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Antagonism by the diacylglycerol kinase inhibitor R59 022 of muscarinic receptor-mediated cyclic GMP formation and binding of [^{3}H]*N*-methylscopolamine

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Protein kinase C (PKC*) activation has been shown to affect a variety of cellular functions, including desensitization of muscarinic receptors [1, 2]. In the presence of concentrations of Ca^{2+} comparable to those found intracellularly in most cells, PKC activation requires diacylglycerol (DAG) which is produced from polyphosphoinositide hydrolysis upon stimulation of neurotransmitter receptors [1]. Therefore, receptor-mediated DAG formation could be a candidate in this signal transduction cascade to mediate cellular responses through its activating effect on PKC. Due to the activity of cellular DAG kinase, the increase in DAG mediated by receptor activation is transient. To observe effects produced by DAG in a receptor-mediated event, a DAG kinase inhibitor should be useful to prolong the elevation of the DAG level and, therefore, the activation of PKC. A commercially available DAG kinase inhibitor, R59 022 (6-[2-[4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl]ethyl]-7-methyl-5*H*-thiazolo[3,2-*a*]pyrimidin-5-one), has been demonstrated to inhibit this kinase in a variety of tissues [3–5]. However, we found that R59 022 possessed potent antimuscarinic properties in inhibiting muscarinic agonist-mediated [^{3}H]cyclic GMP response and antagonist binding in mouse neuroblastoma N1E-115 cells.

Materials and methods

Culturing of N1E-115 cells and measurement of [^{3}H]cyclic GMP formation, as well as [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) binding to intact cells, were done as described previously [6]. R59 022 (Janssen Life Sciences) was dissolved in 95% ethanol:dimethyl sulfoxide (4:1, v/v) to a concentration of 2×10^{-2} M. A 1:5 dilution of this solution was made in 0.005 N HCl and further dilutions were made with water. In the [^{3}H]cyclic GMP assays, the cells were preincubated with various concentrations of R59 022 for 30 min at 37° before 1 mM carbamylcholine (CBC), 0.1 mM histamine, 10 mM NaF or 5 mM NaN_3 (all from the Sigma Chemical Co., St Louis, MO) was added. In [^{3}H]NMS binding experiments, various concentrations of R59 022 were present in the incubation mixture together with 0.2 nM [^{3}H]NMS (80 Ci/mmol, New England Nuclear, Boston, MA) for 60 min at 37°.

Results and discussion

R59 022 inhibited CBC-mediated [^{3}H]cyclic GMP formation in a concentration-dependent manner (Fig. 1). At 3 μM, a concentration reported to be the IC_{50} of R59 022 in inhibiting human red blood cell membrane and intact platelet DAG kinase [3], it inhibited the CBC-mediated cyclic GMP response by ~50%. A series of experiments, therefore, was designed to investigate the possible sites where R59 022 exerts its inhibitory effect on this muscarinic receptor-mediated response.

Figure 1 shows that R59 022 competed for the specific binding of the muscarinic antagonist [^{3}H]NMS in a concentration-dependent fashion. R59 022 inhibited [^{3}H]NMS binding and CBC-mediated [^{3}H]cyclic GMP formation in a similar concentration range (Fig. 1); therefore, this kinase inhibitor interfered with muscarinic receptor response probably by acting as a receptor antagonist. Because of the steepness of the inhibition curve of R59 022 on CBC-mediated cyclic GMP response, the possible interference by R59 022 of cyclic GMP generation beyond the receptor was also studied. Muscarinic receptor-mediated cyclic GMP response in N1E-115 cells is evidently a GTP-binding protein modulated process.† Fluoroaluminate, which combines with cellular GDP to form a GTP analogue [7], increased cyclic GMP formation in these cells probably by activating a GTP binding protein. NaF-induced cyclic GMP formation was inhibited by R59 022 in a concentration-dependent pattern, albeit at higher concentrations than those which inhibited the response to CBC (Fig. 2). Therefore, R59 022 may exert its inhibitory effect partially by interfering with the coupling of GTP binding protein to a component(s) involved in the cyclic GMP formation process. However, this mechanism may not play a major role in the inhibitory effects of the compound on receptor-

