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Biochemical Pharmacology, Vol. 39, No. 12, pp. 2069–2073, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Muscarinic receptor antagonist activity of diacylglycerol lipase and kinase inhibitors

(*Received* 1 *May* 1989; *accepted* 11 *December* 1989)

Diacylglycerol (DG) is a central intermediate in lipid metabolism and is one of the two products resulting from agonist-activated phosphoinositide breakdown [1–3]. One approach used to identify the role of the diacylglycerol–protein kinase C (DG–PKC) pathway in stimulus–response coupling involves the exogenous addition of synthetic diacylglycerols or the tumor-promoting phorbol esters [4, 5]. An alternative approach has been to modify intracellular DG metabolism by the use of inhibitors. Recently, several reports [6–11] have investigated the effect of RHC 80267 [1,6-di(*O*-carbamoyl)cyclohexanone oxime)hexane], a DG lipase inhibitor, and R 59 022 (6-[2-[4-[(4-fluorophenyl) phenylmethylene]-1-piperidinyl]ethyl-7-methyl-5H-thiazolo[3,2-alpha]pyrimidin-5-one) a DG kinase inhibitor, on stimulus–response coupling. The enzyme DG lipase hydrolyzes the fatty acid ester bond at the second carbon of DG, resulting in the formation of monoacylglycerol and free fatty acid. DG kinase converts DG to phosphatidic acid by phosphorylation of the hydroxyl at the third carbon.

We reported earlier [11] that RHC 80267, R 59 022 and phorbol myristate acetate (PMA) all inhibit muscarinic-stimulated responses in guinea pig pancreatic minilobules. This, along with reports from other groups [12, 13], led to the suggestion that the activation of PKC resulting from the elevation of DG produced by these agents was responsible for the inhibition of the muscarinic receptor, presumably by a feedback mechanism. An alternative explanation involves direct inhibition by those drugs of muscarinic responses at the receptor level. Therefore, we examined the effects of RHC 80267 and R 59 022 on caerulein-stimulated cGMP formation and [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) binding to muscarinic receptors.

Materials and Methods

Guinea pig minilobule preparation. Preparation of guinea pig or rat pancreatic minilobules and conditions for cGMP studies were similar to those described earlier [11].

Membrane preparation. Rat heart ventricles and cerebral cortex membranes were homogenized in 10 vol. of 100 mM NaCl, 20 mM HEPES [4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid], 10 mM $MgCl_2$ (Buffer A) using 3 × 6 sec bursts of a Polytron homogenizer (Brinkmann Instrument, Inc., Westbury, NY) for heart and five strokes of a motor-driven Potter–Elvehjem homogenizer for cerebral cortex. Rat and guinea pig pancreatic minilobules were homogenized as described for cerebral cortex. Tissue homogenates were filtered through two layers of muslin, and centrifuged at 40,000 *g* for 20 min at 4°. Pellets were washed an additional two times, resuspended in buffer, and stored at −70° until used.

[^{3}H]*NMS Binding.* Membranes were incubated with [^{3}H]NMS buffer A (1 mL total volume) for 2 hr at 37°. Bound and free [^{3}H]NMS were separated by Millipore filtration or a Scraton cell harvester (Scraton Inc., Sterling, VA) as described previously [14]. Specific binding was defined by 1 μM atropine. Protein content, measured with bicinchoninic acid protein assay reagent (Pierce, Rockford, IL), was shown such that bound [^{3}H]NMS was less than 10% of total radioligand. Inhibition constants (K_i) were calculated from the Cheng and Prusoff equation [15]. RHC 80267 and R 59 022 were dissolved and diluted in dimethyl sulfoxide (DMSO). The final DMSO concentration in the incubation medium was 0.25%, and this had no effect on [^{3}H]NMS binding to membranes.

Chemicals. [^{3}H]NMS, 85–90 Ci/mmol, was purchased from New England Nuclear (Boston, MA). Atropine sulfate, carbamylcholine chloride, and HEPES were purchased from the Sigma Chemical Co. (St. Louis, MO). R 59 022 was bought from Janssen Life Science Products, and RHC 80267 was a gift of Mabel Hokin-Neaverson.

