MOLECULAR PHARMACOLOGY, 14, 246-255

Muscarinic Cholinergic Receptors in Rat Corpus Striatum and Regulation of Guanosine Cyclic 3',5'-Monophosphate

MICHAEL R. HANLEY[1] AND L. L. IVERSEN

Medical Research Council Neurochemical Pharmacology Unit, Department of Pharmacology, Cambridge University Medical School, Cambridge CB2 2QD, England

(Received September 6, 1977)
(Accepted October 21, 1977)

SUMMARY

HANLEY, MICHAEL R. & IVERSEN, L. L. (1978) Muscarinic cholinergic receptors in rat corpus striatum and regulation of guanosine cyclic 3',5'-monophosphate. *Mol Pharmacol.*, 14, 246-255.

Binding of cholinergic agonists and antagonists to muscarinic receptors in slices of rat striatum was assessed by their ability to displace the specific ligand [^{3}H]quinuclidinyl benzilate (QNB), and these results were compared with the effects of these drugs in eliciting changes in cyclic 3',5'-GMP in the same preparation *in vitro*. The agonists oxotremorine, arecoline, and carbachol caused transient increases in cyclic GMP, reaching a maximum after 2 min of incubation. The increase in cyclic GMP amounted to a 6-fold change over basal levels in the absence of the phosphodiesterase inhibitor isobutylmethylxanthine, or a doubling over basal levels in its presence. There was also a significant increase in tissue cyclic AMP, which, like the cyclic GMP response, was antagonized by muscarinic blocking drugs. The EC_{50} values for agonists in increasing cyclic GMP were similar to the estimated K_i values for these compounds in inhibiting QNB binding; thus the cyclic GMP response appears to be linearly related to muscarinic receptor occupancy. Muscarinic antagonists and some neuroleptic drugs antagonized the cyclic GMP response elicited by oxotremorine, with relative potencies as follows: scopolamine > QNB > atropine > clozapine > thioridazine > chlorpromazine > haloperidol. Similar rank orders and relative potencies were seen for these drugs as inhibitors of QNB binding. Pilocarpine increased cyclic GMP at low concentrations, but this effect was no longer seen at higher concentrations. The cyclic GMP response to oxotremorine required the presence of calcium ions in the external medium, and was maximal at calcium concentrations above 1 mM. Sodium azide caused an increase in tissue cyclic GMP which was additive to that elicited by oxotremorine. It is suggested that occupation of muscarinic receptors may activate a soluble intracellular guanylate cyclase indirectly, with calcium possibly acting as the intracellular mediator. The ability of some neuroleptic drugs to inhibit the cyclic GMP response to muscarinic agonists in brain slices adds further weight to the view that such compounds may possess important antimuscarinic actions in mammalian central nervous system.

This work was supported by a grant from the Scottish Rite Schizophrenia Research Program.

[1] Permanent address, Department of Molecular Biology and Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720.

0026-895X/78/0142-0246$02.00/0

INTRODUCTION

The second messenger role of cAMP[2] in hormonal activity has prompted research into its involvement in synaptic transmission (1, 2). Ample evidence has accumulated that some putative neurotransmitters can alter cAMP levels in both intact and cell-free systems (3, 4), and these changes have been linked to the physiological control of postsynaptic potentials (5).

By analogy, a mediator role has also been postulated for cGMP. Cholinergic agonists increased cGMP levels in heart (6), smooth muscle (7), secretory systems (8, 9), cultured neurons (10), and brain tissue slices (11–13). In all instances the response had a drug sensitivity characteristic of muscarinic acetylcholine receptors. Kebabian *et al.* (14) examined in detail the muscarinic cholinergic regulation of cGMP content in the superior cervical ganglion. Muscarinic agonists caused a cGMP increase that was mimicked by preganglionic electrical stimulation (15) and was localized histochemically in the postganglionic neurons (16).

The identification and study of muscarinic receptors has become possible by application of radiolabeled reversible agonists (13) and antagonists (17), or irreversible alkylating antagonists (18). This has allowed a direct comparison of drug effects in terms of muscarinic receptor occupancy and their actions on contraction in smooth muscle (19). We have attempted a similar comparison in the central nervous system, using increased cGMP accumulation as the pharmacological response. The rat corpus striatum was considered to be a particularly suitable tissue, since it has a high density of muscarinic receptors (20) and exhibits various other receptor-linked changes in cyclic nucleotide metabolism (21). A similar comparison of muscarinic receptor occupancy and guanylate cyclase activation has recently been reported in studies on neuroblastoma cells (22).

[2] The abbreviations used are: cAMP, adenosine cyclic 3′,5′-monophosphate; cGMP, guanosine cyclic 3′,5′-monophosphate; IBMX, 3-isobutyl-1-methylxanthine; QNB, quinuclidinyl benzilate.

