.

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† Abbreviations: $Ins(1,4,5)P_3$, inositol (1,4,5) trisphosphate; InsPs, inositol phosphates; GTPγS, guanosine-5′-(3-*O*-thio) trisphosphate; *p*-FHHSiD, *para*-fluoro-hexahydro-sila-difenidol; PMA, phorbol 12-myristate 13-acetate; dBcAMP, dibutyryl cyclic AMP; G protein, guanine nucleotide binding regulatory protein.

Zalcman, National Institutes of Mental Health (Rockville, MD, U.S.A.). Pirenzepine and AF-DX 116 were obtained from Dr Karl Thomae, GmbH (Germany).

Accumulation of [^{3}H]*InsPs*. The method used was essentially that described by Berridge *et al.* [16]. Miniprisms (300 × 300 μm) of olfactory bulbs of male Sprague–Dawley rats (250–300 g) were preincubated for 30 min at 37° in a Krebs-bicarbonate medium containing 25 mM $NaHCO_3$, 10 mM glucose, 1.2 mM $MgSO_4$, 1 mM KH_2PO_4, 5 mM KCl, 120 mM NaCl, 1.2 mM $CaCl_2$ and adjusted to pH 7.3 by gassing with 95% O_2 and 5% CO_2. Thereafter, the tissue was suspended in fresh medium containing [^{3}H]*myo*-inositol (5 μCi/mL) and the incubation continued for 120 min with periodical gassing with 95% O_2 and 5% CO_2. The labeling period was stopped by removing the medium and washing the tissue with ice-cold Krebs-bicarbonate buffer containing 10 mM *myo*-inositol. The tissue was resuspended in an oxygenated medium containing 25 mM HEPES–NaOH (pH 7.3), 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM glucose, 110 mM NaCl, 3.8 mM KCl, 10 mM *myo*-inositol and 1.2 mM $CaCl_2$, and distributed into Bio-vials (Beckman, Ireland). LiCl (final concentration 10 mM) was then added and the samples were incubated for 10 min at 37°. Cholinergic agonists were then added as indicated and the incubation continued for 20 min. Time-course experiments indicated that during this period both basal and carbachol-stimulated [^{3}H]InsPs accumulations were linear. The final incubation volume was 300 μL. To examine the effects of muscarinic antagonists, PMA and dBcAMP, the compounds were added immediately after LiCl. Preincubation with the muscarinic receptor antagonists for a period of time longer than 10 min did not affect the potency of the compounds. The incubation was terminated by adding 940 μL of chloroform–methanol (1:2 v/v). The samples were sonicated for 10 sec and further 310 μL aliquots of chloroform and then water were added. Following centrifugation at 1000 *g* for 10 min, the upper aqueous phase was applied to a column of Dowex 1 × 8 in the formate form and [^{3}H]InsPs were eluted with five bed volumes of 1 M ammonium formate–0.1 M formic acid. The radioactivity present in the eluate and in the organic phase was determined by liquid scintillation counting. Results are expressed as percentages of the total radioactivity incorporated that was converted to InsPs. Experiments were carried out to check the effect of carbachol on the accumulation of the different [^{3}H]InsPs. [^{3}H]Inositol monophosphate ([^{3}H]$InsP_1$), bisphosphate ([^{3}H]-$InsP_2$) and trisphosphate ([^{3}H]$InsP_3$) were eluted from the Dowex 1 × 8 column by the stepwise addition of solutions containing increasing concentrations of formate [16]. The recovery of $InsP_1$ was monitored by the addition of [^{14}C]$InsP_1$ standard (Amersham) and averaged 80%. In agreement with other studies in rat brain [7, 17, 18], the long term exposure to carbachol (20 min) resulted in a predominant increase in the accumulation of [^{3}H]-$InsP_1$, whereas the stimulatory effects on [^{3}H]$InsP_2$ and [^{3}H]$InsP_3$ formation were minimal. Assays were performed in triplicate.

