M2, M3 and M4, but not M1, Muscarinic Receptor Subtypes are Present in Rat Spinal Cord

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ABSTRACT
Muscarinic receptors in the spinal cord have been shown to mediate antinociception and alter blood pressure. Currently, there is much interest in identifying which muscarinic receptor subtypes regulate these functions. Toward that end, this study aimed to identify and localize the muscarinic receptor subtypes present in spinal cord using in vitro receptor autoradiography with [3H]-pirenzepine and [3H]-N-methylscopolamine. The results showed that M2 binding sites were distributed throughout the dorsal and ventral horns, whereas M3 binding sites were localized to laminae I to III of the dorsal horn. Only background levels of M1 binding sites were detected. Saturation binding assays using [3H]-pirenzepine in spinal cord homogenates confirmed the absence of M1 receptors. Competition membrane receptor assays using [3H]-N-methylisocapomoline and the unlabeled antagonists pirenzepine, 11-2-[[(diethylamino)methyl]-1-piperidinyl]-acetyl]-5, 11-dihydro 6H-pyrdo(2, 3-b)(1, 4) benzodiazepine-one, methoctramine, and methoctramine in combination with atropine corroborated the autoradiographic findings and also revealed the presence of M4 binding sites. The finding that M2 and M3 binding sites were localized to the superficial laminae of the dorsal horn where nociceptive Aδ and C fibers terminate suggests the possibility that either or both of these muscarinic receptor subtypes mediate antinociception. The present demonstration of M4 binding sites in spinal cord is consistent with the possibility that M2 and/or M4 receptors are involved in the regulation of blood pressure at the spinal level.

Activation of muscarinic ACh receptors in spinal cord of animals produces antinociception (Abram and O’Connor, 1995; Abram and Winne, 1995; Detweiler et al., 1993; Iwamoto and Marion, 1993; Naguib and Yaksh, 1994) and hypertension (Buccafusco, 1996; Carp et al., 1994; Feldman et al., 1996). Similar responses have been noted in human subjects (Hood et al., 1995; Lauretti et al., 1996). It has been proposed that spinal antinociception is mediated by M1 and/or M2 muscarinic receptors (Iwamoto and Marion, 1993) and that neostigmine-induced hypertension is mediated, in part, by spinal M2 receptors (Lathe et al., 1994).

Muscarinic receptors previously have been demonstrated to be present in spinal cord of human, rat and cat using in vitro autoradiography with a variety of tritiated ligands, including [3H]-QNB (Gillberg et al., 1984; Rayaalp and Neff, 1980; Scatton et al., 1984; Seybold and Elde, 1984; Villiger and Faul, 1985; Yamamura et al., 1983), [3H]-PZ (Villiger and Faull, 1985), [3H]-ACh (Gillberg et al., 1988) and [3H]-NMS (Wamsley et al., 1984). Wamsley and colleagues (1984) showed that high- and low-affinity muscarinic binding sites are present in spinal cord. Villiger and Faull (1985) named these receptors M1 and M2, respectively.

Since the foregoing autoradiographic studies were performed, additional muscarinic receptor subtypes have been identified by molecular cloning, and there are now known to be five muscarinic receptor subtypes (Bonner et al., 1987; Kubo et al., 1986). By convention, molecularly identified subtypes are referred to as m1 to m5, and pharmacologically identified subtypes are designated M1 to M4 (Birdsall et al., 1989). The M1 to M4 subtypes generally correspond to the m1 to m4 subtypes (Caulfield, 1993; Waelbroeck et al., 1990). However, it is important to note that muscarinic antagonists are only relatively selective, not exclusively specific, for individual subtypes. The relative subtype selectivity of many muscarinic antagonists has been evaluated (Caulfield, 1993), and it is now clear that [3H]-NMS (Dörje et al., 1990) and [3H]-QNB (Bolden et al., 1991; Jakubik et al., 1995) label m1 to m5 receptors with equal and high affinity and that PZ has a high affinity for m1/M1 receptors, a relatively lower affinity for m3/M3 and m4/M4 receptors and a low affinity for m2/M2