METHODS

Preparation of striatal slices. Adult Wistar rats were decapitated, and the brains were rapidly dissected on a chilled surface. Striata were trimmed free of surrounding tissue (approximately 100 mg of striatal tissue wet weight, per rat), and 250 × 250 μm slices were cross-cut with a McIlwain tissue chopper. The slices were suspended in 50 ml of oxygenated Krebs-Ringer-bicarbonate (37°, pH 7.4). The suspension was incubated in a shaking water bath at 37° (constantly gassed with 95% O_2–5% CO_2) for 1 hr, with decantation and replacement of the Krebs medium at 20-min intervals. For the final 20 min of this incubation, in most experiments, the slices were resuspended in Krebs medium containing 1 mM 3-isobutyl-1-methylxanthine. At the end of the preliminary incubation period, the medium was decanted and the slices were resuspended at a concentration of 200 mg, wet weight, per milliliter in a calibrated plastic vial.

Drug treatment of slices and assay of cyclic nucleotides. Fifty-microliter aliquots (10 mg, wet weight) of the striatal slice suspension were added to 220 μl of Krebs medium plus 1 mM IMX in small glass centrifuge tubes and equilibrated at 37° for 10 min. Antagonist drugs were added at the beginning of this final equilibration period. Agonists were added after this step in 2.5 μl of medium, and the incubation was continued for a further 2 min, unless otherwise noted. Tissue cGMP was released by heating the samples on a boiling water bath for 10 min (21) and the tissue debris was removed by centrifugation at 4° for 10 min. Duplicate 100-μl samples of the supernatant were taken for cGMP determination using a radioimmunoassay kit (Radiochemical Centre), and 50-μl samples were taken in some experiments for cAMP determination by a sensitive protein-binding assay (23). The tissue pellets were solubilized by heating in 1 ml of 1 N NaOH, and the protein concentration was determined using bovine serum albumin as a standard.

Control experiments established that the measured cGMP content was linearly

related to sample volume. Internal standards of cGMP were quantitatively recovered and were additive to measured tissue levels.

[^{3}H]QNB binding to slices. Twenty-five-microliter aliquots of striatal slice suspension (5 mg, wet weight), prepared and initially incubated as described above, were added to 460 μl of Krebs medium in 15-ml plastic centrifuge tubes. Drug additions were made in 5 μl of Krebs medium. (±)-[3-^{3}H]Quinuclidinyl benzilate (specific activity, 13 Ci/mmole; Radiochemical Centre, Amersham) was added in 10 μl of a diluted stock solution (stored frozen) to give a final concentration of 1.36 nM (unless otherwise noted). Incubations were carried out at 20° for 30 min with gentle agitation of the slices at 5-min intervals. Slices were then pelleted by centrifugation for 2 min at 3500 × *g* with a bench centrifuge at 20°. The tubes were drained, and the slices were washed twice by resuspension in 1 ml of Krebs medium and centrifugation. After the last draining, residual Krebs medium was removed from the walls of the tube with tissue paper. [^{3}H]QNB was extracted by addition of 1 ml of ethoxyethanol to the pellet. The ethoxyethanol-slice suspension was counted by liquid scintillation spectrometry after the addition of 10 ml of 0.4% (2,4″-*tert*-butylphenyl)-5-(4″-biphenyl-1,3,4-oxidazole) (w/v) in toluene. Blank values for nonspecific binding were determined by parallel incubation in the presence of 1 mM oxotremorine or 1 μM QNB. Control experiments showed that more than 99% of tissue [^{3}H]QNB was extracted by ethoxyethanol.

Calculation of IC_{50} and apparent K_i values for [^{3}H]QNB binding. Apparent K_i values for drugs acting as inhibitors of [^{3}H]QNB binding were calculated from the formula

$$K_i = \frac{IC_{50}}{1 + S/K_d}$$

IC_{50} values were determined from log probit plots of percentage inhibition of specific [^{3}H]QNB binding at various test concentrations of drug, each assayed in triplicate. S is the concentration of [^{3}H]QNB (1.36 nM), and K_d is the dissociation constant for specific [^{3}H]QNB binding (3.1 nM). Thus calculated K_i values for inhibition of [^{3}H]QNB binding are 1.44 times lower than measured IC_{50} values and it is assumed that all inhibitors act competitively and obey simple mass-action kinetics.

