Rapid Communication

A Putative M_3 Muscarinic Cholinergic Receptor of High Molecular Weight Couples to Phosphoinositide Hydrolysis in Human SK-N-SH Neuroblastoma Cells

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Abstract: The M_1-selective (high affinity for pirenzepine) muscarinic acetylcholine receptor (mAChR) antagonist pirenzepine displaced both N-[^{3}H]methylscopolamine ([^{3}H]NMS) and [^{3}H]quinuclidinylbenzilate from intact human SK-N-SH neuroblastoma cells with a low affinity (K_i = 869–1,066 nM), a result indicating the predominance of the M_2 or M_3 (low affinity for pirenzepine) receptor subtype in these cells. Whereas a selective M_2 agent, AF-DX 116 {11-2[[2-[(diethylamino)methyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one} bound to the mAChRs with a very low affinity (K_i = 6.0 μM), 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), an agent that binds with high affinity to the M_3 subtype, potently inhibited [^{3}H]NMS binding (K_i = 7.2 nM). 4-DAMP was also 1,000-fold more effective than AF-DX 116 at blocking stimulated phosphoinositide (PPI) hydrolysis in these cells. Covalent labeling studies (with [^{3}H]propylbenzilylcholine mustard) suggest that the size of the SK-N-SH mAChR (M_r = 81,000–98,000) distinguishes it from the predominant mAChR species in rat cerebral cortex (M_r = 66,000), an M_1-enriched tissue. These results provide the first demonstration of a neural M_3 mAChR subtype that couples to PPI turnover. **Key Words:** Muscarinic receptor subtypes—Phosphoinositide turnover—Propylbenzilylcholine mustard—Pirenzepine. **Fisher S. K. and Heacock A. M.** A putative M_3 muscarinic cholinergic receptor of high molecular weight couples to phosphoinositide hydrolysis in human SK-N-SH neuroblastoma cells. *J. Neurochem.* **50**, 984–987 (1988).

Muscarinic acetylcholine receptors (mAChRs) are present in high concentrations in the CNS and are known to couple to a number of biochemical effector systems, such as stimulated phosphoinositide (PPI) turnover, inhibition of adenylate cyclase, and activation of guanylate cyclase (for review, see Nathanson, 1987). Subtypes of the mAChR

have also been proposed on the basis of the differential ability with which pirenzepine binds to the receptor and inhibits the functional responses. Pirenzepine has a high affinity for the M_1 subtype, whereas it has a low affinity for both the M_2 and M_3 subtypes. The latter can be differentiated on the basis of the relative selectivity of AF-DX 116 {11-2[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one} for M_2 and 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methiodide) for M_3 (Doods et al., 1987). The M_2 and M_3 receptors are principally found in heart and exocrine glands, respectively. In neural tissues, the M_1 subtype predominates and couples to PPI turnover. In the present study, we demonstrate the presence of a high density of high-molecular-weight (M_r > 80,000) mAChRs of the M_3 subtype in a neural cell line, human SK-N-SH neuroblastoma. Furthermore, these receptors are shown to couple functionally to inositol lipid hydrolysis.

MATERIALS AND METHODS

myo-[2-^{3}H]Inositol (15 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). N-[^{3}H]Methylscopolamine ([^{3}H]NMS; 80 Ci/mmol), [*propyl*-2,3-^{3}H]benzilylcholine mustard ([^{3}H]-PrBCM; 52 Ci/mmol), and [^{3}H]quinuclidinylbenzilate ([^{3}H]QNB; 46 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). Dowex-1 (100–200 mesh; ×8 in the formate form) was obtained from Bio Rad Labs (Rockville Center, NY, U.S.A.). Pirenzepine and AF-DX 116 were obtained from Boehringer-Ingelheim (Ridgefield, CT, U.S.A.). 4-DAMP was a generous gift from Dr. Raj K. Goyal (Beth Israel Hospital, Boston, MA, U.S.A.).

Cell culture and biochemical determinations

Human SK-N-SH neuroblastoma cells were cultured under conditions similar to those previously described (Fisher and Snider, 1987). In the experiments reported, 14–22-day-old cells were used. Measurement of the binding of radioligands to intact cells and monitoring of PPI hydrolysis were as previously described (Fisher and Snider, 1987). Protein content was determined by the method of Geiger and Bessman (1972).