Results

Figure 1A shows the effects of RHC 80267 and R 59 022 on carbachol-stimulated cGMP levels in guinea pig minilobules. In the absence of inhibitor, carbachol stimulated cGMP dose dependently, with an EC_{50} of 3 μM, N = 3 (Fig. 1A). In the presence of RHC 80267 (75 μM) and

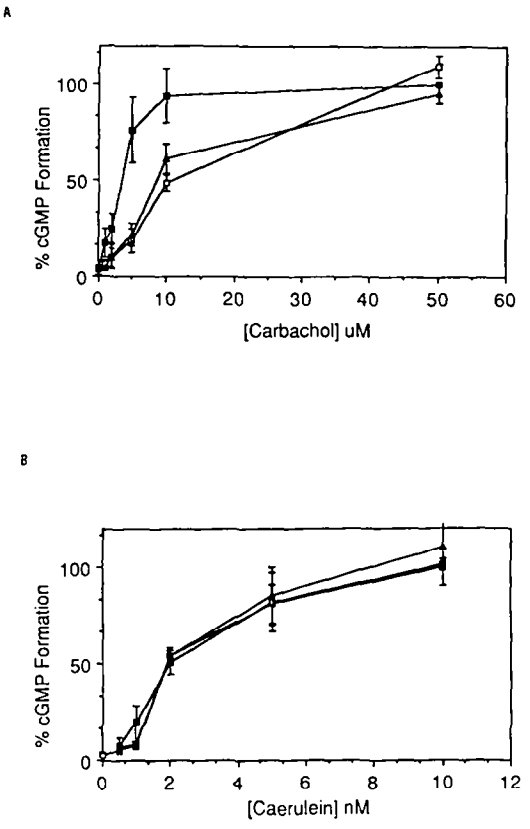

Fig. 1. Effects of RHC 80267 and R 59 022 on carbachol-stimulated cGMP concentration–response curves. Guinea pig pancreatic minilobules were preincubated with 75 μM RHC 80267 (▲), 10 μM R 59 022 (□) or dimethyl sulfoxide vehicle (■) for 15 min. This was followed by a 2-min incubation with different concentrations of carbachol (A) and caerulein (B). Results of representative experiments are expressed as means ± SD of four observations. Similar results were obtained in two other experiments.

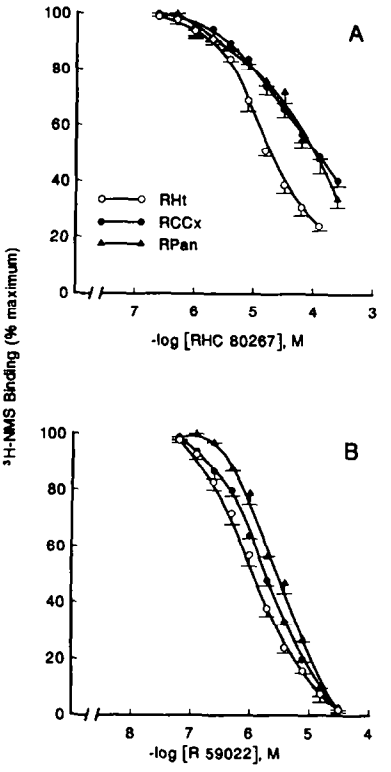

Fig. 2. Inhibition of [^{3}H]NMS binding to rat heart, cerebral cortex and pancreas muscarinic receptors by RHC 80267 and R 59 022. Specific [^{3}H]NMS binding to rat heart (○), cerebral cortex (●) and pancreas (▲) membranes was determined in the presence of increasing concentrations of RHC 80267 (A) or R 59 022 (B) using 500–600 pM [^{3}H]NMS. Results show the mean curves (± SE) of four to six determinations.

R 59 022 (10 μM), the carbachol concentration–response curves were shifted to the right with EC_{50} values of 8.3 and 11.1 μM respectively. Inhibition of carbachol-stimulated responses by these agents could be overcome by higher concentrations of carbachol, and maximum responses to this agonist were not significantly different in the absence or presence of inhibitors ($P > 0.05$).

