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COMMUNICATIONS

Investigation into the effects in-vitro of the 5-hydroxytryptamine reuptake inhibitor, alaproclate, on carbachol-stimulated inositol phospholipid breakdown in the rat cerebral cortex

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The effect of alaproclate in carbachol-stimulated inositol phospholipid (PI) breakdown in rat cerebral cortical miniprisms has been investigated. Carbachol-stimulated PI breakdown was greatly enhanced by increasing the assay potassium concentration from 5·88 to 18·2 mM. Alaproclate, on the other hand, did not influence carbachol-stimulated PI breakdown over the concentration range tested (0–100 μM) at either assay [K^+]. The elution pattern of the inositol phosphates from the Dowex-1 columns was also unaffected by alaproclate both in the absence and presence of carbachol. Thus, the potentiation by alaproclate of tremor and salivation induced by the muscarinic agonist oxotremorine in-vivo reported previously is not seen when muscarinic function is measured in-vitro using carbachol-stimulated PI breakdown.

In a recent study, it was found that in rodents the selective 5-hydroxytryptamine (5-HT) reuptake inhibitor, alaproclate, was able to potentiate tremor and salivation induced by the muscarinic agonists oxotremorine and arecoline and by the anticholinesterase physostigmine (Ögren et al 1985a, b). Although these effects of alaproclate can be blocked by the 5-HT antagonists metitepine and danitracen, they cannot directly be linked to a 5-HT-like action of alaproclate, since metergoline, cinanserin and ketanserin (as well as (−)-alprenolol) were without antagonistic effect (Ögren et al 1985a; Ögren, unpublished). Furthermore, lesion of the 5-HT system with *p*-chloroamphetamine (2 × 10 mg kg^{-1}) did not block the potentiation by alaproclate of oxotremorine-induced tremor (Ögren et al 1987). The effects of alaproclate on oxotremorine-induced tremor are not found with other 5-HT reuptake inhibitors such as fluoxetine, citalopram and norzimeldine (Ögren et al 1985a).

In brain miniprism preparations, muscarinic function can be studied in-vitro by measuring the stimulation of inositol phospholipid hydrolysis ('PI breakdown') produced by cholinergic agonists such as carbachol (Brown et al 1984; Fisher et al 1984; Gonzales & Crews 1984; Jacobson et al 1985). In the present study, carbachol-stimulated PI breakdown has been measured in cerebral cortical miniprisms in order to determine whether the potentiation of muscarinic function produced by alaproclate in-vivo can also be observed in-vitro. Some of the present results were presented at the Xth International Congress of Pharmacology, Sydney, 23–28 August, 1987.

Materials and methods

Male Sprague-Dawley rats (Alab Laboratorietjänst, Sollentuna, Sweden) either 100–120 g (for the experiments shown in Table 1) or 250–300 g (for the experiments shown in Fig. 1) were used. The 100–120 g rats were killed by CO_2 inhalation and the 250–300 g rats by decapitation. On each experimental day, one rat was killed and the cerebral cortex removed and placed in Krebs-Henseleit bicarbonate (KHB) buffer at room temperature (20–23 °C). Miniprisms (0·35 × 0·35 mm) were made by use of a McIlwain tissue chopper and washed three times with KHB buffer at room temperature.

PI breakdown (Fig. 1) was measured by the methods of Berridge et al (1982) and Watson & Downes (1983), with slight modifications. Briefly, miniprism suspensions (2 × 3 mL) were incubated with [^{3}H]myo-inositol (10 μCi mL^{-1} miniprism suspension) for 60 min at 37 °C under an atmosphere of 95% O_2 : 5% CO_2. The samples were then washed three times with KHB buffer (37 °C) and reincubated for 10 min at 37 °C under an atmosphere of 95% O_2 : 5% CO_2. This washing and reincubation procedure was repeated, the samples were washed a further three times with KHB buffer (37 °C) and the washed miniprisms were pooled. Aliquots (25 μL) were added to 15 mL glass centrifuge tubes containing 6 μL alaproclate (dissolved in KHB buffer to give 0–100 μM assay concentrations) and 260 μL of a modified KHB buffer containing 10 mM Li^+ and either 6 or 20 mM K^+ (assay concentrations: Li^+, 8·8 mM; K^+, 5·88 and 18·2 mM, respectively), with the [Na^+] reduced by a corresponding amount. After incubation for 30 min at

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37 °C under an atmosphere of 95% O_2 : 5% CO_2, 10 μL carbachol (dissolved in distilled water to give 0–1000 μM assay concentrations) was added and the samples incubated a further 25 min at 37 °C. Reactions were stopped by the addition of 0·94 mL chloroform –methanol (1 : 2 v/v) and the radiolabelled inositol phospholipid ('Lipid') and combined inositol phosphate ('InsP') fractions were isolated essentially as described by Berridge et al (1982). PI breakdown in Fig. 1 is expressed as InsP/(Lipid + InsP), since this unit is not dependent either upon the number of miniprisms pipetted per aliquot or upon the degree of tritium labelling of the inositol phospholipids (Fowler et al 1987).