Mass measurement of inositol (1,4,5) *trisphosphate* (*Ins*(1,4,5)P_3). The formation of Ins(1,4,5)P_3 in response to muscarinic receptor activation was determined either in intact cell preparation or in homogenates of rat olfactory bulb. In the first case, miniprisms were incubated for 2 min at 37° in Krebs-bicarbonate medium with and without 1 mM carbachol. Preliminary experiments indicated that exposure to carbachol for either 1 or 5 min did not affect the extent of the response to the agonist. In the second case, miniprisms were homogenized in 20 vol. of an ice-cold medium containing 10 mM Tris–HCl (pH 7.0), 1 mM EGTA, 5 mM $MgCl_2$ and 0.1 mM ATP using a teflon–glass tissue grinder (five strokes by hand). The homogenate was diluted 2-fold and centrifuged at 27,000 *g* for 20 min at 4°. The pellet was resuspended in the same volume of medium, homogenized and incubated in an ice bath for 15 min. The tissue was then homogenized and centrifuged as above. The pellet was washed once more and finally resuspended in homogenization medium to a protein concentration of 1.0–1.5 mg/mL. Aliquots of the homogenate (200 μL) were incubated in the presence of the various agents for 60 sec at 37° in a reaction medium (final volume 300 μL) containing 10 mM Tris–HCl buffer (pH 6.8), 5 mM $MgCl_2$, 0.1 mM ATP, 1 mM EGTA and sufficient $CaCl_2$ to give a free Ca^{2+} concentration of 0.1 μM. Sodium orthovanadate (100 μM) was included to inhibit Ins(1,4,5)P_3 degradation [19].

For both tissue preparations, the incubation was stopped by adding 300 μL of ice-cold 1.0 M trichloroacetic acid. The samples were centrifuged at 15,600 *g* for 10 min at 4° and the supernatants mixed with a solution of freon : tri-*n*-octylamine (3:1) to remove trichloroacetic acid. The Ins(1,4,5)P_3 content of the tissue extracts was determined by a radioreceptor assay kit (Du Pont de Nemours, Germany). Assays were performed in triplicate.

The concentration of free Ca^{2+} was calculated with a computer program (EQCAL, Biosoft, U.K.), using the stability constants for metal–chelate and metal–nucleotide complexes reported by Martell and Smith [20]. Protein content was determined by the method of Bradford [21], using bovine serum albumin as a standard.

Statistical analysis. Results are reported as means ± SE. Agonist concentration–response curves were analysed by a least-squares curve fitting computer program (Graph-Pad, ISI Software, Philadelphia, PA, U.S.A.). The antagonist effects were examined according to Schild analysis [22] and the potency of each antagonist was determined from the ratios of the EC_{50} values of the agonist estimated in the absence and in the presence of multiple concentrations of the antagonist. The pA_2 values were determined from the *x* intercepts and calculated by least-squares regression analysis of the Schild plots, where the log of the dose ratios (*DR*)-1 is plotted as a function of the antagonist concentration. Statistical significance of the difference between means was determined by Student's *t*-test.