Figure 1B shows the effects of RHC 80267 and R 59 022 caerulein-stimulated cGMP production in guinea pig minilobules. By contrast to their inhibition of carbachol-stimulated responses, RHC 80267 and R 59 022 showed no significant effect on caerulein-stimulated increases in cGMP. Thus, EC_{50} values (2 nM) were similar in the absence or presence of inhibitors.

These apparent discrepancies between inhibitors of DG metabolism on carbachol- but not caerulein-stimulated cGMP formation could be accounted for by differences in the time course of PKC translocation [16]. Alternatively, an explanation for the shift to the right of the carbachol- but not caerulein-stimulated cGMP concentration–response curve would involve competition by RHC 80267 and R 59 022 for muscarinic but not caerulein receptors. To test for this possibility, we examined the ability of these drugs to inhibit [^{3}H]NMS binding to muscarinic receptors.

Figure 2 and Table 1 show that both RHC 80267 and R 59 022 were able to inhibit [^{3}H]NMS binding to membrane preparation of guinea pig pancreas, rat heart, cerebral cortex and pancreas. These rat tissues were chosen because they contain the three pharmacologically defined muscarinic receptor subtypes. Heart contains a predominance of cardiac-M2 muscarinic receptors [17]; cerebral cortex contains M1, M2 and glandular type receptors in roughly equal proportions [17]; rat pancreas contains predominantly glandular M2 receptors which are pharmacologically distinct from cardiac M2 receptors [18]. We were unable to obtain full competition curves for RHC 80267 because of drug solubility limitations (Fig. 2A). This was not a problem for the more potent drug, R 59 022 (Fig. 2B). The significant cardioselectivity of RHC 80267 seen in Fig. 2A was partly a reflection of assays conducted at similar radioligand concentration, since the K_D of [^{3}H]NMS for heart tissue was higher than values for brain and pancreas (see legend to Table 1). Calculated inhibition constants for RHC 80267 and R 59 022 are shown in Table 1. RHC 80267 had similar K_i values (32–35 μM) for muscarinic receptors of rat cerebral cortex, rat and guinea pig pancreas, and a slightly lower K_i value (2.5-fold) for rat heart. From an estimated K_i value of 33 μM for RHC 80267, we calculated a concentration-ratio of 3.2 at 75 μM RHC 80267. This value agreed with an experimentally determined concentration-ratio of 2.8 for inhibition of the carbachol-stimulated cGMP increase (Fig. 1A). These results suggest that the observed inhibition of carbachol-stimulated cGMP formation by RHC 80267 in guinea pig pancreas was accounted for by competition at the muscarinic receptors.

Table 1. Inhibition constants (K_i) of RHC 80267 and R 59 022 for muscarinic receptors

Tissue	K_i (μM) RHC 80267	N	R 59 022	N
Rat heart	14 ±2	6	0.73 ± 0.15	5
Rat cerebral cortex	35 ±5	6	0.58 ± 0.12	5
Rat pancreas	32 ±7	7	0.94 ± 0.10	3
Guinea pig pancreas	33 ±5	3	ND	

IC_{50} values were calculated from Hill plots of competition data, and inhibition constant (K_i) values were calculated from the Cheng and Prusoff equation [15]. K_D values for [^{3}H]NMS were: rat heart 793 ± 95 pM, N = 4; rat cerebral cortex, 252 ± 42 pM, N = 5; rat pancreas 276 ± 37 pM, N = 3; guinea pig pancreas, 293 ± 50 pM, N = 3. The corresponding binding site maxima were: 138 ± 10, 1215 ± 172, 34 ± 5 and 68 ± 10 fmol/mg protein, respectively (N = 3–5). ND = not determined. Values are means ± SD.

R 59 022 was a more potent muscarinic receptor antagonist (Fig. 2B) than RHC 80267 with K_i values of 0.58 to 0.94 μM for rat muscarinic receptors. Hill slope factors of competition curves for rat heart, cerebral cortex and pancreas were 0.96, 1.02 and 1.05, respectively, which were not significantly different from unity ($P > 0.05$). No evidence of receptor selectivity was apparent for R 59 022.

