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Pretreatment With an Irreversible Muscarinic Agonist Affects Responses to Apomorphine

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CROCKER, A. D. AND R. W. RUSSELL. *Pretreatment with an irreversible muscarinic agonist affects responses to apomorphine.* PHARMACOL BIOCHEM BEHAV **35**(3) 511–516, 1990.—The main aim of the present study was to investigate if responses to the direct dopamine agonist, apomorphine, could be modified by changes in the activity of cholinergic neurones. A novel approach was adopted in which these responses were assessed following reduction of muscarinic receptor concentration (mAChR) in the brain (assessed from [^{3}H] QNB binding) by the alkylating derivative of oxotremorine, N-[4-(2-chloroethylmethylamino)]-2-pyrrolidone (BM 123). Stereotyped responses elicited by apomorphine were significantly reduced when QNB binding was 12% and 50% of control values. No changes in [^{3}H] spiperone binding were found. There was significant hypothermia in the group with 12% QNB binding sites which was significantly increased by apomorphine. Body temperature returned to normal when QNB binding was 50% of control values. There was a significant decrease in activity when QNB sites were reduced to 12% of normal and vertical activity was still significantly reduced at 50% QNB binding, though horizontal activity was not then different from controls. These data are consistent with the hypothesis that changes in the function of mAChR modify responses elicited by dopamine receptor stimulation in both the striatum and other brain regions.

Irreversible muscarinic agonist | N-[4-(2-chloroethylmethylamino)]-2-pyrrolidone (BM 123) | Apomorphine | [^{3}H] QNB binding | Brain | Muscarinic receptors | Dopamine receptors | Stereotypy | Striatum | Body temperature | Activity | Rat

SEVERAL mechanisms, including changes in receptor concentration and coupling to second messengers, have been shown to affect responses to agonists in isolated organ systems (18). However, in determining what mechanisms are involved in modifying agonist responsiveness in the central nervous system it is important to take into account that changes in the activity of neurones downstream of the receptor may also be involved.

We have demonstrated that increased responsiveness to dopamine agonists can occur in the absence of changes in dopamine receptor concentration or dopamine stimulated adenylate cyclase activity (7, 15–17). The end-point assessed in these experiments was the incidence of stereotyped head-down sniffing which we have shown to be the result of stimulation of D2 dopamine receptors in the ventral striatum (4,9). Since it is well accepted that some dopamine neurones in the striatum synapse on cholinergic interneurons (21) and their activation results in an inhibition of acetylcholine release (2) it is likely that changes in the functioning of these cholinergic neurones affect responses to dopamine agonists. This hypothesis is supported by observations that stereotyped responses elicited by dopamine agonists were enhanced by administration of muscarinic antagonists (29). Conversely, these responses were decreased by muscarinic agonists (1) and in rats with increased concentration of striatal muscarinic receptors (5).

The main aim of the present study was to investigate these interactions further using a novel approach in which responses to a direct dopamine agonist, apomorphine, were assessed following reduction of muscarinic receptor (mAChR) concentration to approximately 10% and 50% of normal. Reduction of mAChR was achieved by intravenous injection of an irreversible alkylating derivative of oxotremorine, N-[4-(2-chloroethylmethylamino)]-2-pyrrolidone (BM 123), converted in aqueous solution at neutral pH to an aziridinium ion which binds specifically and covalently to mAChR (10, 26, 27). Previous experiments showed that treatment with BM 123 could reduce the concentration of mAChR in the brain to approximately 10% of normal as shown by [^{3}H] quinuclidinyl benzilate binding (28,32).

In the present study preliminary experiments were carried out to establish the dose response relationship between BM 123 and mAChR concentration so that an appropriate dose of the alkylating agent could be selected. Subsequent experiments were designed so

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that the effects of BM 123 and apomorphine separately could be compared with their combined effects. Besides recording effects of these treatments on stereotyped head-down sniffing, as an index of the modification of striatal dopamine receptor function, spontaneous activity and core body temperature were assessed because these variables are also affected by stimulation of dopaminergic and muscarinic receptors in other brain regions. A preliminary account of these findings has been presented (8).

METHOD

Animals

Male Sprague-Dawley rats were housed in groups of ten in large colony cages and kept in temperature- and humidity-controlled rooms with continuous illumination. Laboratory chow and drinking water were available ad lib. Rats were weighed daily during the experimental period as an index of their general health.