RESULTS

Activation of muscarinic receptors was effective

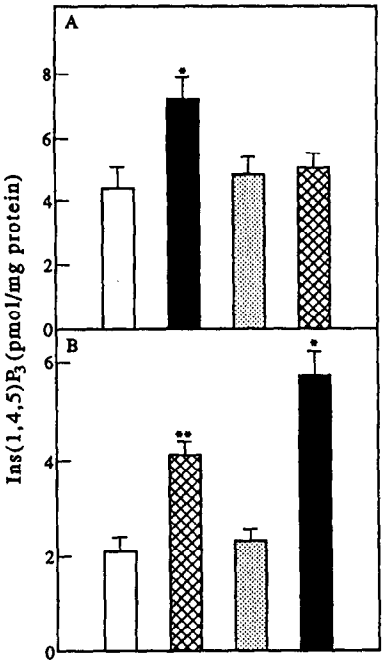

Fig. 1. (A) Stimulation of Ins(1,4,5)P_3 accumulation by carbachol in miniprisms of rat olfactory bulb. Miniprisms were incubated for 2 min with vehicle (□), 1 mM carbachol (■), 10 μM atropine (▨) and carbachol plus atropine (▩). Values are the means ± SE of three experiments. *$P < 0.05$. (B) Stimulation of Ins(1,4,5)P_3 formation by GTPγS and its potentiation by carbachol in membranes of the rat olfactory bulb. Membranes were incubated for 1 min in the presence of vehicle (□), 1 μM GTPγS (▩), 1 mM carbachol (▨) and carbachol plus GTPγS (■). The incubation mixture also contained 100 μM sodium orthovanadate. Values are the means ± SE of five experiments. **$P < 0.01$ vs vehicle; *$P < 0.05$ vs GTPγS alone.

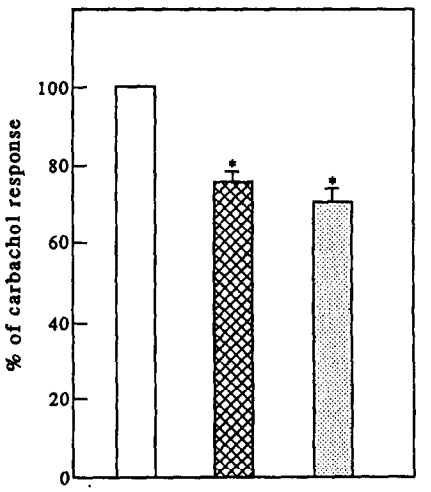

Fig. 2. Inhibition of carbachol-stimulated [^{3}H]InsPs accumulation and dBcAMP in miniprisms of rat olfactory bulb. Miniprisms were prelabeled with [^{3}H]*myo*-inositol for 120 min, washed and then exposed to vehicle (□), 1 μM PMA (▩) and 1 mM dBcAMP (▨). After 10 min, 1 mM carbachol was added and the incubation continued for 20 min. Values are expressed as per cent of the increase in [^{3}H]InsPs accumulation produced by carbachol in the absence of either PMA or dBcAMP and represent the means ± SE of three experiments. Values of [^{3}H]-InsPs accumulation were: basal 14.4 ± 0.6%, carbachol 28.4 ± 0.9%. *$P < 0.05$ versus carbachol alone.

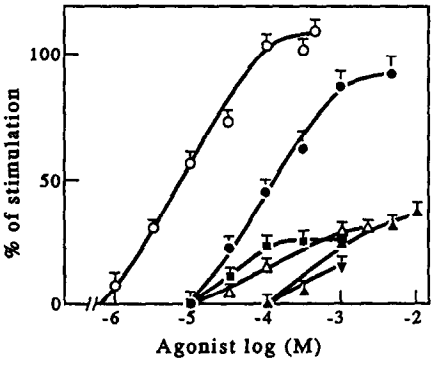

Fig. 3. Concentration-dependent stimulation of [^{3}H]InsPs accumulation by cholinergic receptor agonists in miniprisms of rat olfactory bulb. Miniprisms, prelabeled with [^{3}H]*myo*-inositol and preincubated with 10 mM LiCl, were exposed for 20 min to the indicated concentrations of oxotremorine-M (○), carbachol (●), oxotremorine (△), arecoline (■), bethanechol (▲) and BM5 (▼). Values are expressed as per cent of stimulation of basal [^{3}H]InsPs accumulation and represent the means ± SE of three to five experiments. Basal [^{3}H]InsPs accumulation was 13.9 ± 0.7%.