To examine the characteristics of muscarinic receptor antagonism by RHC 80267 and R 59 022, we conducted saturation binding isotherms in the absence and presence of these drugs. Figure 3A shows binding of [^{3}H]NMS to rat cerebral cortex membranes in the absence and presence of 37.5 and 75 μM RHC 80267. Scatchard analyses (inset) of control binding revealed a single site with an equilibrium dissociation constant, K_D = 267 pM. In the presence of 37.5 and 75 μM RHC 80267, there was an increase in K_D for [^{3}H]NMS to 652 and 901 pM (N = 2), respectively, but no significant change in B_{max} values ($P > 0.05$). Similar data were obtained with guinea pig pancreatic membranes (data not shown).

Figure 3B shows [^{3}H]NMS binding to rat cerebral cortex membranes in the absence and presence of 1 and 5 μM R 59 022. Scatchard analyses indicated that the K_D value of [^{3}H]NMS for muscarinic receptors was increased in the presence of increasing concentrations of R 59 022, whereas B_{max} values were not altered significantly (Fig. 3 inset). Thus, the K_D of [^{3}H]NMS increased from a control value of 274 pM to 506 pM and 990 pM in the presence of 1 and 5 μM R 59 022 respectively (N = 2). These data indicate that RHC 80267 and R 59 022 interact competitively with [^{3}H]NMS for the muscarinic receptors on rat cerebral cortex membranes.

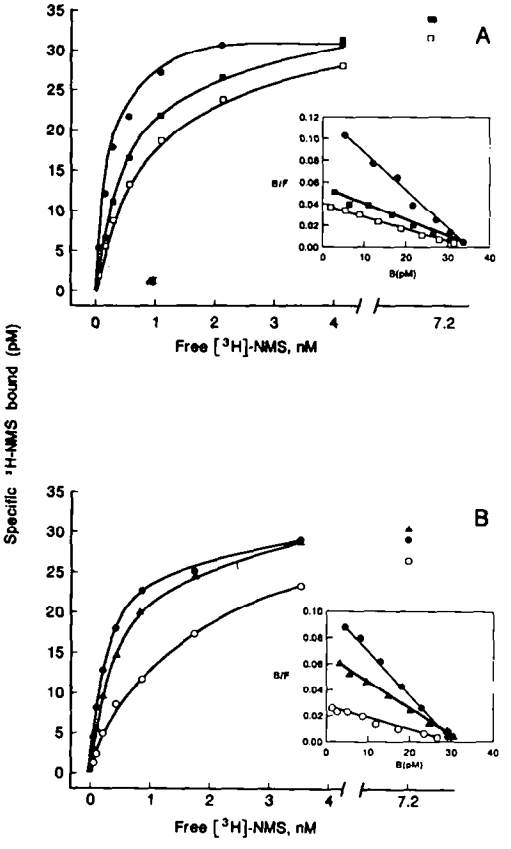

Fig. 3. Inhibition of [^{3}H]NMS binding to rat cerebral cortex membranes by RHC 80267 (A) and R 59 022 (B). Saturation binding isotherms of [^{3}H]NMS binding were performed in the absence (●) and presence of 37.5 μM (■) and 75 μM (□) RHC 80267 (A) or 1 μM and (▲) and 5 μM (○) R 59 022 (B). Scatchard analyses of the specific binding data (insets) gave the following parameters: (A) control, B_{max} = 33.9 pM, K_D = 276 pM; 37.5 μM RHC 80267, B_{max} = 35.9 pM, K_D = 673 pM; 75 μM RHC 80267, B_{max} = 34.1 pM, K_D = 862 pM. (B) control, B_{max} = 30.4 pM, K_D = 287 pM; 1 μM R 59 022, B_{max} = 31.5 pM, K_D = 499 pM; 5 μM R 59 022, B_{max} = 29.4 pM, K_D = 1100 pM. Results are representative of experiments performed twice.