Pharmacological Treatments

N-[4-(2-chloroethylmethylamino)]-2-pyrrolidone (BM 123) was obtained from the Department of Pharmacology, University of California (Los Angeles, CA). It was dissolved in 100% ethanol at 0°C to give a stock solution of 100 mM from which appropriate dilutions were made in ice-cold saline no more than 10 minutes before intravenous injection. The intravenous route (via the tail vein) was selected to maximise the changes of BM 123 reaching the brain before conversion to the quaternary aziridinium ion which does not penetrate the blood-brain barrier. Control rats, hereafter referred to as the saline group, received vehicle.

Apomorphine (Sigma, St. Louis, MO) was dissolved in saline to give a final concentration of 1.6 μmols·ml^{-1} and kept for no longer than 30 minutes at 5°C prior to injection. Apomorphine and saline were injected subcutaneously in volumes of 1 ml·kg^{-1}.

Binding Assays

Rats were sacrificed by cervical dislocation and the brains removed. Frontal cortex, striatum and hippocampus were dissected over ice and homogenised in 20 vol. of Tris-HCl buffer (0.05 M, pH 7.4). Homogenates were centrifuged at 48,000 × g and the pellets resuspended in fresh buffer. This procedure was repeated twice before storage at −80°C for no longer than two weeks.

[^{3}H] QNB Binding

Washed homogenates of striatum, cortex and hippocampus (0.5 mg wet weight of tissue/tube) were incubated with [^{3}H] QNB (Radiochemical Centre, Amersham) ranging in final concentration from 0.01–1.0 nM at 37°C for 15 min as described previously (25). Nonspecific binding was defined using 1 μM atropine. Binding data were analysed using a nonlinear curve fitting programme (19) to determine the B_{max} and K_D. Concentration of mAChR was expressed as pmol·100 mg^{-1} protein and K_D in nM. Protein concentration was assayed using the method of Lowry *et al.* (23).

[^{3}H] Spiperone Binding

Ten mg aliquots of striatal tissue were incubated with concentrations of [^{3}H] spiperone (20 Ci mmol^{-1}, Radiochemical Centre, Amersham) ranging from 0.01–1.0 nM at 37°C for 15 minutes as described previously (6). Nonspecific binding was defined using 1 μM (+)-butaclamol. Binding data were analysed by the method of Holford (19) to obtain B_{max} and K_D.

General Signs

The occurrence of three general signs of muscarinic receptor stimulation was recorded at 5-min intervals starting immediately after an injection of BM 123 or saline and continuing for 30 min. Tremor was selected as a standard general indicator of CNS, and chromodacryorrhea and salivation, of peripheral involvement (28).

Core Body Temperature

Body temperature was determined by inserting a thermistor probe 6–8 cm into the rectum. The probe was attached to a CRL digital recorder and temperatures measured to one decimal place after the reading had been constant for 10 sec.

Spontaneous Activity and Stereotypy

Assessment of activity and stereotypy was carried out in an open field as described previously (3) by an observer blind to the treatments rats had received. Locomotor activity (number of lines crossed) and rearing activity (number of rears) were assessed for 1-min periods at 10, 15, 20 and 25 min after injection of apomorphine or saline.

Stereotypy was also rated during these 1-min periods as the frequency of head-down sniffing which exhibits a clear dose-response relationship with apomorphine (3). The following rating scale was used: 0 = rat asleep; 1 = absence of sniffing; 2 = infrequent bursts of sniffing; 3 = frequent sniffing; 4 = continuous sniffing. The ordinal data were analysed using nonparametric methods (31).

Statistical Analysis

For analyses of all data, except those for general signs and stereotypy, parametric statistics were used and results are expressed as mean ± S.E.M. ANOVAs, when significant at $p<0.05$, were followed by post hoc comparisons (20,30). Fisher's Exact Test was used for analyses of differences in the general signs and Kruskal-Wallis followed by Mann-Whitney tests for the stereotypy data (31).

Selection of Dose of BM 123

Preliminary experiments were carried out to determine what dose of BM 123 could be used in the main experiment to reduce [^{3}H] QNB binding maximally without causing substantial mortality.

First, the relationship between dose of BM 123 and reduction in QNB binding was established for three brain regions, striatum, hippocampus and cortex. Results are shown in Fig. 1. B_{max} decreased as a hyperbolic function as the dose of BM 123 increased, in all three brain regions assayed, approaching asymptotes at approximately 8 pmol·100 mg^{-1} protein. Binding was reduced to less than 20% of normal with doses greater than 23 μmol·kg^{-1} and to below 10% at 63 and 100 μmol·kg^{-1}. There were no statistically significant differences between the three brain regions and no significant effects on the dissociation constant, K_D, which ranged from 0.05–0.11 nM and were similar to those reported previously (25).

