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Evaluation of Quaternized and Neutral Muscarinic Receptor Ligands in Normal and DES-treated Rat

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The localization of quaternized muscarinic receptor (mAChR) antagonists, [^{11}C]methyl tropanyl benzilate ([^{11}C]MTRB) and [^{11}C]methyl quinuclidinyl benzilate ([^{11}C]MQNB), in rat pituitary was compared to that of [^{11}C]tropanyl benzilate ([^{11}C]TRB), a neutral antagonist. The quaternized ligands localize via a mAChR-mediated mechanism as shown by 60% reduction in radioactivity concentrations in the presence of QNB. [^{11}C]TRB appears to localize primarily by a non-mAChR specific mechanism. Induction of pituitary prolactinomas by diethylstilbestrol resulted in a reduction of [^{11}C]MTRB pituitary localization compared to normals. Elevated serum prolactin levels due to prolactinoma presence had no measurable effect on myocardial [^{11}C]MTRB uptake or on K_D values. B_{max} values for myocardial mAChR were similar for controls and for DES exposure of 10 weeks.

Muscarinic receptors (mAChRs) are intrinsic membrane proteins found in many tissues which elicit an array of biochemical responses from control of heart rate to neurotransmission in the brain. Imaging these receptors could yield medically useful data from both the central nervous system and peripheral organs such as the heart. Rather than synthesizing a large number of potential ligands in radioactive form, we developed a two stage assay system to preselect target ligands. The first stage consisted of a competitive *in vitro* binding assay designed to provide information on relative binding affinities. The second stage was an *ex vivo* assay which provided information on both *in vivo* distribution and kinetics (Otto *et al.*, 1989). Application of this screening procedure lead to radiosynthesis and further studies of the following ligands: [^{11}C]-(+)-2α-tropanyl benzilate ([^{11}C]TRB) and [^{11}C]-*N*-methyl piperidyl benzilate ([^{11}C]NMPB) (Mulholland *et al.*, 1988a,b, 1989) (see Fig. 1). Our initial objective in synthesizing [^{11}C]MTRB was to check its feasibility as a myocardial imaging agent. Gibson *et al.* (1979) demonstrated that quaternization of [^{3}H]QNB to form [^{3}H]MQNB lead to better heart/lung ratios than observed with [^{3}H]QNB. Other investigators, using [^{11}C]MQNB, verified that the complications of non-specific lung uptake of neutral mAChR ligands were reduced with quaternization (Maziere *et al.*, 1981; Syrota *et al.*, 1985).

In studying [^{11}C]MTRB *in vivo*, we noted a difference in behavior between [^{11}C]TRB and [^{11}C]MTRB in the pituitary. Pituitary localization of both ligands was expected as mAChRs have been described in the pituitary (Avissar *et al.*, 1981; Shaeffer and Hsueh, 1980; Burt and Taylor, 1980; Mukherjee *et al.*, 1980). The *in vivo* demonstration of mAChR-mediated pituitary localization of [^{3}H]NMPB (Avissar *et al.*, 1981) together with the difference in behavior of the tropanyl benzilate ligands prompted a more thorough evaluation of ^{11}C labeled muscarinic antagonists in the pituitary.

Having an interest in detecting the presence of pituitary prolactin secreting adenomas (prolactinomas), we evaluated both [^{11}C]TRB and [^{11}C]MTRB in a rat model for prolactinoma. Diethylstilbestrol (DES) implanted in the F344 rat lead to increased serum prolactin levels (Wiklund *et al.*, 1981). Further characterization of this model verified the increases in serum prolactin levels and pituitary weight, and documented increases in percent prolactin secreting cells (Otto *et al.*, 1986a). Although the role of the mAChR receptor in the pituitary has yet to be sharply defined, there is evidence for cholinergic control of prolactin secretion (Carmeliet and Denef, 1988; Hall *et al.*, 1984; Sharif, 1988). Comparison of mAChR antagonist localization in normal rat pituitary with localization in a prolactinoma model might

reveal differences in mAChR density that would aid in understanding the role of cholinergic influence in the pituitary.

Our efforts were directed towards (1) comparing uptake of both neutral and quaternized mAChR radioligands in pituitary tissue, heart and brain, (2) evaluating the effects of DES-treatment on radiolabeled mAChR ligand uptake in pituitary, heart and brain and (3) determining the effects of high circulating prolactin levels on the biochemical characteristics (B_{max} and K_D) of myocardial mAChR.