Covalent labeling of muscarinic receptors

Slices of adult rat cerebral cortex (Heacock et al., 1987) or intact SK-N-SH cells were preincubated [in buffer A, minus Ca^{2+} (Fisher and Snider, 1987)] in the presence or absence of 10 μM atropine for 15 min at 37°C, followed by addition of 5–10 nM cyclized [³H]PrBCM (Hunter and Nathanson, 1986). After 30 min at 37°C, tissues were pelleted by centrifugation at low speed, washed twice with cold buffer A (minus Ca^{2+}), and then homogenized at 4°C in 62.5 mM Tris-HCl (pH 6.8) containing the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), phenanthroline (0.5 mM), pepstatin A (0.1 μM), soybean trypsin inhibitor (10 μg/ml), and EGTA (2 mM). Proteins were solubilized in 2% (wt/vol) sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol, and 8 M urea. In some experiments, disulfide bond formation was blocked by alkylation with 0.2 M iodoacetamide (Dadi and Morris, 1984). Samples were electrophoresed on 10% polyacrylamide gels (Laemmli, 1970), which contained 4 M urea. Coomassie Blue-stained gels were dried and then cut into 2-mm slices for determination of radioactivity (Hunter and Nathanson, 1986). Alternatively, labeled proteins were visualized by fluorography.

Data analysis

Results are expressed as mean ± SEM values.

RESULTS AND DISCUSSION

Specific binding of [³H]NMS and [³H]QNB to mAChRs on intact SK-N-SH cells was saturable, with B_{max} values of 379 ± 20 and 429 ± 20 fmol bound/mg of protein, respectively. The corresponding K_D values were 1.21 ± 0.22 and 0.23 ± 0.04 nM (n = 5). The binding of both radioligands could be displaced by pirenzepine, but with low affinity. The K_i values obtained for pirenzepine, 869 ± 133 ([³H]-NMS) and 1,066 ± 263 ([³H]QNB) nM (n = 5), suggest that M_2 and/or M_3 subtypes predominate in the SK-N-SH cell. Evidence that the M_3 rather than the M_2 receptor is present was obtained from studies with AF-DX 116 (M_2 selective) and 4-DAMP (M_3 selective). Both antagonists completely displaced [³H]NMS binding, but 4-DAMP was 1,000-fold more potent (Fig. 1). All three antagonists, i.e., pirenzepine, AF-DX 116, and 4-DAMP, blocked stimulated PPI hydrolysis with potencies that reflected the abilities of these agents to bind to the mAChR. Previously, we have shown that pirenzepine is a weak competitive inhibitor of stimulated PPI hydrolysis in SK-N-SH cells (K_i = 250 nM) (Fisher and Snider, 1987). However, 4-DAMP is ~100-fold more potent than pirenzepine in the inhibition of stimulated PPI turnover, whereas AF-DX 116 is 10-fold less potent (Fig. 2). Inclusion of increasing concentrations of both 4-DAMP and AF-DX 116 resulted in a parallel shift in the dose-response curves to carbamoylcholine, which is a characteristic of a competitive antagonist. Schild regression analysis of
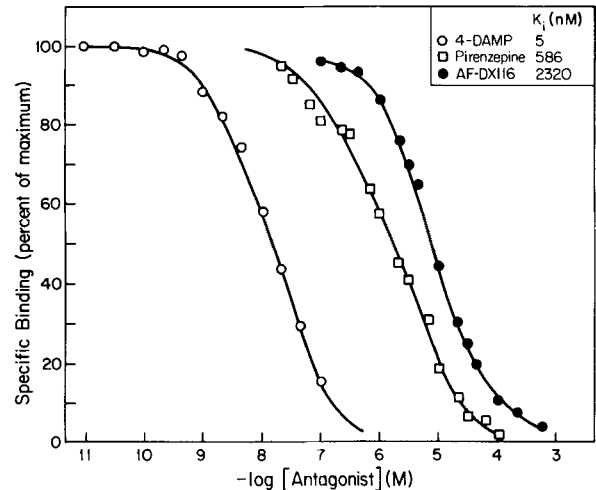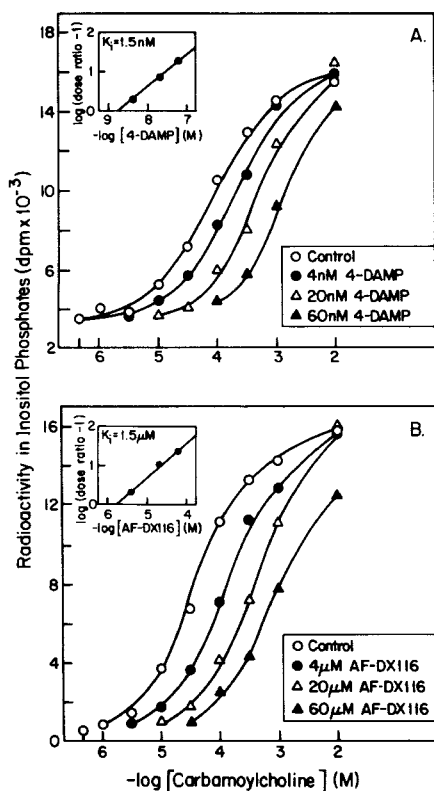