Discussion

Receptor-activated amylase release in pancreas is mediated by two different mechanisms. One class of agonists mediates its response through elevation of cAMP, and the other class, which includes muscarinic, cholecystokinin and bombesin, mediates their responses through inositol lipid breakdown and calcium mobilization [19, 20]. Inositol trisphosphate and DG are the two primary products generated during this process. There is now a general agreement that I(1, 4, 5)P3 mobilizes calcium from intracellular pools [20], but the functional significance of increased DG and the involvement of the DG–PKC pathway in stimulus–secretion coupling is not understood completely. Indeed, DG may be involved in feedback inhibition of the secretory response rather than stimulating amylase secretion [12].

Others have implicated DG–PKC in receptor desensitization [13].

In pancreas, Dixon and Hokin [2] first demonstrated that RHC 80267 even at a concentration of 75 μM selectively inhibits diacylglycerol lipase in caerulein-stimulated tissue, resulting in elevation of diacylglycerol and inhibition of arachidonic acid. RHC 80267 in that study was clearly shown not to inhibit any other step of caerulein-stimulated phosphoinositide cascade.

In this study, where we have compared the effects of RHC 80267 and R 59 022 on carbachol- and caerulein-stimulated cGMP formation, it is clear that the above inhibitors, even at maximal concentrations, only modulated carbachol response, but had no effect on caerulein response. As both carbachol and caerulein have been shown to employ identical signal transduction mechanisms in pancreatic secretion, the selective effect of these inhibitors on carbachol response raised the possibility that the agents may be affecting the carbachol response at the receptor level.

In earlier work [10], Chaffoy de Courcelles *et al.* reported a dopamine D_2, adrenergic α_1 and histamine H_1 antagonistic effect of R 59 022, and have suggested (personal communication, cited with permission) that this compound may also exert some muscarinic antagonistic activity. The present studies provide evidence that both RHC 80267 and R 59 022 inhibited [^{3}H]NMS binding to muscarinic receptors in several tissues and that the inhibition was purely competitive. These findings were somewhat unexpected since RHC 80267 and R 59 022 show little structural similarity to each other, or to classical muscarinic receptor antagonists. RHC 80267 does possess two nitrogen atoms separated by a six-carbon chain which is similar to polymethylene tetramines, a new class of muscarinic antagonists reported recently [21].

We conclude that even though phorbol ester pretreatment produces similar reductions in response to muscarinic stimulation as the two inhibitors of DG metabolism, RHC 80267 and R 59 022, these effects are mediated through different mechanisms. While phorbol ester most likely mediates its effect through protein kinase C activation, the inhibitory effects of RHC 80267 and R 59 022 are the result of inhibition of agonist binding to muscarinic receptors. Recently, Capito *et al.* [22] studied the effect of RHC 80267 on carbachol and glucose-stimulated insulin secretion in mouse islet. They only observed an inhibitory effect of RHC 80267 on glucose but not carbachol-stimulated insulin secretion. Careful review of their results indicates that they used a single supramaximal carbachol concentration of 100 μM which, from our studies, was able to overcome the inhibition by 75 μM RHC 80267. Caution should be exercised in the use of RHC 80267 and R 59 022 in studies involving muscarinic receptor responses because their activities as DG kinase and lipase inhibitors are similar to their affinities for cholinergic muscarinic receptors.

Acknowledgements—This work was supported by NIH Grant HL16318 to Lowell E. Hokin. The authors would like to thank Mabel Hokin-Neaverson for the gift of RHC 80267.