ABBREVIATIONS: AF-DX 116, 11-2-[[diethylamino)methyl]-1-piperidinyl]-acetyl]-5, 11-dihydro 6H-pyrdo(2, 3-b)(1, 4)-benzodiazepine-one; ANOVA, analysis of variance; CV, cresyl violet; H, tritium; IML, intermediolateral nucleus; Kd, dissociation constant; LFB, luxol fast blue; METH, methoctramine; nH, Hill number; NMS, N-methylscopolamine; PZ, pirenzepine; QNB, quinuclidinyl benzilate; RT-PCR, reverse transcriptase-polymerase chain reaction; T, thoracic.
receptors (Waelbroeck et al., 1990). Thus previous in vitro autoradiographic studies using [3H]-NMS, [3H]-QNB and [3H]-PZ may have erroneously classified the muscarinic receptor subtypes present in spinal cord.

Increased knowledge about the multitude of muscarinic receptor subtypes (Caulfield, 1993), the ability to selectively visualize these subtypes (Flynn and Mash, 1993) and the rising interest in muscarinic pharmacology for use in clinical anesthesia (Hood et al., 1995; Lauretti et al., 1996) encouraged us to undertake the present study, which tested the hypothesis that spinal cord contains M1 to M4 muscarinic receptors.

Materials and Methods

Animals and chemicals. This study was approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University, College of Medicine. Male LBNF1 rats (Harlan SPD, Pottsville, AL), 10 weeks of age, were housed in stainless steel cages and had access to food (Harlan Teklad rodent diet (W) 8604, Madison, WI) and water ad libitum. Standardized environmental conditions included lights on between 0700 and 1900, lights off between 1900 and 0700, temperature 21°C to 23°C, humidity 40% to 60%, and 11 to 15 air changes per hour. Animals were given at least 1 week of acclimatization in home cages before use.

Radiolabeled ligands [3H]-PZ (77.9 Ci/mmol) and [3H]-NMS (84 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). PZ and atropine sulfate were obtained from Sigma Chemical Co. (St. Louis, MO). METH was purchased from Research Biochemicals International (Natick, MA). The antagonist AF-DX 116 was provided as a gift by Boeringer-Ingelheim (Ridgefield, CT).

In vitro receptor autoradiography. Five rats were decapitated by guillotine, and the thoracic spinal cords were extracted rapidly and frozen on dry ice. Spinal cords were sectioned serially using a Hacker-Bright cryostat (Bright Instrument Company LTD, Huntingdon, England), and 25-μm transverse sections were thaw-mounted on chrome alum-coated glass slides. Slide-mounted tissue sections were dried under reduced pressure at 4°C for at least 2 h and kept at −70°C until processed according to standard autoradiographic techniques (Kuhar and Unnerstall, 1990).

Consecutive slides were used to label M1, M2 and M3 muscarinic receptors at each level of the thoracic cord in accordance with the binding assays described by Flynn and Mash (1993). These assays take advantage of the selectivity of PZ binding and the kinetic properties of NMS binding and have been shown to be useful for localizing muscarinic receptor subtypes throughout monkey brain (Flynn and Mash, 1993) and in cat brain stem (Baghdoyan et al., 1994; Mallios et al., 1995). Briefly, to visualize M1 binding sites, tissue sections were labeled with 3 nM [3H]-PZ for 60 min. Because [3H]-NMS associates most rapidly to the m2 subtype (Flynn and Mash, 1993; Waelbroeck et al., 1990), M2 binding sites were labeled with a 2-min incubation in 0.5 nM [3H]-NMS, which followed a 60-min preincubation in 0.3 μM PZ to occlude m1, m3 and m4 sites. [3H]-NMS dissociates most slowly from the m3 subtype (Flynn and Mash, 1993; Waelbroeck et al., 1990). Thus M3 binding sites were labeled by preincubating the sections for 3 min in 0.5 nM NMS to occlude m1, m2 and m4 sites and then incubating them in 0.25 nM [3H]-NMS for 2 h. This labeling was followed by a 75-min dissociation of the radioligand by the addition of 1 μM atropine. For all three muscarinic receptor subtypes, nonspecific binding was determined in selected adjacent sections by adding 10 μM atropine to the assay buffer. Radiolabeled sections and [3H] micro-scales (Amersham Corporation, Arlington Heights, IL) were apposed to [3H]-Hyperfilm (Amersham) for 6 weeks at 4°C. Films were developed in Kodak D-19; then tissue sections were fixed in paraformaldehyde vapors (Herkenham and Pert, 1982) before staining with luxol fast blue (LFB; 0.2%) and cresyl violet (CV; 0.1%).