Separation of the inositol phosphates (Table 1) was undertaken using a slight modification of the method of Downes & Wusteman (1983). Briefly, miniprism aliquots (40 μL) were incubated for 30 min at 37 °C under an atmosphere of 95% O_2 : 5% CO_2 with 6 μL alaproclate (assay concentration 0, 5 or 100 μM) and 224 μL of a modified KHB buffer containing 21·2 mM [K^+] (assay concentration 18·2 mM), without added Li^+, but with the [Na^+] reduced by the corresponding amount. Ten μL carbachol (assay concentration 0, 50 or 1000 μM) was added and the samples incubated for a further 4 min at 37 °C. Reactions were stopped by the addition of 200 μL 15% trichloroacetic acid, and the samples were centrifuged at 3000 rev min^{-1} for 5 min in a Wifug bench centrifuge. Aliquots (360 μL) from 4 supernatants were pooled, 2 mL diethyl ether was added, and the samples were vortexed. Aliquots (1200 μL) of the aqueous layer were placed on Dowex-1 (X8, formate form) columns followed by 4800 μL distilled water. This elution (fraction 1) was followed by 2 × 6 mL elutions with distilled water (fractions 2 & 3) and thereafter: I, 3 × 6 mL elutions with 5 mM sodium tetraborate/60 mM ammonium formate (fractions 4–6); II, 4 × 6 ml elutions with 100 mM formic acid/200 mM ammonium formate (fractions 7–10); III, 5 × 6 mL elutions with 100 mM formic acid/400 mM ammonium formate (fractions 11–15); and IV, 5 × 6 mL elutions with 100 mM formic acid/1000 mM ammonium formate (fractions 16–20). Results are given as the total d min^{-1} for 160 μL (i.e. 4 × 40 μL) miniprism aliquots for the combined fractions with the different eluents (see Table 1).

Myo-[2-^{3}H]inositol (specific activity 16 Ci $mmol^{-1}$), in a sterile water solution, was obtained from New England Nuclear GmbH, Dreieich, West Germany. D-Myo-[2-^{3}H]inositol-1-phosphate, D-myo-[2-^{3}H]inositol-1,4-bisphosphate and D-myo-[2-^{3}H]inositol-1,4,5-trisphosphate (specific activity in each case 1 Ci $mmol^{-1}$) were obtained from Amersham International plc, Amersham, UK, and used within two weeks of delivery. Alaproclate hydrochloride was synthesized at Astra Alab AB, Södertälje, Sweden. Carbamoylcholine chloride (carbachol) and Dowex-1 (X8, chloride form) were obtained from the Sigma Chemical Co., St. Louis, Mo., USA. All other reagents were standard laboratory reagents of analytical grade whenever possible.

Results and discussion

PI breakdown in cerebral cortical miniprisms was stimulated by carbachol with an EC50 value of roughly 100 μM (Fig. 1), in agreement with the literature, where

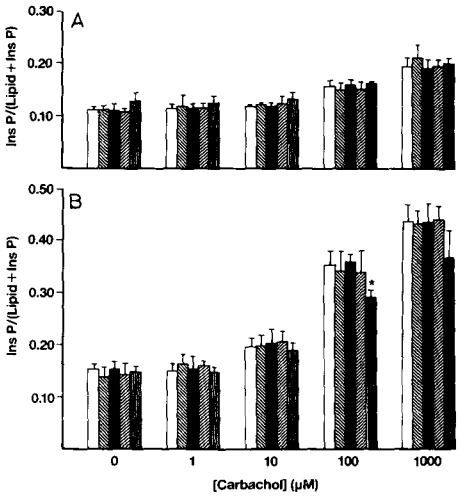

FIG. 1. Effect of alaproclate upon carbachol-stimulated PI breakdown in rat cortical miniprisms. Miniprisms were preincubated at 37 °C with either 0 (□), 0·1 μM (▨), 1 μM (■), 10 μM (▩) or 100 μM (▦) alaproclate followed by a further 25 min in the presence of carbachol, at K^+ concentrations of (A) 5·88 and (B) 18·2 mM. Data are means ± s.d., n = 3–4. At both assay [K^+], the InsP/(Lipid + InsP) values in the presence of 100 and 1000 μM carbachol (and also for 10 μM carbachol at [K^+] = 18·2 mM) were in all cases significantly higher than the corresponding basal (i.e. [carbachol] = 0) values ($P < 0·05$; two-tailed paired t-test). *$P < 0·05$ with respect to the corresponding value in the absence of alaproclate for the same carbachol concentration (two-tailed paired t-test).