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Biochemical Pharmacology, Vol. 39, No. 12, pp. 2073–2076, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Delayed treatment with Nerve Growth Factor (NGF) reverses ECMA-induced cholinergic lesions in rat brain reaggregate cultures

(*Received* 5 *October* 1989; *accepted* 12 *February* 1990)

Low concentrations of the alkylating cholinotoxin, ethylcholine mustard aziridinium (ECMA) produce selective cholinergic lesions in sub-populations of cholinergic neurones in developing rat whole-brain reaggregate cultures *in vitro* [1, 2]. Nerve Growth Factor (NGF) has significant and relatively specific positive neurotrophic effects on cholinergic neurones in the developing, lesioned adult and ageing brain [2–5]. These properties appear to be mediated through activation of phosphoinositide turnover and protein kinase C [6]. We have previously reported that although NGF significantly elevates choline acetyltransferase (ChAT) activity in brain reaggregate cultures after several days exposure (in a thyroid hormone dependent manner) it does not appear to reverse ECMA-induced lesions if added simultaneously with the neurotoxin [2]. It was postulated that this may be due to alkylation of NGF receptor-associated proteins on cholinergic neurones, precluding retrograde NGF transport to, and action on nuclei in the cholinergic perikarya [7]. We have now investigated whether high concentrations of NGF are effective if addition is delayed until several days following ECMA lesioning, when both the ECMA alkylating capacity has diminished and *de novo* receptor protein synthesis has occurred. The effects of GM1 ganglioside have also been examined since it appears to promote the functional recovery of injured neurones and the possible interaction of NGF with its receptor [8].

Methods

Foetal rat whole brain reaggregate cultures were prepared as described previously [2] in a serum-supplemented (S+) DMEM-based culture medium. The freshly prepared cholinotoxin, ECMA, was added at 8 days *in vitro* (8 DIV). B-NGF (7S sub-unit, Sigma Chemical Co., Poole, U.K.) was added by two different protocols by either, Protocol A: three times during the culture period at a final concentration of 50 ng/mL at 8, 10 and 12 DIV (with or without GM1 ganglioside; final concentration 100 μg/mL); or Protocol B: at 10, 12 and 14 DIV (delayed NGF addition experiments). Both treatment protocols involved three NGF additions at 48 hr intervals over a similar period assuming an ECMA degradation time of approximately 6–8 hr [9]. Partial-culture medium changes were performed on alternate days from 8 DIV onwards. Cultures were harvested and washed at 15–16 DIV and assayed for protein, ChAT activity and muscarinic receptor binding (mAChR: defined as the specific binding of [^{3}H]quinuclidinylbenzilate-QNB in the presence of 100 μM atropine) as previously described [2].

Results

NGF at low concentrations of 5 ng/mL produces only fairly modest 45–50% increases in ChAT activity in control reaggregates, an effect which appears to be dependent on the presence of thyroid hormone ($L\text{-}T_3$) in the culture medium serum supplement [2, 4]. Higher concentrations of NGF (50 ng/mL), however, produce much larger increases in ChAT (Figs 1 and 2; NGF treated cultures = approx 100–400 % untreated control reaggregate ChAT activity). Co-treatment with NGF plus GM1 ganglioside did not potentiate this effect (Fig. 1). The absolute level of control ChAT activity varied between culture batches as expected and shown previously [2] but was always of the same order of magnitude.

NGF at 50 ng/mL similarly increased the level of mAChR (approx. 200% of control binding; Fig. 3) with GM1 ganglioside having no additional effect. ECMA lesioning at 8 DIV reduced the reaggregate ChAT activity to around 50% of control activity as previously reported [2] and conversely doubled the level of mAChR (Figs 1 and 3). Addition of 50 ng/mL NGF three times at 48 hr intervals (with or without 100 μg/mL GM1 ganglioside) at the same time as ECMA by protocol A did not produce elevations of ChAT at 15–16 DIV as seen in non-ECMA treated control cultures at this timepoint (Fig. 1) and produced no further enhancement in the level of mAChR (Fig. 3).

However, using protocol B where the first of the three *in vitro* NGF treatments was delayed until 10 DIV (2 DIV after initial ECMA treatment) and cholinergic function similarly investigated at 15–16 DIV, there was a complete reversal of the lesion-induced ChAT reduction produced by the cholinotoxin (Fig. 2). (The combined results as shown were derived from two separate culture runs where statistically significant reductions and reversals were demonstrated in each case.)

Discussion

We have now shown that high concentrations of NGF (50 ng/mL) can produce large elevations in ChAT activity in normal, developing reaggregate cultures of rat whole