RESULTS

[^{3}H]QNB binding to striatal slices. The slice preparation was assessed for its suitability for use in receptor occupancy measurements. Specific binding of [^{3}H]QNB measured in the concentration range 0.1–10.0 nM occurred rapidly and reached saturation within 30 min at 20°, whereas nonspecific binding, in the presence of a high concentration of nonradioactive oxotremorine or QNB, increased linearly with incubation time (Fig. 1). The amount of nonspecific binding was similar whether oxotremorine (1 mM) or QNB (1 μM) was used as the displacing agent. Nonspecific binding accounted for 30–40% of total [^{3}H]QNB binding when 250-μm slices were incubated with 1.36 nM [^{3}H]QNB for 30 min. In other experiments the proportion of nonspecific binding was found to be reduced if smaller slices of striatal tissue (100 μm) were used, and in striatal homogenates under comparable incubation conditions nonspecific binding was approximately 10% of total binding and did not increase with time. The specific binding of

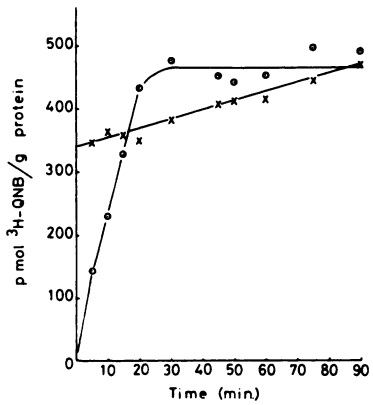

FIG. 1. *Time course of binding of saturating concentration of [^{3}H]QNB (5 nM) in rat striatal slices during incubation at 20°*

⊙, specific binding; X, nonspecific binding. Values are means of quadruplicate determinations.

[^{3}H]QNB, but not the nonspecific component, was saturable, and a Scatchard analysis of these data indicated K_d = 3.1 nM (mean of two separate experiments). In other experiments, using competition with unlabeled QNB, an IC_{50} value of 5.0 nM was obtained, indicating a calculated K_i value of 3.47 nM, similar to the K_d value. The number of specific binding sites for [^{3}H]QNB in striatal slices was calculated from the Scatchard analysis as 0.4 pmole/mg of protein, and this value is similar to that determined in other experiments using striatal homogenates,[3] and to the value reported previously for rat striatal homogenates (20) of 0.48 pmole/mg of protein. Thus it would appear that saturable [^{3}H]QNB binding can be measured in a brain slice preparation, and that the number of binding sites estimated in this way is similar to that observed in cell-free homogenates of brain tissue, although the apparent K_d for QNB in slices indicates a considerably lower affinity for binding in slices than in homogenates, a point that will be developed more fully elsewhere.[3]

To check whether any metabolism of [^{3}H]QNB occurred during incubation with the brain slices, in some experiments striatal slices were rinsed and homogenized after incubation with [^{3}H]QNB, and the labeled material in the tissue extract was subjected to high-voltage electrophoresis. Essentially all the extracted radioactivity moved as a single peak, co-migrating with standard [^{3}H]QNB. Similar checks were applied to stock solutions of [^{3}H]QNB, which were found to remain stable on storage at −20° and with repeated freezing and thawing.

cGMP responses to cholinergic drugs. The incubation conditions were chosen to minimize variations in control levels of cyclic nucleotides. Resting levels of cGMP in the absence of the phosphodiesterase inhibitor IBMX were 0.20 ± 0.03 pmole/mg of protein (n = 10), and for cyclic AMP, 4.20 ± 0.62 pmoles/mg of protein (n = 6). The addition of 1 mM IBMX to the incubation medium raised the basal level of cGMP to 0.72 ± 0.09 pmole/mg of protein (n = 34), and that of cAMP, to 7.80 ± 0.30 pmoles/mg of protein (n = 16).

Figure 2 shows the time course of changes in cGMP concentrations in response to added muscarinic receptor agonists. Oxotremorine at 100 μM produced a 6-fold increase in cGMP in the absence of IBMX, and approximately a doubling in its presence. Arecoline at 100 μM and carbachol at 500 μM (without IBMX) gave comparable results. In all cases the maximal cGMP response was reached within 60–90 sec after drug addition, and declined rapidly thereafter. The addition of IBMX did not affect the time needed to attain the maximum response, but it appeared to prolong the falling phase. For example, without IBMX the oxotremorine-induced cGMP response fell to control values 4 min after drug addition, whereas with IBMX it did not return to the baseline until 10 min. There were slight differences in both the rate of onset of the cGMP response and the decay time for the different agonists. The half-times for decay were 2.5 min for oxotremorine, 2.9 min for arecoline, and 3.4 min for carbachol. The time course of the cGMP response was unaffected by the concentration of agonist used, peak responses being consistently reached at 2 min.

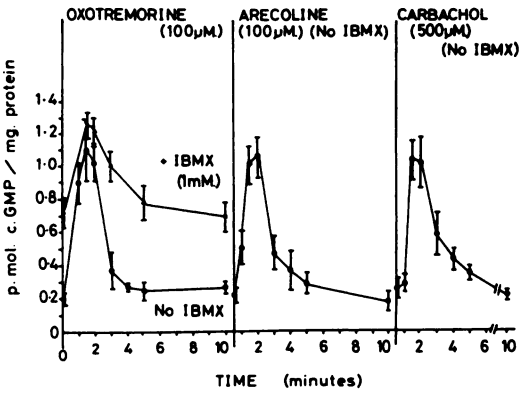

FIG. 2. *Time course of cGMP responses to muscarinic agonists*

Slices were incubated for the indicated periods at 37° and promptly placed in a boiling water bath (10 min) to release cyclic nucleotides. Each data point is the mean ± standard error of duplicate determinations in two separate experiments. Units are picomoles of cGMP per milligram of protein. IBMX, 3-isobutyl-1-methylxanthine.