Next, it was important to determine the LD_{50} for single doses of BM 123 which was done using the up-and-down technique of Dixon (11). Doses of BM 123 beginning at 10 μmol·kg^{-1} and increasing by 0.2 $\log_{10}$ units were chosen. Two independent

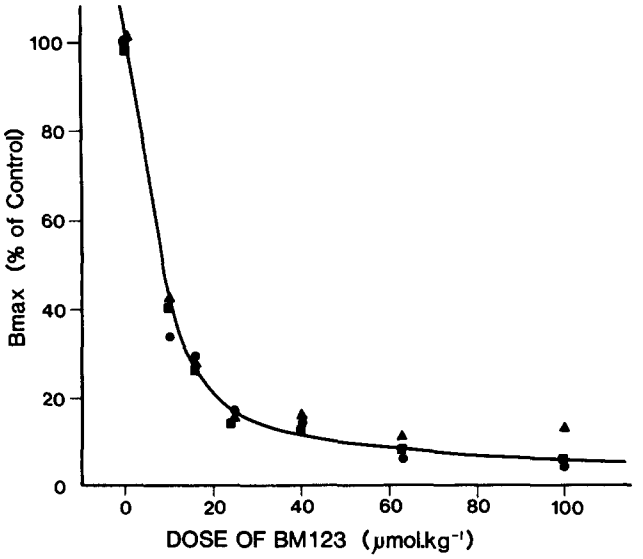

FIG. 1. Dose-effect relationship between the dose of BM 123 administered intravenously and B_{max} for [^{3}H] QNB binding in striatum ■, hippocampus ● and cortex ▲. Results for B_{max} are expressed, for each brain region, as percentage of vehicle-injected controls.

replications were carried out and observations made over a period of 30 min following injection. Analysis of results, using Dixon's tables (11), established the LD_{50} at 77.7 μmol·kg^{-1} for the first replication and 79.3 μmol·kg^{-1} for the second.

Records were kept of general signs induced by BM 123 and saline injections. None of the signs were seen in the rats receiving saline, whereas BM 123 injections were followed in the first 5 min by tremor in all animals. Chromodacryorrhea and salivation also appeared in this latter group at the same time and persisted for 10–15 min.

Experimental Plan

A dose of 63 μmol·kg^{-1} was selected for use in the main experiment to achieve maximum reduction of [^{3}H] QNB binding without substantial mortality, since this dose was 0.1 $\log_{10}$ unit below the LD_{50}. Two deaths occurred in rats (n = 26) receiving this dose, which reduced QNB binding by 90% 4 hr after injection (Fig. 1). A previous study had established the time of recovery of QNB binding (28) and these data were used to determine that 24 hr following injection of 63 μmol·kg^{-1} BM 123, QNB binding would have recovered to approximately 50% normal. Therefore, in the main experiment, rats (BM-apo) were challenged with apomorphine either 4 or 24 hr following BM 123 injection when QNB binding was expected to be reduced by approximately 90% and 50%, respectively.

A four group experimental design was employed at each of the 4-hr and 24-hr time periods so that the responses of BM-apo rats could be compared to those of rats injected first with BM 123 and subsequently challenged with saline (BM-sal) and also to rats injected first with saline and subsequently challenged with apomorphine (sal-apo) or saline (sal-sal), respectively. Assignment to groups (N = 6/group) was made randomly with the single restriction that there should be no significant differences in body weights among the groups at the 4- and 24-hr time periods, which were 307 ± 23.5 g and 295 ± 13.4 g, respectively.

Rats in all four groups were weighed and their core body temperatures recorded before initial injection. They were then injected with either 63 μmol·kg^{-1} BM 123 or an equivalent volume of vehicle into the tail vein. This was followed at either 4 or 24 hr by SC injection of 1.6 μmol·kg^{-1} of apomorphine or an equivalent volume of saline (1 ml·kg^{-1}). Ten min after the apomorphine or saline injection rats were placed individually in the open field apparatus and horizontal (locomotion), vertical (rearing) and stereotyped behaviour observed for 15 min as described earlier. This was followed 30 min after injection by measurement of core body temperature before animals were sacrificed and their brains removed for ligand binding assays.