it has further been shown that the cortical stimulation is antagonized by atropine with a pA_2 value of about 8·8 (see e.g. Brown et al 1984; Jacobson et al 1985). Increasing the assay [K^+] from 5·88 to 18·2 mM had only modest effects on the basal rate of PI breakdown, but greatly enhanced the stimulation by carbachol (Fig. 1), a finding in agreement with the literature (Eva & Costa 1986; Court et al 1986; Baird & Nahorski 1986; Gurwitz & Sokolovsky 1987). Neither basal nor carbachol-stimulated PI breakdown, at either assay [K^+], was affected by alaproclate over the concentration range 0·1–10 μM (Fig. 1). The lack of effect of alaproclate on basal PI breakdown is consistent with previous biochemical studies demonstrating that this compound does not act as a cholinergic agonist or potentiate the release of endogenous acetylcholine (Ögren et al 1985b). The highest concentration of alaproclate tested (100 μM) produced a small decrease in the stimulation of PI turnover by 100 (and possibly also 1000) μM carbachol at

an assay [K^+] of 18·2 mM (Fig. 1), suggestive of a weak antagonist effect at this concentration. This is consistent with the finding that alaproclate can inhibit the binding of 0·5 nM [^{3}H]quinuclidinyl benzilate to muscarinic receptors in cortical membrane preparations with an IC50 value of 80 μM (Hall & Ögren 1981). However, this concentration is very much higher than the IC50 value for inhibition of serotonin uptake (0·37 μM) (Hall & Ögren 1981).

Of the different inositol phosphates, inositol-1,4,5-trisphosphate appears to have second messenger functions (for review, see Berridge 1986). This raises the possibility that alaproclate can potentiate muscarinic function by slowing the rate of breakdown of inositol-trisphosphate to inositol-bisphosphate. Such an effect would not change the total InsP fraction, consistent with the data in Fig. 1, but would change the relative amounts of the different inositol phosphates. In order to investigate this possibility, the method of Downes & Wusteman (1983), with slight modifications (the number, and in some cases the volume, of the elutions was increased), was used to separate the inositol phosphates. The method used produced a good separation between the inositol and the inositol phosphates, and a reasonable separation between inositol-1,4,5-trisphosphate and the bis- and mono-phosphates (Table 1). There was, on the other hand, a considerable overlap of the elution profiles for inositol-1,4-bisphosphate and inositol-1-phosphate (Table 1). In addition, inositol-1,3,4-trisphosphate and inositol-1,3,4,5-tetrakisphosphate (the latter in particular following stimulation by carbachol plus raised [K^+], Baird & Nahorsky 1986), which are produced from inositol-1,4,5-trisphosphate (Irvine et al 1986) are likely to be coeluted with the inositol-1,4,5-trisphosphate under the conditions used in the present study (see Batty et al 1985). Because of these constraints, the d min^{-1} given for the different elution fractions in Table 1 cannot exclusively be ascribed to inositol 1-mono-, 1,4-bis- and 1,4,5-trisphosphates. Nevertheless, a decreased rate of breakdown of the trisphosphate to the bis- and monophosphate forms would be observable under the conditions used as an increased d min^{-1} in fractions 16–20 together with a corresponding decrease in fractions 7–15.

Incubation of the miniprisms (at an assay [K^+] of 18·2 mM) with carbachol for 4 min at 37 °C produced significant increases in the d min^{-1} recovered in fractions 4–6, 7–10, 11–15 and 16–20 (Table 1) indicating that during this period there has been considerable, but *not* complete, metabolism of the extra inositol tris-(and tetrakis-) phosphate produced by activation of the muscarinic receptors coupled to the PI breakdown. Alaproclate (5 and 100 μM) did not affect the d min^{-1} found in fractions 4–6, 7–10, 11–15 and 16–20 for the

Table 1. The effect of alaproclate and carbachol on PI breakdown in rat cortical miniprisms: elution of the individual inositol phosphates from Dowex columns. For the calibration data (top half of Table), columns were loaded with either [^{3}H]myo-inositol (4·4 μCi), [^{3}H]myo-inositol-1-phosphate, [^{3}H]myo-inositol-1,4-bisphosphate or [^{3}H]myo-inositol-1,4,5-trisphosphate (all 0·22 μCi) and the d min^{-1} in the fractions expressed as a percentage of the total d min^{-1} eluted. Values are means (n = 4). For the alaproclate/carbachol data (bottom half of Table), 40 μL cortical miniprism aliquots were incubated for 30 min at 37 °C with alaproclate, then for a further 4 min in the presence of carbachol. The assay [K^+] was 18·2 mM. Reactions were stopped with trichloroacetic acid, the samples centrifuged and supernatants from four samples pooled. The ether extraction and Dowex fractionation phases were then undertaken as described in Materials and methods. Data are given as means ± s.d. (n = 4–5) of the total d min^{-1} in each fraction per 160 μL (i.e. 4 × 40 μL) miniprism aliquot. Significance of differences between groups was determined by two-tailed paired *t*-test: $^*P < 0.05$, $^{**}P < 0.01$ with respect to the corresponding sample with the same alaproclate concentration in the absence of carbachol; in no case was $P < 0.05$ for comparisons between samples in the absence and in the presence of alaproclate for a given carbachol concentration.