[3] R. Gilbert, M. Hanley, and L. L. Iversen, unpublished observations.

The muscarinic nature of the cGMP response was confirmed by the use of selective inhibitors (Table 1). Nicotinic receptors antagonists (α-bungarotoxin and hexamethonium) were ineffective, but muscarinic antagonists (QNB and atropine) were potent blocking agents. Nicotine did not change basal levels of cGMP. Oxotremorine (100 μM) also unexpectedly caused a significant increase (80%) in the concentration of cAMP after 2 min (Table 2). This response was blocked by muscarinic but not by nicotinic antagonists. Antagonists for *beta* adrenoceptors (propranolol), *alpha* adrenoceptors (phentolamine), and H_1-histamine (mepyramine) and H_2-histamine (methiamide) receptors did not affect the cAMP response. The dopaminergic antagonist α-flupenthixol (10 μM). however, completely inhibited the oxotremorine-induced cAMP rise, suggesting that it might represent an indirect response possibly due to release of dopamine. The elevated levels of cAMP were stable for 2–5 min, and declined thereafter to basal levels at 15 min.

Although the percentage increase in cGMP was greater without IBMX, the addition of IBMX was desirable in order to obtain reproducible responses at low agonist concentrations, and to improve the reproducibility with which basal cyclic nucleotide levels could be determined in different slice preparations. In the presence of IBMX the muscarinic cGMP response was sufficiently consistent to allow dose-response curves to be established.

Figure 3 shows such dose-response curves for three agonists, and Table 3 gives the EC_{50} values determined by log probit analysis, together with Hill coefficients. Oxotremorine was more potent than arecoline, which was in turn more potent than carbachol, as has been observed physiologically (19). All these curves yielded Hill coefficients less than unity: 0.75 for oxotremorine, 0.77 for arecoline, and 0.45 for carbachol (Table 3). All three agonists produced identical maximal cGMP levels (Fig. 2). The effect of the partial agonist pilocarpine is illustrated in Fig. 4. The dose-response curve was bell-shaped, with responses diminishing at drug concentrations above 10 μM. The Hill coefficient for the stimulating range of pilocarpine concentrations was 0.80.

TABLE 1

Pharmacology of cGMP response in striatal slices

Values are means ± standard errors for six experiments.

Addition	cGMP
	pmoles/mg protein
None (basal level)	0.72 ± 0.09
100 μM oxotremorine	1.25 ± 0.08
+1 μM atropine	0.71 ± 0.05
+1 μM QNB	0.69 ± 0.06
+10 μM α-bungarotoxin	1.31 ± 0.11
+10 μM hexamethonium	1.27 ± 0.10
100 μM nicotine	0.77 ± 0.09

TABLE 2

Pharmacology of cAMP response in striatal slices

Values are means ± standard errors for six experiments

Addition	cAMP
	pmoles/mg protein
None (basal level)	7.8 ± 0.3
100 μM oxotremorine	13.2 ± 0.7
+10 μM α-bungarotoxin	13.0 ± 0.8
+1 μM atropine	7.4 ± 0.4
+1 μM QNB	7.6 ± 0.2
+10 μM hexamethonium	13.1 ± 0.7
+10 μM propranolol	12.7 ± 0.7
+10 μM phentolamine	13.1 ± 0.6
+10 μM mepyramine	13.6 ± 0.6
+10 μM methiamide	13.1 ± 0.7
+10 μM α-flupenthixol	7.9 × 0.4

Antagonist potencies were determined against maximal stimulation of muscarinic receptors by 100 μM oxotremorine. Figure 5 shows the results obtained with a range of antagonists, including both classical muscarinic drugs and some neuroleptics which have previously been shown to be active on central nervous system muscarinic receptors (24). The antagonists were effective in the order scopolamine > QNB > atropine. All had Hill coefficients near 1.0. The neuroleptics were active in the order clozapine > thioridazine > chlorpromazine > haloperidol. Clozapine and chlorpromazine yielded unusually steep dose-response curves, with Hill values approaching 2.0 (Table 4). In control experiments antagonists were in-

cubated with slices in the absence of oxotremorine. None of the drugs tested caused any change in cGMP or cAMP, except for haloperidol, which sometimes caused a small increase in the levels of both cyclic nucleotides.