Materials and Methods

Drugs

Non-radioactive precursor (+)-2α-tropanyl benzilate (TRB) was prepared by base catalyzed transesterification of (+)-2α-tropanol with methyl benzilate (Atkinson *et al.*, 1977). *N*-Desmethyl-(+)-2α-tropanyl benzilate (nor-TRB) and (+)-2α-tropanol were kindly provided by Dr Edward R. Atkinson. *N*-Methyl-4-piperidyl benzilate and 4-piperidyl benzilate were synthesized by literature procedures (Biel *et al.*, 1961). (−)-Quinuclidinyl benzilate was purchased from Research Biochemicals Inc., Natick, Mass., U.S.A. Carbon-11 syntheses of [^{11}C]TRB, [^{11}C]MTRB (Mulholland *et al.*, 1989), [^{11}C]NMPB (Mulholland *et al.*, 1988a,b) and [^{11}C]MQNB (Syrota *et al.*, 1985) were conducted by reacting the appropriate precursor (1–2 mg in 200 μL of *N*,*N*-dimethyl formamide) with high specific activity [^{11}C]methyl iodide and then isolating products by normal phase chromatography. The radiochemical purities and specific activities of final formulated products were determined by analytical reversed phase HPLC (4.6 × 250 mm C-18 5μ, 3:1:1 acetonitrile:methanol:20 mM $KHPO_4$, pH 6.7, 1 mL/min, u.v. (220 nm)/radioactivity (NaI), lower detection limits: 10–30 ng. Specific activities at the end of synthesis were in the range of 300–4000 Ci/mmol and the radiochemical purities were greater than 98%.

The following compounds were obtained from commercial sources: [^{3}H]-*N*-methyl scopolamine ([^{3}H]NMS) (35–70 Ci/mmol) (New England Nuclear), diethylstilbestrol (DES) (Aldrich Chemical Company), atropine sulfate (Sigma), scintillation cocktail (ICN Biomedical). Fischer F344 and Sprague–Dawley female rats were purchased (Harlan). Animals were exposed to alternating 12 h periods of light and dark and received rat chow and water *ad libitum* during the study.

Tissue distribution studies

Tissue distribution studies were performed in both F344 female rats weighing between 170 and 225 g at 5, 15, 45, 60 and 90 min post injection and in female Sprague–Dawley rats weighing between 150 and 250 g. DES-treated rats were implanted as previously described (Otto *et al.*, 1986) and used in tissue distribution studies at 0–16 weeks post implant. Radiolabeled drugs (50–200 μCi in less than 250 μL total volume) were administered intravenously (except where noted) in the femoral vein to rats anesthetized with ether. Animals ($N = 3$ per time interval) were anesthetized and killed by decapitation. The brains and other tissues were removed rapidly, counted for carbon-11 and then weighed. Tissue concentrations were calculated in terms of percent injected dose per gram (%ID/g) or percent injected dose per organ (%ID/organ). For QNB blocking studies, rats ($N = 3$) were pretreated intraperitoneally (i.p.) with ±QNB (2 or 6.6 mg/kg body weight depending on study) either 10 min or 1 h prior to radiodrug injection. Control rats ($N = 3$) were pretreated with saline vehicle only.

In vitro assay for heart tissue

Hearts from DES-treated rats were excised at 10 and 16 weeks post DES implant and stored at −80°C until used. Hearts from non-DES-treated rats were collected and frozen until used. For the assay, hearts were thawed and homogenized in ice-cold 50 mM Na–K phosphate buffer (pH 7.4) (hereafter referred to as buffer); about 0.6 g tissue were homogenized in 11.5 mL buffer. Protein concentration was determined using the Bradford assay (Bradford, 1976) (standard: bovine serum albumin). Protein concentration of the homogenate was 0.24–0.28 mg/assay volume. To assay the binding of [^{3}H]NMS, triplicate samples of 50 μL of homogenate in buffer containing a minimum of 10 different concentrations of [^{3}H]NMS were used. Nonspecific binding was also determined in triplicate by including 1 μM atropine sulfate in the assay. Each assay was initiated by addition of homogenate to yield a final volume of 1.2 mL and incubated at room temperature for 2 h. The assay was terminated by addition of 3.0 mL of ice-cold buffer followed by immediate vacuum filtration using glass fiber filters (Schleicher & Schuell No. 32) presoaked in 2.5% polyethylenimine. After washing the filters with 3 × 3.0 mL cold buffer, they were transferred to a scintillation vial. Cocktail was added and the vials stored in the dark for 12 h before counting (64% efficiency). The count data was analyzed using the LUNDON-1: Saturation Analysis: Radioligand Binding curve analysis package (Lundon Software Inc., Cleveland, Ohio).