in increasing Ins(1,4,5)P_3 accumulation in miniprisms of rat olfactory bulb (Fig. 1A). Following 2 min exposure to 1 mM carbachol the tissue concentration of Ins(1,4,5)P_3 increased by 65%. This effect was completely blocked by 1 μM atropine. The activation of phosphoinositide-specific phospholipase C was studied in membrane preparations following 60 sec exposure to 1 mM carbachol. The cholinergic agonist failed to affect Ins(1,4,5)P_3 production in the absence of added guanine nucleotide (Fig. 1B). GTPγS (1.0 μM) increased Ins(1,4,5)P_3 formation by 107% and the concomitant addition of carbachol significantly potentiated the stimulatory effect of GTPγS. The synergism of carbachol with GTPγS was also observed 20 and 120 sec after agonist addition (result not shown). In various brain regions and in different cell types muscarinic stimulation of phosphoinositide hydrolysis has been shown to be inhibited by exposure to phorbol esters, which are potent stimulators of protein kinase C [23], and to agents that increase the intracellular levels of cyclic AMP [23–27]. These regulatory mechanisms were therefore investigated in the rat olfactory bulb. In miniprisms prelabeled with [^{3}H]*myo*-inositol, carbachol (1 mM) increased [^{3}H]InsPs accumulation by about 2-fold. Preincubation of the tissue with either 1 μM PMA or the membrane permeant cyclic AMP analog dBcAMP (1 mM) significantly reduced

Table 1. Properties of cholinergic agonists in stimulating phosphoinositide hydrolysis and adenylate cyclase activity in the rat olfactory bulb

Agonist	[^{3}H]InsPs accumulation EC_{50} (μM)	% efficacy*	Adenylate cyclase EC_{50} (μM)	% efficacy*
Carbachol	96.0 ± 5.3	100	0.923 ± 0.09	100
Oxotremorine-M	8.2 ± 0.7	110	0.085 ± 0.004	100
Oxotremorine	98.1 ± 8.8	33	0.103 ± 0.06	72
Arecoline	49.2 ± 3.9	27	1.41 ± 0.17	90
Bethanechol	490.8 ± 15	40	11.10 ± 1.25	70
BM5	1000	15	0.16 ± 0.02	64

Values of agonist-stimulated [^{3}H]InsPs accumulation were obtained from the experiments reported in Fig. 3, whereas the values for adenylate cyclase stimulation were taken from Ref. 28.

* Based on the response obtained at the maximal concentration tested, as compared with carbachol considered as 100%.

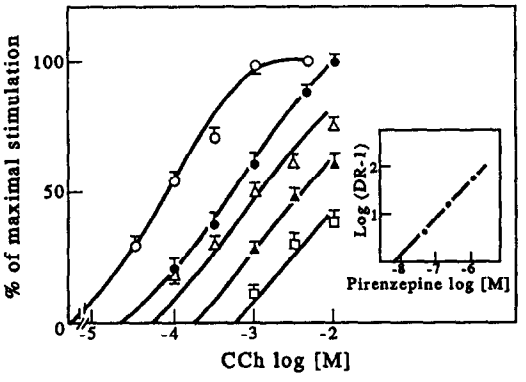

Fig. 4. Antagonism of carbachol stimulation of [^{3}H]InsPs accumulation by pirenzepine in miniprisms of rat olfactory bulb. Miniprisms labeled with [^{3}H]*myo*-inositol were preincubated for 10 min with 10 mM LiCl in the absence (○) and in the presence of 50 nM (●), 200 nM (△), 1.0 μM (▲) and 2.0 μM (□) pirenzepine. Thereafter, carbachol (CCh) was added at the indicated concentrations and the incubation continued for 20 min. Values are expressed as per cent of maximal stimulation of [^{3}H]InsPs accumulation elicited by carbachol in the absence of the antagonist and represent the means ± SE of three experiments. Inset: Schild plot of pirenzepine antagonism.