Comparison of drug potencies toward [^{3}H]QNB binding and cGMP production. The results obtained with the agonists oxotremorine, arecoline, and carbachol in the two systems agreed, and in both instances the Hill values were less than 1.0 (Table 3). Pilocarpine, although classified as a partial agonist (13), behaved as a mixed agonist-antagonist in its effects on cGMP accumulation. It was an order of magnitude more potent in stimulating cGMP accumulation than in displacing [^{3}H]QNB.

Results obtained with antagonists are compared in Table 4. It is difficult to compare directly the values obtained from the antagonism of the cGMP response elicited by a high concentration of an agonist (100 μM oxotremorine) with the results obtained for the same drugs against a low concentration of an antagonist (1.4 nM [^{3}H]QNB). For this reason, we have compared the IC_{50} values from the cGMP experiments with the K_i values for inhibition of [^{3}H]QNB binding. Scopolamine was more effective than QNB in antagonizing the cGMP response, whereas the reverse was true for inhibition of [^{3}H]QNB binding. With this exception, the rank order and relative differences in potency for the two systems were very similar. Apparent K_i values for inhibition of the cGMP response could be calculated, assuming mass-action interaction at a single site, these values being 18.8 times lower than the IC_{50} values. These values indicated that most of the antagonists tested were approximately 10 times more potent in the cGMP system than in the [^{3}H]QNB binding assay (Table 4). Furthermore, the neuroleptics clozapine and chlorpromazine displayed apparent cooperativity in their antagonism of the oxotremorine stimulation of cGMP, whereas their inhibition of

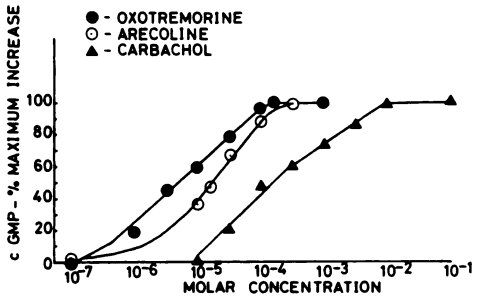

FIG. 3. *Dose-response curves for muscarinic agonists*

Agonists were added at the indicated concentrations, and the slices were incubated for 2 min. Each data point is the mean of triplicate determinations in three separate experiments (oxotremorine) or two separate experiments (carbachol and arecoline). The standard error of the mean was 10–15% of the data point in each case.

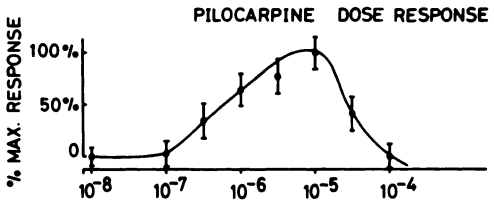

FIG. 4. *Dose-response curve for pilocarpine*

Each data point is the mean ± standard error of triplicate determinations in two separate experiments.

TABLE 3

Comparison of agonist potencies for cGMP and [^{3}H]QNB binding

Agonist	EC_{50} for cGMP increase	Hill coefficient cGMP response	K_i for inhibition of [^{3}H]QNB binding	Hill coefficient for inhibition of [^{3}H]QNB binding
	μM		μM	
Oxotremorine	5.6	0.75	5.7	0.49
Arecoline	22.0	0.77	54.2	0.42
Carbachol	120.0	0.45	102.0	0.31
Pilocarpine	1.2[a]	0.80	18.6	0.75

[a] Displayed mixed agonist-antagonist properties.

[^{3}H]QNB binding gave Hill coefficients near 1, suggesting no such cooperativity.

Characteristics of the muscarinic cGMP response. Previous results (8, 14, 25–28) have indicated that changes in tissue cGMP content are dependent on the presence of extracellular calcium. Figure 6 shows the effect of calcium ion concentration on the basal and oxotremorine-stimulated levels of cGMP in striatal slices. Basal levels of cGMP were increased by elevated calcium concentrations in the incubation medium. The oxotremorine-induced stimulation was abolished by removal of calcium, and was reduced to 30% of maximal values in the presence of 0.25 mM calcium. The inset (Fig. 6) shows the difference between oxotremorine-stimulated and basal levels, plotted as a percentage of the maximum response. The concentration of calcium required for full stimulation was 1 mM and increasing cal-

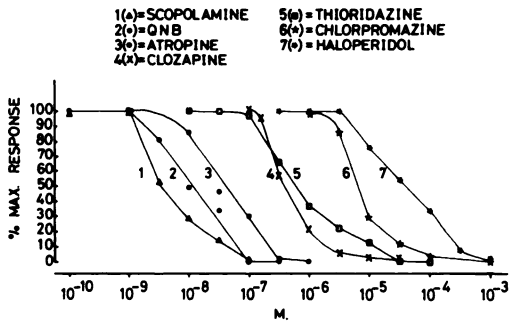

FIG. 5. *Dose-response curves for muscarinic antagonists and neuroleptics against cGMP stimulation by 100 μM oxotremorine*

Antagonists were incubated for 10 min with slices before the addition of oxotremorine. For the neuroleptics, controls were run with the solubilization medium alone (0.1% ascorbic or lactic acid). Each data point is the mean of triplicate determinations in one or two experiments. The standard error of the mean was 10–15% of the data point in each case.