Results and Discussion

Rat biodistribution studies were performed at $t = 30$ min (see Table 1). The expected differences in brain penetration of quaternized ([^{11}C]MTRB and [^{11}C]MQNB) and neutral ([^{11}C]TRB) mAChR ligands was observed: the neutral ligand has a 300-fold higher concentration in brain than the quaternized (positively charged) ligands. However, both neutral and quaternized radioligands had comparable pituitary uptake at 30 min. Heart uptake was higher for [^{11}C]MTRB than for either [^{11}C]MQNB or [^{11}C]TRB

Table 1. Comparison of [^{11}C]MTRB, [^{11}C]MQNB and [^{11}C]TRB in various rat* tissues at $t = 30$ min; data as %ID/g ± SEM

Tissue	[^{11}C]MTRB†	[^{11}C]MQNB†	[^{11}C]TRB‡
Brain	0.009 ± 0.001	0.010 ± 0.000	2.95 ± 0.17
Pituitary	1.03 ± 0.12	0.841 ± 0.138	1.58 ± 0.19
Heart	11.75 ± 0.91	7.71 ± 1.54	4.95 ± 0.11
Blood	0.032 ± 0.005	0.035 ± 0.014	0.111 ± 0.027

*Fischer F344 female rats. †$N = 3$. ‡$N = 10$.

(for further discussion of [^{11}C]MTRB in the myocardium see Mulholland *et al.*, 1988c).

Blockade by QNB pretreatment of mAChR, at $t = 30$ min, was employed in order to demonstrate receptor mediation as the mechanism for tissue localization. Figure 2 shows the effects of QNB pretreatment (2.0 mg/kg of ±QNB 60 min prior to radiotracer administration) on the radioactivity localization in heart, pituitary and brain for [^{11}C]TRB, [^{11}C]MTRB and [^{11}C]MQNB. QNB clearly reduced the tissue concentration of all compounds in the myocardium. Unexpectedly, the effects of QNB on pituitary uptake of neutral ([^{11}C]TRB) and quaternized ([^{11}C]MTRB and [^{11}C]MQNB) mAChR ligands were different. The uptake of [^{11}C]TRB was reduced by 17% when pretreated with QNB (control: 1.59 ± 0.19% ID/g; QNB: 1.31 ± 0.09) whereas the concentration of [^{11}C]MTRB in the pituitary was reduced by 68% and that of [^{11}C]MQNB by 85%. Clearly the localization in normal pituitary of [^{11}C]MTRB and [^{11}C]MQNB can be significantly reduced by QNB pretreatment whereas only a small reduction is observed for [^{11}C]TRB.

The pituitary uptake of [^{11}C]TRB is similar to that reported for [^{3}H]NMPB, a neutral mAChR antagonist (Avissar *et al.*, 1981). Using a different protocol for QNB pretreatment, the uptake of [^{3}H]NMPB was reduced by about 30% in the pituitary. The QNB pretreatment protocol of Avissar *et al.* (1981) was followed except for the use of 6.6 mg/kg body weight of ±QNB in place of 3.3 mg/kg of active QNB. This

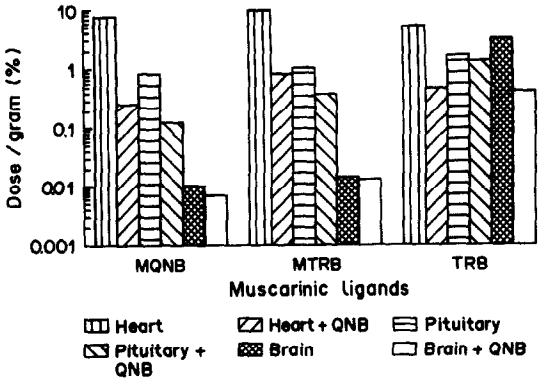

Fig. 2. Comparison of the effect of QNB on pituitary, heart and brain uptake at $t = 30$ min. Fischer F344 rats were injected with vehicle only or with 2.0 mg/kg body weight 60 min prior to radioligand administration. Data are the average of $N = 3$ for [^{11}C]MQNB and [^{11}C]MTRB; $N = 10$ for [^{11}C]TRB.