TABLE 1

EFFECTS ON STRIATAL [^{3}H] QNB BINDING 4 AND 24 HOURS AFTER PRETREATMENT WITH SALINE OR BM 123 FOLLOWED BY SALINE OR APOMORPHINE

	4 hr After Pretreatment		24 hr After Pretreatment	
Group	B_{max} (pmol·100 mg^{-1}) protein	% Control B_{max}	B_{max} (pmol·100 mg^{-1}) protein	% Control B_{max}
Sal-sal	244.3 ± 11.67	100	230.2 ± 10.57	100
Sal-apo	254.2 ± 4.15	104	213.8 ± 10.45	93
BM-sal	28.9 ± 5.93*	12	112.6 ± 7.76*	49
BM-apo	29.8 ± 5.80*	12	114.2 ± 12.47*	50
F(3,20)	284.72		35.38	
p	<0.001		<0.001	

Results expressed as mean ± SEM.

*$p<0.001$ compared with corresponding Sal-sal value.

There were no significant differences in K_D [4 hr, $F(3,20) = 2.20$, $p = >0.05$; 24 hr, $F(3,20) = 0.83$, $p = >0.05$].

RESULTS

Reduction in QNB Binding

Effects of single injections of BM 123 (63 μmol·kg^{-1}) on striatal [^{3}H] QNB binding are summarised in Table 1 which shows there are highly significant differences in B_{max} at 4 and 24 hr. Scheffe comparisons showed that the two groups injected initially with BM 123 4 hr before had essentially identical B_{max} values for striatum which were 12% those of the corresponding saline controls ($p<0.001$). The same held for the groups injected with BM 123 24 hr previously when QNB binding had returned to 49% and 50% of control values ($p<0.001$). Similar results were obtained for cortex and hippocampus where QNB binding was reduced to 21% and 13%, respectively, 4 hr after BM 123 injection and to 49% and 47% of control values at 24 hr.

Effects on Spiperone Binding

ANOVA of striatal [^{3}H] spiperone binding data showed no differences in either B_{max} or K_D between the four treatment groups at either 4- or 24-hr time periods. B_{max} values ranged from 19.7 ± 2.1 to 23.2 ± 1.5 pmol·100 mg^{-1} protein and K_D values from 0.06 to 0.11 nM.

General Signs

General signs of mAChR stimulation were observed in all animals receiving BM 123, but not in those receiving saline injections ($p<0.001$; Fisher's Exact test). Tremor was evident during the first 5 min following BM 123 injection, but never

TABLE 2

EFFECTS OF APOMORPHINE ON HEAD-DOWN STEREOTYPED SNIFFING IN RATS PRETREATED WITH BM 123 OR SALINE

Group	4 hr After Pretreatment	24 hr After Pretreatment
Sal-sal	2 (1–2)	1 (1–2)
Sal-apo	4 (3–4)*	4 (4)‡
BM-sal	1 (0–1)†	0 (0–1)
BM-apo	2 (1–3)	3 (2–3)§

Results expressed as medians (range) for each group.
*$p<0.04$ cf. BM-apo; †$p<0.004$ cf. Sal-sal at 4 hr.
‡$p<0.004$ cf. BM-apo; §$p<0.008$ cf. Sal-sal at 24 hr.

persisted longer than 5 min. Chromodacryorrhea and salivation also appeared during this period, the former lasting for no longer than 10 min, but salivation persisting for 15–20 min following injection. The presence of these signs immediately following injection of BM 123 confirmed its initial action as a muscarinic agonist.

Stereotypy

The effects of the various treatments on stereotypy are summarised in Table 2 as medians and ranges. As expected, apomorphine significantly increased stereotypy in the groups receiving initial treatment with saline at both 4 and 24 hr ($p<0.001$ in each case). However, apomorphine-induced stereotypy was significantly reduced in the groups receiving BM 123 4 hr ($p<0.04$) and 24 hr ($p<0.004$) previously. In addition, BM 123 alone suppressed sniffing behaviour and analyses of the differences between the sal-sal and BM-sal groups showed significant changes at both 4-hr ($p<0.004$) and 24-hr ($p<0.008$) time periods.