The NIH Image program was used to digitize and analyze autoradiographic images and histological sections, as described previously (Baghdoyan et al., 1994; Mallios et al., 1995). For every sheet of film, the autoradiographic image made from one set of [3H] micro-scales was digitized to make possible the calibration of optical density to fmol of binding per mg tissue equivalent (Geary et al., 1985). Autoradiograms of spinal cord sections were magnified four times using a microscope and then were digitized and written to disk. All slide-mounted, LFB/CV-stained spinal cord sections used to generate autoradiograms were also magnified, digitized, and written to disk.

Because of the low resolution of film autoradiography, it was not possible to distinguish between laminae I and II on the computerized autoradiographic images. Therefore, laminae I and II are discussed hereafter as one entity called laminae I/II. The digitized LFB/CV images were used as templates to identify the boundaries of laminae I/II and lamina III. Each of these boundaries was traced on the digitized image of the LFB/CV section using the outlining feature of NIH Image. The outline then was copied to the digitized autoradiogram of the same section. Binding density was measured within the outlined region on the digitized autoradiographic image, and binding density values were pasted to a spreadsheet for subsequent statistical analyses.

Means and standard errors of the means were calculated on 16 to 20 samples from each segmental level of the thoracic spinal cord. Two-way ANOVA was used to test the hypothesis that M2 and M3 binding sites were differently distributed across all 12 segments of the thoracic spinal cord and between laminae I/II and lamina III. A one-way ANOVA with a Tukey’s post-hoc test identified the thoracic cord segments that contributed to any significant differences in muscarinic receptor density. To evaluate quantitatively which laminae contributed to any significant differences in muscarinic receptor distribution, further analyses applied multiple t tests with Bonferroni correction factors (αactual = 0.05/number of comparisons).

Membrane receptor assays. Eleven rats were decapitated by guillotine, and the thoracic spinal cord and hippocampus were extracted rapidly and placed in a glass petri dish on ice. The remaining meninges and blood vessels were removed, and the tissue was weighed and diluted to 50 times its wet weight in ice-cold 20 mM Tris-HCl + 1 mM MnCl2 buffer, pH 7.4 (for [3H]-PZ saturation binding) or 50 mM phosphate buffer + 1 mM MgCl2, pH 7.4 (for [3H]-NMS saturation and competition binding). The tissue was homogenized, centrifuged and resuspended three times. After the second centrifugation, the pellet was resuspended in distilled water, and a 0.2-ml sample was taken to determine the protein content of the homogenate using the BCA Protein Assay Reagent (PIERCE, Rockford, IL).

Saturation binding assays were performed according to the methods of Waelbroeck and colleagues (1986), using six concentrations of [3H]-PZ ranging from 0.2 to 20 nM or six concentrations of [3H]-NMS ranging from 35 to 3500 pM. Competition binding assays also were performed according to procedures described by Waelbroeck et al. (1986; 1990). Homogenates of spinal cord were incubated with 240 pM [3H]-NMS and with PZ alone, AF-DX 116 alone, METH alone or METH in combination with atropine (METH + atropine) as competitors. PZ (0.4 nM–100 μM) was used in order to distinguish between M1 and M3 binding sites. AF-DX 116 alone (0.4 nM–100 μM) and METH alone (0.4 nM–100 μM) were used to distinguish between M2 and M3 binding sites. As described by Waelbroeck et al. (1990), METH (0.4 nM–100 μM) in combination with atropine (1 μM) was used to distinguish between M3 and M4 binding sites. The rationale for using METH + atropine to identify M3 and M4 binding sites is based on the previously demonstrated finding that [3H]-NMS dissociates more slowly from m3/M3 and m4/M4 receptors than from m1/M1 and m2/M2 receptors (Flynn and Mash, 1993; Waelbroeck et al., 1990). Therefore, the addition of 1 μM atropine for the final 35 min of incubation should differentially displace [3H]-NMS from the four muscarinic receptor subtypes, leaving residual [3H]-NMS bind-
Results