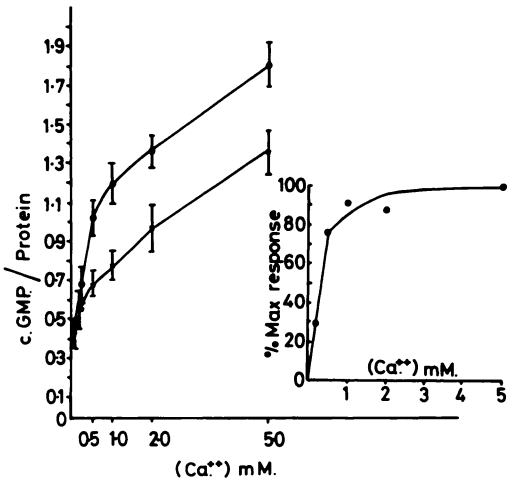

FIG. 6. *Basal and oxotremorine-stimulated levels of cGMP as a function of calcium concentration in the incubation medium*

Slices were transferred for the final 10-min incubation into Krebs solution containing the indicated calcium concentration and then exposed to 100 μM oxotremorine (added in the same medium) for 2 min or incubated for an additional 2 min. The upper curve shows the oxotremorine-stimulated values, and the lower shows the basal values (picomoles per milligram of protein). The inset shows the dependence of the oxotremorine stimulation on calcium, expressed as a percentage of the maximum difference between basal and oxotremorine-induced levels. Each data point is the mean ± standard error of triplicate determinations in two separate experiments.

TABLE 4

Comparison of antagonist potencies for cGMP and [^{3}H]QNB binding

Antagonist	IC_{50} for cGMP response[a]	Hill coefficient cGMP response	K_i for inhibition of [^{3}H]QNB binding	Hill coefficient for [^{3}H]QNB binding
	μM		nM	
QNB	23.0	1.05	3.1	1.46
Scopolamine	5.6	1.10	5.2	1.05
Atropine	54.0	1.10	6.8	1.15
Clozapine	600.0	1.70	311.0	0.80
Thioridazine	1,300.0	1.10	836.0	0.97
Chlorpromazine	9,300.0	1.65	6,850.0	0.85
Haloperidol	64,000.0	1.00	32,900.0	1.06

[a] Against 100 μM oxotremorine.

cium levels beyond this had no further effect.

Sodium azide has been used to differentiate soluble from particulate guanylate cyclase. The former is unresponsive to azide, whereas the particulate enzyme in brain can be activated by azide in a concentration-dependent manner (29). The dose-response curve of cGMP vs. sodium azide concentration, in both the presence and absence of 100 μM oxotremorine, is shown in Fig. 7. Maximal responses to azide were obtained at 5 mM in striatal slices. The oxotremorine-induced response was additive to the azide-induced increases at all azide concentrations.

DISCUSSION

This study demonstrates the feasibility of using a tissue slice preparation for the comparison of binding data and cyclic nucleotide responses. The suitability of the slices for binding experiments was not unexpected, since [^{3}H]QNB binds with high affinity and specificity to muscarinic receptors (17). For the purpose of the present study we felt it important to compare cyclic nucleotide responses and [^{3}H]QNB binding under similar experimental conditions. There are differences between binding data obtained in intact striatal slices and in striatal homogenates, and these will be described in more detail elsewhere.

Muscarinic receptor-linked changes in cGMP levels have been demonstrated in several systems (10, 12, 14, 30, 31). Our results in striatal slices are similar to these previous reports in several respects. One common feature is the transient duration of the changes in cGMP accumulation. In all cases maximal cGMP levels are reached 1–2 min after stimulation of muscarinic receptors. We found the time course of the cGMP response to be relatively independent of the particular agonist used or its concentration. Receptor desensitization is therefore unlikely to explain the transient nature of the response. The decay phase was prolonged by the addition of a phosphodiesterase inhibitor, as reported previously for neuroblastoma responses to carbachol (10). This suggests that phosphodiesterase activity may regulate the rapid removal of intracellular cGMP.

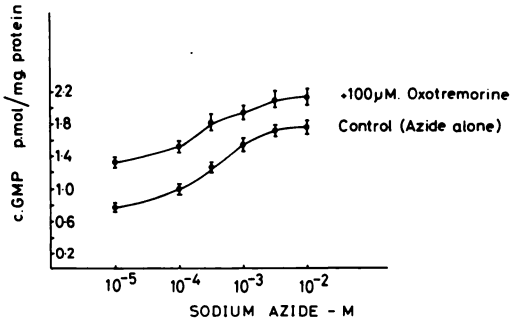

FIG. 7. *Basal and oxotremorine-stimulated levels of cGMP as a function of sodium azide concentration in the incubation medium*

Azide treatment was conducted as were experiments on altered calcium concentration (see legend to Fig. 6). Each data point is the mean ± standard error of triplicate determinations in two separate experiments.