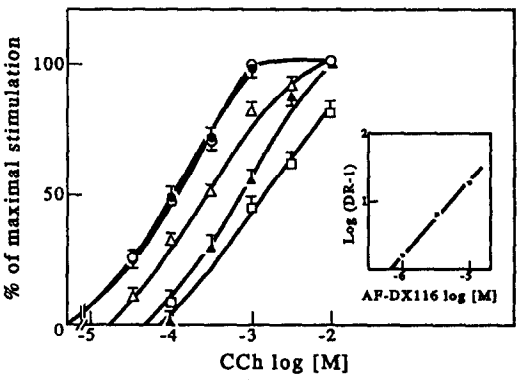

Fig. 5. Antagonism of carbachol stimulation of [^{3}H]InsPs accumulation by AF-DX 116 in miniprisms of rat olfactory bulb. Miniprisms labeled with [^{3}H]*myo*-inositol were preincubated with 10 mM LiCl in the absence (○) and in the presence of 0.3 (●), 1.0 (△), 3.0 (▲) and 10 (□) μM AF-DX 116. Thereafter, carbachol (CCh) was added at the indicated concentrations and the incubation was continued for 20 min. Values are the means ± SE of three experiments. Inset: Schild plot of AF-DX 116 antagonism.

the carbachol stimulation of [^{3}H]InsPs accumulation (Fig. 2). The inhibitory effects of PMA and dBcAMP corresponded to a 25.0 ± 1.2 and 30.1 ± 2.0% decrease in the carbachol stimulation, respectively. Increasing the concentrations of PMA and dBcAMP to 5 μM and 3 mM, respectively, did not produce greater inhibitions of the carbachol response (result not shown). Both PMA and dBcAMP failed to affect basal phosphoinositide hydrolysis.

Various cholinergic agonists were capable of stimulating phosphoinositide breakdown in miniprisms of rat olfactory bulb (Fig. 3). Oxotremorine-M and carbachol enhanced [^{3}H]InsPs accumulation by 110 and 92% of basal value, respectively, whereas arecoline, oxotremorine, bethanechol and BM5 were much less effective. Analysis of the EC_{50} values (Table 1) yielded the following rank order of potency: oxotremorine-M > arecoline > carbachol = oxotremorine > bethanechol > BM5. For comparison, the EC_{50} values and relative efficacies of the cholinergic agonists in stimulating adenylate cyclase activity [28] are reported in Table 1.

Pirenzepine, a muscarinic antagonist with higher affinity for the M1 receptor subtype [29], caused a rightward shift in the concentration–response curve of carbachol (Fig. 4). The pA_2 value of pirenzepine was 8.26 ± 0.03 and the slope of the Schild plot was 0.86 ± 0.05. The M2-selective antagonist AF-DX 116 [30] counteracted the carbachol stimulation of [^{3}H]InsPs accumulation with a pA_2 of 6.12 ± 0.01 and slope value of 1.06 ± 0.04 (Fig. 5). The M3 antagonist *p*-FHHSiD [31], tested at the concentrations of 0.2, 0.5 and 2.0 μM, acted with a pA_2 of 6.70 ± 0.05 and a slope value of 1.10 ± 0.08

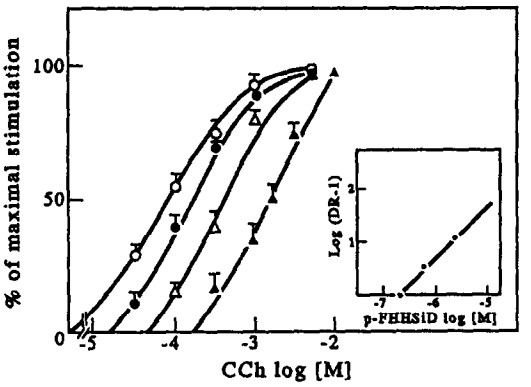

Fig. 6. Antagonism of carbachol stimulation of [^{3}H]InsPs accumulation by *p*-FHHSiD in miniprisms of rat olfactory bulb. Miniprisms labeled with [^{3}H]*myo*-inositol were preincubated with 10 mM LiCl in the absence (○) and in the presence of 0.2 (●), 0.5 (△) and 2.0 (▲) μM *p*-FHHSiD. Thereafter, carbachol (CCh) was added at the indicated concentrations and the incubation was continued for 20 min. Values are the means ± SE of three experiments. Inset: Schild plot of *p*-FHHSiD antagonism.