FIG. 1. Inhibition of specific [³H]NMS binding to SK-N-SH cells by addition of 4-DAMP, pirenzepine, and AF-DX 116. Cells were incubated for 120 min at 37°C with the indicated concentrations of antagonists. The K_i values [calculated by the method of Cheng and Prusoff (1973)] were 7.2 ± 1.5 nM, 869 ± 133 nM, and 6.0 ± 2 μM for 4-DAMP, pirenzepine, and AF-DX 116, respectively (n = 4–5).

the data yielded K_i values of 1–3 nM for 4-DAMP and 1.5–2 μM for AF-DX 116. These values are in good agreement with the affinity constants obtained from the radioligand binding data shown in Fig. 1. The differential abilities of pirenzepine, AF-DX 116, and 4-DAMP to bind mAChRs in SK-N-SH cells are very similar to that observed for the submandibular gland (an M_3-containing tissue) but differs markedly from that observed in either the atrium (M_2 containing) or hippocampus (M_1 containing) (Doods et al., 1987). That these three antagonists also exhibit the same relative affinities for inhibition of stimulated PPI turnover indicates that activation of M_3 receptors can lead to the hydrolysis of inositol lipids.
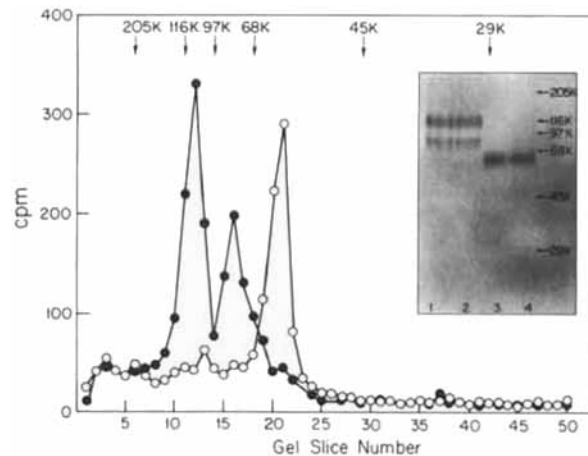
The possibility that the pharmacological characteristics of the mAChRs in SK-N-SH cells might reflect the presence of different molecular species compared with that in an M_1-containing tissue, such as the rat cerebral cortex, was explored. Intact SK-N-SH cells or rat cerebral cortex slices were labeled with [³H]PrBCM, and the labeled peptides were analyzed by SDS-urea gel electrophoresis (Fig. 3). Rat cortex contained a single species of M_r 66,000 ± 1,000 (n = 6), whereas SK-N-SH cells exhibited [³H]PrBCM-labeled peptides of considerably larger size with the major species of M_r 98,000 ± 2,000 (n = 6). The labeling of the less abundant M_r 81,000 ± 1,300 (n = 6) species varied but on the average accounted for approximately one-third of the total. In an experiment in which SK-N-SH cells were directly homogenized in SDS-containing buffer, both species were present; however, the portion of radioactivity in the M_r 81,000 species was reduced to 20% of the total. Thus, the possibility that the lower-M_r species is, at least in part, derived from proteolysis of the M_r 98,000 species cannot be ruled out. Rapid proteolysis of the mAChR in heart cells has been described (Hunter and Nathanson, 1986). Alternatively, because mAChRs are glycoproteins (Rauh et al., 1986), the M_r 81,000 species may represent a reduced glycosylation state of the receptor. Alkylation of sulfhydryl