Eluent:		H_2O	I	II	III	IV
Fraction:		1–3	4–6	7–10	11–15	16–20
		% of total eluted d min^{-1}				
[^{3}H]Inositol		99·6	0·22	0·15	0·02	0·004
[^{3}H]Inositol-1-phosphate		6·9	34·1	58·9	0·09	0·019
[^{3}H]Inositol-1,4-bisphosphate		2·9	4·7	59·5	29·5	3·4
[^{3}H]Inositol-1,4,5-trisphosphate		1·3	3·6	4·4	16·2	74·6
Alaproclate (μM)	Carbachol (μM)	d min^{-1} eluted in each fraction/160 μL miniprism aliquot				
0	0	362 700 ± 61 700	1790 ± 370	1710 ± 310	390 ± 100	330 ± 30
5	0	353 300 ± 71 600	1880 ± 520	1870 ± 600	500 ± 130	340 ± 140
100	0	355 900 ± 77 500	2260 ± 620	1950 ± 590	530 ± 140	340 ± 30
0	50	374 700 ± 92 300	2420 ± 520*	2980 ± 1060*	770 ± 240	640 ± 340
5	50	385 400 ± 102 500	2450 ± 800	3110 ± 1230*	700 ± 260	550 ± 180**
100	50	349 300 ± 100 700	2640 ± 960	3180 ± 1230*	790 ± 260*	550 ± 200
0	1000	397 200 ± 100 000	3660 ± 1010**	6460 ± 2060**	1800 ± 420**	1400 ± 380*
5	1000	403 000 ± 62 200*	3550 ± 1310*	6310 ± 1600**	1740 ± 310**	1400 ± 250**
100	1000	351 100 ± 69 400	3590 ± 1190**	6840 ± 2690**	2050 ± 570**	1300 ± 280**

carbachol-stimulated miniprisms (Table 1), consistent with the suggestion that under the conditions used alaproclate did not alter the rate of breakdown of inositol tris- (and tetrakis-) phosphate to inositol bis- and monophosphates.

It can thus be concluded that under the conditions and in the concentration range used, alaproclate does not influence carbachol-stimulated PI breakdown in rat cortical miniprisms, in contrast to its effects on oxotremorine-induced tremor and salivation in-vivo (Ögren et al 1985a) and upon the duration of the cellular responses to carbachol in NIE-115 neuroblastoma cells in-vitro (Hedlund 1987). The properties of the muscarinic receptors coupled to PI breakdown appear to vary both between the brain and the periphery and between different brain regions. Thus, for example, whilst oxotremorine is 40–100% as effective (in terms of maximal response) as carbachol as a stimulator of PI breakdown in the rat parotid gland (Gil & Wolfe 1985; Jacobson et al 1985), it is a poor stimulator of brain PI breakdown (Fisher et al 1984; Gonzales & Crews 1984; Jacobson et al 1985). Gurwitz & Sokolovsky (1987) have reported that in cerebral cortical slices, muscarinic agonists increase PI turnover by both tetrodotoxin (TTX)-sensitive and TTX-resistant mechanisms, and that whilst carbachol exerts its effects via both pathways, oxotremorine stimulates PI breakdown by the TTX-sensitive pathway alone. A regional variation in the potency of the M1-selective antagonist pirenzepine as antagonist of carbachol-stimulated PI breakdown has also been found, with the IC50 value being lower for rat cortex than for the pons-medulla (Rooney & Nahorski 1986; see also Brown et al 1985; Gil & Wolfe 1985). The finding of a Hill slope less than unity (0·69) for the antagonism by pirenzepine of carbachol-stimulated PI breakdown in the rat cortex (Rooney & Nahorski 1986) would, however, suggest that more than one muscarine receptor subtype is linked to PI turnover in this brain region. Nevertheless, the present findings raise the possibility that the differences between in-vitro and in-vivo actions of alaproclate on muscarinic function are due to qualitative (and regional) differences in the muscarinic receptors involved in the salivation and tremor responses in-vivo and those coupled to the PI breakdown response in cortical miniprisms. An alternative explanation is simply that alaproclate exerts its effects on muscarinic function at the post-receptor level, but with a cellular mechanism unrelated to PI breakdown.

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