(Fig. 6). The affinity constants of pirenzepine, AF-DX 116 and *p*-FHHSiD in blocking the muscarinic stimulation of cyclic AMP formation in the rat olfactory bulb were determined previously [32]. The potencies of the antagonists in counteracting the two muscarinic responses are compared in Table 2.

DISCUSSION

The present study describes some biochemical and pharmacological properties of the muscarinic stimulation of phosphoinositide hydrolysis in the rat olfactory bulb. The first issue addressed by our investigation was whether in the olfactory bulb muscarinic receptors can directly promote the breakdown of phosphatidylinositol(4,5)bisphosphate through coupling to phospholipase C. This enzyme catalyses the hydrolysis of phosphatidylinositol(4,5)bisphosphate in diacylglycerol and Ins(1,4,5)P_3. Using a radioreceptor assay for Ins(1,4,5)P_3 mass determination, we showed that in miniprisms of olfactory bulb carbachol produces a rapid increase in the formation of Ins(1,4,5)P_3 and this response is completely blocked by atropine, indicating that the cholinergic agonist acts on muscarinic receptors. The resting levels of Ins(1,4,5)P_3 and the extent of the carbachol stimulation are close to those determined previously in rat cortical slices [33].

As the response observed in tissue miniprisms may be triggered indirectly through the release of endogenous stimulatory neurotransmitters, we investigated whether the muscarinic stimulation of Ins(1,4,5)P_3 formation could also be detected in a cell-free tissue preparation. As observed in other brain areas and different cell types, in membranes of the rat olfactory bulb the production of Ins(1,4,5)P_3 is markedly stimulated by GTPγS, probably through activation of a guanine nucleotide binding regulatory protein (G protein) which stimulates phospholipase C [13]. In the absence of the guanine nucleotide, carbachol fails to increase Ins(1,4,5)P_3 formation, but potentiates the GTPγS stimulation when the two compounds are combined. These data are consistent with the idea that in rat olfactory bulb muscarinic receptors stimulate phosphoinositide hydrolysis through a G protein-mediated activation of phospholipase C.

Previous studies have demonstrated that muscarinic stimulation of phosphoinositide hydrolysis is modulated by activation of different second messenger-regulated biochemical pathways. Thus, cyclic AMP analogs and agents that increase intracellular levels of cyclic AMP reduce the [^{3}H]-InsPs accumulation stimulated by muscarinic receptor activation [26, 27]. A similar result is also obtained in the rat olfactory bulb, where the cyclic AMP analog dBcAMP significantly reduces the carbachol-stimulated phosphoinositide hydrolysis without affecting the basal activity. The extent of the dBcAMP inhibition (30%) is close to the values reported by other investigators [26, 27]. The mechanism by which cyclic AMP can cause a partial inhibition of muscarinic stimulation of phosphoinositide hydrolysis has not yet been elucidated. The G protein–phospholipase C interaction and the phospholipase C molecule have been proposed as the possible sites of action of cyclic AMP-mediated inhibition (for review, see Ref. 13). With regard to the muscarinic stimulation of adenylate cyclase activity, we found that this response is not affected by the addition of dBcAMP to tissue homogenate (Olianas and Onali, unpublished observation).

Table 2. Antagonism of muscarinic stimulation of phosphoinositide hydrolysis and of adenylate cyclase activity in the rat olfactory bulb

Antagonist	[^{3}H]InsPs accumulation		Adenylate cyclase	
	pA_2	Schild slope	pA_2	Schild slope
Pirenzepine	8.26 ± 0.03	0.86 ± 0.05	6.45 ± 0.04	1.04 ± 0.05
AF-DX 116	6.12 ± 0.01	1.06 ± 0.04	6.84 ± 0.05	0.98 ± 0.04
p-FHHSiD	6.70 ± 0.03	1.10 ± 0.06	7.13 ± 0.04	0.92 ± 0.06

Values for antagonism of muscarinic-stimulated [^{3}H]InsPs accumulation were obtained from the experiments reported in Figs 4–6, whereas the values for the antagonism of muscarinic-stimulated adenylate cyclase activity were taken from Ref. 32.