Core Body Temperature

Effects of apomorphine on core body temperature are presented in Table 3. There were no significant pretreatment differences between the four groups. However, ANOVA for measures after apomorphine or saline challenge showed highly significant differences at both 4- and 24-hr time periods: $F(3,20)=32.17$, $p<0.001$ at 4 hr and $F(3,20)=20.63$, $p<0.025$ at 24 hr. Post hoc analyses established that, when compared with the control group treated only with saline (sal-sal), there was significant hypothermia ($p<0.01$) in the three groups receiving BM 123 initially and/or challenged 4 hr later with apomorphine. The sal-apo and BM-sal group exhibited the same degree of hypothermia, whereas the BM-apo group was significantly ($p<0.01$) more hypothermic than all other groups. Thus, stimulation of either cholinergic or dopaminergic systems produced hypothermia and stimulation of both together was additive. Twenty-four hr after BM 123 injection, the BM-sal group had returned to control levels and only groups injected with apomorphine (BM-apo and sal-apo) differed significantly ($p<0.01$) from saline controls (sal-sal), but not from each other.

Spontaneous Activity

The total locomotor and rearing activity observed during the 15-min sampling period at 4 and 24 hr are shown in Table 4, together with ANOVAs for differences between the groups. Three of the four ANOVAs show the existence of highly significant differences between the groups, the exception being the ANOVA for locomotor activity at 24 hr after initial injections. At this time the levels of locomotor activity in the groups receiving BM 123 (BM-sal; BM-apo), although reduced were not significantly different from control (sal-sal) levels.

Post hoc comparisons of the data for locomotor activity at 4 hr established that the two groups treated initially with BM 123 were

TABLE 3

EFFECTS OF APOMORPHINE FOLLOWING BM 123 OR SALINE PRETREATMENT ON CORE BODY TEMPERATURE

Group	Core Body Temperature (°C) Pretreatment Mean SEM	Postchallenge Mean SEM	% Control Body Temp.
Sal-sal			
4 hr	38.05 ± 0.10	37.95 ± 0.11	100
24 hr	37.57 ± 0.12	37.77 ± 0.07	100
Sal-apo			
4 hr	37.83 ± 0.20	36.80 ± 0.26*†	96
24 hr	37.80 ± 0.11	36.48 ± 0.27‡	97
BM-sal			
4 hr	37.97 ± 0.15	36.37 ± 0.21*†	96
24 hr	37.48 ± 0.16	37.60 ± 0.14	100
BM-apo			
4 hr	38.00 ± 0.22	34.63 ± 0.33*	91
24 hr	37.72 ± 0.22	35.85 ± 0.25‡	95

Results expressed as mean ± SEM.
*$p<0.01$ cf. 4 hr Sal-sal group; †$p<0.01$ cf. 4 hr BM-apo group; ‡$p<0.01$ cf. 24 hr Sal-sal group.

TABLE 4

EFFECTS OF APOMORPHINE CHALLENGES ON LOCOMOTOR AND REARING ACTIVITY FOLLOWING BM 123 OR SALINE PRETREATMENT

Group	Locomotion	Rearing
Sal-sal		
4 hr	58.8 ± 6.39	15.3 ± 3.23
24 hr	70.5 ± 7.61	16.5 ± 2.40
Sal-apo		
4 hr	56.6 ± 9.93	3.0 ± 1.29‡
24 hr	42.5 ± 10.04	4.6 ± 1.36§
BM-sal		
4 hr	3.6 ± 0.80*†	1.0 ± 0.63‡
24 hr	37.1 ± 3.53	6.0 ± 0.68§
BM-apo		
4 hr	8.3 ± 1.67*	1.33 ± 0.42‡
24 hr	54.6 ± 12.00	4.50 ± 0.88§
ANOVA		
4 hr	*F = 25.77 $p=<0.001$	*14.72 <0.001
24 hr	*F = 2.55 $p=>0.05$	*20.92 $p<0.001$

*All $df=3,20$.

Results expressed as means ± SEM.
*$p<0.01$ cf. Sal-sal at 4 hr; †$p<0.01$ cf. Sal-apo and BM-apo at 4 hr; ‡$p<0.01$ cf. Sal-sal at 4 hr; §$p<0.01$ cf. Sal-sal at 24 hr.

very significantly hypoactive ($p<0.01$), whereas the apomorphine challenges had no effect on activity.

Rearing activity was significantly reduced ($p<0.01$) in groups receiving either BM 123 or apomorphine at both 4- and 24-hr time periods and Scheffe comparisons showed no differences between these treatments. However, since either treatment produced almost total reduction in rearing, it was not possible to determine if the effects of BM 123 and apomorphine were additive.