In vitro receptor autoradiography reveals M2 and M3 binding sites in dorsal horn. The main finding to emerge from the autoradiographic data was that the thoracic spinal cord contains M2 and M3, but no M1, binding sites. The distribution of binding sites is shown in figures 1 and 2. Only background levels of M1 binding (fig. 1) were observed, whereas M2 sites (figs. 1 and 2) were distributed throughout the gray matter of the thoracic spinal cord. Relatively high M2 densities were found in the superficial layers of the dorsal horn corresponding to laminae I/II and III of Rexed (Rexed, 1952), the intermediolateral nucleus (fig. 1, IML), laminae VI and VII, lamina IX in the ventral horn and lamina X. M3 binding sites (figs. 1 and 2) were localized mainly to laminae I/II, with a lower M3 density in lamina III. The ventral horn was devoid of M3 binding sites.

Saturation binding assays confirm the absence of M1 binding sites in spinal cord. Saturation binding with [3H]-PZ was used to confirm the autoradiographic finding of no M1 binding sites in spinal cord. The saturation binding protocol first was verified using tissue from hippocampus, where a high density of M1 receptors has been demonstrated (Waebroeck et al., 1986). Assays performed using hippocampal homogenates showed saturable binding with a dissociation constant ($K_d$) of 5.5 nM and a maximal binding ($B_{max}$) of 100.8 fmol/mg tissue. These results agree with previously published values ($K_d = 2–8$ nM, $B_{max} = 94.3$ fmol/mg tissue, Watson et al., 1983; $K_d = 4–6$ nM, Waebroeck et al., 1986). Having thus demonstrated that the saturation binding protocol was working, the assay then was applied to tissue from spinal cord. Specific binding of [3H]-PZ to homogenates of spinal cord was negligible, which strongly indicates that M1 receptors are not present in this tissue.

Competition binding assays provide evidence for M2, M3 and M4 binding sites in spinal cord. The presence of M2, M3 and M4 binding sites in spinal cord was determined by competition binding measured with [3H]-PZ and PZ, AF-DX 116, METH and atropine as unlabeled competitors. Figure 5 shows the competition curves obtained from these experiments, and table 1 reports the $K_d$ and $B_{max}$ values calculated for the competing ligands. Table 1 $K_d$ values were compared with previously published dissociation constants for PZ, AF-DX 116 and METH (table 2) in order to identify which muscarinic receptor subtypes were present in spinal cord homogenates.

Competition of [3H]-NMS binding with PZ (fig. 5) showed a normal competition curve with a Hill coefficient ($n_H$) of 0.95 ± 0.03, which indicates that PZ detected one binding site. Comparison of the $K_d$ value obtained for PZ in the present study (table 1, 197.1 ± 29.9 nM) with $K_d$ values for PZ obtained using known muscarinic receptor subtypes (table 2) suggests that PZ labeled M2 and/or M3 receptors in the spinal cord. Table 2 indicates that the M2 and M3 $K_d$ values for PZ are not sufficiently different to distinguish between these two subtypes.

Competition of [3H]-NMS binding with METH alone (fig. 5) produced a steep competition curve with a Hill coefficient ($n_H$) of 1.21 ± 0.06. This $n_H$ was significantly greater than unity ($t = 3.4$; df = 8; $P < .001$), which indicates positive cooperativity. This positive cooperativity probably resulted from the METH-induced allosteric inhibition of [3H]-NMS displacement (Waebroeck et al., 1990). To minimize the effects of this allosteric inhibition, only data obtained with concentrations of METH that were below 1 μM were used to calculate $K_d$ and $B_{max}$. Comparison of the METH $K_d$ value (table 1, 24.5 ± 1.8 nM) with previously reported $K_d$ values for METH (table 2) suggests that the spinal cord $K_d$ value might represent both M2 and M4 receptors. The relatively high density of receptors obtained from the fit of the present METH data ($B_{max} = 391.7 ± 61.0$ fmol/mg protein) suggests the possibility that the $K_d$ value derived from the METH competition represents M2 and M4 receptors in spinal cord.