protocol resulted in a reduction of [^{11}C]TRB localization in brain and heart by >79% but a reduction in the pituitary of less than 36%. These results again demonstrate the inability of QNB to reduce pituitary localization of [^{11}C]TRB to the extent observed in other tissues containing mAChR receptors. We conclude that neutral mAChR antagonists behave differently in the pituitary than the quaternized antagonists. It is possible that the localization of [^{3}H]NMPB and [^{11}C]TRB in the pituitary is a combination of a non-mAChR specific uptake mechanism and a receptor-mediated mechanism with non-mAChR uptake predominating. Differences in uptake between neutral and quaternized antagonists have been reported in the lung (Gibson *et al.*, 1979). Similar results, i.e. largely non-receptor-mediated localization, were reported for the pituitary uptake of [^{3}H]-spiroperidol, a dopaminergic D_2 antagonist (Otto *et al.*, 1986b). Spiroperidol is similar to TRB and NMPB in that all are neutral amines. These results

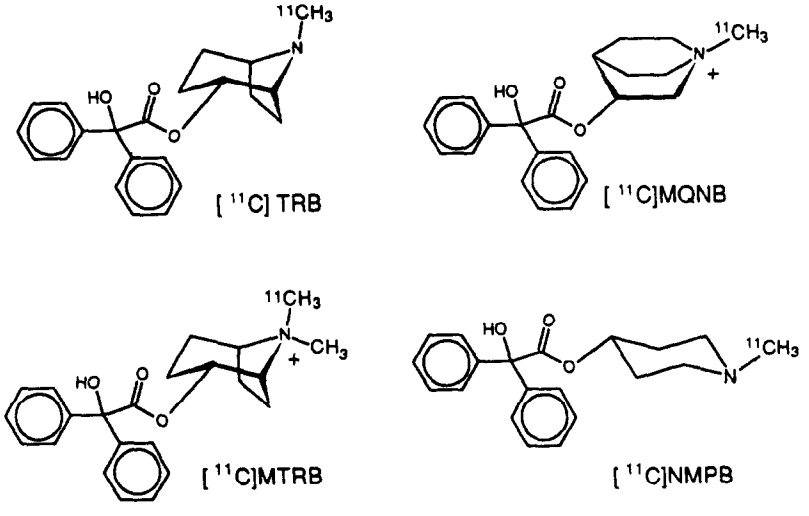

Fig. 1. Structures of mAChR antagonists. [^{11}C]TRB and [^{11}C]NMPB are neutral ligands; [^{11}C]MTRB and [^{11}C]MQNB are quaternized.

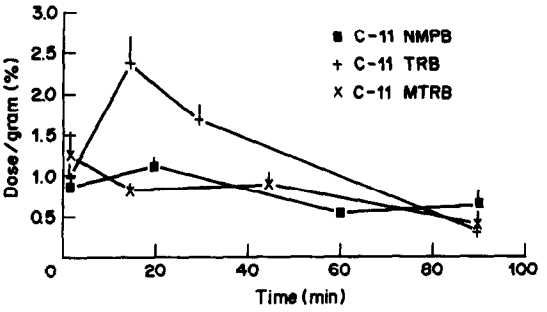

Fig. 3. Time course of radioactivity concentration of [^{11}C]TRB, [^{11}C]MTRB and [^{11}C]NMPB in pituitary tissue of Sprague–Dawley rats. Data as %ID/g ± SEM; $N = 3$ for each data point.

suggest that a general mechanism for uptake of neutral amines operates in the pituitary.

We also compared the time course of [^{11}C]MTRB, [^{11}C]TRB and [^{11}C]NMPB in the pituitary of Sprague–Dawley rats (see Fig. 3). [^{11}C]TRB has higher pituitary localization values than either [^{11}C]NMPB or [^{11}C]MTRB. Despite the differences in effects of QNB pretreatment (about 30% for [^{3}H]NMPB and 68% for [^{11}C]MTRB), the radioactivity concentration of both ligands is similar at each time point.