DISCUSSION

During the past fifteen years experimental evidence has accumulated to support various hypotheses about interactions between dopaminergic and cholinergic system in the brain. The present experiments were designed to study such interactions using a novel approach in which the DA system was challenged by acute administration of the agonist, apomorphine, at mAChR concentrations of 12 and 50% of control values which were obtained using the irreversible muscarinic agonist, BM 123. The active species of BM 123, its aziridinium ion, which is formed by cyclization, has potent and selective muscarinic agonist effects (27).

The reaction sequence is as follows:

$$A + R \rightleftarrows AR \rightarrow AR^* \rightarrow E1 \rightarrow E2 \rightarrow E3 \rightarrow En$$

where A is the aziridinium ion: R, mAChR; AR, the initial, reversible ion-receptor complex; AR*, the irreversible alkylated complex; and the Es, various end-points in the sequence of events between AR* and the physiological and behavioural variables we have measured. Because the rate constant for $AR \rightarrow AR^*$ is 0.02–0.3 min^{-1} (13), agonist effects lasting more than 1–2 hr cannot be due to AR and must be associated with AR*.

Thus, in the present studies, the short-lived effects seen in the first 15 min following BM 123 injections, i.e., tremor, chromodacryorrhea and salivation, are consistent with central and peripheral mAChR stimulation by the aziridinium ion and confirm previous findings (28). They are also consistent with the findings of Ehlert *et al.* (12) and Ehlert and Jenden (13) who observed that the initial effects of BM 123 on guinea pig ileum were due to stimulation of mAChR. However, the effects observed on the dependent variables at 4 and 24 hr after BM 123 injection, when 88% and 50%, respectively of mAChR were in the alkylated state, were due to AR*.

The reduction of striatal QNB binding to 12% or 50% of control values was associated with a significant reduction in stereotyped head-down sniffing responses elicited by apomorphine. These effects were not due to changes in the concentration or affinity of striatal D2 dopamine receptors because these were unchanged following BM 123 treatment. Since the head-down sniffing response has been shown to be elicited by stimulation of D2 dopamine receptors located in a distinct area of the ventral striatum (4), these findings provide direct support that the cholinergic system can modulate responses mediated by striatal dopamine receptors.

Treatment with cholinergic antagonists have been reported to increase (29), while cholinergic agonists decrease (1) stereotyped responses to apomorphine. From these latter reports it would be predicted that reducing mAChR concentration would result in increased stereotyped responses to apomorphine, not the decrease observed in the present study. The reason for the decreased stereotyped sniffing response is unknown, although one possibility is that the alkylated receptor complex, AR*, is functionally active in terms of initiating prolonged cellular responses. This suggestion, although unlikely in terms of the effects reported using other alkylating agents (24), has not been excluded by experiments (13,27) carried out in isolated systems. For example, Ehlert and Jenden have reported that in the guinea pig myenteric plexus preparation, BM 123 caused inhibition of the evoked release of acetylcholine that persisted after extensive washing and was not reversed by atropine (13). These data are consistent with a sustained and possibly irreversible agonist effect of the alkylated receptor complex.

In this context it is interesting that when QNB binding sites were reduced by 88%, 4 hr after BM 123 injection, the BM-sal group exhibited significant hypothermia, and significant reductions in spontaneous locomotor activity, both effects observed after systemic administration of a cholinergic agonist (22,25).

Further support for a prolonged agonist effect of AR* comes from the combined effects of BM 123 (4 hr after injection) and apomorphine on body temperature which, although reduced significantly by BM 123 and apomorphine alone, was further significantly reduced by a combination of the two treatments. Both cholinergic (22) and selective D2 dopaminergic agonists (14) have been reported to produce hypothermia in rats through stimulation of hypothalamic receptors and the effects of activating both receptor types are additive (5).

The data also give an indication of the receptor reserve available for the different functions assessed. For example, apomorphine-induced stereotypy and rearing activity were significantly reduced at both 4 hr and 24 hr after BM 123 treatment, whereas by the time QNB binding sites had recovered to 50% of control values, at 24 hr following BM 123 injection, hypothermia had disappeared and locomotor activity, although reduced, was not significantly different from control levels. The differences in the recovery of these dependent variables may well reflect differences in receptor reserve for these functions.

In conclusion, the data are consistent with the hypothesis that changes in the function of mAChR modify responses elicited by dopamine receptor stimulation in both the striatum and other brain regions, such as the hypothalamus. However, since the alkylating agent was administered systemically in these experiments it was not possible to determine the location of these mAChR. Preliminary experiments investigating the effects of intrastriatal injections of the aziridinium ion of BM 123 are in progress to clarify their location.

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