Competition with AF-DX 116 alone and METH in combination with atropine (fig. 5) produced shallow competition curves, a result that indicates the presence of more than one binding site. A two-binding-site model provided a significantly ($P < .001$) better fit than a one-site model when these competition curves were analyzed. For AF-DX 116, the fitted $K_d$ values (table 1) and the shallow competition curve with $n_H$ of 0.77 ± 0.04 (significantly different from unity, $t = 6.3$; df = 8; $P < .001$) indicate that AF-DX 116 recognized M2 and M3 receptors (compare table 1 $K_d$ values with table 2 $K_d$ values).
After addition of 1 μM atropine to the METH incubation, [3H]-NMS was still bound to the homogenate (fig. 5), which indicates the presence of slowly dissociating (non-M1, non-M2) binding sites. Taken together, the $n_H$ of 0.60 ± 0.03 (significantly less than unity, $t = 12.5; df = 6; P < .001$) and the affinity constants fitted to the METH + atropine competition curve (table 1) indicate the presence of both M3 and M4 muscarinic receptors (table 2) in spinal cord homogenates.

**Discussion**

These results provide new information about the distribution of muscarinic receptor subtypes in spinal cord. No M1 binding sites were found in the present study. Instead, the data offer evidence for the presence of M3 receptors localized to the same areas where M1 receptors previously had been suggested to be present (Gillberg et al., 1988; Villiger and
The distribution of M2 binding sites corresponds well to previous results (Villiger and Faull, 1985). The present study also provides evidence for the existence of M4 receptors in spinal cord.

Absence of M1 muscarinic receptors in spinal cord. Spinal cord M1 receptors were reported by earlier studies, yet the present study found only background levels of M1 binding (fig. 1). How can this apparent discrepancy be explained? Previous autoradiographic studies (Villiger and Faull, 1985; Yamamura et al., 1983) used a 20 nM concentration of [3H]-PZ to visualize M1 receptors, whereas the present study used 3 nM [3H]-PZ. According to the formula

\[
\text{% occupancy} = 100 \times \frac{[\text{PZ}]}{[\text{PZ}] + K_d}
\]

where [PZ] is 3 nM or 20 nM and \(K_d\) for PZ is 6 nM or 85 nM, respectively (\(K_d\) values from Flynn and Mash, 1993), it can be shown that 3 nM PZ would occupy 33% of M1 sites but only 3.4% of M3 sites. Likewise, 20 nM PZ would occupy 77% of M1 sites and 19% of available M3 sites. Although these percentages are calculated under the assumption that M1 and M3 receptors are available in equal concentrations, these calculations help explain the differences between the present results and previous findings. At a concentration of 20 nM (Villiger and Faull, 1985; Yamamura et al., 1983), PZ would label enough M3 sites to be visible by autoradiography. Because only M1 and M2 muscarinic receptors were known to exist in the early 1980s, the [3H]-PZ binding sites originally found in spinal cord were identified as M1.

Autoradiographic data revealing an absence of spinal cord M1 binding sites were confirmed in spinal cord homogenates. A recent study using RT-PCR and HPLC demonstrated that only background levels of m1 mRNA are present in spinal cord (Wei et al., 1994). Most recently, m1-toxin only minimally displaced [3H]-NMS binding in the lumbar region of
human spinal cord (Borenstein et al., 1996). Thus multiple techniques now support the conclusion that the spinal cord does not contain M1 receptors.

Evidence for the presence of M2, M3 and M4 receptors in spinal cord. The present data provide autoradiographic evidence for M2 and M3 receptors in thoracic cord (figs. 1 and 2). In spinal cord homogenates, competition of $[^3H]$-NMS binding with AF-DX 116 revealed two binding sites (fig. 5; table 1), and the $K_d$ values corresponded to M2 and M3 receptors (table 2). $K_d$ values obtained from competition binding with METH and with PZ (table 1) also are consistent with the presence of M2 and M3 receptors. The existence of m2 and m3 mRNA in spinal cord has been demonstrated by RT-PCR (Wei et al., 1994). Taken together, these findings confirm the presence of M2 and M3 receptors in thoracic spinal cord.