To study the effect of DES exposure, the time course of [^{11}C]MTRB in rats exposed to DES for 10 weeks, the effects of QNB pretreatment and the effects of differing lengths of exposure to DES on [^{11}C]MTRB localization were determined. In contrast to the time course of [^{11}C]MTRB in Fischer F344 rats (1.49 ± 0.31 %ID/g at $t = 2$ min to 1.71 ± 0.42 at $t = 60$ min), the time course in DES-implanted animals showed a steadily decreasing %ID/g tumor over the same time frame (0.392 ± 0.037 to 0.134 ± 0.002). Receptor-mediated localization was demonstrated as above. QNB pretreatment of rats exposed to DES for 6 weeks reduced [^{11}C]MTRB tumor localization by 77% (%ID/g for controls was 0.442 ± 0.107; for QNB treated rats, 0.100 ± 0.025). As the length of exposure increases from 0 to 15 weeks, the %ID/organ of [^{11}C]MTRB increases from 0.009 ± 0.002 for controls to 0.209 ± 0.008 at 15 weeks of DES, a 23-fold increase. However, when corrected for increasing tumor weight the %ID/g tumor decreased from 1.03 ± 0.12 (control rats) to 1.28 ± 0.22 at 6 weeks DES to 0.830 ± 0.00g at 15 weeks DES.

Histological characterization of the DES-induced tumors after 4 weeks of DES exposure in female F344 rats showed (1) that the percent of prolactin (PRL) secreting cells increased from 32.3 ± 1.4% to 79.7 ± 1.0, a 2.5-fold increase, and (2) that there was a 5-fold increase in serum PRL levels (Otto *et al.*, 1986a). Since the %ID/g tumor decreases while the percent of PRL secreting cells increases, it appears that there may be changes in either B_{max} or K_D relative to normals. It is not clear whether these results are due to DES or to high circulating levels of prolactin. The presence of estrogen affected *in vitro* agonist binding to mAChR by reducing the proportion of high affinity binding sites and by decreasing the dissociation constant (Sokolovsky *et al.*, 1981). If estrogen effects occur *in vivo*, the changes in localization of [^{11}C]-MTRB, albeit an antagonist instead of an agonist, may be related to DES presence. Efforts are underway to determine these binding characteristics in DES-treated pituitary tissue.

The heart uptake of [^{11}C]MTRB appears to be constant from 6 to 15 weeks of exposure to DES, suggesting that minimal change in myocardial mAChR density or in kinetic behavior results from high circulating PRL levels (Otto *et al.*, 1986a). Competitive binding assays using hearts from the DES treated rats at 10 and 16 weeks post implant were compared to data from normal hearts. [^{3}H]NMS (Brown and Goldstein, 1986; Aguilar and De Robertis, 1987) was employed as radioligand in this assay as it is a quaternized ligand and thus similar to [^{11}C]-MTRB. In each case, analysis of binding data using LUNDON-1 was performed. Comparison of a model for one site with a model for one site plus non-saturable binding (the next most complex binding model), yielded F test scores of <0.01 and runs test scores of >0.05. Both scores support the one site model as the best fit for the data (Lundeen and Gordon, 1986). A minimum of 14 different ligand concentrations were included in each curve analysis. Non-linear analyses were used as opposed to Scatchard–Rosenthal analysis to determine K_D and B_{max} values. Values of K_D did not change: for normal hearts, K_D was $5.8 \pm 0.3 \times 10^{-11}$ mol; for 10 week DES hearts, $4.8 \pm 0.4 \times 10^{-11}$; for 16 week DES, $5.0 \pm 0.25 \times 10^{-11}$. Values for B_{max} were similar for control hearts ($8.6 \pm 0.4 \times 10^{-14}$ mol/mg protein) and 10 week DES hearts ($7.9 \pm 0.2 \times 10^{-14}$). These data are in agreement with a study which showed that the density of mAChR, as measured with [^{3}H]QNB, was constant in rats implanted with the MtTW15 adenoma for 5 weeks (Nelson *et al.*, 1987). B_{max} values in 16 week DES hearts increased to $16 \pm 2 \times 10^{-14}$. Further study is needed to verify and determine the significance of this increase.

In conclusion, these studies have shown that [^{11}C]TRB localization in the pituitary is not primarily receptor-mediated whereas the localization of the quaternized ligands, [^{11}C]MTRB and [^{11}C]MQNB is receptor-mediated as demonstrated by reduction of radioactivity levels in the presence of QNB. The presence of DES-induced prolactinomas decreased tissue concentration of [^{11}C]MTRB in the pituitary. DES treatment did not affect [^{11}C]MTRB localization in the myocardium as evidenced by tissue concentration values and by similarity in K_D and B_{max} value in normal and 10 week DES-treated rat hearts.

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