A second consistent feature of muscarinic cGMP responses is their dependence on calcium. The striatal slice response to various calcium concentrations in the medium (Fig. 6) is similar to that obtained for cell-free guanylate cyclase (32). It has been suggested that calcium may function in the intact cell as an intermediate messenger between stimulation of cell surface receptors and activation of guanylate cyclase (25–28). The calcium activation of the oxotremorine-induced cGMP response suggests that a soluble form of guanylate cyclase is involved, since the particulate enzyme is inhibited by calcium (33). The results obtained with sodium azide also support the view that a soluble guanylate cyclase is the source of the increased cGMP. Cholinomimetic and azide-induced stimulations were additive irrespective of the azide concentration (Fig. 7). Since the particulate and not the soluble enzyme is activated by sodium azide (29), the muscarinic response appears to be coupled to activation of a soluble guanylate cyclase, possibly mediated by changes in intracellular calcium (27, 28).

In spite of the indirect nature of the receptor-cyclic nucleotide coupling, there was nonetheless good agreement between measures of receptor occupancy and the cGMP response. EC_{50} values for the ago-

nists oxotremorine, arecoline, and carbachol agreed well with the K_i values for antagonist ([^{3}H]QNB) displacement (Table 3). Thus receptor occupancy appeared to be linearly related to the magnitude of the cGMP change over the effective concentration range for each agonist. This appears to rule out the possible existence of "spare receptors" (13), which have been invoked to explain the discrepancy between drug potencies in binding assays and biological test systems (13, 19).

Agonist dose-response curves for both binding and cGMP experiments displayed apparent negative cooperativity. The possible origins of this effect have been discussed in some detail (13). The possibilities are receptor heterogeneity, true negative cooperativity, receptor desensitization, and competing agonist-agonist interactions. The available evidence would suggest that the first of these is the most likely (13).

Muscarinic antagonists were active in both systems, although estimated K_i values suggested that they were approximately one order of magnitude more potent in blocking the cGMP response than in displacing [^{3}H]QNB. The reasons for this disparity are unknown, and it may be more apparent than real, since the validity of such estimates of absolute potency in either system may be questioned. The finding that the rank order of potencies of these drugs in the two systems were nearly identical supports the notion that both assays involve the same receptor system.

We have also extended the observations of antimuscarinic activity in some neuroleptic drugs (24) to show that these drugs can block the cGMP elevation coupled to muscarinic receptor stimulation. The rank order of potency of the neuroleptics tested agrees well with previous data (24, 34). Richelson (35) has also recently reported that these and other neuroleptic drugs were able to antagonize the muscarinic receptor-mediated stimulation of cGMP formation in cultured neuroblastoma cells.

One difference between the binding and cGMP results obtained with the neuroleptics clozapine and chlorpromazine was that the antagonism of cGMP production was apparently cooperative, unlike the simple mass-action curves obtained for the other muscarinic antagonists, or for these drugs as inhibitors of [^{3}H]QNB binding. These discrepancies are difficult to interpret, but they suggest that the interaction of neuroleptics with cGMP is complex.

Pilocarpine acted as a mixed agonist-antagonist for cGMP, with the agonist character being expressed at lower concentrations than those expected from [^{3}H]QNB displacement. The ability of pilocarpine to produce a full cGMP response was unexpected in view of its partial agonist classification (13), but this has also been seen in its stimulation of phosphatidylinositol turnover (36).

In their similar studies of muscarinic responses in neuroblastoma cells, Strange *et al.* (22) failed to find any activation of guanylate cyclase activity by pilocarpine, although the drug did antagonize the activation caused by carbachol and was able to displace [^{3}H]QNB binding. Their results were also somewhat different from ours in that muscarinic agonists yielded steeper dose-response curves for activation of guanylate cyclase (Hill coefficient >1.0) than for displacement of [^{3}H]QNB. Their suggestion that muscarinic activation of cGMP formation corresponds only to the occupancy of a relatively low-affinity subpopulation of muscarinic receptors in neuroblastoma cells does not appear to be the case in brain slice preparations.

In conclusion, the present results indicate that the transient increase in cGMP observed in brain slices following muscarinic receptor stimulation is a reliable indicator of drug interactions for both agonists and antagonists at central nervous system muscarinic receptors. Nevertheless, the importance of the cGMP change in determining the over-all physiological response of the tissue to muscarinic agonists remains unclear. The finding that certain neuroleptic drugs are able to antagonize this response in brain slices adds further support to the hypothesis that some drugs in this category possess important central nervous system muscarinic antagonist activity (24).