The autoradiography protocol used in the present study (Flynn and Mash, 1993) did not permit localization of M4 receptors. The existence of M4 receptors in the spinal cord, however, can be suggested based on the reinterpretation of previous autoradiographic data using 20 nM $[^3H]$-PZ to label muscarinic receptors (Villiger and Faull, 1985; Yamamura et al., 1983). According to the % occupancy formula described above, 20 nM $[^3H]$-PZ (Villiger and Faull, 1985; Yamamura et al., 1983) may have labeled a substantial proportion of M4 sites (31%, assuming a $K_d$ value of 44 nM; Flynn and Mash, 1993). In the present study, the 3 nM $[^3H]$-PZ concentration used for competition binding was too low to be detected. Thus competition binding in spinal cord homogenates was performed to determine whether M4 receptors were present.

METH + atropine discriminated between two slowly dissociating (non-M1, non-M2) receptors (fig. 5; table 1). $[^3H]$-NMS dissociates slowly from genetically defined m3 and m4 muscarinic receptors (Flynn and Mash, 1993; Jakubik et al., 1995), and METH has a higher affinity for m4 than for m3 receptors (Dörje et al., 1990) (table 2). The METH + atropine competition data revealed $K_d$ values (table 1) consistent with the assumption that METH + atropine distinguished between M3 and M4 receptors (table 2). The competition bind-

**TABLE 1**

Dissociation constants ($K_d$) and receptor densities ($B_{max}$) obtained from fitted competition binding data

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>M2 (fmol/mg protein)</th>
<th>M3 (fmol/mg protein)</th>
<th>M4 (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>METH</td>
<td>24.5 ± 1.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PZ</td>
<td>197.1 ± 29.9</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>AF-DX 116</td>
<td>64.0 ± 6.2</td>
<td>1642.6 ± 344.9</td>
<td>—</td>
</tr>
<tr>
<td>METH + atropine</td>
<td>—</td>
<td>875.2 ± 99.5</td>
<td>16.9 ± 4.0</td>
</tr>
<tr>
<td>B$_{max}$ (fmol/mg protein)</td>
<td>—</td>
<td>—</td>
<td>8.4 ± 0.9</td>
</tr>
</tbody>
</table>

Means ± S.E.M. were calculated from four or five individual experiments performed in duplicate. These $K_d$ values were used to identify the muscarinic receptor subtypes in spinal cord. The competition curves for METH and PZ were fit to a one-binding-site model, and the calculated $K_d$ values indicate the presence of M2 receptors. A two-binding-site model provided a significantly ($P < .001$) better fit than a one-site model when analyzing the competition curves for AF-DX 116 and METH + atropine. Competition binding with AF-DX 116 demonstrated the presence of M2 and M3 receptors in spinal cord homogenates. The METH + atropine competition binding protocol provided evidence for the existence of M3 and M4 receptors in spinal cord. $K_d$ values reported in this table are within the published range (table 2). Thus the spinal cord muscarinic receptor subtypes identified in the present study are pharmacologically similar to known muscarinic subtypes found in other tissues (Waebroeck et al., 1990) and similar to cloned muscarinic receptor subtypes expressed in vitro (Caulfield, 1993).
ing assays using METH alone identified only one binding site (fig. 5; table 1). It is likely that this binding site represents both M2 and M4 receptors, because METH alone cannot distinguish between these two muscarinic receptor subtypes (table 2). Thus these competition binding assays provide evidence for the presence of spinal cord M4 receptors. A new autoradiographic study using m4-toxin to displace [3H]-NMS binding has now localized m4 receptors to lamina II of human spinal cord (Borenstein et al., 1996).

Muscarinic receptor subtypes in spinal cord: functional implications. The present data did not address the functional roles of M2 and M3 receptors localized to laminae I to III of the dorsal horn. However, given the current interest in the spinal cord as a site of anesthetic action (Kendig, 1993), and given the widespread use of intrathecal drug administration in clinical practice (Collins, 1995), it is relevant to discuss the functional implications of the present data. Neostigmine injected intrathecally has been shown to enhance blood pressure, an effect that can be inhibited by atropine or METH (Buccafusco, 1996; Carp et al., 1994; Williams et al., 1993). This indicates that M2 receptors and, in light of the present findings (fig. 5; table 1), M4 receptors might be involved in the spinal regulation of blood pressure. Antinociception elicited by intrathecal injection of neostigmine, on the other hand, can be prevented by the administration of PZ but not AF-DX 116 (Bouazziz et al., 1995). The administration of (+)-cis-methylthioxolane (Iwamoto and Marion, 1993) produced antinociception that could be prevented by PZ or METH. The present findings are consistent with the possibility that cholinergically induced antinociception (Smith et al., 1989; Yaksh et al., 1995; Zhuo and Gebhart, 1991) is mediated by the activation of M2, M3 and/or M4, but not M1, receptors. PZ does antagonize M3 and M4 receptors with a relatively high affinity (table 2), and M2, M3 (figs. 1 and 2) and m4 (Borenstein et al., 1996) receptors have been localized to the superficial laminae of the dorsal horn.