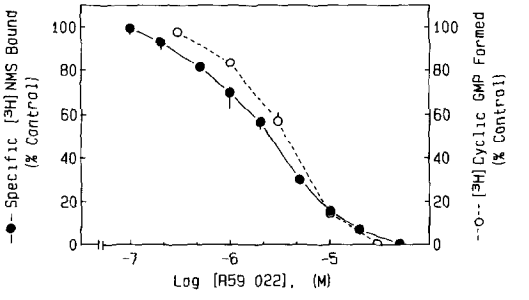

Fig. 1. Effect of R59 022 on muscarinic receptors. N1E-115 mouse neuroblastoma cells were prelabeled with [^{3}H]guanosine for [^{3}H]cyclic GMP measurements as described [6] and incubated in triplicate with increasing concentrations of R59 022 for 30 min at 37°. CBC (1 mM) was then applied, and the reaction was terminated after 30 sec. The average [^{3}H]cyclic GMP levels for basal and CBC-stimulated cells were 3,663 ± 807 and 91,422 ± 12,632 dpm/10^6 cells (mean ± SE) respectively. R59 022 at the concentrations tested had no effects on the basal cyclic GMP levels. For [^{3}H]NMS binding assay, cells were incubated in triplicate with 0.2 nM [^{3}H]NMS and increasing concentration of R59 022 at 37° for 60 min. Nonspecific binding was defined using 2 μM atropine, and the assay was terminated by rapid filtration. The average binding (mean ± SE) was 1184 ± 57 dpm/mg protein for total and 253 ± 22 for nonspecific. Results presented are averages from three independent experiments; the bars are SE.

* Abbreviations: PKC, protein kinase C; DAG, diacylglycerol; [^{3}H]NMS, [^{3}H]*N*-methylscopolamine; CBC, carbamylcholine; and IC_{50}, concentration at which 50% of the response or receptor binding is inhibited.

† Lai WS, Surichamorn W, Forray CC and El-Fakahany EE, manuscript in preparation.

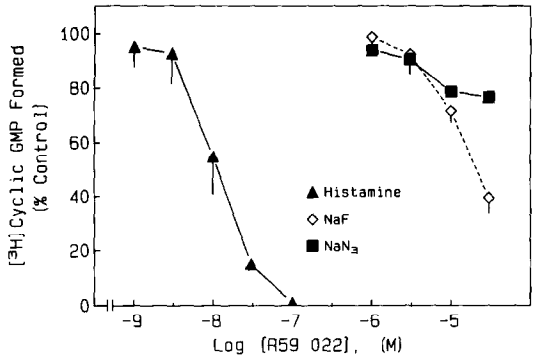

Fig. 2. Effects of R59 022 on histamine-, NaF-, and NaN_3-induced [^{3}H]cyclic GMP formation. After prelabeling of N1E-115 cells with [^{3}H]guanosine, cells were incubated in triplicate with increasing concentrations of R59 022 for 30 min at 37°; then histamine (0.1 mM), NaF (10 mM), or NaN_3 (5 mM) was applied. The reaction with histamine was terminated 30 sec after the addition of the agent, whereas the reactions with NaF or NaN_3 were terminated at 6 and 5 min respectively. The average basal [^{3}H]cyclic GMP level was 3,663 ± 807 dpm/10^6 cells; histamine-, NaF-, and NaN_3-induced [^{3}H]cyclic GMP synthesis averaged 100,044 ± 7,578, 57,834 ± 13,990 and 102,536 ± 15,691 dpm/10^6 cells respectively (means ± SE). Data are averages of three independent experiments; bars are SE.

mediated responses, particularly at low concentrations. Muscarinic receptors mediate cyclic GMP synthesis in N1E-115 cells by stimulating a soluble guanylate cyclase which can be activated directly by NaN_3 [8]. At 30 μM R59 022, a concentration which inhibited ~100% of CBC-mediated cyclic GMP formation, there was suppression of NaN_3-mediated [^{3}H]cyclic GMP synthesis by only ~20% (Fig. 2). Thus, it seems that a direct effect of R59 022 on guanylate cyclase contributed little, if any, to the inhibition of receptor-mediated cyclic GMP synthesis.

In summary, the DAG kinase inhibitor R59 022 was a potent antagonist of muscarinic receptor-mediated cyclic GMP response. In fact, it has also been reported that this kinase inhibitor acts as an antagonist at dopamine D_2, adrenergic α_1 and histamine H_1, in addition to serotonin S_2, receptors [3]. R59 022 exerts its inhibitory effects by its ability to interact directly with muscarinic receptors, as well as by interfering with the regulatory function of GTP binding proteins coupled to these receptors. R59 022 slightly affected guanylate cyclase, but only at high concentrations. Direct interactions with the receptor binding sites appear to be the primary mechanism mediating its antimuscarinic effects, since R59 022 demonstrated a 300-fold higher potency in inhibiting histamine-induced cyclic GMP formation (Fig. 2), suggestive of receptor-specific antagonistic effects which take place with different potencies. From this study, we conclude that when a DAG kinase inhibitor such as R59 022 is employed to investigate the involvement of PKC in a receptor agonist-mediated response, care must be taken in order to rule out the possibility that such an agent may act as an antagonist to the receptor concerned or may influence other components that are involved in the receptor-mediated response.

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