groups by iodoacetamide treatment had no effect on migration of [³H]PrBCM-labeled peptides (Fig. 3, inset); thus, the higher molecular weight of the SK-N-SH mAChRs does not appear to result from intermolecular disulfide bond formation. Differences in proteolytic activity between rat cortex and SK-N-SH cells do not appear to be involved, because mixing of unlabeled rat cortex with labeled SK-N-SH cells before sample preparation had no effect on electrophoretic migration of the mAChRs. It may be that the SK-N-SH mAChR is more heavily glycosylated than that in the cortex, a possibility that can be addressed by endoglycosidase treatment (Rauh et al., 1986; Liang et al., 1987). Alternatively, the molecular and pharmacological characteristics of the mAChR expressed by SK-N-SH cells may reflect the presence of a receptor protein distinct from the species that predominates in the cerebral cortex.

Muscarinic receptors thus far identified by [³H]PrBCM-labeling appear to fall into two groups: (a) those of M_r 65,000–80,000, found in brain (Venter, 1983; Dadi and Morris, 1984; Large et al., 1986), heart (Venter, 1983; Hunter and Nathanson, 1986), and NG108-15 cells (Hootman et al., 1985; Liang et al., 1987) and (b) those of M_r

FIG. 3. SDS-urea gel electrophoresis of [³H]PrBCM-labeled mAChRs. Proteins (300–350 μg) from labeled SK-N-SH cells (●) or rat cortex slices (○) were subjected to electrophoresis as described in Materials and Methods. The ratio of specific to nonspecific (plus 10 μM atropine) labeling in the homogenate sample was 8.5:1 for SK-N-SH cells and 3.5:1 for cortex. No radioactive peaks were detected in lanes containing tissue labeled in the presence of atropine (data not shown). **Inset:** Fluorograph of a duplicate gel (exposed for 20 days) with (lanes 1 and 2) SK-N-SH cell homogenate and (lanes 3 and 4) cortical homogenate. Samples in lanes 2 and 4 were iodoacetamide treated. The molecular weight markers, in order of decreasing M_r, were the following: myosin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, and carbonic anhydrase.

> 80,000, found in SK-N-SH cells, exocrine glands (Hootman et al., 1985), and 1321N1 astrocytoma cells (Liang et al., 1987). Data presently available are suggestive of but insufficient to conclude whether or not the pharmacologically defined mAChR subtypes can be definitively assigned to either of these groups. Of the mAChRs thus far sequenced (Bonner et al., 1987), three are of similar size, whereas one (termed m_3) is considerably larger. Screening of SK-N-SH cell mRNAs with the appropriate molecular probes will be necessary for determining whether its mAChR belongs to the latter category.

The present results implicating the M_3 subtype in PPI hydrolysis, together with previous reports implicating both M_1 and M_2 subtypes (Fisher and Agranoff, 1987), indicate that multiple mAChR subtypes with distinct pharmacological and molecular characteristics are able to couple to the same functional response.

FIG. 2. Schild analysis of inhibition by **(A)** 4-DAMP and **(B)** AF-DX 116 of stimulated PPI turnover in SK-N-SH cells. Dose-response curves for stimulated inositol phosphate release were determined in the absence or presence of either 4-DAMP or AF-DX 116 at the concentrations indicated. Values shown are the means of triplicate replicates from a single experiment. **Insets:** The log of (dose-ratio − 1) is plotted as a function of 4-DAMP or AF-DX 116 concentration. The calculated K_i values were 1.5 nM and 1.5 μM for 4-DAMP and AF-DX 116, respectively. In a second experiment using the same experimental conditions, K_i values of 3.1 nM and 1.9 μM were calculated for these two antagonists.

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