Limitations, conclusions and future directions. One potential problem for studies using in vitro receptor autoradiography to localize muscarinic receptor subtypes is that the muscarinic antagonists used as probes are only relatively subtype-selective, not exclusively subtype-specific (reviewed in Caulfield, 1993). Thus care must be taken to minimize the cross-labeling of subtypes. Selective autoradiographic labeling of muscarinic receptor subtypes has been achieved using a variety of ligand binding approaches (Araujo et al., 1991; Aubert et al., 1992; Flynn and Mash, 1993; Frey and Howland, 1992; Quirion et al., 1993; Vilaro et al., 1993; Zubieta and Frey, 1993). As described in detail elsewhere (Flynn and Mash, 1993; Mallios et al., 1995), the binding assays used in the present study to visualize muscarinic receptor subtypes took advantage of the selectivity of [3H]-PZ binding to label M1 receptors and exploited the distinct kinetic binding properties of [3H]-NMS (Flynn and Mash, 1993; Waeltbroeck et al., 1986) to label M2 and M3 receptors. Minimal cross-labeling of muscarinic receptor subtypes also was achieved by labeling only a fraction of the receptors present. Flynn and Mash (1993) demonstrated that the binding assay for M1 autoradiography labels 29% of m1 receptors, 0.5% of m2 receptors, 3% of m3 receptors and 6% of m4 receptors; the M2 binding assay labels 5%, 59%, 11% and 7% of m1, m2, m3 and m4 receptors, respectively; and the M3 binding conditions provide a 1%, 0%, 18% and 1.7% occupancy of m1, m2, m3, and m4 sites, respectively. It is the unique kinetic binding properties of [3H]-NMS to m1 to m4 muscarinic receptors that permit this minimal amount of autoradiographic cross-labeling.

The above values for percent occupancy of muscarinic receptors were derived using cloned muscarinic receptor subtypes expressed in A9L cells and were verified for native muscarinic receptors using primate brain and rabbit peripheral tissue (Flynn and Mash, 1993). It is possible that the dissociation rates and occupancy values for muscarinic receptor subtypes expressed in rat spinal cord may differ from those published by Flynn and Mash (1993). The pharmacological characteristics of muscarinic receptors, however, have been noted to be quite similar between species (Caulfield, 1993). In addition, differential kinetics of [3H]-NMS binding to muscarinic receptors expressed in rat brain have been demonstrated (Waeltbroeck et al., 1986; 1990). Therefore, it is likely that the kinetic parameters of [3H]-NMS binding that were applied in the present study are relevant for muscarinic receptors expressed in rat spinal cord. Characterizing the kinetics of [3H]-NMS binding in rat spinal cord will be of interest for future studies.

The present data revealed that M2 and M3 receptors are localized to laminae I to III. The limitations of film autoradiography made it impossible to distinguish between laminae I and II. The present results encourage the use of muscarinic receptor subtype-specific antibodies to identify which cell types in the spinal cord express muscarinic receptors and to determine the pre- and/or postsynaptic localization of spinal cord muscarinic receptor subtypes (Levey et al., 1995).

In conclusion, this study provides new insights into the distribution of muscarinic receptor subtypes in spinal cord. Until now, the only muscarinic receptors thought to be present were M1 and M2. The data presented here show that M2, M3 and M4, but not M1, receptors exist in spinal cord. Given that intrathecal injections of cholinergic agonists and acetylcholinesterase inhibitors produce antinociception, an exciting opportunity for future research is to determine which spinal muscarinic receptor subtypes modify nociceptive input.

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