Activators of protein kinase C have also been shown to cause a partial inhibition of muscarinic stimulation of phosphoinositide hydrolysis in different cell systems [23–25]. The ability of PMA to inhibit carbachol-stimulated [^{3}H]InsPs accumulation in the rat olfactory bulb indicates that this regulatory mechanism may also be operative in this brain area. As for the cyclic AMP-induced inhibition, the molecular mechanisms underlying the phorbol ester effect are not yet clear, because protein kinase C activators have been shown to phosphorylate both G proteins and muscarinic receptors [34, 35]. We have reported previously that phorbol esters stimulate brain adenylate cyclase activity in a Ca^{2+}- and GTP-dependent manner [36]. In membranes of the olfactory bulb the PMA stimulation of adenylate cyclase activity was additive with that produced by carbachol [8]. Thus, the different sensitivity to the effect of phorbol esters represents an important feature differentiating the muscarinic stimulation of [^{3}H]InsPs accumulation from the effect on cyclic AMP formation in the rat olfactory bulb.

The results obtained examining the effects of different agonists on phosphoinositide hydrolysis show that oxotremorine-M and carbachol behave like full agonists whereas oxotremorine, BM5, arecoline and bethanechol were partial agonists. This agonist profile is similar to that reported for muscarinic stimulation of phosphoinositide hydrolysis in other brain regions [37–39]. However, when compared with the muscarinic stimulation of adenylate cyclase, notable differences can be observed. In fact, in eliciting the latter response arecoline is a full agonist and oxotremorine and BM5 appear much more potent and effective. The low potency and efficacy of BM5 in stimulating phosphoinositide hydrolysis are consistent with the previous observation that the compound possesses some selectivity for m2 and m4 receptors coupled to adenylate cyclase and no activity at m1 and m3 receptors linked to phosphoinositide hydrolysis [40].

The M1 antagonist pirenzepine counteracts the carbachol stimulation of phosphoinositide hydrolysis with a potency ($pA_2 = 8.26$) about 140- and 36-fold higher than those displayed by the M2 antagonist AF-DX 116 ($pA_2 = 6.12$) and the M3 antagonist *p*-FHHSiD ($pA_2 = 6.70$), respectively. The absolute potencies of the antagonists correlate with their reported affinities for the M1 receptor subtype [41], indicating that in the rat olfactory bulb the muscarinic stimulation of phosphoinositide hydrolysis mostly involves the activation of M1 receptors. A predominant role of M1 receptors in the stimulation of phosphoinositide breakdown has previously been documented in the rat cerebral cortex, although a minor contribution by M3 receptors has also been proposed [42]. Pirenzepine has been shown to block the muscarinic stimulation of adenylate cyclase activity of the rat olfactory bulb with a high and a low affinity component [32]. The low affinity component ($pK_i = 6.31$) constituted the major portion of the response. Schild analysis also yielded a pA_2 value of 6.45 for this antagonist. These and other data on the sensitivity to various antimuscarinic drugs indicate that the predominant receptor involved in adenylate cyclase stimulation does not belong to the M1 subtype, but pharmacologically resembles the m4 gene product [32]. The rank order of potency of the three antagonists in inhibiting the carbachol stimulation of phosphoinositide hydrolysis (pirenzepine > *p*-FHHSiD > AF-DX 116) is markedly different from that shown by these compounds in counteracting the muscarinic stimulation of adenylate cyclase activity (*p*-FHHSiD > AF-DX 116 > pirenzepine).

In conclusion, the characterization of the muscarinic stimulation of phosphoinositide hydrolysis in the rat olfactory bulb provides new information on the functional activity of muscarinic receptors in the olfactory bulb and may help in the understanding of the role of these receptors in olfaction. Moreover, the different biochemical and pharmacological properties indicate that muscarinic stimulation of phosphoinositide hydrolysis and of adenylate cyclase activity constitute two separate signal transduction pathways in the olfactory bulb.

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