ACKNOWLEDGMENTS

We thank Drs. Ken Minneman and Piers Emson for valuable advice and assistance with methodology. In addition, we are grateful to Dr. Michael Young for critical discussions and supplies of cholinergic drugs, and to Susan Gardiner for excellent technical assistance. Chlorpromazine and thioridazine were kindly supplied by May and Baker, Ltd; clozapine, by Wander (Sandoz) Laboratories; and haloperidol, by Janssen Pharmaceutica.

REFERENCES

1. Greengard, P., McAfee, D. A., & Kebabian, J. W. (1972) *Adv. Cyclic Nucleotide Res.* **1**, 337–355.
2. Drummond, G. I. (1973) *Neurobiol.* **2**, 119–176.
3. Daly, J. W. (1976), *Life Sci.*, **18**, 1349–1358.
4. Iversen, L. L. (1977) *J. Neurochem.* **29**, 5–12.
5. Greengard, P. (1976) *Nature*, **260**, 101–108.
6. George, W., Polson, J. B., O'Toole, A. G. & Goldberg, N. (1970) *Proc. Natl. Acad. Sci. U.S.A.*, **66**, 398–403.
7. Schultz, G., Hardman, J. G., Schultz, K., Davis, J. W. & Sutherland, E. W. (1973) *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 1721–1725.
8. Smith, R. J. & Ignarro, L. J. (1975) *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 108–112.
9. Haymovits, A. & Scheele G. A. (1976) *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 156–160.
10. Matsuzawa, H. & Nirenberg, M. (1975) *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 3472–3476.
11. Ferrendelli, J. A., Steiner, A. L., McDougal, D. B., Jr. & Kipnis, D. M. (1970) *Biochem. Biophys. Res. Commun.*, **41**, 1061–1067.
12. Lee, T. P., Kuo, J. F., & Greengard, P. (1972) *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 3287–3291.
13. Birdsall, N. J. M. & Hulme, E. C. (1976) *J. Neurochem.*, **27**, 7–16.
14. Kebabian, J. W., Steiner, A. L. & Greengard, P. (1975). *J. Pharmacol. Exp. Ther.*, **193**, 474–488.
15. Weight, F., Petzold, G. & Greengard, P. (1974) *Science*, **186**, 942–944.
16. Kebabian, J. W., Bloom, F. E., Steiner, A. L. & Greengard, P. (1975) *Science*, **190**, 157–159.
17. Snyder, S. H., Chang, K. J., Kuhar, M. J. & Yamamura, H. (1975) *Fed. Proc.*, **34**, 1915–1921.
18. Burgen, A. S. V., Hiley, C. R., & Young, J. M. (1974) *Br. J. Pharmacol.*, **51**, 279–285.
19. Yamamura, H. & Snyder, S. H. (1974) *Mol. Pharmacol.*, **10**, 861–867.
20. Yamamura, H. & Snyder, S. H. (1974) *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 1725–1729.
21. Forn, J., Krueger, B., & Greengard, P. (1974) *Science*, **186**, 1118–1120.
22. Strange, P. G., Birdsall, N. J. M. & Burgen, A. S. V. (1977) *Biochem. Soc. Trans.* **5**, 189–191.
23. Brown, B. L., Ekins, R. P. & Albano, J. D. M. (1972) *Cyclic Nucleotide Res.*, **2**, 25–40.
24. Miller, R. J. & Hiley, C. R. (1974) *Nature*, **248**, 596–597.
25. Schultz, G., Hardman, J. G., Schultz, K., Baird, C. & Sutherland, E. W. (1973) *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 3889–3893.
26. Ferrendelli, J. A., Rubin, E. H. & Kinscherf, D. (1976) *J. Neurochem.*, **26**, 741–748.
27. Ohga, Y. & Daly, J. W. (1977) *Biochim. Biophys. Acta*, **498**, 46–60.
28. Ohga, Y. & Daly, J. W. (1977) *Biochim. Biophys. Acta*, **498**, 61–75.
29. Kimura, H., Mittal, C. K. & Murad, F. (1975) *Nature*, **257**, 700–702.
30. Palmer, G. C. & Duszynski, C. R. (1975) *Eur. J. Pharmacol.*, **32**, 375–379.
31. Diamond, J. & Hartle, D. K. (1976) *J. Cyclic Nucleotide Res.*, **2**, 179–188.
32. Wallach, D. & Pastan, I. (1976) *Biochem. Biophys. Res. Commun.*, **72**, 859–865.
33. Kimura, H. & Murad, F. (1975) *Life Sci.*, **17**, 837–843.
34. Sayers, A. & Burki, H. (1976) *J. Pharm. Pharmacol.*, **28**, 252–253.
35. Richelson, E. (1977) *Nature*, **266**, 371–373.
36. Jafferji, S. & Michell, R. (1976) *Biochem. J.*, **154**, 653–657.